Analysis of Conformation and Dynamics of Membrane-Associated Myristoylated HIV-1 Nef

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My godmother, Sylvie, for taking care of my cats while I was away

My sister, Elizabeth, for love and support

My mother, Lucy, for compassion and understanding regardless of the situation

My father, Frank, for courage and determination against all odds

God for keeping my on the right path and giving me strength when I had nearly none
Abstract of Dissertation

The accessory protein HIV-1 Nef is crucial for the progression of AIDS following HIV infection. Anchoring of HIV-1 Nef to the inner leaflet of the cell membrane is essential for many of Nef major functions, including cell signaling disruption and internalizing cell surface receptors. Membrane anchoring is accomplished via N-terminal myristoylation, a covalent acylation modification and electrostatic attraction of select N-terminal residues. Disruption of this modification has been shown to dramatically impair Nef’s functions and reduce HIV infectivity.

The shape profile of membrane bound on full-length myristoylated Nef was determined using Neutron Reflectometry (NR) and Langmuir monolayers combined with molecular modeling. Isotopically labeled myristoylated Nef was expressed and purified to increase the fidelity of the final neutron reflectometry model. The overall membrane bound profile of Nef was probed at various lipid packing densities, including 20 to 35 mN/m. It was found that at high (35 mN/m) lipid packing densities, the myristate moiety was unable to insert into the lipid membrane and Nef adopted what was believed to be a more “closed conformation” where the majority of the protein was suspended roughly 50 Å from the lipid head groups. Conversely, at low (20 mN/m) lipid packing densities Nef adopted a more “open conformation”. In this state, the myristate and hydrophobic residues on the N-terminal arm was able to insert into the membrane and the core of Nef was suspended roughly 100 Å from the lipid headgroups.

In order to better refine details of membrane associated Nef, a new method combining the Langmuir monolayer system (employed in NR measurements) and hydrogen deuterium exchange mass spectrometry (HDX MS) was developed. Method development and validation were completed to assess the utility and applicability as an analytical technique. In order to
confirm that this new method could be used for HDX, the deuterium recovery had to be monitored. There was no significant loss of deuterium label using the Langmuir monolayer HDX MS method when compared to a more conventional, solution based format. The method was further validated by observing known lipid-induced structural changes in the peptide melittin and the myristoyl-switch protein Arf-1. In both experiments, the reproducibility and quality of the data remained high and were equivalent with conventional solution HDX MS methods.

After validation of this HDX MS method involving Langmuir monolayers, the conformation of full-length myristoylated Nef at the membrane was investigated. At low lipid packing densities, Nef peptides in the N-terminal arm and the C-terminal loops showed significant protection upon membrane association, whereas the core was exposed. The deuterium incorporation of Nef at a high and low lipid packing density was compared and showed that Nef was significantly more exposed to labeling in the low lipid packing environment. Both observations are consistent with the previously determined neutron reflection models with regards to the observed “open” and “closed” conformations. The new HDX MS method can be applied to other peripheral membrane proteins to resolve their membrane bound dynamics. This novel method can also be combined with other orthogonal techniques to further expand the conformational details of membrane-bound proteins.
# Table of Contents

Title Page .......................................... i
Acknowledgements .................................. ii
Abstract ............................................ iii
Table of Contents ................................... v
List of Figures ...................................... x
List of Tables ....................................... xiv
Glossary of Terms .................................. xv

Chapter 1 - Introduction, Background and Significance: HIV-1, Nef’s Function and Membrane Association

1.1 Background and Significance ........................ 1
   1.1.a AIDS and the HIV Global Epidemic ............ 1
   1.1.b Introduction to Nef and Nef Functions ......... 3
   1.1.c Recruitment to the Membrane and Theoretical Model of Association 9

1.2 Approaches to Analysis of Nef
   1.2.a Combining HDX-MS and NR for Investigating Protein Conformation 14
   1.2.b Membrane Proteins Analysis Using Langmuir Monolayers 17

1.3 References ...................................... 20

Chapter 2 – Expression and Purification of Myristoylated Nef ........................................ 26

2.1 Introduction ..................................... 26
   2.1.a N-terminal Myristoylation of Proteins .......... 26
2.1.b Expression of Myristoylated Proteins 28

2.2 Methods 29

2.2.a The Duet Vector System 29

2.2.a.1 Expression Vectors 29

2.2.a.2 Expression in LB 32

2.2.a.3 Expression in M9 32

2.2.a.4 Ni-affinity Purification 33

2.2.a.5 Ni-affinity Purification with Triton X-100 33

2.3 Production of Myrsitoylated Nef for HDX-MS and NR Experiments 34

2.3.a Expression and Purification using the Duet Vector System 34

2.3.a.1 Production of Stable Isotopically Labeled Nef 40

2.4 Conclusions 46

2.5 References 47

Chapter 3 – Conformation of HIV-1 Nef as Determined by Neutron Reflection 50

3.1 Introduction 50

3.1a Neutron Reflection for Protein Analysis 51

3.2 Methods 54

3.2.a Production of Hck SH3 55

3.2b Langmuir Trough System used for Neutron Reflection 57

3.3 Analysis of Nef by Neutron Reflection 59

3.4 Discussion of the NR Results 64

3.5 Conclusions 70
Chapter 4 – Hydrogen Deuterium Exchange Mass Spectrometry for the Analysis of Protein Conformation and Dynamics

4.1 Introduction

4.1a Monitoring changes in conformation and dynamics in proteins with amide hydrogens

4.1b Detecting hydrogen exchange with mass spectrometry.

4.2 Hydrogen Exchange

4.2a Hydrogen Exchange in Folded Proteins

4.2b Types of Hydrogens

4.2c Proton Transfers

4.2d Base Catalyzed Amide Hydrogen Exchange

4.2e Parameters Influencing Amid Hydrogen Exchange

4.2e.1 The Effect of pH

4.2e.2 The Effect of Temperature

4.2e.3 The Effect of Primary Structure

4.2f Mechanisms of into Proteins

4.3 Experimental Methods

4.3a Materials

4.3b Buffers

4.3c Peptide Peptides

4.3d Sample Preparation and Analysis
4.3.d.1 Deuterium Labeling 93
4.3.d.2 Chromatography 95
4.3.d.3 Mass Spectrometry 98
4.3.d.4 Data Analysis and Processing 99
4.3.d.5 Error and Replication 103

4.4 References 105

Chapter 5 – Hydrogen Exchange Mass Spectrometry of Peripheral Membrane Proteins Using Langmuir Monolayers 110
5.1 Introduction 110
5.2 Methods 114
   5.2.a Production of Myristoylated ARF-1: Expression and Purification 114
   5.2.b Digestion Optimization of Membrane Associated Samples 115
   5.2.c Hydrogen Exchange Mass Spectrometry Using Langmuir Monolayers 118
5.3 Method Optimization 122
   5.3.a Assessing Deuterium Recovery from Trough Labeled Samples 122
   5.3.b Validating Protein-Lipid Interactions using Melittin 125
   5.3.c Lipid-binding Induced Conformational Changes in ARF-1 127
5.4 Conclusions 153
5.5 References 154

Chapter 6 – Hydrogen Exchange Mass Spectrometry of Nef at Langmuir Monolayers 160
6.1 Introduction 160
6.1.a Investigating Membrane-Associated Nef 160
6.1.b Nef Structure and Functions at the Membrane 162

6.2 Methods 163
6.2.a Studying Liposome-Bound Nef 163
6.2.b Optimization of Labeling pH 190
6.2.c Method for Solution Hydrogen Exchange of myrNef using Langmuir Monolayers 192

6.3 Effects of Membrane Association on Nef Conformation 194
6.3.a Peptides followed for Hydrogen Exchange of myrNef 194
6.3.b Comparison of solution and 20 mN/m monolayers 194
6.3.c Effects of lipid packing density on conformation 229

6.4 Conclusions 235
6.5 References 236

Chapter 7 – Perspectives and Future Plans 240
7.1 Introduction 240
7.2 Future Directions 243
7.2.a Altering Lipid Monolayer Environment 244
7.2.b Probing Conformational Changes and Partner Binding with Fluorescence 245
7.2.c Quantifying Penetration into Membranes 246

7.3 Brining the Langmuir Trough System to a Neutron Source 249
7.4 References 250
Appendix I 253
List of Figures

| Figure 1.1 | Global Distribution of AIDS | 2 |
| Figure 1.2 | Overview of Nef Functions | 5 |
| Figure 1.3 | Nef Variants | 6 |
| Figure 1.4 | Nef Structure | 8 |
| Figure 1.5 | Myristoylated Protein Membrane Interactions | 11 |
| Figure 1.6 | Theoretical Nef Binding Model | 12 |
| Figure 1.7 | Experimental Aims of Project | 16 |
| Figure 2.1 | Duet Vector Plasmid Map | 30 |
| Figure 2.2 | Workflow for Expressing myrNef | 31 |
| Figure 2.3 | Ni-NTA Purification of Nef | 35 |
| Figure 2.4 | Intact Mass Spectra of Nef | 36 |
| Figure 2.5 | SDS-PAGE of Nef | 37 |
| Figure 2.6 | Modifications of Nef | 39 |
| Figure 2.7 | Intact Mass Spectra of Myristoylated Nef | 41 |
| Figure 2.8 | Workflow for Expression of Stable Isotopically Labeled Proteins | 43 |
| Figure 2.9 | Intact Mass Spectra of Stable Isotopically Labeled Nef | 44 |
| Figure 3.1 | Intact Mass Spectra of Stable Isotopically Labeled Hck SH3 | 58 |
| Figure 3.2 | Langmuir Trough Assembly | 60 |
| Figure 3.3 | Surface Pressure Readings from Langmuir Trough | 61 |
| Figure 3.4 | Neutron Reflectometry Results for Nef | 65 |
| Figure 3.5 | Nef Membrane Binding Process | 68 |
List of Tables

Table 2.1 Stable Deuterium Labeling Sites for Nef 42
Table 3.1 Stable Deuterium Labeling Sites for Hck SH3 56
Table 5.1 Test Peptide Information 123
Table 6.1 Nef Peptide Information 195
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>AnEx</td>
<td>Anion Exchange</td>
</tr>
<tr>
<td>BPI</td>
<td>Base Peak Intensity</td>
</tr>
<tr>
<td>CA</td>
<td>HIV Capsid Protein</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>DDA</td>
<td>Data Dependent Acquisition</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DPPG</td>
<td>1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast Atom Bombardment</td>
</tr>
<tr>
<td>FA</td>
<td>Formic Acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic Interaction Chromatography</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HDX</td>
<td>Hydrogen Deuterium Exchange</td>
</tr>
<tr>
<td>IN</td>
<td>HIV Integrase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LUV</td>
<td>Large Unilamellar Vesicle</td>
</tr>
<tr>
<td>M9</td>
<td>Minimal Media</td>
</tr>
<tr>
<td>MA</td>
<td>HIV matrix protein</td>
</tr>
<tr>
<td>mN/m</td>
<td>Milinewtons per meter</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar Vesicle</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MyrNef</td>
<td>Myristoylated Nef</td>
</tr>
<tr>
<td>NC</td>
<td>HIV Nucleocapsid Protein</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NR</td>
<td>Neutron Reflectometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NMT</td>
<td>N-myristoyltransferase</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PLGS</td>
<td>ProteinLynx Global Server</td>
</tr>
<tr>
<td>PP-II</td>
<td>Polyproline type II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PR</td>
<td>HIV protease</td>
</tr>
<tr>
<td>RT</td>
<td>HIV Reverse transcriptase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3</td>
</tr>
<tr>
<td>SLD</td>
<td>Scattering Length Density</td>
</tr>
<tr>
<td>SU</td>
<td>HIV surface glycoprotein (gp120)</td>
</tr>
<tr>
<td>SUV</td>
<td>Small Unilamellar Vesicle</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIC</td>
<td>Total Ion Chromatogram</td>
</tr>
<tr>
<td>TM</td>
<td>HIV transmembrane glycoprotein (gp41)</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra performance liquid chromatography</td>
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</table>
Chapter 1

Introduction, Background and Significance:

HIV-1, Nef’s functions and Membrane Association

1.1 Background and Significance

1.1.a AIDS and the HIV Global Epidemic

Acquired Immunodeficiency Syndrome (AIDS) was identified in the early 1980s and the AIDS epidemic has resulted in over 25.3 million deaths (UNAIDS 2015). The World Health Organization estimates that 36.9 million people are living with the human immunodeficiency virus (HIV) worldwide (Figure 1.1) and that over 2 million individuals were newly infected in 2014 (UNAIDS 2015). The discovery that AIDS is caused by infection by this retrovirus was made in 1983 [1]. HIV infects CD4+ lymphocytes and causes apoptosis in less than two days [2]. Over the course of infection, this results in a severe depletion of the host’s immune system. An infection with HIV leaves most individuals vulnerable to various other secondary infections, which are generally the immediate cause of death.

The human immunodeficiency virus consists of fifteen proteins and one RNA molecule [3]. The HIV genome contains nine open reading frames. The Gag, Pol and Env polyproteins (which are common to all retroviruses) are cleaved into individual proteins after translation. The structural components of the HIV virion are made up of the four Gag proteins, MA (matrix), CA (capsid), NC (nucleocapsid) and p6 and the two Env proteins, SU (gp120) and TM (gp41). The
Figure 1.1. Global Status of the AIDS epidemic. The estimated population infected with HIV for each country as of 2014. Adapted from UNAIDS report, 2015.
enzymatic functions, for converting and merging the viral genetic code with that of the host, of
the virus are encoded within the three Pol proteins, PR (protease), RT (reverse transcriptase) and
IN (integrase). The additional six proteins are commonly called accessory proteins lack structural
or enzymatic functions. Vif, Vpr and Nef are packaged within the viral particle and all assist in
the establishment of infection and/or immune evasion. Tat and Rev are gene regulatory proteins
and Vpu assists in virion assembly. Lastly, the entire viral genome is encoded within a 9-kb
RNA molecule, which is packaged up in each mature virion [3].

1.1.b Introduction to Nef and Nef Functions

The HIV-1 accessory protein Nef has been shown to be critical for HIV function [4-6].
Interestingly, progression to AIDS can be delayed for individuals infected with an HIV strain
containing a non-functional nef gene [7-9]. Nef is required for maintaining high viral levels in vivo
[10] and is expressed at very high levels early in the viral life cycle [11]. Several animal models
have confirmed the importance of Nef function for viral infection. SIV+ rhesus monkeys infected
with a nef deleted viral strain do not develop an immune deficiency [12]. In contrast, mice
engineered to express HIV Nef alone [13] develop an immune deficiency-like syndrome which
leads to death. All of these observations clearly indicate that Nef is a very important protein for
the viral life cycle. Therefore is equally as important for us to investigate and understand.

Dissimilar to HIV protease, reverse transcriptase and integrase, the accessory proteins
have no known catalytic activity. Accessory proteins act primarily by interacting with host
proteins, hijacking the function of the host proteins and causing them to act in a manner that is
detrimental to the host but beneficial to the virus. Nef in particular has been shown to interact
with over 30 host proteins \cite{14}. The HIV accessory protein Nef has three main functions (Figure 1.2): (1) altering signaling pathways, (2) down regulation of cell surface receptors and (3) infectivity enhancement \cite{15-18}. Nef interacts with many cellular signaling proteins \cite{19} and as a result enhances the responsiveness of T cells to stimulation \cite{20}. These interactions are thought to induce a variety of signaling pathways and activate transcription factors \cite{21} which in turn enhance viral and cellular gene expression. Interestingly, these major Nef functions are impaired when membrane association is inhibited \cite{22, 23}.

HIV-1 Nef affects the surface expression of several receptors. First, down modulation of MHC-I \cite{24, 25} protects infected primary T cells from being destroyed by CD8+ cytotoxic T lymphocytes (CTL) \cite{15}. As a result of MHC-I depletion, natural killer (NK) cells would normally destroy infected cells; however Nef selectively down modulates HLA which reduces the ability of NK cells to kill HIV infected cells \cite{26}. Another major function of Nef is endocytosis and lysosomal degradation of CD4 receptors \cite{27}. CD4 assists T cell activation and is the primary receptor for HIV and SIV entry. Down regulation of CD4 limits superinfection (reinfection of a cell already infected with HIV) and enhances mature viral budding and release. Nef recruits the AP-2 clathrin adaptor complex to CD4 resulting in trafficking to lysosomes for degradation \cite{17}. A sequence alignment \cite{28} of several Nef allelic variants \cite{29} and one LTNP variant (long term non-progressor) are shown in Figure 1.3. Nef proteins from the various strains display very high sequence similarity even though their ability to enhance HIV replication may be vastly different. Despite the wealth of knowledge of Nef function very little is known about its full-length myristoylated structure.

Experimentally derived structural data for Nef is incomplete. To-date, a full length X-ray crystallography or NMR structure has yet to be solved. However, high resolution structures for
Figure 1.2. Overview of Nef functions. Nef interacts with a variety of host proteins to achieve its range of functions. 1) Nef utilizes the AP/Clathrin pathways to internalize cell surface receptors such as MHC-1 and CD4; thus, preventing communication with cytotoxic T lymphocytes (CTLs) and antigen presenting cells (APCs). 2) Nef promotes viral replication, release and works to limit superinfection to promote infectivity. 3) Nef influences downstream signaling events by interacting with signaling factors, such as cellular kinases. Adapted from Kirchhoff et al. 2008.
<table>
<thead>
<tr>
<th>Nef Construct</th>
<th>Theoretical Mass (Da)</th>
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<tbody>
<tr>
<td>ELI</td>
<td>23,481.39</td>
</tr>
<tr>
<td>LTNP4</td>
<td>23,305.37</td>
</tr>
<tr>
<td>NL4-3</td>
<td>23,446.80</td>
</tr>
<tr>
<td>Consensus</td>
<td>23,343.36</td>
</tr>
<tr>
<td>SF2</td>
<td>24,121.38</td>
</tr>
</tbody>
</table>

### Figure 1.3
Theoretical masses and sequence alignment for several Nef constructs. Sequences were aligned using ClustalOmega (Sievers, Wilm et al. 2011).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>* (asterisk)</td>
<td>Positions which have a single, fully conserved residue</td>
</tr>
<tr>
<td>: (colon)</td>
<td>Conservation between groups of strongly similar properties</td>
</tr>
<tr>
<td>. (period)</td>
<td>Conservation between groups of weakly similar properties</td>
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**Table**

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</tr>
<tr>
<td>. (period)</td>
<td>Conservation between groups of weakly similar properties</td>
</tr>
</tbody>
</table>
portions of the Nef molecule have been determined (Figure 1.4). The Nef core domain (residues 54-205) was co-crystallized with Fyn SH3 domain \[^{30}\]. The internal loop (residues 149-177) and the N-terminal 16 residues remained highly disordered even as a crystal and as a consequence no electron density was determined for those regions. In the same year that the X-ray structure of Nef core domain was published, a separate group determined an NMR structure of the core and loop (residues 40-158 & 174-206) of Nef. In the following year a refined structure from the same experiment was published \[^{31}\]. Both structures reveal a similar fold. The N-terminus of the core domain consists of a polyproline type II (PP-II) helix (critical for binding to SH3 domains) followed by two alpha helices which pack against four anti-parallel beta strands. On the opposing face of the strands, two short alpha helices make up the C-terminal region (Figure 1.4). The N-terminal 25 residues of Nef have also been studied by NMR \[^{32}\]. These data reveal the presence of significant helical content spanning over residues 6-22. This helix was also seen when the N-terminus was myristoylated. However, the myristoylated peptide had poor solubility in aqueous solution so had to be solved under a variety of other conditions (trifluoroethanol, methanol, sodium dodecyl sulfate micelles). A more complete view of the Nef N-terminal region was solved by NMR in aqueous buffer at pH 7.1 both myristoylated and non-myristoylated \[^{33}\]. Comparison of the two forms showed that myristoylation stabilized two alpha helices near the N-terminus. The lack of structural details about the intact molecule and how the N-terminal arm, core domain and loop potentially interact with one another leaves a gap in the understanding of Nef. A model of full-length myristoylated Nef has been proposed (Figure 1.4) \[^{34}\]. This model combines the NMR data of the myristoylated N-terminus (2-57) and the NMR data of the core domain and uses molecular modeling to fill in any gaps.
Figure 1.4. Structure of HIV-1 Nef. 

A) Crystal structure of the core domain (residues 58-205) of NL4-3 Nef in complex with Fyn SH3. 

B) NMR structure of the myristoylated, N-terminal Nef arm (residues 2-57). 

C) Theoretical model of full-length, myristoylated Nef. This model was generated using data from the N-terminal arm and core domain and modeling the C-terminal loop and N-terminal linker. 

D) Distribution of surface charge on myr-Nef with regions of negative charge displayed in red and regions of positive charge displayed in blue.
Arold and Baur hypothesized that myristoylated Nef acts like a ‘myristoyl-switch’ protein. This category of proteins normally occludes the attached myristate moiety into a hydrophobic pocket while in solution. Once these proteins are allowed to associate with a membrane, the myristate moiety inserts into the lipid tails and this induces a structural rearrangement of the protein. Given the model by which ‘myristoyl-switch’ reversibly bind and reorganize at membrane, Arold and Baur proposed two Nef conformations. The “closed” conformation, where the myristate associates with the core domain of Nef, is dominant when Nef is in the cytosolic fraction and is incompetent for interacting with host cellular proteins. Nef then undergoes a conformational change to an “open” conformation when the myristate inserts into the plasma membrane allowing Nef to interact with its many cellular partners. As shown in the hydrophobic surface map (Figure 1.4) the core domain of Nef lacks a deep pocket where the myristate could insert. There are two small hydrophobic patches on the core where the myristate could potentially associate. A non-myristoylated full-length form of HIV-1 Nef was previously interrogated by hydrogen exchange mass spectrometry and the results generally match the proposed Arold and Baur model. The results from these experiments showed that most regions away from the Nef core were highly exposed and dynamic, suggesting a global lack of intrinsic structure.

1.1.c Recruitment to the membrane and theoretical model of association

Myristoylation of Nef allows for the reversible binding to lipid membranes. Similar to other acylated proteins, the myristate moiety on the N-terminus of Nef can insert into the phospholipid bilayer and act as a membrane anchor. Myristoylation is a weak membrane
interaction compared to other methods of membrane anchoring (Figure 1.5) \cite{40}. Myristoylation of HIV-1 Nef is catalyzed by the enzyme N–myristoyltransferase (NMT) \cite{41} and humans have two isoforms of this enzyme \cite{42}. Nef was found to be preferentially myristoylated by NMT-1 \cite{43}, and the complex of Nef and NMT-1 has been characterized \cite{44}.

Nef is found both membrane associated and in the cytosolic fraction \cite{45-47}. Membrane association has been reported to be accomplished by an initial association of an N-terminal basic cluster on Nef to negatively charged phospholipid head groups (Figure 1.6) \cite{32, 48} followed by insertion of the N-terminal myristoyl moiety into the phospholipid \cite{49, 50}. The myristoylation consensus sequence, [Met-Gly-X-X-(S/T)], where X represents any amino acid, is conserved in all Nef strains \cite{33}. Disruption of Nef’s N-terminal myristoylation results in greatly reduced infectivity \cite{10}, inhibits Nef:membrane interaction and the downregulation of CD4 and MHC-1 \cite{10, 19, 50, 51}, and eliminates progression to an AIDS-like syndrome in transgenic mice \cite{13}.

Myristoylation of Nef is believed to alter the tertiary and quaternary structure of Nef \cite{52, 53}. Circular dichroism, differential proteolysis, gel filtration, ultracentrifugation, fluorescence emission and fluorescence autocorrelation (for determination of diffusion time) comparing myristoylated vs. nonmyristoylated Nef all suggest that myristoylated Nef exists predominantly as a monomer in vitro (whereas non-myristoylated Nef can form higher order structures) and that the myristoylated monomer is more compact than the non-myristoylated form. A small myristoylated peptide mimic of the Nef N-terminus was shown to bind to Nef \cite{54}, supporting a theory that when in the cytosolic fraction, myristoylated Nef adopts a “closed” conformation with the myristoyl moiety tucked into a binding pocket in the core of Nef, and when Nef is membrane associated (with the myristate now embedded in the lipid bilayer), it assumes an “open” conformation which enables access to Nef’s many binding partners \cite{14, 52, 53}.
Figure 1.5. Various means by which myristoylated proteins interact with lipid membranes. Insertion of the myristate alone (i) is not sufficient for membrane anchoring. Additional interactions from electrostatics (ii), additional hydrophobic interactions (iii), and protein-protein interactions (iv) enhance the binding strength of membrane-bound, myristoylated proteins. Adapted from Resh 2009.
Figure 1.6. Theoretical binding model for Nef. Nef is initially attracted to the membrane via electrostatic attraction between the basic cluster region on the arm and the negatively charged lipid headgroups. This process is accompanied by the formation of an N-terminal amphipathic helix. The myristate and hydrophobic residues of the arm insert into the lipid tails of the membrane, anchoring Nef to the membrane. Adapted from Gerlach et al. 2012.
This is contention in the field with regards to how Nef associates with the membrane and with its binding partners. Recent findings from Jia et al. dispute the above mentioned “open” and “closed” conformations of Nef. In that work, a crystal structure of the Nef fused with the cytoplasmic domain of MHC-1 was resolved and showed that the N-terminus of Nef was located on the core through interactions with Trp 13 and Met 20. It was proposed that complexation occurs before membrane binding, and positions the Nef core closer to the membrane.

Membrane anchoring points located on MHC-1 and Nef are coplanar in the model generated by Jia and coworkers, and this point provided as evidence to validate the Nef:MHC-1 complex. Anchoring of the N-terminal arm to the Nef core is in contrast to previous claims that the N-terminus is detached. However, the model that Jia and coworkers proposed was generated using a fused construct, was unable to resolved flexible regions of the N and C terminal loops, and lacked a membrane component during the analysis. Additionally, positioning the core in close proximity to the lipid membrane is at odds with more recent reports of the Nef core being displaced approximately 100 Å from the membrane and observed exposure of the Nef core upon lipid association. Ren et al. published a model of Nef in complex with AP-1 also showing the Nef in close proximity to the membrane. However, the details of the contacts between the membrane and Nef differ from the model proposed by Jia et al. and the authors suggested that they may be multiple ways for Nef to interact with the membrane surface.

Discrepancies regarding how Nef interacts with AP-1 also exist in the literature. While it is accepted that Nef must ‘hijack’ AP-1 to downregulate cell surface receptors, but the details on this process are incomplete. One report suggests that Nef drives membrane association in an Arf-1 dependent fashion, and a second report demonstrated that Arf-1 is needed for downregulation of MHC-1 by Nef. These reports are in direct contradiction with a
publication suggesting that Arf-1 is not required for membrane localization \[^{[62]}\]. More recent studies involving the Nef:AP-1 binding have been reported by Shen et al. In that work, the interactions AP-1:Arf-1 with a fused tetherin-Nef construct were characterized by Cryo-EM \[^{[63]}\]. These results showed that the AP-1:Arf-1 complex is more organized than previously thought, and the Nef takes advantage of the organization to influence the clathrin mediated endocytosis. Similar to the models describe above, this study involved non-naturally occurring fusion constructs, lacked a lipid component in the analysis and utilized techniques that are not capable of capturing the dynamics of the proteins in question.

1.2 Approaches to Analysis of Nef

The biology of Nef is well-documented, but there are conflicting reports on how Nef realizes its functions. The findings discussed above offer conflicting details on Nef’s interaction with host binding partners and recruitment to the membrane. Furthermore, many of these studies utilized techniques that cannot capture the full-length protein or give insight into protein dynamics. It’s accepted that membrane binding plays a crucial role in facilitating Nef’s roles, but the data provided in the literature are incomplete with respect to the conformation of full-length, membrane bound myristoylated Nef. To address this issue, the principal goal of the research cataloged in this dissertation was to develop a new method of studying membrane proteins. This is able to probe the membrane bound conformation of full-length myristoylated Nef by combining neutron reflectometry (NR) and hydrogen deuterium exchange mass spectrometry (HDX MS). NR is used to monitor an overall shape profile of Nef with respect to the membrane, and finer details on dynamics and conformation are observed using HDX MS.
1.2.a Combining HDX MS and NR for Investigating Protein Conformation

The primary goals of the research were to (Figure 1.7):

(a) Study membrane-bound conformation of full-length, myristoylated Nef

(a) Develop a new method for studying membrane associated proteins with a hybrid HDX MS-NR technique.

(b) Validate the new HDX method with prototypical membrane-binding proteins.

(c) Determine the effects of membrane-binding on Nef conformation.

A high resolution (<5Å) structure of full-length myristoylated Nef at the membrane is not currently experimentally viable and due to the abundance of highly disordered regions in Nef, one may never be available. These disordered regions do not, however, limit the use of hydrogen deuterium exchange (HDX) to probe the structural dynamics and conformational changes of Nef, especially at the membrane. The details of hydrogen exchange will be explained in Chapter 4. Hydrogens in proteins are constantly exchanging with hydrogens in the solvent. The rate of this exchange of hydrogens is modulated by the presence of secondary, tertiary, quaternary structure and solvent occlusion. By comparing the rate of exchange of the same protein under two states (native vs. ligand bound, denatured, mutated, chemically modified, myristoylated, complexed with another protein or membrane associated) one can determine changes in local unfolding, solvent occlusion and/or backbone dynamics in each state.

Monitoring hydrogen exchange with mass spectrometry (MS) has several key advantages over other structural techniques (such as NMR of X-ray crystallography) which make HDX MS exceptionally well suited for Nef. NMR typically requires high concentrations in
Figure 1.7. The goals of this research. The overall aim of the project was to investigate the membrane bound conformation of myrNef. Multiple phases were needed to achieve this goal. First, a new method for studying membrane proteins that combines hydrogen exchange mass spectrometry (HXMS) and neutron reflection (NR) was developed. Second, this hybrid method was validated by studying the lipid bound conformations of two prototypical membrane proteins. Lastly, this validated method was applied to probe the conformational changes of Nef upon membrane association and changes in lipid packing density.
solution, while the ability to concentrate proteins in the chromatography step before elution into the mass spectrometer allows for routine analysis of dilute samples. HDX MS provides access to low expression or difficult to purify proteins. NMR requires much more protein per sample than what was available, while the sensitivity of mass spectrometry allows for a full HX time course to be analyzed with only a few hundred pmol of Nef. Further advances in instrumentation (ultra-performance liquid chromatography, highly sensitive mass spectrometers) allow for much less material to be used per injection in an HDX MS experiment \cite{68} than is required for more traditional structural techniques.

1.2. b Membrane Protein Analysis using Langmuir Monolayers

As stated above, NR is a biophysical technique able to resolve the membrane-bound shape profile of proteins. While this technique is capable of studying systems in near-native conditions, it lacks the power to probe peptide level dynamics. Utilizing HDX MS can provide details on dynamics and conformation that are otherwise missed with NR. The combination of these orthogonal techniques is the basis to achieve the goals listed in Figure 1.7. Initially, global shape profiles of full-length, myristoylated Nef were observed at two lipid packing densities using NR. The finer details on dynamics and conformation of the observed Nef shape profiles were investigated in the same system using HDX MS.

The expression and purification of myristoylated Nef (MyrNef) and other proteins needed for method development and validation will be described in Chapter 2. This expression protocol used a single Duet vector \cite{44,69} (containing both the N-myristoyl transferase and Nef DNA in one plasmid), expression in minimal media, and a single step purification with detergent to
increase yields of MyrNef while limiting production of unwanted modified variants. To obtain increase the quality of NR data, a robust protocol for expressing stable isotopically labeled Nef was developed and is described in Chapter 2.

In Chapter 3, NR in conjunction with Langmuir monolayers was used, as an alternative to X-ray crystallography and NMR, to study the membrane-bound conformation of Nef. Lipid packing density influences the membrane-bound conformation of Nef and two distinct shape profiles were modeled based on NR data. Nef adopted an elongated profile when bound to loosely-packed lipid monolayers and a compact profile when bound to tightly packed lipid monolayers [56]. While these data were the first direct observation of full-length, myristoylated Nef at the membrane, peptide level dynamics were not resolved with this technique and this system had to be integrated with HDX MS (described in Chapter 4) to study the conformation of membrane bound proteins, including Nef.

The lack of details on membrane protein structure using NR necessitated the development of a new method to study membrane proteins. A novel hybrid method using Langmuir monolayers as a lipid mimetic and HDX MS as a technique was integrated into the same NR system previously described. Unlike other lipid mimetics, Langmuir monolayers allow for precise control over the lipid packing density. The method development and validation of this hybrid method is described in Chapter 5. Deuterium recoveries for samples labeled in the HDX trough system were comparable with recoveries from conventional HDX labeling techniques. Melittin and Arf-1 were analyzed using the HDX trough method to probe for expected lipid-induced conformational changes [70]. The HDX results for these two model system were in agreement with results in the literature, and served to validate the HDX trough. Furthermore, the reproducibility, signal intensity and quality, and peptide coverage were comparable to
conventional solution labeling techniques. The newly validated HDX trough method can be applied to any membrane-anchored peripheral protein and was employed to address the membrane-bound conformation of Nef.

In Chapter 6, the newly validated HDX tough method was applied to full-length, myristoylated Nef while at the membrane. Two different lipids packing densities were interrogated. The results showed significant changes in the N-terminal arm and the C-terminal disordered loop. Furthermore, at high lipid packing densities, Nef adopted a more protected state than when associated with loosely packed lipid membranes. These data agree with and expand upon the results on Nef obtained using NR. Although HDX MS was applied to membrane-bound D123ANef, a dimerization deficient mutant of Nef, there were no significant differences between the mutated and wild-type proteins. This suggests that dimerization might be driven via interactions with host cell proteins.

Completion of the goals described in the beginning of this section and Figure 1.7 are a major step forward in the understanding of the Nef protein and most importantly, the Nef protein fully functional (full-length and myristoylated) and localized to a membrane (where it is required to be for function). This understanding may allow future scientist to develop treatments which interfere with Nef function or lipid interactions. As a result of this work, methods for the application of HDX-MS to membrane proteins were developed and can be used as a template for investigating other biologically relevant membrane binding proteins that have previously been challenging to study.
1.3 References


61. Wonderlich ER, et al. (2011) ADP ribosylation factor 1 activity is required to recruit AP-1 to the major histocompatibility complex class I (MHC-I) cytoplasmic tail and disrupt MHC-I trafficking in HIV-1-infected primary T cells. *Journal of virology* 85(23):12216-12226.


Chapter 2

Expression and Purification of Myristoylated Nef

2.1 Introduction

Recall from Chapter 1 that a major goal of this work was to determine the effect of N-terminal myristoylation on the structure and dynamics of Nef by comparing the myristoylated form to the non-myristoylated form of Nef. The first step in this process was to express and purify both versions of the protein. Production of non-myristoylated Nef was routinely accomplished in our lab by over-expression in *E. coli* and affinity purification. This was not the case for myristoylated protein. Despite previously published protocols, production of myristoylated Nef was not straightforward. Several impediments, including low expression levels, incomplete modification, two unwanted N-terminal chemical modifications, a high degree of instability, and co-purification with N-myristoyl transferase made the acquisition of this protein a significantly difficult task. This chapter will describe in detail the steps that were taken, both successful and unsuccessful, to develop a method for reliably producing myristoylated Nef.

2.1.a N-Terminal Myristoylation of Proteins

Membrane proteins can be integral, with a large portion of the sequence projecting into or through the membrane or peripheral, only associating with either one surface of the phospholipid bilayer (peripheral) or both surfaces (transmembrane)\(^{[1]}\). Acylation of proteins is a common modification which can act as a membrane anchor for peripheral membrane proteins.
One of the most common forms of protein acylation is the addition of an N-terminal myristate, a 14 carbon saturated fatty acid. All myristoylated proteins have the N-terminal consensus sequence [Met\textsubscript{1}-Gly\textsubscript{2}-X\textsubscript{3}-X\textsubscript{4}-X\textsubscript{5}-(S/T)\textsubscript{6}], where X represents any amino acid. The initiator methionine is removed by methionine aminopeptidase co-translationally leaving Gly\textsubscript{2} as the N-terminal amino acid. The myristate is then bonded to the N-terminal glycine through an amide bond by an N-myristoyl transferase (NMT) \cite{3}.

In eukaryotic cells, myristoylated proteins are primarily signaling proteins (i.e. G\textsubscript{a} subunits, ADP-ribosylation factors, serine/threonine and tyrosine kinases and EF-hand calcium-binding proteins) involved in regulating cellular structure and function \cite{4-6}. Some bacterial and viral proteins are also myristoylated but lack endogenous N-myristoyltransferases of their own. As a result, these proteins are myristoylated by the NMTs of their respective hosts. At least 19 viral and 4 bacterial proteins have been identified experimentally with many more predicted through the presence a myristoylation consensus sequence \cite{7}. Examples include the VP2 coat protein of the Polyomavirus, v-src from the Rous sarcoma virus and the effector protein AvrB in \textit{pseudomonas syringae} \cite{7}. Interestingly, some of these proteins are predicted to be myristoylated on the N-terminus following exposure of the glycine after proteolytic cleavage \cite{8}.

Myristoylation itself is the weakest membrane anchor, and myristoylated proteins often require additional interactions to supplement and maintain their membrane association \cite{9}. Additional interactions may include palmitoylation of nearby cysteine residues, phospholipid-binding domains, transmembrane regions or protein-protein interactions or clusters of positive charges. For HIV-1 Nef, where myristoylation, a basic cluster of amino acids and several tryptophan residues near the N-terminus are thought to collectively play a role in membrane anchorage. Although primarily a membrane targeting motif, myristoylation of viral proteins can
play a role in interactions with other or act as conformational switches\textsuperscript{[10-13]}. Myristoylation may also serve to protect Nef from ubiquitin-mediated degradation\textsuperscript{[14]}. Myristoylation and other N-terminal modifications can block ubiquitination and decrease the turnover rate of such modified proteins. Therefore, it is imperative to study Nef in its myristoylated form.

2.1.b Expression of Myristoylated Proteins

As mentioned above, myristoylation is a characteristic of eukaryotic cells. As such, production of myristoylated proteins can be achieved through over-expression in mammalian, yeast, and insect cells because of the presence of endogenous NMTs\textsuperscript{[15]}. However, slow growth and the higher nutrient requirements, make expression in eukaryotic cells more difficult than protein expression in prokaryotic systems such as \textit{E. coli}\textsuperscript{[16]}. While expression in \textit{E. coli} is quicker and simpler, the trade-off is that these cells lack the cellular machinery (N-myristoyl transferases) required for myristoylation\textsuperscript{[17]}. Furthermore, the production of stable isotopically labeled myristoylated Nef (discussed later in this chapter) was required for neutron reflectometry experiments, and this is not routinely or easily accomplished without expression in \textit{E. coli}.

Expression of myristoylated proteins in \textit{E. coli} can be accomplished if the expression host is co-transformed with plasmids containing DNA encoding the target protein and the appropriate N-myristoyl transferase\textsuperscript{[18]}. Since this technique was first reported, many human, myristoylated proteins have been successfully produced this way including ADP-riobsylation factor (Arf)\textsuperscript{[19]} and recoverin\textsuperscript{[20]}. Similar methods have been used to produce myristoylated Nef\textsuperscript{[21-24]}. An alternative method of producing myristoylated Nef using a pET-Duet vector has also been used\textsuperscript{[25]}. Duet vectors (see Figure 2.1) have multiple cloning sites, both of which are
under control of the T7 lac promoter. With these plasmids, two different proteins can be expressed simultaneously and be maintained with a single antibiotic, increasingly the overall transformation efficiency while decreasing the stress on the expression host. Prior work by Morgan et al. established a robust protocol for expression and purifying full-length myristoylated Nef using Duet vectors [24]. Due to the advantages of the Duet expression system over the two-vector approach, all myristoylated Nef for this work was produced by using a Duet vector containing both NMT-1 and Nef DNA and follows the protocol established by Morgan and coworkers. An overview of the Duet expression protocol is shown in Figure 2.2.

2.2 Methods

This section will cover the procedures for producing myrNef for HDX MS labeling experiments. The production of stable isotopically labeled Nef will be introduced and discussed in this chapter.

2.2.a The Duet vector system

2.2.a.1 Expression vectors.

A pET-Duet-1 vector containing h-NMT-1 (residues 81-496) in the first cloning site and SF2 Nef (C-terminal 6x His tag) in the second cloning site was obtained from D. Willbold [25]. A modified version of the plasmid was supplied by T. Smithgall (University of Pittsburgh) where the Consensus Nef (C-terminal 6x His tag) was present in the second cloning site. A plasmid map for this construct is shown in Figure 2.1. Both Nef plasmids carried ampicillin resistance [24].
Figure 2.1. Plasmid map of the pET Duet vector for Consensus Nef. N-myristoyltransferase-1 is in the first cloning site and the Consensus Nef is in the second cloning site. (Gluck et al. 2010)
Expression and Purification of Myristoylated Nef

Figure 2.2. Workflow of the expression and purification of myristoylated Nef. A pET Duet vector containing both NMT-1 and HIV-1 Nef was transformed into Rosetta 2 competent cells, which were grown in M9 (minimal media).
2.2.a.2 Expression in LB.

The Duet vector was transformed into Rosetta2 (DE3)pLysS cells using the manufacturer’s instructions and maintained by ampicillin antibiotic selection. Expression in rich media was carried out as described before\textsuperscript{[24, 25]} with slight modification. A fresh colony was selected and grown overnight in 15 mL LB at 37 °C and 200 rpm. The following day, this starter culture was diluted to 500 mL LB with the appropriate antibiotics and allowed to grow at 37 °C and 200 rpm until OD\textsubscript{600} = 0.6-0.8. The cultures were then moved to a 16 °C incubator, 200 rpm, and allowed to grow to OD\textsubscript{600} = 0.8. 10 mL of myristic acid solution (0.5 mM myristic acid, 0.6 mM bovine serum albumin, pH 9.0, heated to 60 °C) were added to the culture and 10-15 min later expression was induced with 1 mM IPTG. After 16 hours of induction at 16 °C and 200 rpm, cells were collected by centrifugation and stored at -80 °C until purification.

2.1.a.3 Expression in M9.

Myristoylated Nef was also expressed with the Duet vector in a modified minimal (M9) media (200 mL 5 x M9 salts, 100 mL HEPES pH 7.4, 2 mL 1 M MgSO\textsubscript{4}, 0.1 mL 1 M CaCl\textsubscript{2}, 10 mL 10% casamino acids, 0.05% glucose per 1 L culture). A single colony was picked to inoculate a 50 mL starter culture in modified M9, and was grown overnight at 37 °C, with shaking at 200 rpm. The following morning, the starter culture was diluted to 500 mL of modified M9 and grown at 37 °C to OD\textsubscript{600} = 0.6-0.8. The culture was moved to 16 °C and supplemented with 10 mL of myristic acid solution (as described above). 10-15 minutes later, IPTG was added to a 1 mM working concentration and the culture was allowed to express protein for 16 hours at 16 °C with shaking at 200 rpm. Cells were collected by centrifugation (3000 rpm, 4 °C) and the pellet stored at -80 °C until purification.
2.1.a.4 Ni-affinity purification.

The purification protocol followed that of Morgan et al. with slight modification. The frozen pellet was thawed on ice and resuspended in 10 mL of lysis buffer (20 mM Tris, 100 mM NaCl, 20 mM imidazole, 3 mM dithiothreitol, 10% glycerol, pH 8.3). Cells were lysed by sonication in the presence of lysozyme and PMSF and then centrifuged (20,000 rpm, 4 °C, 1 hr). The supernatant of the clarified lysate was added to 1.0 mL of a Ni-NTA slurry and mixed end-over-end for 1.5 hr at 4 °C. The clarified lysate/Ni bead mixture was loaded into the gravity flow column (Bio-Rad) equipped with a UV detector system (Pharmacia Biotech) and extensively washed with lysis buffer. The beads were then washed with lysis buffer including increasing amounts of imidazole (50, 100, 150, and 250 mM). Nef began to elute at 100 mM imidazole (Figure 2.3, Panel A). The fractions were checked with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray mass spectrometry for purity and to confirm myristoylation.

2.2.a.5 Ni-affinity purification with Triton X-100.

A different purification protocol using detergent designed to prevent Nef loss, washed away NMT-1 and increase yield was also used. Triton X-100 in the lysis buffer decreased the amount of Nef lost in the pellet during centrifugation; thus, increasing the total yield per purification. The frozen pellet was thawed on ice and resuspended in 10 mL of lysis buffer (20 mM Tris, 100 mM NaCl, 20 mM imidazole, 3 mM dithiothreitol, 1% Triton X-100, 10% glycerol, pH 8.3). Cells were lysed by sonication on ice in the presence of lysozyme and PMSF and then centrifuged (20,000 rpm, 4 °C, 1 hr). The supernatant of the clarified lysate was added to 1.0 mL of a Ni-NTA bead slurry mixed end-over-end for 1 hour at 4 °C. The
clarified lysate/Ni bead mixture was loaded into the gravity flow column/UV detector system as described above and washed with 25 mLs of lysis buffer. Triton X-100 was removed by a second wash with 40 mLs of Cholate buffer (20 mM Tris, 100 mM NaCl, 20 mM imidazole, 3mM dithiothreitol, 50 mM cholate, 10% glycerol, pH 8.3). Cholate was removed by another wash of 40 mLs of 20 mM Tris, 100 mM NaCl, 20 mM imidazole, 3mM dithiothreitol, 10% glycerol, pH 8.3). A step gradient (50 mM, 100 mM, 150 mM, 250 mM imidazole) was used to elute the protein from the beads; Nef began to elute at 100 mM imidazole (Figure 2.3, Panel A). Nef eluted as one uniform peak if an elution buffer of 250 mM imidazole was used in the purification (Figure 2.3, Panel B). The collected fractions were checked with SDS-PAGE and electrospray mass spectrometry for purity and to confirm myristoylation.

2.3 Production of Myrsitoylated Nef for HDX-MS and NR Experiments

2.3.a Expression and purification using the Duet vector system

The Duet vector system was used to produce non-MyrNef (from the Duet vector lacking NMT-1 in the first coding region) as well as myrNef expressed in rich (LB) and minimal (M9) media. A pET 21-a vector to express non-myristoylated Nef was also utilized in the early stages of this project. Mass spectra for these proteins are shown in Figure 2.4. Expression of C-terminally histidine tagged SF2 Nef (both non-myristoylated and myristoylated) in LB was carried out as described in section 2.2.a.1. Induction gels for this expression (Figure 2.5, panel A) are similar to those from the duet vector system (Figure 2.5, panel B). One noted exception is a band for NMT (around 50 kDa) can be seen to over-express in the MyrNef cultures (Figure 2.5, panel B). This band is not present in the non-Myr constructs as the vector is lacking NMT-1. Looking at the purification gels, Ni-affinity purification
Figure 2.3. Purification of myristoylated Nef using Ni-NTA batch purification. A) Myristoylated Nef was eluted with a step gradient of imidazole. B) Myristoylated Nef was eluted in a single step with 250 mM imidazole and was isolated in a much smaller volume.
Figure 2.4. Intact mass spectra of HIV-1 Nef (strains SF2 and Consensus) with and without myristoylation. The desired protein is indicated with a star.
Figure 2.5. Expression and purification of Nef monitored by SDS-PAGE. A) Expression gel of non-myristoylated Consensus Nef. B) Expression gel of myristoylated SF2 and Consensus Nef. C) Purification gel of myristoylated SF2 and Consensus Nef. The desired band is indicated by a red arrow.
(section 2.1.a.5) resulted in high yield and purity for the both the non-myristoylated and myristoylated constructs (Figure 2.5, panel C). Furthermore, both SF2 and Consensus constructs were isolated with a single Ni-affinity purification step (Figure 2.5, panel C). However, the resolution of the SDS-PAGE is limited and what are not readily apparent are the small modifications on Nef itself. Transformed spectra of Nef show a collection of modifications to the desired protein. These spectra for myristoylated Nef (Figure 2.6) show successful myristoylation (+210 Da) but also include three other major species which were not seen in SDS-PAGE. The first is non-myristoylated Nef and the other two are non-myristoylated Nef +178 Da and +258 Da. These modifications reduced the amount of myristoylated Nef and presented a challenge for purification.

Gluconoylation (+178) and phospho-gluconoylation (+258) are known chemical modifications of typically N-terminally histidine tagged proteins [27]. Gluconoylation (+178) results from an endogenous phosphatase hydrolyzing the phosphate (-80 Da) from the sugar. While these modifications are typically observed for N-terminally tagged proteins, these additions still were present for the C-terminally tagged Nef constructs, after the tag had been moved to allow for N-terminal myristoylation. Regardless, these modifications reduced the overall myristoylation efficiency and difference expression strategies were used to reduce gluconylation [24].

In order to reduce gluconoylation and phospho-gluconoylation, MyrNef was expressed in minimal media as described in section 2.1.a.3. The reduced amount of glucose in minimal media should result in less gluconolactone available to chemically modify Nef [24]. The
Figure 2.6. Advantages of using minimal (M9) media for Nef overexpression. The use of minimal (M9) media increases the myristoylation efficiency while reducing undesired gluconylation and phospho-gluconylation additions.
transformed spectra of Nef purified from cells using this expression system are shown in Figure 2.7. Expression in minimal media resulted in high levels of MyrNef with little unmodified or gluconoylated and phosphogluconoylated Nef (Figure 2.7 and 2.6, bottom spectra).

The above purification protocols all resulted in some losses of Nef to the pellet during centrifugation (Figure 2.5, panel C). This is not surprising as Nef is a membrane associated protein and is most likely partitioning to the membrane while it is being expressed. It is common to purify membrane proteins with the aid of detergent in order to solubilize them from the membrane. As stated above, Triton X-100 in the lysis buffer was used to recover at least some of this membrane-bound fraction during Nef purification (described in section 2.1.a.5) \[^{24}\].

2.3.a.1 Production of stable isotopically labeled Nef

As introduced in Chapter 1, neutron reflectometry experiments are enhanced by analyzing stable isotopically labeled proteins (see also Chapter 3). The production of stable isotopically labeled proteins is accomplished through the use of enriched growth media where resources for proteins synthesis (i.e. amino acids, peptides, sugars, etc.) have been labeled with an isotope (i.e. N\(^{15}\), H\(^2\), C\(^{13}\)). These isotopes become incorporated into an overexpressed protein during synthesis by the bacteria \[^{28}\]. For the case of deuterium enriched media, aliphatic hydrogen positioned along the protein structure are replaced with deuterium. Unlike labile hydrogens (see chapter 4), aliphatic hydrogens are retained in aqueous solvents; thus, aliphatic deuterons will also be retained during analysis. Use of enriched growth media for stable isotopic labeling of proteins has been utilized routinely for mass spectrometry studies \[^{29, 30}\] as well as NMR studies \[^{31, 32}\].
Figure 2.7. Intact mass spectra of myristoylated Consensus Nef. Each protein was purified using different concentrations of imidazole in the elution buffer. Myristoylated Nef begins to elute at concentrations of 100 mM, and is still present in fractions eluted with 250 mM imidazole. The desired protein is indicated with a star.
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<th>Nef</th>
<th>Total sites per residue</th>
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</tbody>
</table>

Max D 1330

Table 2.1. Table of stable deuterium labeling sites for SF2 myrNef. The amino acid, number of aliphatic hydrogen sites for each amino acid, number of each amino acid in the Nef protein and the total sum of aliphatic hydrogen sites per each residue is shown. The maximum deuterium content for myr SF2 Nef is tabulated at the bottom.
Figure 2.8. Isotopically labeled protein production workflow.
Figure 2.9. Intact mass spectra of proteins used for Neutron Reflectometry experiments. A) Transformed spectra comparing unlabeled SF2 myrNef (upper panel) and isotopically labeled SF2 myrNef (bottom panel). B) Raw m/z spectra of unlabeled SF2 myrNef (upper panel) and isotopically labeled SF2 myrNef (bottom panel).
A protocol for producing stable isotopically labeled Nef was developed and optimized. The workflow of producing heavy proteins is shown in Figure 2.8. In brief, an agar plate is streaked with a fresh transformation of the respective vector and allowed to grow over night. The following morning, a single colony is selected from the plate and used to inoculate a 50 mL starter culture (LB media). This starter culture is grown over night at 37 °C shaking at 200 rpm. The next day, 5 mL of the starter culture is diluted to 250 mL of BioExpress D-media (Cambridge Isotope Laboratories, Andover MA), a growth media where the essential nutrients for bacteria have been enriched with deuterium. The BioExpress media comes as a 10X concentrate and must be prepared in D$_2$O. The expression culture was grown at 37 °C and shaking at 200 rpm until the OD$_{600}$ is between 0.6-0.8. The expression culture is then induced with 1 mM (working concentration) of IPTG moved to a shaker at 16 °C and allowed to expressed overnight shaking at 200 rpm. The following day, cells were pelleted by centrifugation (3000 rpm, 4 °C and the resulting pellet was stored at -80 °C until purification.

Table 2.1 shows the possible positions for deuterium labeling for every amino acid and was used to determine the theoretical maximum deuterium content for SF2. The maximum deuterium content was used to determine the extent of stable isotope labeling using the following equation:

\[
\frac{(\text{observed mass}) - (\text{unlabeled mass})}{\text{maximum deuterium}} \times 100 = \%D \text{ Label}
\]

where the observed mass is of the stable isotope labeled protein and the unlabeled mass is of the completely undeuterated protein. Examples of the mass spectra showing the difference in the observed mass and light mass for SF2 MyrNef shown in Figure 2.9.


2.4 Conclusions

In order to accomplish the experimental aims presented in Chapter 1, a robust production of MyrNef needed to be established. Despite the optimized expression protocol of MyrNef that had been previously reported \[24\] several unpredictable roadblocks (partitioning with the membrane, ensuring high myristoylation efficiency and the chemical modifications) made this process more complicated.

MyrNef has been produced using a two vector system. Given the reported low yields and multistep protocol of the two vector system, a simpler method which yielded greater quantities of MyrNef was desirable for this project. Expressing MyrNef with the Duet vector system in LB resulted in greater yields of MyrNef \[24, 25\], but the chemical modifications and low myristoylation efficiency proved problematic. Moving this expression to minimal media resulted in a drastic reduction of gluconoylation and phosphogluconoylation \[24\]. The final optimized protocol (as shown in Figure 2.8) should be a general procedure for the expression and purification of MyrNef (any of the Nef strains) in the future.

Expression of MyrNef in SF9 (insect) cells would have been an alternative route to acquiring this protein. Other groups \[33-35\] have utilized this method for steady MyrNef production, despite the challenging nature and cost of maintaining insect cell lines. This method would have been pursued as well if not for the rather large requirements of protein needed of neutron reflectometry (NR) experiments (covered in Chapter 3) or the Langmuir monolayer HXMS experiments (described in Chapters 5 and 6). As that is the case, expression of MyrNef was limited to the bacterial system.
2.5 References


Chapter 3

Conformation of HIV-1 Nef as Determined by Neutron Reflectometry

Parts of this chapter have been published and are available in Appendix I


3.1 Introduction

As mentioned in Chapter 1, one of the major goals was to investigate the structure of membrane-associated HIV-1 Nef. The first experimental evidence of this was obtained using a biophysical technique called Neutron Reflectometry (NR). NR is an orthogonal technique to HDX-MS as it obtains the overall shape profile of proteins with respect to a film or membrane. In this chapter, the membrane-bound conformation of Nef as interrogated by NR will be discussed. These data provided an insight into the structural changes of Nef upon membrane binding as well as influences that lipid packing density had on Nef conformation. My role for this work was to provide protein stable isotopically labeled samples for analysis (discussed in part in Chapter 2) and to operate the instruments used during the NR experiments. The data covered in this chapter provide a foundation for future work with Nef and Langmuir monolayers discussed in Chapters 5 and 6.
3.1.a Neutron Reflection for Protein Analysis

There is abundant evidence that enzyme activity or protein-protein interactions can be dependent upon association with lipid membranes \(^{[1-4]}\). The positioning of proteins and protein motifs relative to either the membrane or to other membrane-bound proteins is, for some proteins, critical and may depend upon conformational changes induced upon membrane association.\(^{[5-12]}\) Lipid modifications to the protein serve to target many proteins to specific membranes locations. Hundreds of proteins are known to be modified with covalently bound lipid groups, the most common of which are fatty acids, isoprenoids, and glycosylphosphatidylinositol anchors \(^{[2, 13, 14]}\). Many of these proteins are involved in signaling, and require membrane association to signal efficiently. In addition to intra cellular membrane location, the structure adopted by proteins at membranes is highly critical for some functions and may play a role in the localization of binding partners \(^{[15]}\). As one example, myristoyl or farnesyl switch mechanisms are known for more than a dozen proteins, with Arf GTPase being a hallmark example \(^{[14, 16]}\). Arf will be further discussed in Chapter 5. These mechanisms cause proteins to switch between conformational states in which the myristoyl or farnesyl moiety is either sequestered or exposed, and can promote membrane binding \(^{[17, 18]}\), facilitate release from the membrane-bound state, and regulate protein-protein interactions \(^{[19, 20]}\).

Despite the importance of acylated proteins in biology (e.g., protein kinases, T cell receptors, GPCRs, pro-apoptotic proteins), standard approaches for studying structure are not amenable to traditional structural determination techniques, such as X-ray crystallography due to the inherent propensity for membrane proteins to aggregate and the overall challenges with dealing with membrane mimetics. In addition, understanding mechanisms involving these proteins at the molecular level has been limited by the absence of structural detail for these
proteins in the membrane-bound state. Several structural studies of membrane-associated 
proteins have consisted of crystallization of soluble proteins with and without a bound small-
molecule ligand or in complex with other proteins but in absence of a membrane \cite{17-19, 21}. Other 
biophysical techniques have been employed to study membrane-bound protein conformation. 
Examples include nuclear magnetic resonance, electron paramagnetic resonance and infrared 
spectroscopy. Nuclear magnetic resonance (NMR), which monitors the resonant frequencies of 
nuclei in an applied magnetic field \cite{22}, has been applied to study transporter and receptor 
proteins \cite{23, 24}. Electron paramagnetic resonance (EPR) is analogous to NMR, but it is electron 
spins that are excited and monitored instead of the spins of nuclei \cite{25}. EPR has been utilized to 
investigate transporters \cite{26} and intrinsically disordered proteins \cite{27}. Infrared (IR) spectroscopy 
monitors the vibration of molecular bonds via excitation from infrared radiation and is sensitive 
to changes to structural changes within proteins \cite{28, 29}. While NMR, EPR and IR spectroscopy 
provide some gross conformational details for membrane-bound proteins, they are unable to 
report full residue distribution with respect to the membrane.

To address the lack of methodology and understanding of membrane-associated proteins, 
NR was used to resolve the membrane-bound location of regions of acylated proteins. The 
method is demonstrated in this Chapter by studying the conformational change of N-terminal 
myristoylated HIV-1 Nef upon membrane insertion. Recall from Chapter 1, Nef is one of 
several HIV-1 accessory proteins and is essential for progression to AIDS \cite{30, 31}. Nef is 
expressed in high concentrations shortly after viral infection, \cite{32} and is required for achieving 
and maintaining high viral loads \textit{in vivo} \cite{33}. Membrane-association is achieved by an N-terminal 
myristoylation essential for the virus \textit{in vivo} \cite{34} as well as a cluster of basic residues (17-22) 
within the N-terminal arm (covered in detail in Chapter 1) \cite{35-37}. 

52
It has been theorized that Nef undergoes a transition from a solution conformation to a distinct membrane-associated conformation, and this transformation enables membrane-associated Nef to interact with host proteins\textsuperscript{[38-41]}. In particular, it has been suggested that insertion of the N-terminal arm and subsequent displacement of the Nef core from the lipid membrane, upon association, will expose binding sites on the core, facilitating interaction with host proteins\textsuperscript{[38]}. On the other hand, based on the crystal structure of Nef with the cytoplasmic tail of MHC-I others have suggested that association of Trp13 and Met20 on the N-terminal arm with the core domain persists upon membrane binding, and that this positions the Nef core close to the membrane for optimal interaction with the cytoplasmic domain of the MHC-I receptor\textsuperscript{[41]}. Others have proposed that association of the Nef core with negatively-charged membranes through its basic surface serves to orient Nef and provide optimal exposure of the di-leucine motif in the flexible loop (residues 152-184) that mediates interactions with adaptor protein complexes\textsuperscript{[42]}. Furthermore, Nef is known to upregulate several Src family kinases through interaction with their SH3 domains,\textsuperscript{[43]}, disrupting cellular signaling pathways. These kinases are also bound to the membrane through N-terminal acylation and positioning of the Nef core domain relative to the SH3 domains may play a role in the varying binding affinities. Despite the importance to the pathogenicity of Nef, to date there is little information regarding the membrane-bound structure of Nef upon membrane association due to the limitations of current structural methods.

Neutron reflectivity is one of few methods that can resolve structural details of membrane-associated proteins under physiological conditions, and may be unique in the ability to directly resolve location of folded domains for full membrane-bound protein conformation.
NR involves measuring the ratio of reflected to incident intensity as a function of momentum transfer defined in equation 3.1:

\[ q_z = \frac{4\pi \sin \theta}{\lambda} \]  

(3.1)

where \( \theta \) is the angle of incidence with respect to the plane of the membrane and \( \lambda \) is the wavelength. The form of this curve is determined by the in-plane averaged scattering length density (SLD) profile normal to the surface. In both cases the SLD is directly related to the atomic composition and the density. Unlike in X-ray experiments where the SLD is determined by the electronic density, the neutron SLD is determined by the properties of the nuclei present. Therefore, for a protein bound to a planar lipid membrane, NR determines the in-plane averaged distribution of amino acid residues normal to the membrane. The NR signal for protonated (light) myrNef was relatively weak; thus, the majority of NR experiments were performed using stable isotopically labeled protein (expression protocol covered in Chapter 2) as the nuclear density is greater.

### 3.2 Methods

This section will cover the protocols for producing Hck SH3 domain, but as a light (all hydrogen) and heavy (deuterium labeled in non-exchangeable positions). The production for Nef (light and heavy) was discussed in detail in Chapter 2. The Langmuir monolayer trough equipment used for these experiments will also be introduced and described in this section.
3.2.a Production of Hck SH3

The Hck SH3 vector was transformed into *E. coli* strain Rosetta2 (DE3)pLysS and maintained by ampicillin (pET-14b, Hck SH3) antibiotic selection. Expression Hck SH3 was carried out in rich media (LB). The SH3 domain did not contain an affinity tag and was purified in two steps: (1) anion exchange and (2) gel filtration. Fractions containing the SH3 domain were pooled and concentrated with Centricon Ultracel YM-3 (3,000 molecular weight cut-off) devices to less than 1 mL. The concentrated pools were loaded onto a 1.5 x 75 cm column packed with Sephadex G75 Superfine (GE Healthcare) resin. The running buffer consisted of 20 mM tris, 100 mM NaCl, 3 mM dithiothreitol, pH 8.3 and was flowed at 0.12 mL/min. Fractions containing the SH3 domain were identified by SDS-PAGE and electrospray mass spectrometry and again concentrated on Centricon Ultracel YM-3 devices. Purity was checked by electrospray mass spectrometry. Concentration was determined by Bradford analysis. The protein was then divided into the appropriate volume to give a concentration of 1 μM per aliquot in the Langmuir trough and stored at -80 °C. As mentioned above, the detailed protocol for expression of isotopically labeled proteins was covered in Chapter 2 and production of stable isotopically labeled Hck SH3 was performed in a similar fashion. Table 3.1 shows the possible positions for deuterium labeling for very amino acid and was used to determine the theoretical maximum deuterium content for Hck SH3.

The maximum deuterium content was used to determine the extent of stable isotope labeling using the following equation (3.2):

\[
\frac{(\text{observed mass}) - (\text{unlabeled mass})}{\text{maximum deuterium}} \times 100 = \%D \text{ Label}
\]  

(3.2)
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**Max D** 425

Table 3.1. Table of stable deuterium labeling sites for Hck SH3. The amino acid, number of aliphatic hydrogen sites for each amino acid, number of each amino acid in the Hck SH3 domain and the total sum of aliphatic hydrogens sites per each residue is shown. The maximum deuterium content for Hck SH3 is tabulated at the bottom.
where the observed mass is of the stable isotope labeled protein and the unlabeled mass is of the completely undeuterated protein. Examples of the mass spectra showing the difference in the observed mass and light mass for Hck SH3 shown in Figure 3.1.

3.2.b Langmuir Trough System used for NR experiments

Specialized equipment for generating and manipulating a Langmuir monolayer was required for the NR experiments with myrNef. Figure 3.2 displays a cartoon representation of the Langmuir trough system. The trough itself is comprised of a Teflon block that has been machined to hold 18 mLs of aqueous buffer. It is onto of this buffered solution (referred to as a subphase) that a Langmuir monolayer, generated from lipids for these experiments, can be generated. These monolayers are generated by adding lipids (typically dissolved in a mixture of methanol/chloroform) to the top of the buffered subphase \[45\]. Upon evaporation of the organic solvent, the lipids will self-assemble into a monolayer that resembles in the inner leaflet of a cell membrane. This monolayer can be compressed or relaxed by a movable, Teflon barrier (Figure 3.2). This barrier is precisely controlled by computer aided servos and is used to accurately maintain a specified lipid packing density. The Langmuir trough used for these experiments had been plumbed to a peristaltic pump, which was used to exchange the buffered subphase. Subphase exchanges were performed after the injected protein samples were allowed to associate with the monolayer (approximately 60 minutes). Exchanging the subphase washed away any unbound protein, ensuring that NR scans were interrogating only monolayer-associated samples and improving the overall quality of the data. The peristaltic pump was later modified for
Figure 3.1. Intact mass spectra of proteins used for Neutron Reflectometry experiments. A) Transformed spectra comparing unlabeled Hck SH3 (upper panel) and isotopically labeled Hck SH3 (bottom panel). B) Raw m/z spectra of unlabeled Hck SH3 (upper panel) and isotopically labeled Hck SH3 (bottom panel).
deuterium labeling experiments. The protocols and experiments using the modified trough will be discussed in detail in Chapter 5.

3.3 Analysis of Nef by Neutron Reflection

MyrNef (strain SF2) was injected underneath a Langmuir monolayer of deuterated dipalmitoylphosphatidylglycerol (dDPPG) and its conformation was resolved by NR as a function of membrane conditions. The structural details of membrane-bound Nef as a function of solution concentration, membrane pressure, and Nef coverage are described below. The data demonstrate a large conformational change from a closed to an open form that displaces the Nef core 100 Å from the lipid headgroups. This large conformational change is likely to affect its ability to interact with host proteins by exposing binding motifs on the core domain or by optimally positioning the core domain for interaction with motifs of membrane-associated host proteins.

NR data indicated that soon after myrNef was introduced to a lipid membrane, there was a process of insertion into the membrane accompanied by a large conformational transition. Because the electrostatic attraction of myrNef for lipid membranes is known to increase with the percentage of negatively-charged lipids, \(^{37}\) lipid monolayers for these experiments were composed entirely of DPPG in order to bias membrane association. When myrNef was circulated under the monolayer, insertion of myrNef into the membrane was evidenced by the backward movement of the trough barrier maintaining the monolayer pressure (increase in surface area at fixed number of lipid molecules, see Figure 3.3). Due to the larger area occupied by the core domain relative to that of the myristate group, insertion of the myristate moiety alone
Figure 3.2. Cartoon representation of the side view of the Langmuir Trough system used for NR experiments. After lipid is spread on the subphase, a motor-driven barrier compresses the monolayer to a set lipid packing density as monitored with the pressure sensor. The computer control system (not shown) integrates the pressure sensor and barrier position and can be set to move the barrier to maintain constant pressure. Protein samples are injected underneath the monolayer after it is in place and associated with the monolayer. Unbound protein is washed away by exchanging the subphase using a peristaltic pump. NR scans of membrane-bound Nef were taken after subphase exchange. From Akgun et al. 2013.
Figure 3.3. Change in surface pressure (black) and barrier position (red) in the trough during myr-Nef at low lipid packing (A) and at high lipid packing monolayers (B). For low lipid packing experiments, the surface pressure was held constant after addition of protein. The barrier gradually moved back as more protein associated and inserted residues into the monolayer. The barrier was stopped within a few millimeters before reaching the end of the trough. The surface pressure rose slightly after the barrier was stopped indicating that protein was still associating with the monolayer. For high lipid packing experiments, Nef was unable to insert residues into the membrane. As a result, the barrier did not gradually move back as in the low packing experiments. In all cases, aspiration dropped the surface pressure quickly and the barrier rapidly moved forward in order to reestablish the preset surface pressure. For all plots, “I” represents when protein was injected underneath the monolayer, “E” represents the subphase exchange, and “A” represents aspiration of the sample after analysis. From Akgun et. al 2013.
can account for an increase in surface area of at most 5%; increases in surface area greater than 5% therefore indicate insertion of residues of the protein in addition to the myristate group \[^{45}\]. Prior work by others has indicated membrane insertion and formation of an amphipathic helix within the N-terminal 27 residues of Nef \[^{37}\].

Upon binding and insertion, myrNef remained associated with the membrane upon extensive exchange (~100 mLs) of the subphase underneath the surface layer to remove non-inserted or loosely-bound Nef. The rate and extent of insertion of residues varied both when membrane pressure was held constant and the concentration of myrNef was changed or vice versa. At a myrNef concentration of 1.0 \(\mu\)M a different conformation was observed for Nef. This conformational state (hereafter referred to as the “open” form) was one in which the core domain was displaced 100 Å below the lipid headgroups.

Further NR studies were performed in which Nef conformation and insertion were controlled by adjusting the surface pressure of the lipid membrane. The reflectivity (Figure 3.4) and surface area data (Figure 3.3) both indicated little insertion of residues at 35 mN/m, but substantial insertion of residues at 20 mN/m. The NR data show distinctly different patterns, and hence different conformations of myrNef, at the two membrane pressures. The density profiles show that the core domain of myrNef is directly adjacent to the lipid headgroups at 35 mN/m, but is displaced 70 Å below the lipid headgroups at 20 mN/m.

Recall from earlier, that deuterium enrichment of the protein being analyzed in NR substantially increases the contrast, allowing for higher resolution data and more precise fitting with molecular models \[^{45}\]. MyrNef in which 80% of the non-exchangeable hydrogen atoms were replaced by deuterium (deuterated myrNef) was prepared and NR data were collected. The
best-fit SLD profile using a free form slab model is shown in Figure 3.4 panel D. The freeform model consists of one layer each for the deuterated lipid tails and the lipid headgroups, and a single layer to describe membrane-bound deuterated myrNef with a variable gradient between each layer. In the fit, the thickness and SLD of the lipid tail layer, after adsorption of deuterated myrNef were constrained to the same values as determined for the data taken prior to adsorption.

Figure 3.4 panel B compares the NR data for a monolayer of DPPG at 20 mN/m on buffer compared with a scan initiated ~4 hours after injecting 0.28 μM myr-dNef. The DPPG monolayer was initially spread to a pressure of ~10 mN/m and then compressed to 20 mN/m. Much larger changes in the NR data were observed upon adsorption compared with the data for myrNef at 35 mN/m. A large increase in area again resulted, similar to that in Figure 3.3, indicating strong insertion of residues into the lipid membrane. The best-fit SLD profile using a free form slab model is shown in Figure 3.4. The freeform model consists of one layer each for the deuterated lipid tails and the lipid headgroups, and two layers to describe membrane-bound deuterated myrNef with a variable gradient between each layer. In the fit the thickness of the lipid tail layer after insertion of deuterated myrNef was constrained to be 4 Å less than that measured for DPPG alone, based on prior data\textsuperscript{[45]}. Based on the relative areas occupied by the core domain of Nef and a DPPG molecule, and the fact that the myristate group has only a single aliphatic chain whereas DPPG has two aliphatic chains, the SLD of the lipid tail layer was constrained to be greater than or equal to 0.95 x SLD\textsubscript{tails} dDPPG + 0.05 x SLD\textsubscript{myr}\textsuperscript{[45]}. The profile band indicates a broad maximum, indicating the core domain, again displaced roughly 100 Å from the lipid headgroups.

As prior work by others\textsuperscript{[46]} indicated that a cluster of basic residues within the N-terminal arm (17-22) interacts with negatively-charged lipid membranes to facilitate Nef
adsorption, in addition to the myristate group, only molecular models in which residues 2-22 resided on or within the lipid headgroups were considered. Molecular structures in which the core domain was located at varying distances from residue 22 were examined. In these calculations a single orientation of the core domain was chosen arbitrarily, as it is not possible to resolve the distribution of core domain orientation from the present NR data alone. The structure giving the best agreement with the data is shown in Figure 3.4. In that case the core domain was separated from residue 22 by 100 Å. The peak in the SLD corresponding to residues 2-22 is located within the lipid headgroups. The calculated SLD profile contains a maximum that is considerably narrower than that of the profile from the free-form fit, and the best-fit curve contains greater oscillations at higher q values than are present in the data. These results indicate that the core domain is distributed over a range of depth. Combining the myr-Nef structure shown in Figure 3.4 with structures in which the core domain is displaced 20 Å closer and also 20 Å further from residue 22 resulted in a good fit to the data as, shown in Figure 3.4.

### 3.4 Discussion of NR Results

Resolving the structure of membrane-associated proteins is quite challenging, but important as positioning of residues and motifs relative to the membrane can strongly impact function, ligand binding/engagement. Little is known about the precise location of domains within membrane-associated proteins with respect to lipid membranes due to a lack of adequate tools and methods. The NR data have revealed that membrane-bound myrNef adopts a very different conformation depending upon the ability of residues to insert into the lipid membrane. In the absence of insertion, membrane-bound Nef adopts a closed form with the core domain
Figure 3.4. Summary of neutron reflectometry results. A) NR data for a deuterated DPPG monolayer at 20 mN/m (black) and a deuterated DPPG monolayer bound to deuterated myrNef at 0.28 uM (red). Fits to the data are shown using a model where residues 2-22 of Nef are embedded in the membrane (blue) and using an ensemble of conformations (yellow). The respective SLD profiles are shown in panel B. C) NR data for a deuterated DPPG monolayer at 35 mN/m (black) and with bound myr dNef at 1.0 uM (red). The respective SLD profile in panel D. E) NR results for dDPPG monolayer at 20 mN/m (black) and scans of bound myr dNef at 0.67 uM after 2 hours (red) 3 hours (purple) and 4 hours (cyan). The respective SLD profiles are shown in panel F. From Akgun et al. 2013.
directly against the lipid headgroups. When residues insert, Nef adopts an open form in which the core domain is displaced into solution 100 Å from the lipid headgroups. It is clear that a substantial number of residues, presumably on the N-terminal arm, insert in addition to the myristate group.

As it has been speculated previously,\textsuperscript{[35-37]} that myrNef adsorbs through a combination of electrostatic interactions between basic residues in the N-terminal arm and the negatively-charged lipid headgroups combined with insertion of the hydrophobic myristate group. The results show that the rate and extent of insertion, are speculated to involve residues 12-22 in the N-terminal arm. These are known to form an amphipathic helix,\textsuperscript{[37]} and this insertion is determined by the density of adsorbed Nef as well as by the membrane pressure. In conditions where the N-terminal arm residues insert (high adsorbed density of Nef and/or low membrane pressure), Nef rapidly adopts an open form. Fitting the NR data with molecular models of Nef indicates that the 100 Å average distance of the core domain from the membrane in the open conformation is fully consistent with residues 2-22 residing within the lipid headgroup region.

The adsorption and insertion processes are each affected by several factors. Both electrostatics and the myristate group affect adsorption. The binding affinity is substantially lower with decreasing fraction of negatively-charged lipids or in absence of the myristate group. Insertion of residues into the membrane is affected by membrane pressure as well as by Nef coverage. At a membrane pressure of 30 mN/m, the “open” conformation resulted only at higher surface coverages whereas at 20mN/m, the open form resulted at low surface coverages. This shows that the open form is not generated by high coverage, but rather insertion of arm residues is the key step leading to the open form. Indeed, a high coverage (f=0.65) and very little
insertion resulted in a prior study involving His-Nef adsorption to lipid monolayers containing a synthetic metal chelating lipid, and in that case Nef remained in the closed form \cite{47}.

Extensive evidence indicates that at least some functions of Nef in vivo require dimerization \cite{48}, yet others \cite{42, 49} have found no evidence for dimerization of free Nef in solution at 1 \muM (concentration in the trough). Reflectivity methods are unable to detect structural changes that occur in the plane of the membrane; thus our results do not directly inform the dimeric status of myr-Nef at the membrane. It was shown elsewhere using analytical gel filtration that in solution, truncated Nef lacking N-terminal residues 2-44 contains significant dimeric and multimeric fractions, whereas myr-Nef and non-myr-Nef exist primarily as monomers \cite{49}. This suggests that dimerization of Nef may be inhibited by association of the N-terminal arm with the core domain. Residues on \alpha-helix 4 and the adjacent loop (R109-D127) have been identified as promoting Nef core domain dimer and trimer association \cite{14}. Others have proposed that membrane insertion of the myristate group causes the N-terminal arm to separate from the core domain and thereby promotes Nef dimerization \cite{38, 40}. These NR data are consistent with this hypothesis in that substantially higher coverages were obtained with Nef in the “open” form.

Gerlach et al. reported a kinetic study of myr-Nef binding to fluid phase membranes of DOPC and DOPG using fluorescence resonance energy transfer (FRET) \cite{37}. As mentioned above, strong myr-Nef binding required the presence of negatively-charged lipids, as observed in the present study. The kinetic data indicated two processes: a fast process that was attributed to electrostatic-driven association followed by myristate insertion, and a slower process that was attributed to formation of an amphipathic helix within the N-terminal 27 residues as outlined in Figure 3.5. Non-myrNef bound only weakly to the membranes and exhibited mainly the slow
Figure 3.5. Nef membrane binding process. Nef is initially attracted to the membrane via electrostatic interactions. For membranes of lower packing density (20 mN/m), the N-terminal arm and the myristate moiety insert into the hydrophobic lipid tails and the core of Nef is displaced 70 angstroms away from the membrane. The exposed core is believed to interact with host cell proteins and may be crucial to dimerization. This structural rearrangement is unable to occur in tightly packed membranes (35 mN/m), and Nef remains unchanged after electrostatic attraction. From Akgun et al. 2013.
process. Both processes occurred on time scales much faster than the events observed in the present study. This is due to the fact that fluid phase lipids were used in their study whereas gel phase lipids were used in the present study. These conformations observed using NR and Langmuir monolayers correspond to those following the fast and slow processes, respectively, in the study of Gerlach et al. They showed that the rate of the fast process increased with membrane curvature, consistent with more rapid insertion of myristate into more loosely packed lipids. This is analogous to and entirely consistent with the present data, where surface pressure and packing density were used to alter the ease of insertion.

Jia et al. determined the crystal structure of a complex of Nef with the cytoplasmic domain of MHC-I using a construct in which the MHC-I cytoplasmic domain was fused to the N-terminus of Nef \[^{[41]}\]. In the crystal structure the N-terminal helix of Nef (residues 6-22) was attached to the core domain of Nef by Trp13 and Met20. The authors speculated that that this association persists upon membrane binding, and that this positions the Nef core close to the membrane for optimal interaction with the cytoplasmic domain of the MHC-I receptor. However, the NR data shows that this speculation is incorrect, since the N-terminal arm separates from the core and inserts into lipid membranes and the core domain is displaced 100 Å from the membrane, a distance much greater than the proposed model from Jia et al. \[^{[50]}\]. Jia et al. also showed that either W13A or M20A mutations abolished Nef-induced downregulation of MHC-I in human T lymphocytes, and this was presented as further support for their assertion that interaction of Trp 13 and Met20 with the core domain of Nef is critical for downregulation of MHC-I. However, the effect of these mutations observed in T lymphocytes could instead be due to decreased membrane association or altered insertion and helix formation. Residues 5-22 form an amphipathic helix with hydrophobic residues Trp5, Trp13, Ile16, and Met20 located on
one side of the helix. Gerlach et al reported significantly decreased binding affinity to 
negatively-charged lipids and impaired helix formation upon mutation of Trp5 and Trp 13[37].

3.5 Conclusions

In summary, this was the first reported measurement of the precise location of the folded 
domain of a terminally acylated protein with respect to a lipid membrane. However, the overall 
shape profile of membrane associated HIV-1 Nef and the striking differences between its 
structure at different lipid packing densities lacked peptide level details on dynamics. As a result, 
a new HDX MS method was developed utilizing a modified version Langmuir trough in order to 
capture finer details on membrane protein conformation [51]. The method validation, 
development and application to membrane-associated proteins are discussed in detail in Chapter 
5. Once the HDX MS method with Langmuir monolayers was developed, it was applied to 
进一步研究膜结合的Nef的构象并扩展NR的发现。The HDX MS results for Nef using the modified Langmuir trough are covered in Chapter 6.

3.6 References


2. Johnson DR, Bhatnagar RS, Knoll LJ, & Gordon JI (1994) Genetic and biochemical 


Chapter 4

Hydrogen Deuterium Exchange Mass Spectrometry

for the Analysis of Protein Conformation and Dynamics

Parts of this chapter have been published and are included in Appendix II:


4.1 Introduction

4.1.a Monitoring changes in conformation and dynamics in proteins with amide hydrogens

Many proteins undergo structural changes that may range from small scale breaking and reforming of hydrogens bonds to larger scale localized unfolding. These protein motions, referred to as dynamics, can be biologically relevant\textsuperscript{[1-5]} and as such, characterizing their scales, locations and rates are important. The dynamics of proteins have been measured for many years by monitoring the exchange of amide hydrogens along the protein backbone with either tritium or deuterium in the solvent\textsuperscript{[6-9]}. Any technique which is sensitive to the different isotopes of hydrogen may be used to monitor hydrogen exchange (density, radioactivity, infrared spectroscopy, NMR or mass spectrometry). This exchange can be followed by NMR because deuterium is NMR silent due to its ‘spin’\textsuperscript{[10]}. As hydrogens are replaced by deuterium, and gradually the NMR signal intensity for those hydrogens disappears. Exchange rates can be
calculated for each assigned amide hydrogen.

For size and solubility issues, not all proteins are amenable to NMR analysis. Monitoring hydrogen deuterium exchange (HDX) with mass spectrometry (MS) is possible because the monoisotopic mass for deuterium (2.014102 amu) is greater than the monoisotopic mass for hydrogen (1.007825 amu)\[10\]. HDX MS can achieve higher resolution (peptide level, typically 10-20 residues) than more traditional biophysical techniques such as circular dichroism, fluorescence, laser light scattering, infrared spectroscopy, gel filtration and analytical ultracentrifugation. Recent advances in “non-ergot” fragmentation techniques (electron capture dissociation and electron transfer dissociation) indicate that single residue resolution are more routinely achievable with HDX MS\[11, 12\].

4.1.b Detecting hydrogen exchange with mass spectrometry.

The monitoring of hydrogen exchange (with tritium) at the peptide level was pioneered in the 1970s and 1980s\[13-15\]. The amount of tritium labeling could be calculated by measuring the radioactivity (specific activity) of the peptides. Labeling with deuterium (a stable isotope of hydrogen, compared to the radioactive tritium) offers a much safer experimental procedure, however a new method of detection needed to be developed.

The earliest combinations of hydrogen exchange and mass spectrometry involved the analysis of intact bovine ubiquitin\[16\]. Although these studies may yield some information about conformation, folding and ligand binding, they suffered the same drawbacks as the biophysical methods listed above; namely resolution (where on the protein(s) hydrogen exchange is occurring). The analysis of peptides derived from labeled proteins affords HDX MS an advantage over many biophysical techniques.
In 1993, expanding on the previous work with tritium\textsuperscript{14}, Zhang and Smith reported the use of mass spectrometry to measure the extent of deuterium labeling in peptides derived from a labeled protein using fast atom bombardment mass spectrometry (FAB-MS) (Zhang and Smith 1993). The following year, Johnson and Walsh reported hydrogen exchange measurements on myoglobin acquired with electrospray ionization mass spectrometry (ESI-MS)\textsuperscript{17}. Since then, HDX MS has become widely used to monitor the biophysical characteristics of proteins and peptides (Figure 4.1). Examples include: protein folding mechanisms\textsuperscript{18}, large protein complexes such as chaperones\textsuperscript{19} and histone binding proteins\textsuperscript{20}, epitope mapping\textsuperscript{21}, viral proteins\textsuperscript{22}, small molecule binding\textsuperscript{23}, biopharmaceuticals\textsuperscript{24} and membrane proteins\textsuperscript{25}.

An introduction to the theory and practice of hydrogen exchange mass spectrometry are presented in this chapter. The specific details as applied to individual experiments will be described as needed in the corresponding chapters.

### 4.2 Hydrogen Exchange

The details of hydrogen exchange have been reviewed numerous times previously\textsuperscript{3, 6, 8, 10, 26-31}. The following sections will provide background necessary to understand the data presented in Chapters 5 and 6.
Figure 4.1. Global snapshot of publication using HDX MS between January 2012 and June 2014. A) Total number of publication categorized based on the author’s affiliation. B) Global map displaying the number of HXMS studies published in each country. C) Total US publications categorized based on the author’s affiliation. D) Breakdown of the non-US countries publications according to country and affiliation. E) Six major applications of HXMS shown a pie graphs. The size of the pie graphs are related to the overall number of publications within that application. Each graph is subdivided into subjects within the major application. This figure is reprinted with permission from Pirrone et al. 2015.
4.2.a Hydrogen exchange into folded proteins

Several types of hydrogens in proteins are in constant exchange with hydrogens in solution. This process is spontaneous, and hydrogens added onto a protein from the solvent can readily “back-exchange” and return to the solvent \(^4, 32\). This rate of this exchange is modulated by several factors including pH and temperature (described below), as well as solvent accessibility and hydrogen bonding. The first two factors can be controlled experimentally and commercial solutions have been developed to address this point \(^33\). The last two factors are dictated by the protein’s higher order (secondary, tertiary and quaternary) structure and suggest that by monitoring hydrogen exchange, one can gain information about the structure of a protein. It should be noted that isotope and solvent effects can influence the exchange process, but are less influential than the solvent accessibility and hydrogen bonding of the protein \(^34\). The presence of structure can slow the exchange of amide hydrogens by as much as \(10^8\) when compared to a denatured form of the same protein \(^6, 32\). Furthermore, neighboring side-chains can influence the rates of exchange through inductive and steric effects \(^35\). By diluting a protein in a deuterated buffer, exchange replaces hydrogen from the protein with deuterium from the solvent, effectively labeling each exchange competent position along the molecule.

4.2.b Types of Hydrogens

There are several types of hydrogens in a protein or peptide (Figure 4.2). Hydrogens covalently attached to carbon do not exchange with solvent under HDX labeling conditions. Hydrogens bonded to nitrogen, oxygen or sulfur in the side chains of residues posses the fastest exchange rates. As a result, these hydrogens can exchange back to hydrogen during the LC step and are not followed. However, the backbone amide hydrogens exchange slowly enough that
they are not completely washed away during the analysis and can be monitored. It has been shown that for folded proteins, amide hydrogens exchange on the order of seconds to days \(^{[6, 32]}\). Every residue (with the exception of proline) has an amide hydrogen, effectively providing a sensor of the local environment at each position along the backbone of the protein.

Amide hydrogens are integral parts of secondary structure. The major force holding \(\alpha\)-helices and \(\beta\)-sheets together are the hydrogen bonds between the backbone carbonyl oxygen and amide hydrogen, and amide hydrogens are also commonly hydrogen bonded to water \(^{[36]}\). In order for one of these occupied amide hydrogens to be competent for exchange, the existing hydrogen bond must be broken, and the catalyst OH\(^-\) (under basic conditions) or H\(_3\)O\(^+\) (under acidic conditions) as well as a source of a new proton, (H\(_2\)O) must both be present.

**4.2.c Proton Transfer**

Proton transfer (Figure 4.3, Panel A) involves first, the formation of a hydrogen bonded complex between the acceptor and donor. Second, the proton rapidly reaches equilibrium within the complex. Lastly, the hydrogen-bonded complex dissociates \(^{[34, 37]}\). Productive transfer (when the proton is carried away by the acceptor) is guaranteed when the acceptor is a weaker acid than the donor (\(pK_{\text{acceptor}} > pK_{\text{donor}}\)). In the opposite situation (\(pK_{\text{acceptor}} < pK_{\text{donor}}\)), hydrogen transfer is productive in only a small percentage of collisions. Because of the low \(pK\) values of most exchangeable hydrogens on side chains, those positions exchange very rapidly and cannot be measured by HDX MS; the \(pK\) for OH\(^-\) protonation is 15.7 \(^{[34]}\). Amide hydrogens have a much larger \(pK\) value (approximately 18) \(^{[38]}\). In this case transfer occurs from a weaker to a stronger acid, making the amide positions well suited for monitoring as the rate of exchange is on a time scale (seconds to days) which can be followed by HDX MS.
Figure 4.2. Hydrogens in protein and peptides. Acidic and basic hydrogens (green) exchange too quickly with the solvent to be followed using mass spectrometry. Aliphatic hydrogens (blue) are too stable and do not readily exchange in solution. Backbone amid hydrogens (red) exchange at a rates that are possible to follow using mass spectrometry. Every amino acid, except proline, has a backbone amide hydrogen and these amide hydrogens are followed during HX MS. Adapted from Morgan and Engen, 2009.

Tyr-Gly-Gly-Phe-Leu
4.2.d Base Catalyzed Amide Hydrogen Exchange

The exchange of amide hydrogens can proceed via base-catalyzed exchange or two acid-catalyzed mechanisms [6]. Under physiological conditions (~pH 7.0) exchange is dominated by base-catalysis. Therefore, the acid-catalyzed mechanisms will not be discussed here but is discussed in elsewhere [34]. The base-catalyzed exchange mechanism [39] proceeds via two steps (Figure 4.3 Panel B). First, extraction of an amide hydrogen by OH forms an imidate ion along the peptide/protein backbone. Second, the imidate ion is reprotonated, completing the proton transfer reaction. In the case of a deuterium labeling experiment, D_2O is typically in excess of H_2O, so every successful proton transfer results in the replacement of a hydrogen on the protein backbone with a deuterium.

4.2.e Parameters Influencing Amide Hydrogen Exchange

4.2.e.1 The Effect of pH

Understanding the effect of pH on hydrogen exchange is crucial for the success of HDX-MS. The rate of exchange can be controlled by adjusting pH (higher pH translates to faster base catalyzed exchange) and this becomes a critical component of the hydrogen exchange MS experimental protocol. The exchange rate constant \( k_{\text{ex}} \) is simply the sum of the acid- \( k_{\text{acid}} \), base- \( k_{\text{base}} \) and water- \( k_{\text{water}} \) catalyzed reactions (Equation 4.1) [6, 40].

\[
k_{\text{ex}} = k_{\text{acid}}[\text{H}_3\text{O}^+] + k_{\text{base}}[\text{OH}^-] + k_{\text{water}}
\]  

(4.1)

The pH dependence of exchangeable hydrogens (amide, side chain and termini) is shown in Figure 4.4. The average exchange for a backbone amide hydrogen reaches its minimum at around pH 2.6 (~4-fold reduction in exchange rates when compared to pH 7) and occurs where the acid- and base- catalyzed rates are equal [34]. The exchange rate is nearly first-order with
Figure 4.4. Effects of pH on exchange rates for various kinds of hydrogens. Ranges under quench and labeling conditions are highlighted in gray. This figure is reprinted with permission from Morgan and Engen 2011.
respect to OH− at pH values greater than pH_{min} and with respect to H_3O^+ at pH values less than pH_{min}; (i.e. each deviation of one pH unit from the pH_{min} results in an approximately 10-fold increase in the exchange rate).

All other exchangeable hydrogens, on average, have exchange rates at least 2 orders of magnitude greater than the backbone amide hydrogens at pH 2.5[^6]. During analysis of deuterium incorporation by MS, this allows for any labeled side chain, N- and C-terminal positions to revert back to hydrogen, greatly simplifying data analysis as this leaves a maximum of 1 deuterium label per residue (except for proline as previously mentioned). As can be seen in Figure 4.4, arginine ε-NHs have an exchange minimum very close to that of amide hydrogens. Because of this, peptides which are rich in arginine can sometimes retain deuterium at the sidechain, making data analysis of such peptides challenging.

4.2.e.2 The effect of temperature.

In addition to pH dependence, hydrogen exchange, like other chemical reactions, follows the Arrhenius equation and is influenced by temperature. Plainly, increasing the temperature, increases the rate of hydrogen exchange. More specifically, the temperature dependence of \( k_{\text{acid}} \), \( k_{\text{base}} \) and \( k_{\text{water}} \) can be calculated using Equation 4.2.

\[
k(x)_T = k(x)_{298} e^{-\frac{E_a(x)}{R} \left(\frac{1}{T} - \frac{1}{298}\right)}
\]

where \( k(x)_{298} \) is the reference rate constant \( k_{\text{acid}} \) (41.7 M^{-1} min^{-1}), \( k_{\text{base}} \) (1.12 \times 10^{10} M^{-1} min^{-1}) or \( k_{\text{water}} \) (3.16 \times 10^{-2} M^{-1} min^{-1}) at 20 °C and low salt conditions[^35], \( R \) is the gas constant (8.134 J mol^{-1} K^{-1}) and \( E_a(x) \) is the reference activation energy for acid- (14 kcal mol^{-1}), base- (17 kcal mol^{-1}) or water-catalyzed exchange (19 kcal mol^{-1})[^35]. The effect of temperature for an average
Figure 4.5: Effects of temperature on amide hydrogen exchange for a poly-alanine peptide at pH 2.5 as determined by equation 4.2.
amide hydrogen exchange rate, pH 2.5, has been calculated and plotted in Figure 4.5 for an unstructured poly-alanine peptide.

The effect of temperature is a result of altering the concentration of OH⁻ via the modulation of the ionization constant of water [6, 34]. Other factors most likely are affected as well, including decreasing diffusion-collision rate constant, which results in the formation of fewer hydrogen acceptor – donor complexes and lowering the probability of an exchange event.

4.2.e.3 The effect of primary structure.

Neighboring side chains can influence backbone amide hydrogen exchange through inductive (charge) and steric (blocking) effects [35, 38, 41]. Polar side chains increase the base-catalyzed reaction rate by making the amide hydrogen more acidic while aliphatic and aromatic residues reduce the exchange rate in a pH-independent manner [35] by obstructing access to the amide position. Correction factors for all 20 naturally occurring amino acids have been measured in model peptides and can be applied to predict exchange rates for individual amide hydrogens in disordered peptides [35, 38]. Primary structure effects can alter the exchange rate of a neighboring amide hydrogen by as much as 10-fold. This effect is significant for determining the amount of time allowed for analysis of a labeled sample due to back-exchange (D to H, in the protonated chromatography buffers), but is less significant in the labeling reaction, where the structure of the protein can affect the hydrogen exchange rate by as much as 10⁸-fold.

4.2.f Mechanisms of Hydrogen Exchange into Proteins

Exchange into proteins is believed to proceed via two mechanisms: (1) exchange into the folded form (Equation 4.3) and (2) exchange into a partially or globally unfolded form (Equation 4.4), where some degree of unfolding of the protein occurs; a hydrogen bond breaks
and the amide hydrogen is present to the solvent and catalyst for exchange:

\[
F_H \xrightleftharpoons[k_{-1}]{k_1} U_H \xrightarrow{k_{ex}} U_D \xrightarrow[k_1]{k_{-1}} F_D
\]  

where \( F \) and \( U \) refer to the folded and unfolded forms of the protein, respectively and \( H \) and \( D \) refer to hydrogen and deuterium. The rate constants \( k_1 \) and \( k_{-1} \) are the rate of unfolding and refolding, respectively. The exchange reaction is only shown in the forward direction because excess deuterium (\( \geq 90\% \)) in the labeling solutions biases the reaction to proceed in one direction. The overall rate constant of exchange (\( k_{obs} \)) is a sum of the two mechanisms (Equation 4.5):

\[
k_{obs} = k_f + k_u
\]

where \( k_f \) and \( k_u \) are the rate constant for exchange in the folded and unfolded states, respectively.

Exchange into the folded form (Figure 4.6, Panel A), has been explained by two different mechanisms; (1) the solvent penetration model \([34, 42, 43]\) and (2) the relayed imidic acid model \([44, 45]\). Regardless of the specific mechanism, the rate of exchange into the folded state (\( k_f \)) can be described in Equation 4.6.

\[
k_f = \beta k_{ex}
\]

where \( \beta \) is the probability that the particular amide is exposed to both catalyst and water simultaneously and \( k_{ex} \) is the rate of exchange into unstructured peptides \([46]\).

Molecular motions, from small segments to the entire protein, give rise to localized unfolding exchange mechanism (Figure 4.6, Panel B). First, the folded form of the protein
Figure 4.6: Models of hydrogen exchange into proteins for folded (A) and unfolded (B) forms. The rates of folding and unfolding are defined as $k_1$ and $k_{-1}$ respectively. Adapted from Wales and Engen (2006)a.
must undergo an unfolding event; second, exchange occurs as would for an unstructured peptide; and third, the protein refolds into its native conformation.

The rate of exchange for this model \((k_u)\) is shown in Equation 4.7.

\[
k_u = \frac{k_1 k_{\text{ex}}}{k_1 + k_{-1} + k_{\text{ex}}}
\]  

(4.7)

where \(k_1\) and \(k_{-1}\) are the rate constants for unfolding and refolding, and \(k_{\text{ex}}\) is the rate of exchange into an unstructured peptide.

There are two kinetic regimes, termed EX2 and EX1, which were first described in 1966 by Hvidt and Nielsen \([7]\). Most proteins are stable under labeling conditions, and the rate of refolding is much faster than the rate of exchange \((k_{-1} \gg k_{\text{ex}}, \text{see Equation 4.4})\). Under this EX2 regime, the protein undergoes many unfolding and refolding events before the backbone amide hydrogen is exchanged. Less common, is the opposite, EX1 condition \((k_{-1} \ll k_{\text{ex}})\), when the protein unfolds (locally or globally) and become exchange completely during one unfolding event \([31]\). EX2 is the predominant kinetic regime in HDX-MS and results in a single isotopic distribution that increase in mass as labeling time increases (Figure 4.7). EX1 kinetics can be differentiated from EX2 because, in HDX-MS, EX1 kinetics results in a bimodal pattern; the isotopic distribution at lower mass is the unlabeled population which has not yet unfolded and the isotopic distribution at the higher mass is the population which has unfolded and become completely labeled.
Figure 4.7: Labeling kinetics observed in hydrogen exchange mass spectrometry experiments. Spectra from EX2 and EX1 are displayed. Reprinted with permission from Weis et al., 2006b.
**4.3 Experimental Methods**

**4.3.a Materials**

Each protein described in the subsequent chapters required slightly different protocols for expression and purification. For that reason the detailed methods for preparation of each protein will be discussed individually in each chapter.

Reagents were obtained from either Sigma-Aldrich or Research Products International. HPLC grade solvents were obtained from Fisher Scientific. D$_2$O was supplied as, 99.96% D from Cambridge Isotope Laboratories.

**4.3.b Buffers**

Buffer preparation is critical for the success of HDX MS experiments. Given the effect of pH on HX, proper control of pH in both labeling and quenched samples must be maintained to ensure reproducible deuterium uptake and back exchange. Each experiment required slightly different buffers as a result of the individual proteins and the specifics will be discussed in each chapter. Generally, proteins are purified in a buffer which is compatible with hydrogen exchange mass spectrometry. This typically means moderate buffering capability, low ionic strength and neutral pH. For quenching, a buffer with a pK$_a$ near 2.5 is desirable and one with a much higher buffering capacity than the labeling solution buffer. A strong phosphate buffer (150 mM) is commonly used because its pK$_1$ is 2.15. Quench buffer containing denaturant, such as guanidine hydrochloride, can also be utilized to further disrupt the analyte protein prior to proteolysis.
4.3.c Peptic peptides

Historically, porcine pepsin has been used to generate peptides in HX MS studies \[^{[47]}\]. Pepsin is a non-specific aspartic protease normally found in the guts of humans and other animals. As such, it is well suited to operate at acidic pH (Figure 4.4). Although pepsin is non-specific, it is highly reproducible under identical conditions (i.e. pH, concentration, temperature, time, etc.) \[^{[48]}\]. Another advantage of pepsin is that it produces overlapping peptides which can increase resolution to single amino acid positions \[^{[49, 50]}\]. Peptic peptides were identified in samples prepared in an identical manner to labeled samples but without including the deuterated buffer (undeuterated controls). Peptides were identified using CID fragmentation on a Q-Tof based instrument in either data dependent analysis using MS\[^E\]\[^{[51]}\].

Initial HDX MS experiments utilized an online pepsin column prepared in house \[^{[33, 48, 52]}\]. However, later experiments utilized an offline digestion (further discussed in Chapters 5 and 6). For these offline digestions, pepsin was added to quenched samples at 1:1 pepsin:protein by weight and digested for 5 min on ice. Because the HX samples were dilute (typically ≤5 µM protein) quenched sample volumes were >250 µL. Samples this large cannot be frozen and analyzed at a later date because of the significant and variable levels of back-exchange due to the amount of time required to thaw the quenched peptic digests.

4.3.d Sample Preparation and Analysis

4.3.d.1 Deuterium Labeling.

There are two general schemes for preparation of deuterated samples, continuous or pulsed labeling \[^{[30]}\]. All of the following work was carried out with continuous labeling (Figure 4.8), but pulsed labeling has been used in the field \[^{[19]}\]. Continuous labeling is exposure of the
Figure 4.8: Experimental workflow for continuous labeling hydrogen exchange mass spectrometry experiments.

Protein

pH ~7
25 °C

Equilibrate
Time = T

Quench
pH 2.5
0 °C

Extra
Time point?

Yes

Protease Digestion
pH 2.5, 0 °C

No

Intact or peptide?

Local

Global

UPLC separation
pH 2.5, 0 °C

Mass analysis & data processing

Purified Protein

Dilute 10-20x with D₂O

T = 10s – 240 min
sample to D₂O for a set of predetermined time points (typically 10 sec to 4 hrs) when individual aliquots are then quenched and analyzed. In the alternative labeling technique, pulsed labeling, each sample is labeled for the same length of time (usually short, 10 sec) while another sample parameter (denaturant, ligand, temperature, etc.) is varied.

Typically, one large volume of sample is prepared (master labeling solution) and diluted with deuterated buffer. At predetermined time points, an aliquot is removed, quenched and frozen for analysis at a later date, provided the protein of interest is able to survive at least one freeze-thaw cycle (generally Nef was labeled without freezing and thawing). One consideration in preparing these samples is the amount of protein the mass spectrometer will consume to generate quantifiable signal. This is highly instrument dependent and newer, more sensitive LC/MS systems were acquired during the time period of this work so the requirement for peptide analysis generally decreased over time (from 200 pmol to 50 pmol per injection). For intact protein analysis, approximately 200 pmol was used per injection.

4.3.d.2 Chromatography

A common consideration for HDX MS is the speed of the chromatographic gradient[4, 33]. This is because the deuterium label will be constantly reverting back to hydrogen when the sample is exposed to the protonated solvents during digestion, trapping and LC separation. By taking advantage of the resolution on the mass scale afforded by the MS, chromatography need not be optimized for base-line resolution of peptides but rather for speed. In addition to fast chromatography, maintaining quench conditions through the chromatography step is critical. In all experiments, chromatography buffers contained 0.05% trifluoroacetic acid (TFA) in high performance liquid chromatography (HPLC) or 0.1% formic acid (FA) in UPLC to maintain a
Figure 4.9: HPLC/MS set-up for intact protein mass analysis. A) Valve set-up for trapping, desalting and eluting protein into the mass spectrometer. Red lines signify flow from the HPLC for the load (i.) and inject (ii.) positions. B) The HPLC gradient used for intact protein mass analysis.

Solvent A: Water, 0.05% TFA, pH 2.5
Solvent B: Acetonitrile, 0.05% TFA, pH 2.5
Flowrate: 0.05 mL/min
Figure 4.10: UPLC/MS set-up for peptide mass analysis. A) Two valve set-up for trapping, desalting and eluting peptides into the mass spectrometer. Flow from the UPLC is shown in red for the trapping and inject positions. B) The UPLC gradient used for all peptide mass analysis (Chapters 5&6).

Solvent A: Water, 0.1% FA, pH 2.5
Solvent B: Acetonitrile, 0.1% FA, pH 2.5
Solvent C: Water, 0.1% FA, pH 2.5
Flowrate: 0.06 mL/min
Trapping: 0.10 mL/min for 3 min
pH of 2.5 throughout analysis. Temperature was controlled by submerging the valve, trap, column and transfer lines in an ice bath (in HPLC) or the use of a UPLC cooling chamber maintained at 0 °C specifically designed for HDX MS experiments \[33\].

One advantage of HDX MS is its ability to handle dilute samples (as low as 1 µM). This arises from the fact that after labeling and quenching, the sample is rapidly concentrated as it is injected onto a trap. This was done for both intact and peptide analysis. For work with all intact proteins (intact mass checks, global deuterium uptake) the HPLC was set up with a single valve (Figure 4.9, Panel A). The sample was loaded onto a POROS 20R2 (“C-18 like”) trap \[53, 54\] and manually desalted with (≥4x the volume of the sample) volume of Buffer A (H\(_2\)O, 0.05% TFA, ice cold if for labeled samples). The protein was eluted off the trap and into the mass spectrometer with a 15-95% B (acetonitrile:water, 0.05% TFA) gradient over 3 min (Figure 4.9, Panel B). For the analysis of peptides, pepsin digested samples were trapped and eluted from a C-18 VanGuard trap (Waters) and separated on a C18 reversed phase column \[33\]. The experiments in Chapters 5 & 6 were performed using UPLC/MS and the UPLC setup used there is shown in Figure 4.10. Peptides were eluted with a 5-35% B gradient over 6 min.

### 4.3.d.3 Mass Spectrometry

Intact mass analysis was carried out on Waters LCT Premier\(^{XE}\) mass spectrometer. Analysis of peptides was done on Waters Synapt G2 HDMS mass spectrometer. All of the instruments were tuned and calibrated according to established guidelines. The LCT mass spectrometer was calibrated with an infusion of horse heart apo myoglobin (Sigma-Aldrich). The Synapt G2 HDMS was calibrated using glu-fibrinopeptide (Sigma-aldrich). Periodic introduction and detection of glu-fibrinopeptide during each analytical was also utilized as a
lock mass \[^{55}\] and continuously corrected the m/z calibration of the Synapt G2.

4.3.d.4 Data Analysis and Processing.

Once spectra were obtained for all proteins and all time points, the process of determining the level of deuterium uptake began. The centroid mass of the isotopic distribution was used for all calculations of deuterium incorporation. For global hydrogen exchange (exchange into the intact protein) the combined scans for the protein were smoothed and the spectrum was exported to MagTran \[^{56}\] where the centroid mass (full width at half maximum) was calculated. The extent of labeling was determined by subtracting the unlabeled intact mass from the labeled intact mass at each time point. The data are plotted as labeling time (log scale) versus deuterium level.

Determining deuterium incorporation into peptides is similar to that of intact proteins. However, there are many chromatographic peaks from the peptic peptides (Figure 4.11) instead of one large peak. Within each peak there can be multiple peptides that are, hopefully, resolved on the m/z scale (Figure 4.11, Panel B and C). For each peptide (and all timepoints for that peptide) an extracted ion chromatogram is generated and all of the scans which make that peak are combined and smoothed. This combined spectrum is used to calculate the centroid of the peptide. Modern mass spectrometers have high enough resolution (>5000) that the isotopic peaks of peptides will be resolved.

Historically, data analysis has been the greatest bottleneck for HDX MS. For a large protein, pepsin may reliably produce over 200 peptides \[^{57}\]. Labeling at least 5 time points and an undeuterated control are typical for this type of experiment which results in needing to calculate 1200 (6 x 200) centroid values per protein for each replicate. Determination of the centroid, C, for an isotopic distribution is calculated using equation 4.8 \[^{58}\].
Figure 4.11: Raw peptide data. A) a total ion chromatogram from a UPLC separation of a digested non-myrNef protein. B) Combined mass spectra for one of the eluting peaks in panel A. C) Zoomed-in peptide at m/z 608.24 (+3).
Figure 4.12: Determination of relative deuterium uptake. A) Mass spectra for an ion at m/z 425.7 (black) and the mass spectra for labeled samples (colored spectra) are shown. The centroid (colored lines) of each isotopic distribution is calculated using equation 4.8. B) The centroid for the unlabeled time point is subtracted from the labeled time points, resulting in the relative deuterium level. C) The relative deuterium levels are plotted on the log scale to generate an uptake plot.
Figure 4.13: Major types of replicates for HXMS. The colored boxes display the five major kinds of replicates beginning with freshly purified protein (biological) and ending in data analysis (processing). The amount of error associated with each replicate is displayed to the right, with biological replicates having the highest amount of experimental error, and processing replicates have the least. Adapted from Engen and Wales, 2015.
Several software packages have been developed which aid in speeding up data analysis \cite{58-61}. The vast majority of peptides were resolved well enough on the m/z scale and commercial software, such as Waters’s DynamX was used extensively to process HDX MS data, and was utilized through this work. Example data showing the determination of deuterium content is shown in Figure 4.12.

Lastly, deuterium uptake can be interpreted in light of any known or hypothetical protein structures. Deuterium uptake is commonly plotted on solved X-Ray crystallographic or NMR structures to assist in the interpretation of the HX MS results. Throughout the following chapters, depictions of protein structures, with HX data overlaid onto them, were generated with PyMOL.

\textbf{4.3.d.5 Error and Replication}

As with any measured phenomenon, sources of error in HDX MS must be quantified and minimized. There are three sources of error associated with determining the deuterium uptake into a protein or peptide: (1) error in sample preparation, (2) error in mass measurement, and (3) error in data processing. The largest source of error is from the biological variability of batch to batch protein expression and purification; however, other sources such as buffer preparation or sample handling can influence the experimental error (Figure 4.13). Small deviations in pH, either in the labeling or quench buffers will have large effects on exchange rate. Any samples which were directly compared were prepared in identical buffers and analyzed on the same day.
Replicates were analyzed using the same buffers (which were stored at 4 °C and, for the labeling buffer, in desiccant) as soon as possible. Error in mass measurement is derived from deviations in day-to-day calibration and room temperature within each sample set. Instrument variation was minimized by measuring a calibrant after (myoglobin, intact analysis) or during (glu-fibrinopeptide, peptide analysis) each run. Data processing errors are derived from variations in calculating the centroid of an isotopic distribution repeatedly. The use of software packages greatly reduces this type of variability; however, deviations in combining scans manually will be carried through the centroid calculation, leading to error. It is particularly difficult to reliably calculate the centroid for an ion which is of low abundance and signal intensity.

Peptide analysis by HDX MS on the QTof system has been well characterized; the error of each data point does not exceed ±0.15 Da so differences in the relative deuterium level between two protein states which exceed 0.3 Da are significant [27]. For the work described in the following chapters analyses were done in replicate. Therefore, error bars do not indicate the standard deviation from the mean. Based on the well characterized nature of measurements on this system and in order to err on the side of caution, differences greater than 0.5 Da were considered significant. Error bars on the deuterium uptake plots in the following chapters were set to ±0.5 Da to highlight major differences (≥1.0 Da).
4.4 References


Chapter 5

Hydrogen Exchange Mass Spectrometry of
Peripheral Membrane Proteins Using Langmuir Monolayers

Parts of this chapter have been published and are available in Appendix III


5.1 Introduction

Recall from chapter 1, that one of the major aims of this work was to develop a new HDX MS method for studying membrane proteins and this required several optimization and validation experiments. Membrane proteins are a crucial component in several cellular processes including signaling, structural and transport roles [1]. A portion of these proteins either pass through or are partially embedded in the lipid tails of the membrane. Well-known examples include bacteriorhodopsin and OmpA found in *E. coli*. In addition to transmembrane proteins, proteins can also associated peripherally to lipid membranes and are generally referred to as peripheral membrane proteins. These proteins can shuttle between cytosolic and membrane bound populations [1]. Two well studied examples of peripheral membrane proteins include the phosphoinositide 3-kinases (PI3K) [2-4] and ADP-ribosylation factor 1 (ARF-1) [5-7]. For many peripheral proteins, recruitment to the cell membrane is facilitated by covalently attached fatty acids groups, such as farnesyl, palmitate and myristate moieties [8-10]. This modification is referred to as acylation and is generally facilitated via an endogenous enzyme. For example, myristoylation of proteins is completed by N-myristoyltransferase-1 [8,11-13]. The membrane
bound conformation of these proteins is essential for activity, and it has been shown that membrane association induces structural changes necessary for protein function \[^{14-17}\]. One example includes a myristoyl or farnesyl switching mechanism where the protein shifts between conformations dependent upon if the myristoyl or farnesyl moiety is inserted into the lipid tails of the membrane or sequestered in a hydrophobic pocket of the protein. Recoverin and ARF-1 are examples of myristoyl switch proteins \[^{18-20}\]. Association with membranes allows for the occlusion of the myristate into the hydrophobic lipid tails, triggering a conformational change in the protein (McLaughlin and Aderem 1995). It is by these mechanisms that membrane association can regulate protein function.

Until recently, conventional techniques, such as X-ray crystallography and NMR, have been inadequate for studying the functionally relevant, membrane-bound conformation of peripheral proteins. Within the past two decades, Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS) has become an invaluable tool for the analysis of the higher order structure of proteins not possible with crystallization or NMR techniques \[^{21-24}\]. Recall from Chapter 4, that HDX MS operates on the principle that amide hydrogens of the protein backbone naturally exchange with deuterium upon dilution into D\(_2\)O. The rate of exchange is determined by pH, temperature, solvent accessibility and the hydrogen-bonding network of the protein \[^{25-28}\]. Temperature and pH are controlled experimentally; thus, details on conformation and dynamics of the protein can be investigated based on the rate of deuterium incorporation. In order to overcome the inherent difficulty of studying membrane proteins, membrane mimetics have been used to aid in the characterization of membrane proteins. Examples of these include detergents to help stabilize hydrophobic regions and solubilize proteins \[^{29}\]. Liposomes and nanodiscs have been used as model systems and are comprised of a collection of lipids mimicking the
composition of the cell membrane \[^{30}\]. Groups have begun to utilize these lipid model systems with HDX MS to study membrane bound conformation and dynamics \[^{23,31-34}\]. These lipid mimetics offer the user control over the charge, lipid composition and lipid tail length depending on which types of fatty acids/detergents are used for the analysis. Control over these parameters has been show to influence protein structure \[^{35}\].

In addition to these lipid mimetics, Langmuir monolayers have also been used to study the membrane-bound conformations of peripheral proteins \[^{36,37}\]. Langmuir monolayers are formed from any self-assembled film spread at an air-liquid interface \[^{38}\]. A monolayer comprised only of lipids is analogous to the inner face of the cell membrane. These monolayers are maintained and controlled within a Langmuir trough which allows the user to finely control the lipid packing density of the membrane. Lipid packing density is a parameter that liposomes and nanodiscs cannot provide; however, Langmuir monolayers still allow for control over the charge, lipid tail length and composition. Lipid packing density can alter protein structure, and conformational changes in peripheral membrane proteins have been studied using Langmuir monolayer and neutron reflection \[^{36,37}\].

Neutron reflection (NR) has been used as an alternative to X-ray crystallography and Nuclear Magnetic Resonance (NMR) to study membrane proteins \[^{39-41}\]. While X-ray crystallography and NMR are high resolution (<5Å) structural determination techniques, larger (>50kDa) or highly dynamic membrane proteins often are not amenable to these methods. One of the major advantages of NR is the ability to study dynamic membrane bound systems \[^{42}\], albeit at much lower resolution (>30Å). Recall from Chapter 3, that Langmuir monolayers were used with neutron reflectometry to study the membrane-bound conformation of full-length, myristoylated Nef \[^{37}\]. Only portions of Nef have been resolved using X-ray crystallography \[^{43}\].
and Nuclear Magnetic Resonance \cite{46,47} and NR was initially utilized to study the conformation of the full-length protein \cite{37}. However, these initial findings reported on a one-dimensional shape profile, and specific details on dynamics were not resolved. Hydrogen deuterium exchange mass spectrometry is a technique capable of probing the dynamics and conformation for proteins that cannot be resolved using X-ray crystallography and NMR \cite{25}. Implementing HDX MS together with NR and Langmuir monolayers can provide a more complete picture of protein conformation in a functionally relevant, membrane associated state.

In an effort to replicate the exact conditions of the previous NR experiments, a new method for studying peripheral membrane proteins using HDX MS and Langmuir monolayers was developed and validated using a small peptide and a prototypical myristoyl-switch protein. This hybrid method is capable of investigating protein conformation using a range of lipid packing densities and can be applied to peripheral or membrane anchored proteins. The method described in this chapter is intended to be integrated into a combined experimental workflow, where one can observe a membrane bound shape profile using NR and resolve the finer details of dynamics and conformation of a membrane-bound state using HDX MS, all in the same system. The development and validation of this method will be discussed in this chapter.
5.2 Methods

This section will cover the procedures for producing myrARF-1 for HDX MS labeling experiments. The integration of Langmuir Monolayers (also referred to as a Langmuir trough) will be introduced and the method development and validation will be discussed in this chapter.

5.2.a Production of Myristoylated ARF-1: Expression and Purification

Myristoylated human ARF-1 was expressed as previously described with slight modification. Briefly, the ARF-1 plasmid with a polyhistidine tag (Addgene plasmid 28168) was cloned into a pET-Duet vector containing hNMT1, and transformed into Rosetta2(DE3)pLysS cells for expression. The protein was isolated using Ni-NTA agarose beads (QIAGEN, Valencia, CA). Purity and myristoylation were confirmed by polyacrylamide gel electrophoresis (PAGE) and electrospray mass spectrometry (ESI-MS).

5.2.b Digestion Optimization of Membrane Associated Samples

Prior to any HDX MS with lipid associated proteins, a series of experiments were performed to validate the digestion efficiency and subsequent peptide coverage of the analyte protein when bound to membranes. The summary of these optimization experiments is shown in Figure 5.1 and the resulting peptide coverage maps are shown in Figure 5.2. As referred to in Chapter 4, an acid quench (250 mM potassium phosphate, pH 2.5) is used to retard the exchange process during an HDX-MS experiment. In many cases, this is sufficient to denature the protein and enable efficient proteolytic digestion of the sample. This digestion is most commonly
Figure 5.1. Overview of the digestion and quench conditions utilized to improve peptide coverage of membrane associated samples. The final optimized conditions were determined to be a combination of pepsin, factor XIII and a denaturing quench.
Figure 5.2. Peptide coverage maps resulting from various quench and digestion conditions.
C. Nef 1:1 aspergillopepsin:pepsin column digestion, Gdn quench

D. Nef 1:1 aspergillopepsin:pepsin offline digestion, denaturing quench

Total: 47 Peptides, 61.0% Coverage, 2.88 Redundancy

Total: 68 Peptides, 96.0% Coverage, 4.81 Redundancy
accomplished by passing the sample through a column packed with pepsin functionalized to 20 µM POROS beads (Chapter 4). The standard approach for proteolysis has not been acceptable for most membrane associated samples for two major reasons: inability to follow myristoylated peptides (retained in pepsin column), poor sequence coverage (~50%) resulting from inadequate digestion of protein samples. To avoid possible retention of myristoylated peptides on the stationary phase of the POROS column, acid proteases were prepared to 10 mg/mL in milli-Q water and immediately added to quenched samples for digestion. To improve the digestion efficiency, a denaturing quench buffer (0.8 M guanidine hydrochloride, 0.8% formic acid, pH 2.4) was used to halt the labeling reaction. The addition of guanidine hydrochloride aids to disrupt the analyte protein structure and allows proteases better access to facilitate digestion. Pepsin and aspergillopepsin will continue to function provided that concentration of guanidine hydrochloride doesn’t exceed 3 µM \[48\]. Further improvements resulted from the combined use of two acid proteases: pepsin and aspergillopepsin (Factor XIII) (Figure 5.2). The use of a denaturing quench and two acid proteases resulted in the best peptide coverage for membrane associated samples.

5.2.c Hydrogen Exchange Mass Spectrometry Using Langmuir Monolayers

For monolayer hydrogen exchange experiments a modified Langmuir trough system was utilized. Cartoon representations of the Langmuir trough system is shown in Figures 5.3 and 5.4, and the procedure for generating a monolayer has been previously described in detail in Chapter 3. Briefly, DPPG was spread from a mixture of methanol and chloroform (70/30 by volume) onto the surface of a buffered subphase (50 mM citric acid, 50 mM sodium phosphate, 100 mM sodium chloride, pH 7.0, H₂O). Once the chloroform and methanol had evaporated, the deposited lipids were compressed to a set pressure (between 20 & 35 mN/m) using a movable
Figure 5.3. Cartoon representation of the top-down view of the Langmuir Trough system used for labeling experiments. The design was adapted from the trough previously used in neutron reflection studies (Akgun, Satija et al. 2013) where the beam footprint was a square 25 nm in length on each side. The area in blue represents the aqueous buffered subphase (18 mL total). A movable barrier is used to compress the monolayer that is spread on top of the subphase. The flow of buffer through the trough is indicated by red arrows. From Pirrone, Vernon et al. 2015.
Figure 5.4. Cartoon representation of the side view of the Langmuir Trough system used for labeling experiments. This system has been modified from the trough system used for NR experiments (Akgun et al. 2013). Red lines indicate the flow of buffer through the trough, driven by the peristaltic pump, during subphase exchange and deuterium labeling. A vacuum pump is used to aspirate labeled samples directly from the monolayer into a cold collection tube. From Pirrone, Vernon et al. 2015.
barrier. After the pressure had stabilized at the specified pressure, protein was injected into the subphase beneath the lipid monolayer using a micropipette and gel-loading tips. The freshly injected protein was allowed to adsorb to the monolayer. The monolayer pressure was fixed during this time, and adsorption was monitored by the gradual backwards migration of the barrier (Figures 5.9). Once the barrier had moved to within 5 mm of the back of trough, the subphase was rapidly exchanged for labeling buffer (50 mM citric acid, 50 mM sodium phosphate, 100 mM sodium chloride, pH 7.0, D$_2$O) using a peristaltic pump and Teflon tubing. The subphase exchange process lasted 10 seconds $^{[49, 50]}$. Adsorbed samples were labeled with deuterium for predetermined times ranging from 10s to 4 hours before the reaction was quenched. To quench samples, portions of the lipid monolayer and the membrane-associated peptide or protein were quickly aspirated into a pre-chilled 1.5 mL sample tube, mixed with a solution of 0.8% formic acid and 0.8M guanidine hydrochloride (quench buffer) to drop the pH to ~2.6, immediately transferred to ice and, digested for 5 minutes with pepsin (60 µg) and aspergillopepsin (70 µg) before being injected for UPLC-MS analysis.

Digested peptides were separated at 0 °C using a nanoAcquity UPLC with HDX technology $^{[51]}$ (Milford, MA). Peptides were trapped on a Waters UPLC BEH C18 1.7 µm VanGuard column and desalted with 0.1% formic acid in water for 3 minutes at 100 µL/min. An additional VanGuard column was placed directly in line with the analytical column (Waters HSS T3 1.8 µm C18, 1.0 mm x 50 mm) to reduce the amount of lipids, downstream of the trap, entering the mass spectrometer. Peptides were eluted over 6 minutes using a 5-35% gradient of acetonitrile/water with 0.1% formic acid flowing at a rate of 60 µL/min. Mass measurements were performed with a Waters Synapt G2 HDMS instrument equipped with a standard ESI source and continous lock-mass correction using Glu-fibrinogen peptide. The instrument was
operating with a capillary voltage of 3.20 kV, a cone voltage of 40 V, a source temperature of 80 °C, and desolvation gas flow of 600 L/hour. Mass spectra were acquired using MS\textsuperscript{E} and spanned a range of 50-2000 m/z \[^{[52]}\]. Peptides were identified using ProteinLynx Global Software 2.5 (Waters), and relative deuterium incorporation curves were generated using DynamX 3.0 (Waters).

5.3 Method Optimization

5.3.a Assessing Deuterium Recovery from Trough Labeled Samples

For HDX experiments, great measures are taken to maximize the deuterium recovery of each sample (Wales and Engen 2006). HDX MS data are less revealing if the deuterium recovery is poor. Thus, it was essential to assess the deuterium recovery from the Langmuir trough system prior to additional labeling experiments. As previous studies have done, \[^{[53, 54]}\] we utilized maximally deuterated peptides to probe and assess deuterium recovery. Deuterium loss was monitored using a mixture of leucine enkephalin, angiotensin I and bradykinin. The sequences for these peptides are shown in Table 5.1. The loss of deuterium for each peptide was compared between samples taken directly from D\textsubscript{2}O (maximally deuterated), samples mixed with quench buffer in a 1.5 mL sample tube (to mimic a solution labeling format), and samples run through the Langmuir trough system including aspiration and quenching steps (to mimic a HDX MS trough labeling experiment). For all peptides, equivalent losses were observed between the Langmuir trough experiments and a conventional HDX MS protocol (Marcisin and Engen, 2010) (Figure 5.5). It is important to note that there was no evidence of insertion of these peptides into the lipid monolayer as the measure surface pressure did not increase and the barrier
Table 5.1. Sequences and average molecular masses for small test peptides.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Sequence</th>
<th>Average Molecular Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine Enkphalin</td>
<td>YGGFL</td>
<td>555.62</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>NRVYIHPFHL</td>
<td>1296.48</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>RPPGFSFPR</td>
<td>1060.21</td>
</tr>
</tbody>
</table>
Figure 5.5. Relative deuterium recovery for a mixture of maximally deuterated test peptides. Recovery was measured for bradykinin, angiotensin, and leucine enkephalin following the Langmuir monolayer labeling protocol (red), a conventional solution labeling protocol (blue), and direct injection into the UPLC without the addition of quench buffer (grey). The average of three independent measurements is displayed with error bars indicating the spread of the data. For angiotensin I, the +2 charge state is shown on the left, while the +3 is shown on the right. From Pirrone, Vernon et al. 2015.
did not move during equilibration. Between these two conditions, the average deuterium loss never differed by more than 3%. Furthermore, losses for peptides analyzed from the trough system never differed more than 8% when compared to samples taken directly from D$_2$O. These results strongly indicate that the extra time needed to aspirate and quench samples from the trough does not significantly reduce deuterium recovery, thus, making Langmuir trough labeling experiments feasible.

5.3.b Validating Protein-Lipid Interactions using Melittin

In addition to quantifying the deuterium recoveries of trough samples, sample binding to the lipid monolayer also had to be tested. If there was no evidence of any protein-lipid interactions in our Langmuir trough system, this new method would be less useful. We elected to use a well characterized and simple peptide, melittin, to test and monitor protein-lipid binding in our system $^{[55]}$. It is a hemolytic peptide and the major toxic component in the venom of the honey bee *Apis mellifera*. Like other peptides that interact with lipid membranes, it is largely hydrophobic with the N-terminal region consisting of mostly non-polar residues. However, the C-terminal portion of the peptide contains a string of charged, basic residues. This amphipathic nature allows melittin to associate with lipid membranes while also being water soluble $^{[56]}$. While melittin has been shown to be unstructured in solution, its membrane bound conformation is largely α-helical with the hydrophobic N-terminal residues interacting with lipid groups (Figure 5.6). The charged hydrophilic groups interacting with the surrounding water molecules, and at high concentrations will aggregate to form pores in bilayers $^{[57-61]}$.

Taking advantage of previous work, melittin was an ideal peptide to validate this system by comparing the deuterium incorporation of the peptide in solution to the incorporation in the
Figure 5.6. Cartoon representation of melittin interacting with DPPS monolayers. Melittin can adopt a parallel orientation (A) (Raghuraman and Chattopadhyay 2007) and/or a perpendicular orientation (B) (Gimenez, Sanchez-Munoz et al. 2015) depending upon concentration. From Pirrone, Vernon et al. 2015.
Langmuir trough in order to probe for membrane interaction. Differences between the deuterium incorporation from samples labeled in solution contrasted with samples labeled in the trough, would reveal any lipid-induced conformational changes. First, melittin was labeled with deuterium for 10 and 30 seconds in the absence of any lipids and analyzed. Next, melittin was injected underneath a Langmuir monolayer and allowed to adsorb for approximately 15 minutes. Melittin insertion was evidenced by the backward movement of the barrier indicating an increase in surface area of the monolayer (Figure 5.7). Once, the barrier had reached the back of the trough, melittin was labeled for 10 and 30 seconds and analyzed. Figure 5.8 shows the deuterium incorporation of both states. As predicted, the deuterium uptake for melittin in solution for both time points is significantly higher. In the absence of a lipid membrane, the peptide is nearly maximally deuterated (+21 Da) in 30 seconds, which agrees with observations indicating a lack of structure in solution. There is a significant reduction in deuterium uptake when melittin is allowed to adsorb to the monolayer. There is a ~4 and 6.8 Dalton difference between solution and monolayer samples for 10 and 30 seconds respectively. These data coupled with the trough barrier movement indicate that the peptide is gaining structure and inserting into the lipid monolayer, protecting it from exchange. This observation concurs with data from previous studies [57-61] and validates this method for observing conformational changes as a result of occlusion from solvent and/or formation of secondary structure upon lipid association.

5.3.c Lipid-binding Induced Conformational Changes in ARF-1

To further validate this method, a larger protein was studied using this novel hybrid system. We elected to use ADP-ribosylation factor 1 (ARF-1) as a model protein for studying
Figure 5.7: Change in surface pressure (black) and barrier position (red) in the trough following introduction of Melittin (I). The surface pressure was held constant after addition of protein. The barrier gradually moved back as more protein associated and inserted residues into the monolayer. The barrier was stopped within a few millimeters before reaching the end of the trough and the subphase was exchanged (E). The sample was collected by aspiration using a vacuum pump (A).
Figure 5.8. Relative deuterium incorporation at times indicated for melittin following the Langmuir monolayer labeling protocol (red) and a conventional solution protocol (blue). The average of three independent measurement is displayed with error bars indicating the spread of the data. From Pirrone, Vernon et al. 2015.
structural changes upon membrane association. ARF-1 is a well-studied 20 kDa guanine-nucleotide-binding protein that is involved in vesicle formation and trafficking across the Golgi membrane \[62\]. Most importantly, ARF-1 and all ARF family proteins are myristoylated on the glycine residue at position 2, and this modification is crucial for membrane association. In addition to the myristoyl moiety, membrane anchoring is also influenced by the presence an N-terminal amphipathic helix, which enables membrane association \[63\].

Similar to many other proteins in the Ras superfamily, ARF-1 transitions between an “inactive” GDP-bound state and an “active” GTP-bound state. This well-studied cycle is regulated by a network of GTPase activating proteins and guanine nucleotide-exchange factors \[64\]. Membrane binding and nucleotide exchange are closely coupled through a myristoyl switching mechanism. This mechanism is characterized by the transition between different conformations in which the myristoyl group is either hidden within a hydrophobic pocket of ARF-1 or inserted into the lipid tails of a membrane \[5\]. Franco and coworkers observed that only myristoylated ARF-1\textsubscript{GDP} could be activated by nucleotide exchange factors, and that the rate of GDP dissociation was significantly greater in the presence of lipid membranes \[65, 66\]. These findings indicate that the membrane is an important element for nucleotide exchange, and that insertion of the myristoyl group induces a structural rearrangement of the N-terminal arm, the switch 1 and switch 2 regions of the protein. This conformational change is conducive for interacting with exchange factors \[65, 66\]. More recently, Liu and coworkers reported that movement of the myristoyl group from a hydrophobic pocket (residues 12, 20, 37, 58, 61, 89, 170, 173, and 177) in the protein to the lipids tails of a membrane was required to prevent steric clashing with portions of the interswitch region (residues 57-61) of ARF-1. A membrane must be available to sequester the myristate before exchange can take place \[6\]. Additional work by
Liu and coworkers has shown that while the N-terminal portion of myristoylated ARF-1 rests against the membrane, residues 17-177 of the protein are situated in the aqueous area away the lipid head groups \[^7\]. These structural details are further evidence of the unique conformation myristoylated ARF-1 adopts to interact with other proteins at the membrane.

Given the previous characterization of the membrane-bound conformation of ARF-1, this protein was elected as an ideal model to validate this new HDX MS labeling method. Continuous labeling of membrane-bound myristoylated ARF-1 was performed in the Langmuir trough system. The deuterium incorporation of the membrane-bound state was compared to an unbound state. In this study, digestion produced in 32 peptides that resulted in 92% sequence coverage (Figure 5.10). All peptides identified by MS\(^{E}\) fragmentation and followed for the entire labeling time course, for both states, are listed in Table 5.2. Overlapping peptides were identified in nearly all areas of the protein, and similar deuterium exchange trends were observed in areas of redundancy (Figure 5.13). Deuterium incorporation was recorded in at least triplicate biological replicates \[^{67}\] with a fresh monolayer spread in the trough for every labeling data point.

An example of the raw data acquired for these peptides is shown in Figure 5.11 and all the followed spectra are shown in Figure 5.12. These data highlight the comparable quality of data that can be obtained from the Langmuir trough compared to a conventional solution labeling format. The N-terminal lipid binding region (residues 1-21) had substantially lower deuterium incorporation when in the presence of the lipid monolayer for time points, with observed differences in deuteration exceeding 5.0 daltons at 10 seconds (Figure 5.14, panel A). These data strongly suggest that the N-terminal residues are gaining structure and/or inserting into the lipid membrane, occluding them from solvent exchange. This supports previously published data describing the formation and insertion of an N-terminal amphipathic helix (Liu, Kahn et al.)
Figure 5.9. Change in surface pressure (black) and barrier position (red) in the trough following introduction of ARF-1 (I). The surface pressure was held constant after addition of protein. The barrier gradually moved back as more protein associated and inserted residues into the monolayer. The barrier was stopped within a few millimeters before reaching the end of the trough and the subphase was exchanged (E). The sample was collected by aspiration using a vacuum pump (A). From Pirrone, Emert-Sedlak et al. 2015.
Figure 5.10. Coverage map of identified peptides (green bars) from myrARF-1 generated using a mixture of pepsin and aspergillopepsin, and followed during HDX-MS experiments. Reproducible peptides were found at least three times in quadruplicate runs. The sequence coverage and redundancy are indicated below the map. From Pirrone, Vernon et al. 2015.
Figure 5.11. Deuterium incorporation in Arf-1. Mass spectra for peptide 1-21 (A) and 166-170 (B) following a solution labeling protocol (black) and the Langmuir Trough labeling protocol (red). From Pirrone, Vernon et al. 2015
Figure 5.12: Raw spectra of Arf-1 peptides. The labeling times are listed to the left, and the labeling state, peptide residues and charge states are listed above.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
110-115, no lipid, +1

110-117, no lipid, +1

110-117, no lipid, +2

110-115, 20 mN/m, +1

110-117, 20 mN/m, +2

Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
Figure 5.12: cont.
166-171, no lipid, +1

166-171, 20 mN/m, +1

Figure 5.12: cont.
Figure 5.13. Deuterium uptake plots for myristoylated ARF-1 comparing the incorporation following the Langmuir monolayer labeling format (red) and a conventional solution labeling format (blue). From Pirrone, Vernon et al. 2015.
Additional peptides with substantial protection upon lipid association were observed in the switch 1 (residues 41-50) and switch 2 (residues 63-89) regions (Figure 5.14, Panels A, C). Interestingly, these regions display significant protection at early time points with differences greater than 2.0 daltons for overlapping peptides in both regions (Figure 5.14, panel A). However, this difference gradually wanes. Peptides in the switch 1 region of membrane bound ARF-1 incorporate comparable amounts of deuterium when compared to the solution state after 10 minutes of labeling. At longer time points, peptides in the switch 2 region incorporate more deuterium (greater than 0.5 Da) in the membrane bound state compared to the solution state. This trend suggests that these regions of the protein are initially protected in ARF-1 as it begins to transition from solution to a membrane bound structure. Once associated, the protein progressively changes conformation, and these regions become more exposed. Indeed it has been shown that the switch regions must be accessible to facilitate effector-adaptor interactions necessary for ARF-1’s role in membrane traffic and our HDX data support these findings [5-7, 65, 66].

Aside from those three regions of protection, the majority of the protein becomes much more exposed upon membrane association (Figure 5.15). Interestingly, areas involved in binding to GTP were some of the most exposed portions of membrane bound ARF-1. Overlapping peptides identified for residues 121-135 and peptide 160-170, all have an increase in deuterium uptake exceeding 2.0 daltons at the 240 minute time point. This signifies substantial exposure of these residues upon association with the monolayer. This contrasts with the switch 1 and switch 2 regions, which are initially more protected following monolayer association. This observation is rationalized by the known contact between ARF-1 and guanine-nucleotide exchange factors at the membrane. It has been shown that these residues must be exposed in order to interact with
Figure 5.14. Effects of monolayer association on Arf-1. A) Deuterium incorporation plots for peptides (i) 1-21, (ii) 41-50, (iii) 63-89 and (iv) 166-170 for monolayer associated (─●─) and solution (−Δ−) labeling. Error bars represent the spread of triplicate measurements. B) Cartoon model of the membrane anchoring Arf-1 via the myristoylated N-terminal helix following nucleotide exchange (Donaldson and Jackson 2011). The helical wheel highlights the residues within the N-terminal helix that interact with the membrane (adapted from Donaldson and Jackson 2011). C) Structural location in myrArf-1*GDP and myrArf-1*GTP of peptides shown and color coded (red, magenta, blue, orange) in panel A. From Pirrone, Vernon et al. 2015.
Figure 5.15. Difference map comparing the deuterium uptake of myrArf-1 in the trough (monolayer associated) and in solution (no monolayer). Average deuterium levels from solution labeling were subtracted from the average deuterium levels of the monolayer associated state. Positive values (exposure) are shown in red and negative (protection) values are shown in blue. From Pirrone, Vernon et al. 2015.
exchange factors to regulate ARF-1 activity (Donaldson and Jackson 2011). The trends observed using our Langmuir monolayer system agree with the prior literature \cite{5,7,65,66} and further validates this system as a means to interrogate structural rearrangements of myristoyl-switch proteins and other peripheral membrane proteins.

5.4 Conclusions

A new method for analyzing peripheral membrane proteins by utilizing HDX-MS with Langmuir monolayers was developed and validated. The deuterium recoveries for this new hybrid method were investigated using small test peptides and found to be comparable to more conventional solution, labeling workflows. No excess deuterium was lost during analysis. A model peptide, melittin, and a model protein, ARF-1, were used to validate this system by monitoring the expected lipid-induced conformational changes upon monolayer association. The reported lipid induced structural changes observed in these two model systems agree with previous findings in the literature. These results validate that our system can monitor conformational changes in proteins upon membrane binding. It is possible to apply this new technique to other proteins, including HIV-1 Nef, a protein previous studied by neutron reflection and Langmuir monolayers. The HDX MS monolayer labeling method was designed to be integrated into a neutron reflection workflow using a Langmuir trough, and by combing these two powerful, orthogonal techniques, a specific shape profile of a protein can be monitored in real time and granular structural details on dynamics and conformation would be captured in one experiment.
5.5 References


Chapter 6

Hydrogen Exchange Mass Spectrometry of Nef at Langmuir Monolayers

Parts of this chapter have been published and are available in Appendix IV


6.1 Introduction

As mentioned in Chapter 1, an important goal of this work was to probe the membrane-associated conformation and dynamics of full-length, myristoylated Nef. After expressing and purifying myristoylated Nef (MyrNef), as described in Chapter 2, the protein was interrogated at monolayers of various lipid packing densities using the validated method described in Chapter 5. The results of these experiments will be described in detail in this chapter.

6.1.a Investigating Membrane-Associated Nef

As discussed in Chapter 5, probing conformational changes in proteins upon interaction with a lipid membrane is highly desirable. A well-known challenge to such structural characterization is the membrane itself, as it is generally not amenable with structural studies and many biophysical measurements. Commonly, when high resolution structural methods such as X-ray crystallography or NMR are unable to provide information, alternative methods have been utilized that are less susceptible to interference from the membrane. In addition to the membrane itself, the parameters of the membrane also need to be considered. As discussed in Chapter 5,
control over some parameters (lipid composition, head-group charge, lipid tail chain length, etc.) is not difficult to achieve, other parameters are much more challenging to control reproducibly. The lipid packing density of a membrane is a parameter that is not easy to control, and in many cases fine control over this parameter is absent for the most common membrane mimetics (e.g., detergents, liposomes and nanodiscs) used for biophysical analyses. While it is true that the additional of cholesterol can influence the rigidity of liposomes/nanodiscs, it is impossible to freely change the lipid packing density of these models after preparation. This is not the case with Langmuir monolayers.

As mentioned in Chapter 3, Neutron reflectometry (NR) has been used to study membrane-associated peptide and proteins. This technique is able to resolve and model the two-dimensional shape profile, distance from the membrane, and nuclear density of membrane-associated proteins, but is incapable of revealing any details on peptide level conformation and dynamics. As explained Chapters 3 and 5, initial NR findings reported on a one-dimensional shape profile for full-length HIV-1 Nef, but specific details on dynamics were not resolved. Hydrogen deuterium exchange mass spectrometry (HDX MS) is capable of probing the dynamics and conformation for proteins that cannot be otherwise cannot be resolved with other structural elucidation techniques. Thus, a portion of this work was dedicated to integrating NR with HDX MS in order to capture finer details on dynamics while also probing an overall shape profile for membrane proteins with the same system used for the initial NR experiments. Shape, distance from the membrane, and nuclear density are determined by the NR measurements while HDX MS provides information on protein dynamics, and location of protected/unprotected backbone amide hydrogen. As mentioned above, HDX MS Langmuir monolayer (also referred to as HDX MS Trough) data are obtained using the same Langmuir
tough as NR, and this allows for very fine and highly reproducible control over the packing
density of the lipid layer. Such control is essential for monitoring conformational changes in
proteins that are sensitive to lipid packing.

6.1.b Nef Structure and Functions at the Membrane

Recall from Chapter 3 that Nef conformation is influenced by lipid packing density. The
effects of lipid packing density on protein conformation were also shown in a recent publication
[7]. There have been several studies that shown that many of Nef’s functions are dependent upon
membrane association including internalization of CD4 and MHC-1 from the cellular surface and
interaction with various cellular kinases located on the inner leaflet of the plasma membrane [9-
13]. The lack of a full length structure for Nef is due to its intrinsic flexibility and propensity
towards aggregation. Sections of the protein have been characterized by either NMR or X-ray
crystallography, and a model of Nef has been assembled based upon these data [14]. Nef consists
of a well folded, ordered core with two highly flexible regions: the N-terminal arm and the C-
terminal disordered loop [15]. The flexible regions account for nearly half of the protein and have
been proposed to have critical roles for Nef functions [15].

The solution conformations of myrNef [16] and non-myristoylated Nef [17] have been studied
before using HDX MS; however, there was no lipid component in either of the studies. It had
been hypothesized that membrane insertion of hydrophobic residues present on the N-terminal
arm alters Nef conformation [18, 19], and the NR work in Chapter 3 was the first direct observation
of this phenomenon [7]. In that work, that the N-terminal myristic acid and hydrophobic residues
on the N-terminal arm inserted into the lipid membrane. Following this, the core of myrNef
transitioned from being directly adjacent to the membrane to a position approximately 100
angstroms from the lipid membrane. Very importantly, however, was the discovery and
characterization with neutron reflection of the conformational transition that was dependent on
lipid packing density. Preliminary membrane optimization work was done using liposomes. The
methods for generating liposomes and studying liposome-bound Nef with HDX MS will be
described below. While never published, the HDX MS results for liposome-bound Nef were used
to streamline the HDX MS Langmuir monolayer labeling method. The novel HDX MS method
[8] described in Chapter 5 was utilized to investigate the dynamics and conformation of
membrane-associated myrNef and the effects of lipid packing density on Nef structure.

6.2 Methods

Purified myrNef was produced as described in Chapter 2. Methods for deuterium
incorporation into myrNef in a solution or in monolayer-associated formats are described in
Chapter 4 and 5, respectively. Methods to generate liposomes and study liposomes-bound Nef
are described in the following section.

6.2.a Studying Liposome-bound Nef

Previous studies [16] have shown that myristoylation alters some binding interactions of Nef
but has an immeasurable effect on overall protein conformation. Previous work also showed no
interaction between myristoylated Nef and POPC-containing nanodiscs could be observed.
Initially this suggested that either Nef did not interact with lipid membranes or that
rearrangement of the lipids must be required for interaction. Recall from Chapter 3 that neutron
reflection (NR) studies [7] provided direct experimental that full-length myristoylated Nef
associated with lipid membrane and changed conformation as a result. The nature of association
Figure 6.1. Coverage map of identified peptides (orange bars) from myrNef and liposomes generated using the method outlined in Figure 5.1, and followed during HDX MS experiments. Reproducible peptides were found at least three times in quadruplicate runs. The sequence coverage and redundancy are indicated below the map.
Figure 6.2. Raw spectra of Nef peptides with and without liposomes. The labeling times, labeling state, peptide residues and charge states are listed above.
Figure 6.2. cont.
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Figure 6.2. cont.
Figure 6.2. cont.
Figure 6.2. cont.
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Figure 6.2. cont.
Figure 6.2. cont.
Figure 6.2. cont.
Figure 6.3. Deuterium incorporation plots of preliminary trough labeling data for myrNef alone (red) and myrNef with liposomes (blue) at pH 8.3.
Figure 6.3 cont.
and structural arrangement was strongly influenced by the lipid packing density. The NR data suggest that Nef was unable to associate with nanodiscs because the lipid packing density of these nanodiscs was too great and could not be altered once produced. As a result, liposomes were utilized as an alternative lipid mimetic to study the membrane-bound conformation of Nef.

Unilamellar liposomes (100 nm in diameter) were generated via extrusion. For Nef HDX MS experiments, liposomes were prepared using a 3:2 ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) to 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1-rac-glycerol (POPG). Following extrusion, liposomes were stored as 20 mM aliquots in 20 mM Tris HCl, 100 mM sodium chloride, pH 8.3 and 4°C. To refine the location of Nef interactions, and measure potential conformational changes upon Nef interaction with lipids, myristoylated Nef bound to liposomes was labeled with deuterium and the location and extent of labeling was measured by mass spectrometry (Chapter 4). Aliquots of full-length myristoylated Nef (refer to Chapter 2 for storage conditions of Nef) were added to liposome aliquots using a 1:200 protein to lipid ratio and the protein: lipid mixture was allowed to incubate for 30 minutes at room temperature. Continuous deuterium labeling was initiated by diluting the Nef: liposome mixture with a deuterated labeling buffer (20 mM Tris HCl, 100 mM NaCl, 3 mM DTT, pD 8.3) and labeled for predetermined timepoints. Labeling was quenched by a 1:1 addition of quench buffer (0.8 M Gdn HCl, 0.8% formic acid, pH 2.5, 0°C). Quenched samples were digested offline on ice for 5 minutes using a 1:1 ratio of aspergillopepsin and pepsin. Digested samples were then immediately analyzed via LC-MS.

The coverage map of the peptides identified and followed in these experiments is shown in Figure 6.1. The raw spectra for all the Nef monitored during the HDX MS labeling time course
Figure 6.4. Effects of liposome binding on Nef at pH 8.3. A) Difference map comparing the deuterium uptake of myrNef with and without liposomes. B) Deuterium incorporation plots for peptides (i) 1-9, (ii) 39-57, (iii) 146-173 and (iv) 206-211 for Nef alone (solid line) and with liposomes (dashed line). C) Structural location in MyrNef of peptides shown and color coded (red, magenta, blue, orange) in panel B.
Figure 6.5. Relative deuterium uptake plotted on a model of myrNef with and without liposomes. The labeling time is noted above and the relative percent deuterium is colored according to the legend shown.
are shown in Figure 6.2. The deuterium incorporation plots resulting from these spectra acquired during HDX MS experiments on liposome-bound Nef are shown in Figure 6.3.

Preliminary results showed lower levels of deuterium incorporation in the N-terminal arm for Nef bound to lipid (Figure 6.4 and Figure 6.5), as compared to Nef alone, and essentially no changes in deuteration in the rest of Nef. Any observations of decreased deuterium uptake are only noted for earlier timepoints (i.e. 10 sec and 1 min). These HDX MS data for the N-terminal corroborated analyses with neutron reflection where part of Nef inserts into the lipids upon association; however, the lack of differences for the rest of the protein did not agree with the NR model of Nef conformation at the membrane (Chapter 3), where the core of Nef is positioned 100 angstroms from the lipid membrane \[7\]. The elongated conformation of Nef after lipid association would likely result in increased deuterium uptake for the regions of the Nef core, and this was not observed with preliminary liposome HDX MS experiments (Figure 6.4). Recall from Chapter 3 that NR experiments with Nef utilized a monolayer comprised of 1, 2-dipalmitoyl-sn-glycero-3-phospho-1-rac-glycerol (DPPG), a negatively charged lipid with two aliphatic carbon tails. The composition used for preliminary liposomes experiments was a 3:2 mixture of POPC: POPG, a mixture of neutral and negatively charged lipids with a single aliphatic carbon tail. Considering the influence of charge and curvature of Nef conformation \[22\], the discrepancies between the preliminary liposome and NR results are perhaps attributed to the different lipid mimetics used for each protocol.
6.2.b Optimization of Labeling pH

Historically, all HDX MS Nef labeling experiments were performed at pH 8.3 \(^{16,17}\). Recall from Chapter 2, all Nef purification buffers as well as long-term storage buffers were adjusted for pH 8.3. Furthermore, biochemical assays for Nef characterization, such as a Z-lyte Hck activation assay \(^{23}\), have been run at pH 8.3 to probe for Nef function and ensure that the protein is folded correctly. All Nef HDX MS trough experiments were initially analyzed at pH 8.3 as well.

While labeling at a pH of 8.3 was amenable for solution labeling experiments, the data obtained using the Trough labeling format was not consistent and difficult to explain (Figure 6.6). The deuterium incorporation into myrNef at 20 and 35 mN/m showed little difference for the majority of identified peptides. This is inconsistent with the previous NR model for membrane-associated myrNef \(^{7}\). Further inconsistencies arose when comparing the solution and membrane associated states for the N-terminal peptides. Considering that the myristoylated N-terminal peptides have been shown to be inserted in the lipid tails of the membrane \(^{7}\), a difference in deuterium uptake is expected for this region. It was theorized that the inability to resolve any significant differences in deuterium incorporation using the Trough labeling format was because myrNef was simply being labeled too rapidly at pH 8.3 and much of the deuterium incorporation into Nef may have already occurred before the first time point. Coupling Nef intrinsic lack of structure with the additional sample handling steps required for Trough labeling experiments required a retardation of the labeling process. Lowering the labeling pH by one unit can delay the exchange rate approximately 10 fold for an exchanged competent amide hydrogen on a unstructured peptide \(^{24}\). Taking this into consideration, it was decided to drop the labeling buffer pH for all myrNef experiments to 6.0 to expand the labeling time window in order to
Figure 6.6. Deuterium incorporation plots of preliminary trough labeling data for myrNef comparing the incorporation with a Langmuir monolayer at 20 mN/m (red), a monolayer at 35 mN/m (green) and a conventional solution labeling format (blue) at pD 8.3.
capture information on conformation rearrangements that may be labeled too quickly at pH 7 or 8.3 \cite{25, 26}.

However, a lower equilibrating and labeling buffer pH presented a new question: would dropping the pH alter Nef function and therefore conformation? To address this question, a Z-lyte Hck activation assay was utilized to determine Nef function at a range of pHs. In this assay, Hck activation is dependent upon Nef and reports directly Nef function \textit{in vitro} \cite{23}. High Hck activation, reported by percent phosphorylation, is indicative of high Nef function. Through this assay it was determined that Nef retains function at pH 6.0 and 7.3 (Figure 6.7). The observed Hck activation by Nef validates that labeling at pH 6.0 is a viable labeling condition \cite{27}.

\textbf{6.2.c Method for Solution Hydrogen Exchange of myrNef using Langmuir Monolayers}

Labeling experiments involving the Langmuir trough were performed as described in Chapter 5. For solution hydrogen exchange, experiments were carried out at 22 °C by diluting myrNef stock solutions in equilibration buffer (50 mM citric acid, 50 mM sodium phosphate, 150 mM sodium chloride, pH 6.0, H$_2$O) 15-fold with labeling buffer (50 mM citric acid, 50 mM sodium phosphate, 150 mM sodium chloride, pD 6.0, 99.8% D$_2$O). Both Nef buffers contained 1 mM dithiothreitol. Following dilution into D$_2$O, samples were continuously labeled for predetermined times ranging from 10 seconds to 1 hour before being quenched to pH 2.6 using a 0 °C solution of quench buffer (0.8% formic acid and 0.8M guanidine hydrochloride in H$_2$O). Quenched samples were digested on ice for 5 minutes by adding pepsin and aspergillopepsin (60 µg and 70 µg, respectively, dissolved in water, pH 4.5). Digested samples were injected into a Waters (Milford, MA) nanoAcquity UPLC with HX technology \cite{28} for desalting, separation.
Figure 6.7. Nef retains function at both pH 6.0 and pH 7.3. The activity of Hck-YEEI was measured in the Z’Lyte in vitro kinase assay as a reporter for Nef functionality, as described in Emert-Sedlak et al. (2009). (a) Hck activation at pH 6.0. Hck-YEEI was titrated into the assay alone or with a ten-fold molar excess of Nef (myr or non-myr). Maximum activation was realized at 64 ng/well with both constructs. (b). The same assay was repeated at pH 7.3 to mimic the conditions used for some previous solution HDX experiments. Maximum Hck-YEEI activation was also realized at 64 ng/well for both constructs. These data were obtained by Prof. Lori Emert-Sedlak. From Pirrone, Emert-Sedlak et al. 2015.
Deuterium incorporation was measured using a Waters (Milford, MA) Synapt G2 mass spectrometer instrument.

6.3 Effects of Monolayer Association on Nef Conformation

6.3.a Peptides followed for Hydrogen Exchange of myrNef

Peptide separation, mass analysis and data processing were performed as described in Chapter 5. In this work, digestion produced in 31 peptides resulting in a 90.2% sequence coverage (Figure 6.8). The peptides from myrNef identified by MS\textsuperscript{E} fragmentation and followed for the entire labeling time course are shown in Figure 6.9 and additional information on each peptide is provided in Table 6.1. Overlapping peptides were identified in nearly all areas of the protein, and similar trends were observed in areas of redundancy (Figure 6.8, 6.9). Deuterium incorporation was recorded in at least triplicate biological replicates\textsuperscript{[29]} with a fresh monolayer spread for every labeling data point. As all experiments were performed under very similar experimental conditions, all deuterium levels were reported as relative and there were no corrections for back exchange\textsuperscript{[30]}.

6.3.b Comparison of solution and 20 mN/m monolayers

Recall from Chapter 5 that the reproducibility and quality of the data obtained from the Trough labeling format is comparable to that obtained from more conventional solution phase experiments. Examples of the raw data acquired for myrNef using the Langmuir monolayer labeling format are shown for two regions of the protein in Figures 6.10 and 6.11. All mass spectra are shown in Figure 6.12.
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<td>4.25</td>
</tr>
<tr>
<td>185</td>
<td>192</td>
<td>8</td>
<td>VWRFDSDLK</td>
<td>7</td>
<td>2</td>
<td>525.79</td>
<td>5.35</td>
</tr>
<tr>
<td>186</td>
<td>192</td>
<td>7</td>
<td>WRFDSKL</td>
<td>6</td>
<td>2</td>
<td>476.26</td>
<td>5.34</td>
</tr>
<tr>
<td>200</td>
<td>205</td>
<td>6</td>
<td>ELHPEY</td>
<td>4</td>
<td>2</td>
<td>394.19</td>
<td>3.52</td>
</tr>
</tbody>
</table>

Table 6.1. Peptic peptides from myristoylated Nef. Each peptide is labeled with the sequence, length, retention time in minutes, charge (z), mass to charge ratio (m/z), and the maximum allowable deuterium label. Myristoylated peptides are shown in red.
Figure 6.8. Coverage map of identified peptides (blue bars) from myrNef generated using a mixture of pepsin and aspergillopepsin, and followed during HDX MS experiments. Reproducible peptides were found at least three times in quadruplicate runs. The sequence coverage and redundancy are indicated below the map. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.9. Deuterium uptake plots for myristoylated Nef comparing the incorporation with a Langmuir monolayer at 20 mN/m (red), a Langmuir monolayer at 35 mN/m (green) and a conventional solution labeling format (blue) for pD 6.0. Error bar represent the spread of triplicate measurements. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.10. Deuterium incorporation in MyrNef. Mass spectra for residues 1-14 (A) labeled in solution (black), labeled with a monolayer at 20 mN/m (red) and with a monolayer at 35 mN/m (blue). (B) Peptide 1-14 is shown in a model of myrNef and the corresponding deuterium incorporation plot for this peptide is shown. Error bars represent the spread of triplicate measurements. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.11. Deuterium incorporation in MyrNef. Mass spectra for residues 159-173 (A) labeled in solution (black), labeled with a monolayer at 20 mN/m (red) and with a monolayer at 35 mN/m (blue). (B) Peptide 159-173 is shown in a model of myrNef and the corresponding deuterium incorporation plot for this peptide is shown. Error bars represent the spread of triplicate measurements. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.12. Raw spectra of Nef peptides with and without monolayers. The labeling times are listed to the left. There are two 60 minute timepoints shown for 35 mN/m. The lipid packing density, peptide residues and charge states are listed above.
Figure 6.12 cont.
Figure 6.12 cont.
69-84, no lipid, +3

Und
10s
1 min
10 min
60 min

69-84, 20 mN/m, +3

Und
10s
1 min
10 min
60 min

69-84, 35 mN/m, +3

Und
10s
1 min
10 min
60 min

69-88, no lipid, +4

Und
10s
1 min
10 min
60 min

69-88, 20 mN/m, +4

Und
10s
1 min
10 min
60 min

69-88, 35 mN/m, +4

Und
10s
1 min
10 min
60 min

Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
Figure 6.12 cont.
186-192, no lipid, +2

186-192, 20 mN/m, +2

186-192, 35 mN/m, +2

200-205, no lipid, +1

200-205, 20 mN/m, +1

200-205, 35 mN/m, +1

Figure 6.12 cont.
Figure 6.12 cont.
For the case of association with membranes at a pressure of 20 mN/m, HDX in myrNef increased in some regions and decreased in others relative to that measured for myrNef in solution, as shown by the deuterium incorporation curves in Figure 6.9. HDX data represented as relative percent exchange into Nef for the monolayer free state is shown in Figure 6.13 and is shown for the monolayer bound state in Figure 6.14. The differences between both states was calculated (see Chapter 4 for details) and plotted in Figures 6.15 and 6.16.

Increased deuterium incorporation upon lipid association was observed principally in the core domain and some parts of the C-terminal region (Figure 6.15, Figure 6.16). In solution, the core of myrNef did not incorporate much deuterium, exchanging no more than 30% after one hour of labeling (Figure 6.13). This observation is consistent with prior HDX MS studies on myrNef \(^{16, 17}\). Association with the monolayer resulted in increased deuterium uptake in several regions in the Nef core. Peptide 89-100 showed an increase of ~1.0 Daltons (Figure 6.16, panels A & B) and peptide 116-142 showed an increase exceeding 2.5 Daltons at 10 seconds (Figure 6.16, panel A). In solution these residues are less exposed and may bear resemblance to the “non-signaling conformation” of Nef that has been previously theorized (Arold and Baur 2001). Decreased deuterium incorporation indicates that these regions may be occluded from solvent exchange by the disordered loops that are present on Nef. Regardless, peptides in the core region appear to have similar dynamics in the solution and monolayer-bound conformation (Figure 6.16, Panels A & B). As an example, the lines for the deuterium incorporation plot for peptide 89-100 have similar slopes, indicating that the dynamics has not been altered by lipid binding.

Both the N-terminal myristoylated region and the C-terminal disordered loop of myrNef showed significant reduction in deuterium incorporation when the protein was associated with the Langmuir monolayer at 20 mN/m (Figure 6.16, Panel A). This protection from labeling
suggests contact with and burial into the membrane, structural creation and stabilization, or a combination of both. Protection from exchange in the first ~20 residues is consistent with the hypothesis that both the myristic acid and residues from the N-terminus including a positively charged region between residues 17-22 \[15, 22, 31\] insert into the membrane. In solution, residues next to the lipid binding region (peptide 35-51 and 35-58) were deuterated rapidly at ten seconds (Figures 6.16), suggesting high solvent accessibility or a lack of structure, but upon lipid association displayed 2-3 Dalton reductions in deuterium incorporation at ten seconds (Figure 6.16). An α-helical sequence (Figure 6.16, Panel C) is located within that span of residues, and it is speculated that this helical region becomes stabilized when the arm separates from the core upon membrane binding. Interestingly, this region remains dynamic when associated with lipids, as the difference in relative deuterium uptake is reduced at later time points (Figure 6.16 panels A&B). This suggests that while this region is more protected from exchange from the formation of an α-helix, this helix remains dynamic after membrane association. Reduced deuteration of the C-terminal disordered loop of membrane-associated myrNef (e.g., residues 147-173) suggests that some backbone amide hydrogens in the C-terminal loop become protected, perhaps by hydrogen bonding with the core of Nef, or become more stabilized (Figure 6.15, 6.16). However, the C-terminal disordered loop remains highly dynamic as many observed difference between monolayer-associated and solution states at early time points have been reduced later in the time course (Figure 6.16).

Recall from Chapter 1, that Nef adopts a “signaling conformation” upon membrane binding, where the C-terminal loop wraps around the core domain \[15\]. It was predicted that the interactions with the core domain would avoid rapid endocytosis and removal from the membrane while other events such as such as phosphorylation or binding to another protein
Figure 6.13. Relative deuterium uptake plotted on a model of myrNef in the absence of lipids. The labeling time is noted above and the relative percent deuterium is colored according to the legend shown. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.14. Relative deuterium uptake plotted on a model of myrNef with a Langmuir monolayer at 20 mN/m. The labeling time is noted above and the relative percent deuterium is colored according to the legend shown. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.15. The effect of lipid association on myrNef deuteration. The average deuterium incorporation for myrNef in solution was subtracted from the average deuterium incorporation for myrNef with a monolayer at 20 mN/m. The labeling time is noted above and the relative percent deuterium is colored according to the legend shown. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.16. Effects of monolayer association on Nef. A) Difference map comparing the deuterium uptake of myrNef in the trough (monolayer associated) and in solution (no monolayer). Average deuterium levels from solution labeling were subtracted from the average deuterium levels of the monolayer (20 mN/m) associated state. Positive values (exposure) are shown in red and negative (protection) values are shown in blue. B) Deuterium incorporation plots for peptides (i) 1-14, (ii) 6-19, (iii) 89-100 and (iv) 147-173 for monolayer associated (–●–) and solution (−Δ−) labeling. Error bars represent the spread of triplicate measurements. C) Structural location in MyrNef of peptides shown and color coded (red, magenta, blue, orange) in panel B. From Pirrone, Emert-Sedlak et al. 2015.
could trigger the loop to become exposed (Arold and Baur 2001). The observed protection of the C-terminal loop in the HDX MS data agree with this early prediction. Furthermore, a recent structure of HIV-1 Nef in complex with the AP-2 clatherin adaptor complex was reported that contained the loop region \[32\]. In that structure two helical regions within the loop are apparent, a helix from residues 150-157 (\(\alpha H4\)) and another single turn helix from 167-170 (\(\alpha H5\)). \(\alpha H5\), along with a series of turns at the C-terminal end of the loop (residues 171-179) are located between AP-2 \(\alpha-\sigma2\) and the Nef core, with \(\alpha H5\) packing against the \(\beta\)-sheet of the core and the turn-rich section of the loop from 171-179 anchored by a hydrogen bond between Asp174 and Glu104 and by a salt bridge between Asp175 of the loop and Nef Arg134 of the core \(\beta\)-sheet. It was concluded that these interactions between loop and core play an important role in organizing this region of the loop. The HDX MS for myrNef from this work suggest that these interactions between loop and core occur upon membrane binding when the N-terminal arm separates from the core.

6.3.c Effects of lipid packing density on conformation

Recall from Chapter 5, that the Langmuir trough system, the pressure of the monolayer is measured continuously and can be controlled by manipulating the barrier position. HDX can therefore be done at various pressures that can be generated very reproducibly. This strategy was used to measure HDX in myrNef when associated with a DPPG monolayer with a pressure of 20 mN/m (as above) and compared to a monolayer with a pressure of 35 mN/m. As described in Chapter 3, at this higher lipid packing densities, the arm of myrNef is unable to insert into the monolayer and myrNef remains in a closed conformation directly against the lipid head groups, as shown by neutron reflection \(^7\). A model of myrNef conformation at a membrane with the
lower and higher lipid packing density, based on the neutron reflection data, is shown in Figure 6.17. Insertion into the monolayer is evidenced by the movement or lack thereof for the barrier in the trough (Figure 6.18). Recall from Chapter 3 that at higher lipid packing density residue insertion at the membrane was prohibited and this resulted in an closed and compact conformation of Nef, where the core of the protein was located approximately 50Å from the lipid monolayer. While the NR data was able to resolve the gross conformation of Nef at highly packed lipid monolayers, the specifics on dynamics were more captured. Following development and validation of the novel HDX MS labeling method, the conformation and dynamics of Nef could be probed further. Association with highly packed lipid monolayers greatly altered deuterium uptake of myrNef; deuterium incorporation was significantly higher throughout the protein at 20 mN/m vs 35 mN/m (Figures 6.19, 6.20). Despite the lack of residue insertion, the deuterium incorporation of myrNef at the 35 mN/m monolayer was not identical to that of myrNef in solution (data shown in Figure 6.9). There was less deuterium in residues 1-83 and 146-184 of myrNef associated with the 35 mN/m monolayer compared with myrNef in solution, implying stabilization of these parts of the structure, protection by the membrane, conformational rearrangements, or all three (Figure 6.20). The deuteration levels of residues 84-145 and 185-210 were largely the same at the two membrane pressures (Figure 6.19). MyrNef with the 20 mN/m membrane had deuterium levels in 1-83 and 146-184 that were intermediate between the 35 mN/m monolayer and solution HDX measurements. These results suggest that when myrNef associates with the membrane at higher pressures (35 mN/m) through interaction of the positively-charged residues (17-22) with the negatively-charged head groups, the arm partially dissociates from the core allowing the C-terminal loop to associate with the core. However, the membrane pressure is too high for residues in the N-terminal arm to insert, myrNef remains in a
Figure 6.17. Cartoon model of the myristoylated Nef structure upon association with (i) a low lipid packing density and (ii) a high lipid packing density membrane. The black arrows represent the distance between the Nef core and the lipid headgroups. The myristate is highlighted in green. This model is based upon the data in Akgun et al. 2013.
Figure 6.18. Change in surface pressure (black) and barrier position (red) in the trough during myr-Nef at low lipid packing (A) and at high lipid packing monolayers (B). For low lipid packing experiments, the surface pressure was held constant after addition of protein. The barrier gradually moved back as more protein associated and inserted residues into the monolayer. The barrier was stopped within a few millimeters before reaching the end of the trough. The surface pressure rose slightly after the barrier was stopped indicating that protein was still associating with the monolayer. For high lipid packing experiments, Nef was unable to insert residues into the membrane. As a result, the barrier did not gradually move back as in the low packing experiments. In all cases, aspiration dropped the surface pressure quickly and the barrier rapidly moved forward in order to reestablish the preset surface pressure. For all plots, “I” represents when protein was injected underneath the monolayer, “E” represents the subphase exchange, and “A” represents aspiration of the sample after analysis. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.19. Effects of lipid-packing density on Nef. Difference map comparing deuterium levels in myrNef at monolayer pressures of 20 or 35 mN/m. Deuterium incorporation was determined from the average of triplicate measurements. The average deuterium incorporation for myrNef with 35 mN/m was subtracted from the average deuterium incorporation for myrNef with 20 mN/m and the value was colored according to the key. From Pirrone, Emert-Sedlak et al. 2015.
Figure 6.20. The effect of lipid packing density on myrNef deuteration plotted onto a model of myrNef. The average deuterium incorporation for myrNef with a monolayer at 35 mN/m was subtracted from the average deuterium incorporation for myrNef with a monolayer at 20 mN/m. The labeling time is noted above and the relative percent deuterium is colored according to the legend shown. From Pirrone, Emert-Sedlak et al. 2015.
closed conformation directly against the lipid layer in an orientation such that the N-terminal portion (residues 30-84) and the C-terminal loop are occluded from solvent by proximity to the membrane. Despite the compact conformation that Nef adopts with high lipid packing density monolayers, the entirety of the protein remains dynamic and the majority of the differences observed between the 20 mN/m and 35 mN/m states are gone later in the time course (Figure 6.19 and 6.20). When the monolayer pressure is lower (20 mN/m) and residues on the N-terminal arm can insert into the monolayer, the arm releases completely from the core and the conformation becomes extended (Figure 6.19). This extended conformation is theorized to expose potential binding sites for partners to interact with Nef in order to propagate its function \[9, 15\]. In that case more deuterium can be incorporated into the N-terminal region and the C-terminal loop (Figure 6.19), albeit not as much as for the solution form due to interaction of the loop with the core in the membrane bound state.

### 6.4 Conclusions

The newly developed method for analyzing conformational features of membrane associated peptides and peripheral membrane proteins combines HDX MS and Langmuir monolayers. An advantage of using the Langmuir monolayer system is that the lipid packing density can be controlled and reproduced from monolayer to monolayer. For proteins that undergo conformational changes as a result of the membrane lipid packing density, this is a very valuable feature. These results showed how packing density could be altered and result in different conformations of the myrNef protein. The HDX MS data for myrNef is consistent with the data obtained in prior NR studies \[7\]. Furthermore, these data provide the first direct evidence
that the C-terminal loop is occluded from solvent in the open form, as predicted for the “signaling” form \[15\].

One of the principle advantages on HDX MS at Langmuir monolayers with the same system that is was initially used to study Nef via NR. The HDX MS Langmuir trough system is capable of combining global structural information from other techniques such as neutron or X-ray reflection with more local information from HDX. The new HDX MS method was engineered to be integrated with a neutron reflection workflow. In such a scheme, protein association with monolayers can first be investigated with the same trough described here using neutron or X-ray reflection, the profile of the protein with respect to the membrane obtained, packing density of the monolayer modulated (if desired) and the impact on conformation monitored. Once an interesting conformation is identified by neutron or X-ray reflection (or at any point, conformation or condition such as packing density), the protein could be labeled with deuterium right at the reflectometer and the sample passed to a UPLC-MS system for HDX measurement. Future steps will be required to fully realize this goal.

6.5 References


Chapter 7

Perspectives and Future Directions

7.1 Introduction

The work outlined in this dissertation (Chapters 3, 5 & 6) represents an advance in the structural understanding of full-length, myristoylated, membrane-bound Nef. The method development and application described in this dissertation were done towards this single objective. Using the novel HDX MS Langmuir system (also referred to as a Langmuir trough), many future experiments that will expand upon this knowledge and understanding of other membrane-bound proteins are possible.

As discussed in Chapter 3, one conclusion of this work was that lipid packing density greatly influences the global conformation of HIV-1 Nef\(^1\). This was the first experimental evidence of a structural rearrangement in membrane-bound Nef. Using neutron reflection (NR) and Langmuir monolayers, the position of the Nef core relative to the lipid membrane was measured. Loosely packed monolayers allowed for portions of the N-terminal arm inserted into the lipids of the monolayer. This insertion was characterized by the formation of an amphipathic N-terminal helix, insertion of hydrophobic residues and occlusion of the myristate into the monolayer. Following membrane association and insertion, Nef adopted an “open” conformation, where the Nef core was displaced approximately 100 Å for the lipid headgroups. Insertion of the myristate and hydrophobic residues in there N-terminal arm was prevented when the lipid packing density of the monolayer is increased. In this situation, Nef adopts a “closed”
conformation the Nef core was displaced approximately 50 Å for the lipid headgroups. In this conformation, portions of the N-terminal arm and the C-terminal disordered loop interacted with the core. Recall that it has been proposed that Nef shifts between conformations in order to interact with host cell protein after HIV infection\(^2\). Insertion of the N-terminal arm and the displacement of the Nef core, would expose potential binding sites and facilitate interactions with host cell proteins. However, if Nef occupies the “closed” conformation, the N-terminal arm and the portions of the C-terminal loop interact with the core and obscuring potential binding sites and possible interactions with host cell proteins. These NR data support this hypothesis.

While the NR experiments advanced our comprehension of Nef structure, this technique provided a global conformation and was unable to resolve finer details on local dynamics and conformation of the membrane bound state. For this reason, a novel method was developed to resolve these details (Chapter 5). The same Langmuir trough system that was utilized for the NR experiments was outfitted to incorporate HDX MS. This involved plumbing to enable the rapid exchange of buffer and a system for harvesting the lipid-associated samples. These modifications necessitated additional sample handling steps to the labeling protocol, and several control experiments were performed to optimize sample and deuterium recovery. A mix of leucine-enkephalin, bradykinin and angiotensin I showed that there is no significant loss of deuterium when samples are analyzed using the HDX MS Langmuir system when compared to a more conventional solution labeling format. Furthermore, the spread of the data collected from the Langmuir trough was comparable to those from conventional solution labeling formats. The amphipathic peptide melittin was used to assess if the HDX Langmuir system could monitor membrane induced conformational changes. Melittin has been well characterized in lipid-binding experiments\(^3-5\) and served as an ideal control for this system. Deuterium incorporation
of melittin was compared following labeling in the absence of lipids and labeling in the trough. Deuterium incorporation for melittin labeled in the trough was found to be significantly lower. Taken together with evidence of melittin insertion into the monolayer (Chapter 5), these data indicated that the HDX MS Langmuir trough system was able to monitor expected conformational changes within a control peptide \cite{6}.

To further validate this method, the system had to monitor expected membrane-induced conformational changes in a protein. The myristoylated protein Arf-1 was elected as a control for this system as its lipid-associated state has been characterized previously \cite{7, 8}. Arf-1 was labeled in a similar fashion to melittin, and the lipid-bound and lipid-free states were compared. In the absence of a monolayer, residues 1-21 incorporated significantly more deuterium, indicating a lack of structure. However, when a monolayer was available, there was a nearly 5 Dalton reduction in deuterium uptake. This signifies significant protection from the formation of secondary structure and interaction with the lipids. Select areas towards the C-terminus of the protein (see Chapter 5) incorporated more deuterium in the monolayer-associated state. The observed results from labeling in the trough agreed with the published data on the membrane-bound structure of Arf-1, and served as further validation that this system could be used to probe the conformation and dynamics of membrane proteins.

By developing and optimizing a new method for studying membrane proteins, the structural details of full-length, membrane-associated Nef could be probed (Chapter 6). Myristoylated Nef was labeled both in the HDX MS Langmuir trough system and in solution, in absence of any lipids \cite{9}. Furthermore, Nef labeling experiments in the trough were performed using loose (20 mN/m) and a tight (35 mN/m) lipid packing densities. For loosely packed monolayers, deuterium incorporation increased for regions of the Nef core and decreased in the
N-terminal arm and C-terminal disordered loop when compared to the solution labeling format. Decreased deuterium uptake into the N-terminal arm and C-terminal loop indicates protection or stabilization from contact with the lipids or the formation of secondary structure. These data taken together with the increased deuterium incorporation observed in the Nef core support the “open” conformation first reported using NR. Deuterium uptake into myristoylated Nef was also greater when associated with loosely packed instead of tightly packed monolayers. This increase in labeling was noted throughout the entirety of Nef and supports the “closed” conformation of Nef that was first reported using NR (Chapter 4). The finer conformational details resolved with this method expand upon the global conformational information previously obtain through NR. Furthermore, this method can be applied to any peripheral membrane protein to simultaneously capture a global conformation using NR and determine local dynamics of a membrane-bound protein, all within the same experiment.

7.2 Future Directions

Recall from Chapter 1, that several membrane proteins are challenging to study with traditional structural elucidation techniques, such as X-ray Crystallography and Nuclear Magnetic Resonance. The work described in this dissertation has addressed the challenge of studying membrane proteins and has laid the foundation for a method that can be used to probe the membrane-bound conformation of proteins that are not amenable to X-ray Crystallography and Nuclear Magnetic Resonance experiments. The novel HDX MS Langmuir method[^10] was originally conceived to study the membrane-bound conformation of HIV-1 Nef[^11], and was the first HDX MS study on full-length, membrane-bound Nef altering conformation following lipid
association. In similar fashion to Nef, the HDX MS Langmuir method can be applied to any peripheral membrane or membrane-anchored protein, especially proteins that have little known structural information. While, the HDX MS Langmuir system was developed in parallel with neutron reflection for the study of membrane proteins, other orthogonal techniques can be integrated into this system to expand the information obtained for a protein.

7.2.a Altering Lipid Monolayer Environment

One of the major advantages of using Langmuir monolayers is the control over the lipid packing density. It was shown that lipid packing density can greatly influence the overall conformation of membrane-bound Nef (Chapter 3). Lipid packing density could also influence the conformation of other membrane-anchored proteins and using the HDX MS Langmuir system would be a method to study this conformational transition. A second major advantage of using Langmuir monolayers is the ability to alter the lipid composition of the membrane. Recall from Chapter 6 that Nef appeared to behave differently when bound to monolayers instead of liposomes. While the labeling pH was different in each of these cases, this does not explain all of the inconsistencies. It is possible that the difference between the compositions of each lipid mimic influenced the conformation of membrane-bound Nef. Altering the makeup of the monolayers used in the HDX MS Langmuir monolayer method could be employed as a procedure to study the effects of lipid composition on other proteins. For example, phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) represent less than 1% of membrane phospholipids, yet they function in a remarkable number of crucial cellular processes.[12] Generating monolayers with varying amounts of PIP2
and PIP3 could be applied to study proteins that have been shown to interact with PIP2/3, such as Btk tyrosine kinases or Arf-6, in their membrane-bound states. Furthermore, adding cholesterol or increasing the diversity of the lipids used in each monolayer or transitioning to supported bilayers \[13\] can be used to study membrane-anchored proteins in a more “native-like” environment.

7.2.b Probing Conformational Changes and Partner Binding with Fluorescence

Recall from Chapter 3, stable isotopically labeled Hck SH3 was produced for NR studies. The intent was to observe SH3 binding to membrane-bound Nef. SH3 binding would have increased the nuclear density around the Nef core, where the PxxP motif is located, and would be increased the spallation of neutrons bombarding the membrane anchored complex. While this phenomenon was observed once, attempts to replicate SH3 binding were unsuccessful. Given the modular nature of the HDX MS Langmuir system, it would be possible to incorporate a separate orthogonal technique to observe SH3 binding. For example, Bimolecular Fluorescence Complementation (BIF-C) could be used to rapidly detect protein-protein interactions of two membrane bound species \[14\]. Example applications of this technique include observing interactions with other binding partners for p53 \[15\], the transcription factor Hoxa1 \[16\] and Arf-1 \[17\]. BIF-C could be combined with the HDX MS Langmuir system to observe and confirm protein-protein interactions between binding partners. Following protein binding, NR could be applied to determine an overall shape profile of the complex, and HDX MS could be used to probe for granular details on the alteration of dynamics upon protein: protein binding.
Employing BIF-C would also be useful to probe for protein oligomerization. For example, BIF-C has been employed to observe dimerization of Nef in vivo \cite{18}. Recall from Chapter 6, that the HDX MS data acquired for Nef did not suggest any evidence of dimerization for WT Nef or for a dimerization deficient mutant, D123A \cite{11}. Furthermore, prior HDX MS solution data has not provided any evidence of dimerization for nonmyristoylated Nef \cite{19} or myristoylated Nef \cite{20}. Nef dimerization has been shown to be important for the internalization of extracellular receptors \cite{21}, and crystal structures of Nef core dimers in complex with SH3 and SH2-SH3 domains of Src family kinases have been published \cite{22, 23}. The differences between these structures suggest that host protein interactions influence dimerization and the HDX MS data support this. However, the addition of BIF-C to study Nef or any other membrane anchored protein would expand upon the HDX MS Langmuir method for the analysis of protein oligomerization.

7.2.c Quantifying Penetration into Membranes

While the work outlined in this dissertation focused on studying the conformation of membrane-bound proteins, there are still details of the membrane interaction that remain elusive. The data presented in Chapters 5 and 6 revealed regions of protection and exposure, but the distance traveled by these residues inserting into the membrane was never quantified. However, these granular details can be resolved with an additional technique known as Electron Paramagnetic Resonance (EPR). EPR is a spectroscopic technique that detects unpaired electrons, such as free radicals \cite{6}. Unpaired electrons are sensitive to local environments and small changes within that environment can be detected in the resulting EPR spectra. EPR has
been used before to probe the depth of lipid-binding residues that are inserted into a membrane and this technique could be used with Nef to study the interactions of the N-terminal arm with a lipid membrane.

Preliminary data towards this goal was acquired in collaboration with Dr. Likai Song from Florida State University. These data were acquired for a synthetic myristoylated peptide consisting of the first 15 residues of the Nef N-terminal arm. A cysteine at position 10 had been coupled to a spin-label for the EPR analysis. Differences in the collected spectra for the peptide in the presence and absence of POPC/POPG liposomes indicated a change in hydrophobicity of the spin-labeled cysteine (Figure 7.1, Panel A). The immersion depth of the spin-labeled resides into the liposomes was determined to be approximately 10 Å (Figure 7.1, Panel B), signifying a depth roughly the length of 6.5 carbon-carbon bonds.

While these data were only acquired for a portion of the N-terminal arm, it is possible to acquire EPR data for full-length myristoylated Nef. Recall from Chapter 1 that the Nef allelic variants used for this work have four cysteines in their sequence. This presents an issue for site-direct spin labeling as the spin-label may not be localized to the N-terminal arm. To overcome this issue, all four must be mutated into serines (termed C4S Nef). Following that a second construct where only the serine at position 10 is mutated back into a cysteine (termed S10C Nef) and spin labeled. Site-directed mutagenesis had been attempted to produce these Nef constructs; however, the resulting proteins had a lysine to arginine misincorporation and were not useful for any further experiments. Future work to optimize mutagenesis and analyze the C4S and S10C constructs with HDX MS, would reveal any conformation changes induced by the mutagenesis. Furthermore, purified S10C Nef could be spin-labeled and used to probe the immersion depth of
Figure 7.1. Preliminary EPR results of myr-Nef peptide. The peptide (shown above) consists of the first 15 residues of the N-terminal arm. Cysteine 10 (red) was spin-labeled. A) EPR spectra of the peptide with and without liposomes. B) Immersion depth of spin-labeled cysteine into lipid membrane.
the N-terminal arm when associated with liposomes and expand the information of the
membrane-bound Nef conformation.

7.3 Bringing the Langmuir Trough system to a Neutron Source

Recall from Chapter 1 that one of the principal objectives of this project was to
completely integrate HDX MS and NR into the same Langmuir monolayer system. While the
two orthogonal techniques of this system were optimized and utilized for characterizing
membrane-associated proteins, the experiments were done at different times and the data were
collected in different locations. In order to acquire both NR and HDX data in the same
experiment, the Langmuir trough system and a mass spectrometer equipped for HDX MS
analysis must be brought to a neutron source.

This goal presents significant cost and logistic challenges. First, shipping the instruments
to the beam is a complicated process. The Langmuir trough, the UPLC system and the mass
spectrometer are large, heavy and delicate instruments that would need to be shipped by ground
transportation. This could cost tens of thousands of dollars and take 4-5 days to travel. Second,
these instruments require time to be setup and tested. The mass spectrometer would be vented
before shipping and as a result would have to pump down to a low vacuum before use.
Generally, this process is done overnight. While the mass spectrometer is returning to a low
vacuum, the UPLC pumps and the HDX cold box would need to be connected, reintroduced to
the mobile phases and allowed to pump solvent overnight. The following day the instrument
would have to be tuned and calibrated using a peptide standard before acquiring any spectra.
The Langmuir trough would have to be set-up and calibrated. The calibration is crucial for
accurately measuring the lipid packing density of the monolayer. The computer controlling all of these instruments would be connected and used for data processing and analysis. All of the setup can likely be achieved in ~24 hours barring any setbacks, and only after this could any experimental data be collected. This initial time investment is significant when considering the limited amount of time researchers are afforded at a national laboratory. All of these challenges can be overcome and would be the first reported instance of using both techniques in the same system, but it would require a significant cost and time investment.

7.4 References


Appendix I

Publication

Conformational Transition of Membrane-Associated Terminally Acylated HIV-1 Nef

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SUMMARY

Many proteins are posttranslationally modified by acylation targeting them to lipid membranes. While methods such as X-ray crystallography and nuclear magnetic resonance are available to determine the structure of folded proteins in solution, the precise position of folded domains relative to a membrane remains largely unknown. We used neutron and X-ray reflection methods to measure the displacement of the core domain of HIV Nef from lipid membranes upon insertion of the N-terminal myristate group. Nef is one of several HIV-1 accessory proteins and an essential factor in AIDS progression. Upon insertion of the myristate and residues from the N-terminal arm, Nef transitions from a closed-to-open conformation that positions the core domain 70 Å from the lipid headgroups. This work rules out previous speculation that the Nef core remains closely associated with the membrane to optimize interactions with the cytoplasmic domain of MHC-1.

INTRODUCTION

There is abundant evidence that enzyme activity or protein-protein interactions can depend upon association with lipid membranes. The positioning of proteins and protein motifs relative to the membrane or to other membrane-bound proteins is, for some proteins, critical and may depend on conformational changes induced upon membrane association (Kim et al., 2009; Osterhout et al., 2003; Schlessinger, 2000; Subramanian et al., 2006; Xue et al., 2004; Zha et al., 2000). Lipid modification serves to target many proteins to specific membranes or submembrane locations. Hundreds of proteins are modified with covalently bound lipid groups, the most common of which are fatty acids, isoprenoids, and glycosylphosphatidylinositol anchors (Farazi et al., 2001; Jeromin et al., 2004; Perinpanayagam et al., 2013; Resh, 2006; Steinhauser and Treisman, 2009). Many of these proteins are involved in signaling and require membrane association to signal efficiently. In addition to intracellular membrane location, the structure adopted by proteins at membranes is also critical for certain functions. As an example, myristoyl or farnesyl switch mechanisms are known for more than a dozen proteins, with Arf GTPase being a hallmark example (Goldberg, 1998; Resh, 2006). These mechanisms cause proteins to switch between conformational states in which the myristoyl or farnesyl moiety is either sequestered or exposed and can promote membrane binding (Ames et al., 1996; Goldberg, 1998), facilitate release from the membrane-bound state (Ames et al., 1996; Goldberg, 1998; Hantschel et al., 2003; Matsubara et al., 2004; McLaughlin and Aderem, 1995; Resh, 2006), and regulate protein-protein interactions (Hantschel et al., 2003; Matsubara et al., 2004).

Despite the obvious importance of acylated proteins in biology (e.g., kinases and phosphatases [Kim et al., 2009; Resh, 2006; Schlessinger, 2000], G proteins [Resh, 2006], GPCRs [Resh, 2006], morphogens [Steinhauser and Treisman, 2009], neuronal calcium sensors [Jeromin et al., 2004], pro- and anti-apoptotic proteins [Perinpanayagam et al., 2013]), standard approaches for studying their structure at membranes are neither adequate nor appropriate. In addition, understanding signaling mechanisms involving these proteins at the molecular level and developing pharmaceutical interventions have been limited by the absence of structural detail for these proteins in the membrane-bound state. Many structural studies of membrane-associated proteins have consisted of crystallization of soluble proteins with and without bound ligand or in complex with other proteins but in the absence of a membrane (Ames et al., 1996; Flaherty et al., 1993; Goldberg, 1998; Matsubara et al., 2004). Nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), fluorescence resonance energy transfer (FRET), Fourier transform infrared-attenuated total reflection spectroscopy (FTIR-ATR), and other methods can be applied to provide some structural details for proteins associated with membranes or membrane mimics, but do not give the full residue distribution with respect to the membrane. A case in which it is essential to define the structural details of a protein at the membrane is the Nef protein from HIV-1. Nef is one of several HIV-1 accessory proteins and is essential for AIDS progression (Baur, 2004; Das and Jameel, 2005). Nef is expressed in high concentrations shortly after viral infection (Klotman et al., 1991) and is required for achieving and maintaining high viral loads in vivo (Goldsmith et al., 1995). Nef lacks catalytic activity but instead realizes its functions by interacting with host proteins—more than 30 proteins that interact with Nef have...
been identified (Arold and Baur, 2001; Baur, 2004; Renkema and Sakse, 2000). Nef exists in both membrane-associated and cytosolic fractions (Coates et al., 1997) and shuttling may occur between the cytosolic fractions and the membrane-associated form (Figure 1A; Bentham et al., 2006). Membrane association is achieved by an N-terminal myristoylation essential for the virus in vivo (Harris, 1995) as well as a cluster of basic residues (17–22) within the N-terminal arm (Figure 1B; Bentham et al., 2006; Curtain et al., 1998; Gerlach et al., 2010). The myristoylation motif (residues 2–7) is essential and highly conserved in Nef alleles from both laboratory HIV-1 strains and in primary isolates from AIDS patients (Geyer et al., 1999). Deletion of the myristate group from Nef dramatically reduces infectivity (Goldsmith et al., 1995), cripples downregulation of CD4 and MHC-I (Goldsmith et al., 1995; Peng and Robert-Guroff, 2001), and prevents formation of an AIDS-like disease in mice transfected with Nef (Hanna et al., 2004). Both the myr group and the basic cluster are required for Nef virion incorporation (Welker et al., 1998). Nef residues 5–22 form an amphipathic helix with hydrophobic residues Trp5, Trp13, Ile16, and Met20 located on one side of the helix. Gerlach and colleagues reported significantly decreased binding affinity to lipid membranes and impaired helix formation upon mutation of Trp5 and Trp 13 (Gerlach et al., 2010).

It has been postulated that Nef undergoes a transition from a solution conformation to a membrane-associated conformation (Figure 1A), and this conformational rearrangement enables membrane-associated Nef to interact with host proteins (Arold and Baur, 2001; Geyer and Peterlin, 2001; Jia et al., 2012; Raney et al., 2007). In particular, it has been suggested that insertion of the N-terminal arm and subsequent displacement of the core domain from the lipid membrane will expose binding sites on the core, facilitating interaction with host proteins (Arold and Baur, 2001). On the other hand, based on the crystal structure of Nef with the cytoplasmic tail of MHC-I, others have suggested that association of Trp13 and Met20 on the N-terminal arm with the core domain persists upon membrane binding, and that this positions the Nef core close to the membrane for optimal interaction with the cytoplasmic domain of the MHC-I receptor (Jia et al., 2012). Others have proposed that association of the core domain of Nef with negatively charged membranes through its basic surface (Figure 1B) orients Nef to provide optimal exposure of the dileucine sorting motif in the flexible loop (residues 152–184) that mediates interactions with adaptor protein complexes (Horenkamp et al., 2011). Nef is known to upregulate several but not all Src family kinases through interaction with their SH3 domains (Narute and Smithgall, 2012), critical to many downstream functions. These kinases are also bound to the membrane through N-terminal acylation, and positioning of the Nef core domain relative to the SH3 domains may play a role in the varying binding affinities. Despite the vital importance to the pathogenicity of Nef, there is no information regarding the position of the core of Nef upon membrane association due to the limitations of current structural methods.

FRET has been used to detect membrane binding and insertion of Nef by Gerlach and colleagues (Gerlach et al., 2010). From kinetic studies, they identified two processes and
proposed a model for the stages of interaction, but structural information for full Nef was absent from their model. Prior structural work, while providing critical insights, has been confined to solution-based analyses even though membrane association is critical for Nef function (Arold et al., 1997; Grzesiek et al., 1996, 1997; Jia et al., 2012; Jung et al., 2011; Lee et al., 1996). While methods such as circular dichroism (Gerlach et al., 2010) and FTIR-ATR are available to assay changes in secondary structure upon membrane binding, full global conformational characterization including distances of motifs relative to the membrane are not provided by these methods.

In the current work, neutron and X-ray reflectometry (NR and XR) were used to resolve conformational changes in myristoylated Nef (myr-Nef) upon membrane insertion. Reflectivity is one of very few methods that can resolve structural details of membrane-associated proteins in physiological conditions and may be unique in the ability to directly resolve details of the full membrane-bound protein structure, in contrast to techniques that probe only labeled residues or secondary structural elements. NR and XR involve measuring the ratio of reflected to incident intensity as a function of momentum transfer \( q_z = \frac{4\pi \sin \theta}{\lambda} \), where \( \theta \) is the angle of incidence with respect to the plane of the membrane and \( \lambda \) is the wavelength (Penfold and Thomas, 1990). The form of this curve is determined by the in-plane averaged scattering length density (SLD) profile normal to the surface. The neutron SLD is determined by the properties of the nuclei present, whereas the X-ray SLD is determined by the electronic properties. In both cases, the SLD is directly related to the atomic composition and the density. Therefore, for a protein bound to a planar lipid membrane, NR and XR determine the in-plane averaged distribution of amino acid residues normal to the membrane in a complementary way. Typically, XR covers a \( q_z \) range that extends to higher values than achievable by NR, and hence XR provides greater insight into the effect of myr-Nef binding and insertion on the structure of the lipid layer. The contrast for the protein in buffer with XR is comparable to, but slightly weaker than, that for NR with protonated myr-Nef, yet is still sufficiently high to resolve large changes in the residue profile. The NR contrast for deuterium-enriched myr-Nef in buffer is substantially greater than that with XR and NR with protonated myr-Nef. Langmuir monolayers and lipid bilayers supported on a solid substrate can both be used as model lipid membranes in NR/XR studies of membrane-bound proteins (Chen et al., 2009; Datta et al., 2011; Kent et al., 2010; McGillivray et al., 2009; Nanda et al., 2010; Shenoy et al., 2012). For biophysical studies, Langmuir monolayers provide an advantage in that the membrane pressure can be controlled and are especially suitable when proteins are known to insert into only the outer leaflet of lipid bilayers.

Myc-Nef (strain SF2) was injected underneath a Langmuir monolayer of deuterated dipalmitoylphosphatidylglycerol (dDPPG) and its conformation was resolved by NR and XR as a function of membrane conditions. The structural details of membrane-bound Nef as a function of solution concentration, membrane pressure, and Nef coverage are described below. The data demonstrate a large conformational change from a closed to an open form that displaces the Nef core domain 70 Å from the lipid headgroups upon insertion of the myristate group and residues of the N-terminal arm. This large conformational change is likely to affect its ability to interact with host proteins by exposing binding motifs on the core domain or by optimally positioning the core domain for interaction with motifs of membrane-associated host proteins.

**RESULTS**

The NR and XR data in this study indicated that soon after myr-Nef was introduced underneath the lipid membrane, a process of insertion into the membrane occurred accompanied by a large conformational transition. Because the affinity of myr-Nef for lipid membranes increases with the percentage of negatively charged lipid, lipid monolayers composed entirely of dDPPG were used in most of this work to maximize the binding affinity, although some experiments were performed using a more biologically relevant ratio of 30% negatively charged lipid to 70% neutral lipid. When myr-Nef was circulated underneath the monolayer, insertion of myr-Nef into the membrane was evident by the backward movement of the trough barrier maintaining the monolayer pressure (increase in surface area at fixed number of lipid molecules, see Figures 1C and 1D and described in more detail in the Experimental Procedures). The insertion was dependent on the membrane pressure, with insertion readily occurring at 25 mN/m and lower but not at 35 mN/m (Figure 1D, and see also below). Due to the larger area occupied by the core domain relative to that of the myristate group, insertion of the myristate moiety alone can account for an increase in surface area of at most 5%; increases in surface area greater than 5% therefore indicate insertion of residues of the protein in addition to the myristate group. Others have reported membrane insertion and evidence for formation of an amphipathic helix within the N-terminal 27 residues of Nef upon association to lipid membranes (Gerlach et al., 2010). Upon insertion, myr-Nef remained associated with the membrane throughout extensive exchange of the subphase underneath the surface layer to remove noninserted, loosely bound myr-Nef. On the other hand, when insertion was inhibited (35 mN/m), membrane-associated myr-Nef was readily removed upon subphase exchange. The rate and extent of insertion of residues varied when membrane pressure was held constant and the concentration of myr-Nef was changed, and vice versa.

To interrogate the membrane-associated conformation of Nef, several NR studies were performed at a fixed membrane surface pressure of 30 mN/m and variable myr-Nef concentration, shown in Figure 2 (NR data in panel a and the SLD profiles resulting from the fitting analysis in panel b). At a myr-Nef concentration of 0.25 μM a fractional surface coverage (\( f \)) of 0.21 resulted (the fractional surface coverage is arbitrarily defined such that when \( f = 1.0 \), the core domains of all Nef molecules would just come into contact with one another in the open conformation shown in Figure 2B), little change in surface area occurred, and a form of Nef that was compact with the core domain adjacent to the lipid headgroups (hereafter referred to as the closed form) was observed. At a myr-Nef concentration of 1.0 μM, the fractional coverage was 0.62, a large increase in area occurred (25%), and a completely different conformation was observed for myr-Nef. For this conformational state (hereafter referred to as the open form) the core domain was displaced ~70 Å below the lipid headgroups (Figure 2B).
Structure
Conformation of Membrane-Associated HIV-1 Nef

Further XR and NR studies were performed in which insertion and Nef conformation were controlled by adjusting the surface pressure of the lipid membrane. XR (Figures 3 and 4) and surface area data (Figure 1D) both indicate little insertion of residues at 35 mN/m, but substantial insertion of residues at 20 mN/m. In the XR data, the conformation of adsorbed protein is evidenced by the variation at low $q_z$ (expanded in Figures 3B and 4B). In the low $q_z$ region the XR curves show distinctly different patterns, and hence indicate different conformations of myr-Nef (0.83 μM), at the two membrane pressures. As shown in the electron density profiles (Figures 3C and 4C) the core domain of myr-Nef was directly adjacent to the lipid headgroups at 35 mN/m, but was displaced 70 Å below the lipid headgroups at 20 mN/m, similar to the profile for 1 μM myr-Nef in Figure 2. At higher $q_z$, the XR curves primarily reflect the structure of the dDPPG monolayer. In particular, the minimum in the data prior to myr-Nef addition indicates the thickness of the dDPPG layer. At 20 mN/m (Figure 3A), the minimum shifted from 0.28 Å$^{-1}$ to 0.33 Å$^{-1}$ after injecting myr-Nef. This shift in the minimum to higher $q_z$ indicates that the thickness of the lipid layer decreased by 4 Å, consistent with tilting of the tails upon insertion of Nef residues. This is another indication of insertion of Nef residues into the lipid layer. At 35 mN/m (Figure 4A), the minimum was unchanged after injecting myr-Nef. Insertion of residues into the membrane and the open extended form of Nef was also observed at 25 mN/m (Figure S2 available online) and again for adsorption to 70/30 dDPPC/dDPPG membranes at 20 mN/m (Figure 5). Others have shown that myr-Nef only binds with high affinity to membranes containing at least 30% negatively charged lipids (Gerlach et al., 2010). The XR measurements are described further in the Supplemental Information.

Deuterium enrichment of the protein being analyzed in NR substantially increases the SLD contrast, allowing for higher resolution data and more precise fitting with molecular models. Myr-Nef in which 80% of the nonexchangeable hydrogen atoms were replaced by deuterium (myr-dNef) was prepared. Figure 6A compares the NR data for a monolayer of dDPPG at 35 mN/m on H$_2$O buffer compared with a scan after adsorption of myr-dNef. In this case, 0.5 μM myr-dNef was incubated against the monolayer for 8 hr and then the concentration was increased to 1.0 μM for 1 hr, at which point adsorption had slowed dramatically and a full scan was collected. The fractional coverage was 0.15. The best-fit SLD profile using a free-form slab model is shown in Figure 6B. The profile band indicates that the core domain lies directly against the lipid headgroups with an uncertainty of ± 5 Å.

The measurement was repeated for a monolayer of dDPPG at 20 mN/m, formed initially by spreading to a pressure of ~10 mN/m and then compressing to 20 mN/m. Myr-dNef (0.28 μM) was injected under the monolayer and a scan initiated 4 hr later (Figure 7A). A large increase in area resulted, similar to that seen for myr-hNef (Figure 1D), indicating substantial insertion of residues into the lipid membrane. The fractional coverage was 0.61. Relative to the NR data for myr-Nef in Figure 2A, a larger change was observed that allowed a much more refined model to be generated. The best-fit SLD profile using a free form slab model is shown in Figure 7B. The red/black profile band contains a broad maximum, indicating the core domain, again displaced roughly 70 Å from the lipid headgroups. Clearly, in the open conformation of Nef, as now derived from multiple NR and XR experiments, the bulk of Nef does not reside next to the membrane but rather is significantly displaced.

Further analysis was performed using molecular models of Nef in which the fractional area coverage and the position of the core domain normal to the membrane were adjusted as free parameters during fitting. As prior work by others indicated that, in addition to the myristate group, a cluster of basic residues within the N-terminal arm (17–22) interacts with negatively-charged lipid membranes to facilitate Nef adsorption.
Figure 3. XR Results for myr-Nef Adsorbed to a dDPPG Monolayer at 20 mN/m
(A) XR data for a dDPPG monolayer at 20 mN/m (black) and scans initiated 2 hr (red) and 16 hr (cyan) after addition of myr-Nef at 0.83 μM.
(B) Expanded view of the XR data in (A) showing the low q_z region.
(C) Electron density profiles corresponding to XR data in (A). Also included is the profile for a scan initiated 14 hr after the addition of myr-Nef (yellow).
See also Figure S2.

Figure 4. XR Results for myr-Nef Adsorbed to a dDPPG Monolayer at 35 mN/m
(A) XR data for a dDPPG monolayer at 35 mN/m (black) and scans initiated 4 hr (red) and 6 hr (cyan) after addition of myr-Nef at 0.83 μM.
(B) Expanded view of the XR data in (A) showing the low q_z region.
(C) Electron density profiles corresponding to XR data in (A).

See also Figure S2.
only molecular models in which residues 2–22 resided on or within the lipid headgroups were considered. Molecular structures in which the core domain was located at varying distances from residue 22 were examined. In these calculations, a single orientation of the core domain was chosen arbitrarily, because it is not possible to resolve the distribution of core domain orientation from the present NR data. The structure giving the best agreement with the data is shown in Figure 7B (blue line), where the core domain is separated from residue 22.

Figure 6. NR Results for myr-dNef Adsorbed to a dDPPG Monolayer at 35 mN/m

(A) NR data for a dDPPG monolayer at 35 mN/m on Tris-buffered H2O subphase (black) and with bound myr-dNef adsorbed from solution at 1.0 μM (red). Best fit is shown using a free-form slab model.

(B) SLD profiles corresponding to the best-fits in (A). The black/gray and red/black bands correspond to the best-fit profiles for dDPPG alone and dDPPG with bound myr-dNef, respectively, with uncertainty limits using a free-form slab model. The molecular models of dDPPG and of Nef are not drawn precisely to scale but were scaled to coincide approximately with the corresponding features in the SLD profiles.

(Gerlach et al., 2010), only molecular models in which residues 2–22 resided on or within the lipid headgroups were considered. Molecular structures in which the core domain was located at varying distances from residue 22 were examined. In these calculations, a single orientation of the core domain was chosen arbitrarily, because it is not possible to resolve the distribution of core domain orientation from the present NR data. The structure giving the best agreement with the data is shown in Figure 7B (blue line), where the core domain is separated from residue 22.
by 70 Å and the peak in SLD corresponding to residues 2–22 is located within the lipid headgroups. The fit to the data, however, is poor because the best-fit curve shown in Figure 7A (blue line) contains greater oscillations at higher qz values than are present in the data, and the calculated SLD profile contains a maximum that is considerably narrower than that of the profile from the free-form fit. This result indicates that the core domains are distributed over a range of depth. Combining the myr-dNef structure shown in Figure 7B with structures in which the core domain is displaced 20 Å closer and also 20 Å further from residue 22 (weighting of 1:2:1) resulted in a good fit to the data as shown in Figures 7A and 7B (yellow lines). For the same procedure but with the core displaced only ±10 Å, the fit was still poor (Figure S3).

The results shown thus far were obtained after the adsorption process advanced to near completion. To gain insight into the kinetics of the process and how quickly Nef inserts and undergoes conformational change, the conformation of myr-dNef was also studied at early stages of adsorption by injecting myr-Nef at 0.67 μM underneath a monolayer of dDPPG at 20 mN/m and collecting successive scans over a limited qz range during the adsorption process. The NR results given in Figures 8A and 8B reveal that at early stages of adsorption (coverages of f = 0.07, 0.23, and 0.37), membrane-bound myr-dNef was predominantly in the open form. The trough area continued to increase steadily during these scans (Figure 8C), indicating insertion of residues into the membrane.

Finally, the importance of the myristate group to the conformational change was examined by studying adsorption of nonmyristoylated Nef (nonmyr-Nef) to monolayers of dDPPG at 30 mN/m. In this case, a deuterated Nef construct containing a N-terminal His tag was used, as described previously (Kent et al., 2010). The dDPPG monolayer was spread to 16 mN/m and then compressed to 30 mN/m before introduction of nonmyr-Nef. For this construct of Nef lacking the myristate group, the affinity for the dDPPG membrane was substantially reduced compared to myr-Nef, and adsorption to relatively high coverage (f = 0.21) required 9 hr at 1 μM. The NR data and corresponding profiles (Figures S1A and S1B) of nonmyr-Nef indicated no insertion and no movement of the barrier (Figure S1C). The SLD profile is that of a compact form with the core domain against the lipid headgroups. Thus, in the absence of the myristate group, insertion of N-terminal arm residues and the transition to the open form do not occur. Comparison of these results for nonmyr-Nef with the results in Figure 2 for myrNef at the same conditions (1 μM Nef and dDPPG at 30 mN/m) demonstrates that insertion of the N-terminal arm and the transition to the open form is promoted by insertion of the myristate group.

DISCUSSION

Resolving the structure of membrane-associated proteins is extremely challenging yet critically important because positioning of residues and motifs relative to the membrane can strongly affect function. While important progress has been made recently (Chen et al., 2009; Datta et al., 2011; Kent et al., 2010; Kreplkj et al., 2012; McGillivray et al., 2009; Nanda et al., 2010; Shenoy et al., 2012), not much is known about the precise distribution of residues of membrane-associated proteins with respect to lipid membranes due to a lack of adequate tools and methods. The present NR and XR data have revealed that membrane-bound myr-Nef adopts a very different conformation depending upon the ability of residues to insert into the lipid membrane.
Our data are entirely consistent with previous hypotheses (Bentham et al., 2006; Curtain et al., 1998; Gerlach et al., 2010) that myr-Nef adsorbs through a combination of electrostatic interactions between basic residues in the N-terminal arm and the negatively charged lipid headgroups, followed by insertion of the hydrophobic myristate group and amphipathic helix. We observed a substantially lower binding affinity with decreasing fraction of negatively charged lipids or in absence of the myristate group, arguing that both lipid association and electrostatic attraction affect the ability of Nef to associate and insert.

Our results indicate that insertion of residues into the membrane is the key step initiating the transition to the open form. In the absence of insertion, here as a result of high membrane pressure, membrane-bound Nef adopts a closed form with the core domain directly against the lipid headgroups. At lower membrane pressure where the myristate and amino acid residues are readily able to insert into the membrane, Nef adopts an open form in which the core domain is displaced into solution ~70 Å from the lipid headgroups. From the extent of the increase in area, it is clear that a substantial number of residues, presumably residues 5–22 on the N-terminal arm known to form an amphipathic helix (Gerlach et al., 2010), insert in addition to the myristate group. Fitting the NR data with molecular models of Nef indicates that the 70 Å average distance of the core domain from the membrane in the open conformation is fully consistent with residues 5–22 residing within the lipid headgroup region. The data suggest that interactions between the N-terminal arm and the core domain that exist in solution are broken upon insertion of a portion of the N-terminal arm into the lipid monolayer, and that the latter is facilitated by the insertion of myristate group. The rate and extent of residue insertion are influenced by the density of adsorbed Nef, by the membrane pressure (lipid packing density), and by the presence of the N-terminal myristate. At a membrane pressure of 30 mN/m, insertion and the open form resulted only at higher myr-Nef concentration whereas at 20 mN/m, insertion and the open form resulted even at low myr-Nef concentration. Furthermore, at a membrane pressure of 30 mN/m and a concentration of 1 μM, insertion and the open form resulted for myr-Nef but not for Nef lacking the N-terminal myristate.

The open form is not triggered by high coverage of Nef on the membrane. Time-dependent scans collected at early stages of adsorption at 20 mN/m show that Nef is predominantly in the open form even at low coverages. Indeed, high coverage (f = 0.37) and yet very little insertion resulted in a prior study involving His-Nef adsorption to lipid monolayers containing a synthetic metal-chelating lipid (Kent et al., 2010), and in that case Nef remained in the closed form. The transition to the open form does not appear to be triggered by electrostatic repulsion of the core from the membrane because the open form was...
obtained for membranes containing as little as 30% negatively charged lipids, and the displacement distance of the core domain from the lipid headgroups was nearly identical for 30% and 100% of negatively charged lipids. Furthermore, the open form did not result when insertion of residues was blocked by a high membrane pressure, despite a greater lipid packing density and therefore greater electrostatic repulsion than at lower membrane pressures.

Gerlach and colleagues reported a kinetic study of myr-Nef binding to fluid phase membranes of DOPC and DOPG using FRET (Gerlach et al., 2010). Strong myr-Nef binding required the presence of negatively charged lipids, as also observed in the present study. The kinetic data indicated two processes: a fast process that was attributed to electrostatic-driven association followed by myristate insertion, and a slower process that was attributed to insertion and formation of an amphipathic helix within the N-terminal 27 residues. They showed that the rate of the fast process increased with membrane curvature, consistent with more rapid insertion of myristate into more loosely packed lipids. This is analogous to and entirely consistent with this study in which surface pressure and packing density were used to alter the energy barrier for insertion.

While the kinetic study of Gerlach and colleagues revealed two processes, no information was provided on the conformation of Nef corresponding to those processes. The present study provides this insight. While both processes in the study of Gerlach and colleagues occurred on time scales much faster than can be resolved by NR and XR, by increasing the energetic barrier for residue insertion we isolated the membrane-bound conformation in absence of helix insertion. In that case, corresponding to the conformation at the end of the fast process of Gerlach and colleagues, Nef is in the closed form (Figure 9). The present data reveal that upon insertion of the amphipathic helix (the slow process of Gerlach and colleagues), Nef adopts an open conformation in which the core domain is displaced on average 70 Å from the lipid headgroups. In addition to providing insight into the conformation of Nef during the processes elucidated by Gerlach and colleagues, the present data also reveal a much longer time scale process in which the coverage of open form Nef on the membrane increases (Figure 9). The present data thus inform the Gerlach model with respect to the conformations of Nef and also extend it to include a longer time scale process of increasing coverage.

It is interesting to speculate about how the present results may be tied to Nef biology and what role Nef insertion and conformation change may play in the ability of Nef to associate with binding partners that lead to Nef signaling/function. Recently, Jia and colleagues determined the crystal structure of a complex of Nef with the cytoplasmic domain of MHC-I using a construct in which the MHC-I cytoplasmic domain was fused to the N terminus of Nef (Jia et al., 2012). In the crystal structure, the N-terminal helix of Nef (residues 6–22) was attached to the core domain of Nef through interactions involving Trp13 and Met20. The authors speculated that this association persists upon membrane binding and positions the Nef core close to the membrane for optimal interaction with the cytoplasmic domain of the MHC-I receptor. The present data are at odds with the assertion that the N-terminal helix of Nef remains attached to the core domain upon membrane binding. Rather, our results show that the N-terminal arm inserts into lipid membranes and the core domain is displaced 70 Å from the membrane in absence of a binding partner protein. However, this fact is not in any way inconsistent with Nef interaction with the receptor, as each core domain is free to explore the full range of distance from the membrane. The 70 Å distance is the average of the distribution of displacements. Jia and colleagues reported that mutations W13A or M20A abolished Nef-induced downregulation of MHC-I in human T lymphocytes, and this was presented as further support for their assertion that interaction of Trp 13 and Met20 with the core domain of Nef is critical for the downregulation of MHC-I. However, these residues likely play important roles in membrane binding and insertion. Therefore, it is entirely possible that the effects of these mutations observed in T lymphocytes are due to decreased membrane association or altered insertion and helix formation.

The present data also provide strong evidence against the assertion (Horenkamp et al., 2011) that association of the core domain of Nef with negatively charged membranes through its basic surface (Figure 1B) orients Nef to provide optimal exposure of the dileucine sorting motif in the flexible loop (residues 152–184) known to mediate interactions with adaptor protein complexes. As we have shown, because the core domain is displaced 70 Å from the lipid membrane in its final resting position, it is unlikely that the membrane affects Nef orientation.

Extensive evidence indicates that at least some functions of Nef in vivo require dimerization (Poe and Smithgall, 2009), yet we (using glutaraldehyde crosslinking and others (Breuer et al., 2006; Horenkamp et al., 2011) have found no evidence for dimerization of free Nef in solution at 1 μM. It was shown elsewhere using analytical gel filtration that in solution, truncated Nef lacking N-terminal residues 2–44 contains significant dimeric and multimeric fractions, whereas myr-Nef and nonmyr-Nef exist primarily as monomers (Breuer et al., 2006). This suggests that...
dimerization of Nef may be inhibited by association of the N-terminal arm with the core domain. Residues on \( z \) helix 4 and the adjacent loop (R109–D127) have been identified as promoting Nef dimer and trimer association (Lee et al., 1996; Arold et al., 1997). Others have proposed that membrane insertion of the myristate group causes the N-terminal arm to separate from the core domain and thereby promotes Nef dimerization (Arold and Baur, 2001; Geyer et al., 2001). Unfortunately, reflectivity methods are unable to detect structural changes that occur in the plane of the membrane; thus, our results do not directly inform the dimeric status of myr–Nef at the membrane. However, our results with NR and XR show that substantially higher coverages are ultimately achieved with Nef in the open form, and thus are consistent with the hypothesis that the arm must separate from the core of the protein to promote multimerization.

In summary, we report the measurement of the precise location of the core domain of terminally acylated Nef with respect to a lipid membrane. Hundreds of proteins are known to be lipophilic, including many that are related to signaling and disease states, and many are potential targets for therapeutic intervention. The present approach will be useful to resolve the membrane-bound conformations of these proteins and will provide insights into signaling mechanisms. It can also inform on the effects of protein–protein interaction at the membrane and disruption of said interactions with pharmacological agents.

EXPERIMENTAL PROCEDURES

Materials
dDPPG and dDPPC in which the 62 protons in the aliphatic tails were replaced with deuterons were purchased from Avanti Polar Lipids. These deuterated lipids were used for both NR and XR measurements. Tris buffer salts and diethanolamine (DETA) were purchased from Sigma-Aldrich and used as received.

Proteins
Protonated myristoylated-Nef (myr-Nef) was expressed in Escherichia coli as described elsewhere (Morgan et al., 2013) using a pET-Duet-1 vector that contained both h-NMT-1 and SF2 Nef (with a C-terminal histidine purification tag). Expression was carried out in 1 L M9 minimal media until the optical density reached 0.6, supplemented afterward for 10 min with 10 mM of 5 mM myristic acid with 0.6 mM BSA. Cells were induced with 1 mM IPTG overnight at 16°C. Purification was performed with Ni-NTA agarose, as described previously (Morgan et al., 2011) and the final purified species was >95% myristoylated, as determined by mass spectrometry. Deuterated myr-Nef (myr-dNef) was prepared by expression in a modified M9 media made with 99.8% D2O; deuterium incorporation was checked by mass spectrometry and showed that the protein was 80% deuterated (data not shown).

Methods
Adsorption Studies
The Langmuir trough and monolayer system are illustrated in Figure 1C. In a typical adsorption run, dDPPG was spread from a 70:30 (by vol) mixture of chloroform and methanol on the surface of 20 mM Tris-buffered H2O subphase (pH 8.2, 100 mM NaCl) held within the Teflon trough (70 mm x 70 mm x 2 mm; Figure 1C). After allowing the chloroform and methanol to evaporate, the subphase was exchanged with Tris buffer containing 1 mM DTT using a peristaltic pump and Teflon tubing with an inlet and outlet. All tubing and fittings were made of Teflon and cleaned using water and Tris buffer after each experiment. The trough was maintained at 20°C ± 2°C.

Neutron and X-Ray Reflection
NR measurements were performed on the NG7 (NCNR/NIST) and Liquids (SNL/ORNL) reflectometers. Details of these spectrometers and the measurement protocols are given in the Supplemental Experimental Procedures. XR measurements were performed using an X-ray reflectometer (Bruker, D8 Advance) employing Cu Kα radiation at NCNR/NIST (Gaithersburg, MD). The copper source was operated at 40 kV and 40 mA, and the wavelength was 0.154 nm. The beam width was 10 mm and the beam height was 0.1 mm. The NR and XR data were analyzed using the Ga_refl program based on the optical matrix method. Ga_refl is available at http://www.ncnr.nist.gov. Simultaneous fits of the data were performed at different stages of a single adsorption run (for example lipids only, with adsorbed protein, and after subphase exchange). The SLD of the subphase was held constant for all the fits. Analyses were performed with free-form models involving a small number of slabs, as well as using molecular structures. The molecular structure of Nef was generated from 1QA5 and 2NEF and manipulated in NAMD2 using CHARMM22 force field. For fitting NR data, the free-form models consisted of one layer each for the lipid tails and the lipid headgroups, and one to four layers for the protein as required to achieve a good fit to the data. When no insertion occurred, as indicated by little or no movement of the barrier, the thickness and SLD of the lipid tail layer after adsorption of myr-dNef were constrained to the same values as determined for the data taken prior to adsorption. This is based on the XR results (Figure 4), where lack of movement in thickness of the lipid layers is demonstrated by absence of a shift in the fringes. For the free-form fit to the NR data in Figure 7, the thickness of the lipid tail layer after insertion of myr-dNef was constrained to be 4 Å less than that measured for dDPPG alone, based on the XR results in Figure 3.

Based on the relative areas occupied by the core domain of Nef and a DPPG molecule, and the fact that the myristate group has only a single aliphatic chain, the SLD of the lipid tail layer was constrained to be greater than or equal to 0.95 × SLD_{DPPG} + 0.05 × SLD_{water}, where the SLD_{DPPG} × 0.05 × SLD_{water} to account for insertion of the protonated myristate group. In the Ga_refl program, the roughness parameter is the full width at half maximum (FWHM) = 2.35 × σ, where σ is the standard deviation of a Gaussian distribution and was constrained in the fitting to be less than the smallest thickness of the two adjacent layers.

Fitting reflectivity data results in defining a family of SLD curves that are consistent with the data. The uncertainty in the fitted profiles was determined by a Monte Carlo resampling procedure in which a large number (1,000) of statistically independent sets of reflectivity data were created from the original data set and the error bars from the counting statistics. The result is a range of values for each fit parameter that is consistent with the statistics of the original data. The uncertainty in a fitted profile is represented by a color-coded band (Figures 6, 7, and 8). This method has been reported in detail elsewhere (Heinrich et al., 2009). The analysis focuses on the location of structural motifs (N-terminal arm and core domain) relative to the membrane. The positions of individual atoms cannot be determined due to insufficient spatial resolution.

Nef coverages were obtained by first converting the SLD or electron density profiles to amino acid (aa) volume fraction profiles using SLD = \( \rho \cdot \varepsilon \) aa volume fraction and then integrating the aa volume fraction profiles to obtain the number (or mass) of Nef per unit area. A coverage of 1.0 was defined as the area per Nef molecule for the open form configuration shown in Figures 2 and 7. The average neutron SLD values (\( \text{SLD}_{\text{n}} \)) for myr-Nef and myr-dNef (80% deuteration) are 2.02 × 10^{−6} Å^2 and 5.19 × 10^{−6} Å^2, respectively. The X-ray SLD is directly proportional to the electron density, where the constant of proportionality is the classical electron radius (2.82 × 10^{−14} Å). The calculated average electron density (\( \rho_{\text{e}} \)) for X-ray SLD is 1.54 Å for myr-Nef are 0.426 e Å^2 and 1.201 × 10^{−6} Å^2, respectively.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2013.08.008.

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Supplemental Information

Conformational Transition of Membrane-Associated Terminally Acylated HIV-1 Nef

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Inventory of Supplemental Information
Supplemental Data

Figure S1. NR data for 6xHis-dNef associated to dDPPG membrane at 30 mN/m (supports Fig 2 in the main text)

Figure S2. XR data for myr-Nef associated to dDPPG membrane at 25 mN/m (supports Fig 3 in the main text)

Figure S3. NR data for myr-dNef associated to dDPPG membrane at 20 mN/m with fits involving an ensemble of three Nef structures for which the core domain distance from the membrane was adjusted +/- 10 Å relative to that of the best-fit structure. (supports Fig 7 in the main text)

Supplemental Experimental Procedures
Protocol for NR measurements
Supporting information for

Conformational transition of membrane-associated terminally-acylated HIV-1 Nef

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Supplemental Data

NR measurements for nonmyristoylated dNef adsorbed to monolayers of dDPPG at 30 mN/m.

Fig S1 shows NR data for a monolayer of dDPPG at 30 mN/m on Tris-buffered H2O subphase along with a scan collected 9 h after injecting 6xHis-dNef at 1 μM. The 6xHis-dNef construct was described previously (Kent, et al 2010). The results show that in absence of N-terminal myristoylation, adsorbed Nef remains in a compact, closed conformation. The fractional coverage was 0.21.
**XR measurements: myr-Nef insertion controlled by surface pressure.**

Fig 3a and Fig 3b in the main text show XR data for a monolayer of dDPPG at 20 mN/m on Tris-buffered H₂O subphase compared with scans collected after injecting 0.83 μM myr-Nef. The dDPPG monolayer was initially spread to a pressure of ~10 mN/m and then compressed to 20 mN/m. The relatively low final surface pressure facilitated insertion of residues into the lipid monolayer. Insertion was clearly evidenced by a large expansion of the area upon injecting myr-Nef (43% after 6 h at which point the barrier reached the back of the trough). Scans were initiated 2 h, 4 h, and 16 h after injecting myr-Nef, respectively. The XR curve varied with time as adsorption proceeded, the first minimum shifting slightly from 0.06 Å⁻¹ to 0.05 Å⁻¹ between the first and third scans and the amplitude of the oscillation increasing as well. These changes reflect an increase in the thickness and concentration of the adsorbed protein layer. The peak in the electron density profiles (Fig 3c) at a depth of roughly 20 Å due to the elevated electron density of the lipid headgroups decreased slightly in magnitude upon injecting myr-Nef. The 43% increase in area per lipid molecule accounts for a decrease in the normalized electron density of the headgroups from 1.32 to 1.22 (1+0.32/1.43). That effect is offset somewhat by insertion of residues of the N-terminal arm into the headgroups and displacement of water molecules. For the profile corresponding to the first scan, the portion corresponding to adsorbed Nef is adequately described by a single layer extending 70 Å below the lipid headgroups. The profile of adsorbed Nef corresponding to the third scan contains a maximum displaced 73 Å from the lipid
headgroups. The fractional coverages for the three scans were 0.22, 0.82, and 1.07, respectively. (f > 1.0 can result if the core domains are distributed or staggered over a range of depth relative to the membrane). The coverage for the third scan was higher compared to the coverages for the NR data in Fig 2, Fig 7, and Fig 8 of the main text due to a much longer incubation time.

Fig 4a and Fig 4b in the main text show XR data for a monolayer of dDPPG at 35 mN/m on Tris-buffered H₂O subphase compared with scans collected after injecting 0.83 μM myr-hNef into the subphase. The higher pressure and dense packing of the lipids inhibited insertion of residues into the lipid layer. This was indicated by a lack of change in the area (increase of only 2.5% over 15 h), and absence of a shift in the minimum corresponding to the lipid monolayer thickness, after injecting myr-Nef. The fractional coverages for the two scans in Fig 4 were 0.27 and 0.61. Whereas the profiles in Fig 4c show that the myr-Nef residue density is greatest directly adjacent to the lipid headgroups, there is also a weak extended portion that is not observed in the NR data in Fig 2 or Fig 6. This is most likely a sparse second layer of myr-Nef loosely associated with the primary bound layer.

For the run corresponding to the data in Fig 6, 0.5 μM myr-dNef was incubated against the monolayer for 8 hrs and then the concentration was increased to 1 μM for 1 h, at which point adsorption had slowed dramatically and a full scan was collected. This difference in protocol appears to have avoided the slight buildup of a second layer. Another possible explanation for the difference is that myr-Nef may be slightly more prone to aggregation than myr-dNef.
Fig S2a and Fig S2b show XR data for a dDPPG monolayer at 25 mN/m on buffer compared with a scan collected 14 h after injecting 0.83 μM myr-Nef and also a scan collected after subphase exchange. The lower final surface pressure (compared to the run corresponding to the data in Fig 4) again facilitated insertion of residues into the lipid tail region. During the insertion of myr groups the area increased substantially, as was observed at 20 mN/m. The electron density profiles in Fig S2c show that the headgroup density decreased in magnitude upon injecting myr-Nef, indicating insertion into the headgroup layer. The electron density profile at 14 h after injecting myr-Nef shows that myr-Nef is in an extended conformation and contains a maximum displaced 66 Å from the lipid headgroups. After subphase exchange the maximum shifts 10 Å further from the lipid headgroups. This suggests that during subphase exchange some electrostatically adsorbed myrNef in closed form was removed and only inserted myr-Nef in open form remained on the membrane. The fractional coverages before and after subphase exchange were 1.11 and 0.80, respectively.

XR measurement of myr-Nef adsorbed to a 70/30 dDPPC/dDPPG monolayer at 20 mN/m

Fig 5a and Fig 5b in the main text show XR data for a 70/30 dDPPC/dDPPG monolayer at 20 mN/m on buffer compared with the third scan collected 22 h after injecting 0.87 μM myr-Nef and a scan collected after exchanging the subphase with buffer. Little difference is observed in the latter two scans. The area expanded 45% after injecting myr-Nef at which point the barrier reached the back of the trough.
The electron density profile (Fig 5c) shows that myr-Nef is in an extended conformation with the core domain displaced ~ 70 Å from the lipid headgroups. The fractional coverage was 0.84.

**Fit to the data of Fig 7a in the main text using an ensemble of three Nef structures in which the core domain distance from the membrane was adjusted +/- 10 Å relative to that of the best-fit single structure.**

This figure is identical to Fig 7 in the main text except that the fit with an ensemble of three Nef structures involves the best fit single structure combined with structures in which the core domain is displaced 10 Å closer and 10 Å further from residue 22 (weighting of 1:2:1). While providing a better fit than obtained with a single Nef structure, the fit for this ensemble is not nearly as good as for the ensemble in Fig 7b of the main text involving structures with the core domain displaced +/- 20 Å from that of the best fit single structure. The best-fit curve shown for the ensemble in Fig S3a (tan line) contains greater oscillations at higher qz values than are present in the data and the SLD profile contains a maximum that is considerably narrower than that of the profile from the free-form fit.
Figure Captions

Figure S1, related to Figure 2. (a) NR data for a dDPPG monolayer at 30 mN/m on Tris-buffered H$_2$O subphase (black) along with a scan collected 9 h after injecting 6xHis-dNef at 1 µM (red). b) Best-fit profile band corresponding to the data in a). c) Normalized trough area versus time showing little change of the area upon injection of 6xHis-dNef.

Figure S2, related to Figure 3. (a) XR data for a dDPPG monolayer at 25 mN/m (black) compared with scans collected 12 h after injecting 0.83 µM myr-Nef (cyan) and after subphase exchange with buffer (red). (b) Expanded view of the XR data in a) showing the low q$_{c}$ region. (c) Electron density profiles corresponding to XR data in a).

Figure S3, related to Figure 7. (a) NR data for a dDPPG monolayer at 20 mN/m on Tris-buffered H$_2$O subphase (black) and with bound myr-dNef adsorbed from solution at 0.28 µM (red). Best fits are shown using a free-form slab model (red), using a model of Nef with residues 2-22 located in the membrane (blue), and using an ensemble of three Nef structures for which the core domain distance from the membrane was adjusted +/- 10 Å relative to that of the best-fit structure shown in b) (yellow). (b) SLD profiles corresponding to the best-fits in (a).
Figure S1.
Figure S2.
Figure S3.
Supplemental Experimental Procedures

Protocol for NR measurements

Neutron reflectivity (NR) measurements were performed on the NG7 horizontal reflectometer at the National Institute of Standards and Technology (NIST) Center for Neutron Research (NCNR) (Figures 2, 6, 8, and S1) and the Liquids Reflectometer at the Spallation Neutron Source, Oak Ridge National Laboratory (Figure 7). Due to fundamental differences in design for these two spectrometers, the density of sampled data points differed. The measurements on NG7 were performed using a wavelength of 0.475 nm and varying angles of incidence. To maximize the intensity, the sizes of the collimating slits and detector slits were increased during the reflectivity scan and this provided an approximately constant relative q resolution $\Delta q/q$ of 0.04, where $q = 4\pi \sin(\theta) / \lambda$, and $\theta$ is the incident and final angle with respect to the surface of the film. All the measurements were done using a 1D PSD detector that ensures the collection of specular and off-specular scattering simultaneously. To obtain the absolute reflectivity, background scattering was subtracted from the specular scattering and the background-subtracted reflected intensity was normalized against main beam. Fig 8 of the main text reports successive scans collected during the adsorption process. These scans were collected over a limited $q_z$ range such that only 1 hr was required to collect each scan. While this collection time is small compared to the timescale of adsorption (4 hr), a small distortion of the curve is inevitable as the data points are collected successively while the adsorbed layer is
changing. However, on the NG7 spectrometer the majority of the collection time for each scan is taken for the last few data points, and so the distortion is mainly present in only those data points and the location of the minimum at \( q_z \sim 0.04 \text{ Å}^{-1} \), that indicates the open form of the protein, is unaffected.

The measurements on the Liquids Reflectometer were performed using a band of wavelengths from roughly 0.375 to 1.06 nm at 14 angles of incidence. The slits were adjusted at each angle to maintain a constant beam footprint on the sample. To obtain the absolute reflectivity, a reflectivity scan was collected with D\(_2\)O in the trough. The incident and reflected intensities were also measured at one angle below the critical edge to determine the fraction of total reflected intensity collected at the detector. These data were then used to determine the absolute normalization factor. Reflectivity scans with H\(_2\)O buffer were collected using the same slit settings as for the measurements with D\(_2\)O. The background subtracted reflectivity was then normalized using the factor determined from the D\(_2\)O measurement. With this normalization procedure, NR data for a monolayer of dDPPG on buffer was within 5% of that measured on NG7 for the same conditions.

The average SLDs (neutron and X-ray) for Nef were calculated from the atomic composition and the molecular volume. The molecular volume of Nef was determined using a standard table of amino acid volumes (Stephen J. Perkins, X-ray and Neutron Solution Scattering, New Comprehensive Biochemistry 1985, 143-265).
Appendix II

Publication

Applications of Hydrogen/Deuterium Exchange MS from 2012 to 2014

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CONTENTS

Protein Folding 99
Folding Mechanisms 99
Amyloids and Fibrils 100
Chaperones 101
Protein Structural Characterization 101
Nonviral, Nonmembrane Proteins 102
Virus-Related Proteins 105
Interactions 105
Epitope Mapping 105
Protein/Large Molecule Interactions 107
Protein/Small Molecule Interactions 109
Biopharmaceuticals 110
Antibodies 110
Other/Nonantibody Biopharm Proteins 110
Membrane Proteins 110
Peripheral Membrane Proteins 111
Transmembrane Proteins 113
Methods 113
Concluding Remarks 113
Author Information 113
Corresponding Author 113
Notes 113
Biographies 113
Acknowledgments 114
References 114

Hydrogen/deuterium exchange (HDX) detected by mass spectrometry (MS) is extraordinarily useful in the study of many aspects of proteins, especially the analysis of protein conformation and dynamics. While once a challenging and therefore sparingly used method, modern HDX MS is more straightforward, rapid, and routine than in the past. As a result, the breadth of applications of the method has expanded. This Review catalogs applications of HDX MS that have appeared in the literature during the 30 months from January 2012 to June 2014. As penetration of the method into nonacademic sectors where confidentiality is necessary is also at an all-time high, many more applications of this method likely exist that have not been reported in the literature.

A synopsis of the recent applications of HDX MS is shown in Figure 1 where classifications have been made in terms of sector in which the work was performed, geography, and general topic. We elected to categorize the publications in these ways to emphasize that the method is used not only in all sectors but also on nearly every continent. Many different possibilities existed for characterizing the applications by topic, and this was not a perfect task. Some papers belong in multiple topics or could arguably be placed in different categories than we finally decided. An Excel database and an Endnote library of the 234 articles we surveyed, both of which contain the topic groupings, are available from the authors.

Academia is the largest sector contributing to published HDX MS applications (Figure 1). Approximately 25% of the papers surveyed included multiple sectors, which we have classified as mixed. As mentioned above, such categorization is biased against work that has not been published. Industrial research and research from governmental laboratories must/may remain confidential. Therefore, the results in Figure 1A are to be interpreted with this in mind. The United States was the primary source of publications in HDX MS during the January 2012 to June 2014 time period (Figure 1B,C), although significant and important work originated from 22 other countries (Figure 1D). To illustrate the HDX MS applications in much greater detail, we have divided the remainder of the article into six sections that each discuss one of the topical classifications shown in Figure 1E.

PROTEIN FOLDING

The study of protein folding is a small (14% of papers surveyed) but nonetheless an important application of HDX MS. The creation of structure during folding slows exchange, particularly in positions that become hydrogen bonded or solvent protected. Monitoring changes in deuterium during folding can reveal not only what parts fold and when but also what factors may affect folding and various folded states. In addition to HDX MS studies of individual proteins folding and unfolding, HDX MS can also be used to understand how other proteins participate in folding or maintaining folded states.

Folding Mechanisms. Work by Tsutsui et al.† provides a classic example of the utility of HDX MS for studying protein folding (Figure 2A). The protein α1-antitrypsin was placed in denaturant; the denaturant was diluted, and the refolding was allowed to proceed for various amounts of time. A short pulse of deuterium (10 s) labeled parts of the protein that were not yet folded at each folding time and the results were summarized in light of the known crystal structure of the protein. HDX MS can access rates, energies, and pathways of protein folding in vitro, as described for a number of other systems including ubiquitin, staphylococcal nuclease, ribonuclease H, and maltose binding protein.‡−§ Folding and/or unfolding as a result of pressure,¶ chromatography,¶ carboxyl-group modification,¶ mutation,§ or binding¶−‖ were also explored in recent publications.

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Amyloids and Fibrils. Failures in protein folding or other disruptions in protein structure can lead to aggregation and/or fibril formation. HDX MS can probe what parts of proteins associate in fibrils, what regions participate in aggregation, and how various conditions affect conversion of soluble proteins into insoluble forms. $\beta_2$-Microglobulin ($\beta_2$-m) forms amyloid fibrils in dialysis-related amyloidosis when degradation in the kidney is compromised. The intrinsic stability of $\beta_2$-m was studied by HDX.
and it was found that the protein visited a highly unprotected, globally unfolded conformational state (Figure 2B). Various conditions were tested, and the rate of unfolding was remeasured in each set of circumstances (Figure 2C) with the goal of determining if global unfolding dictated $\beta_2m$ aggregation and fibril formation. While the dynamics of $\beta_2m$ were easily measured by HDX MS, there was poor correlation between the rate of EX1 unfolding and fibril formation, interpreted as meaning that other fibril nucleation mechanisms besides global unfolding are at play. Fibril formation was studied for other proteins/peptides known to aggregate including amyloid beta (A$\beta$) peptide, $^{13,14}$ $\alpha$-synuclein, $^{15}$ prions, $^{16-19}$ tau, $^{20}$ insulin, $^{21}$ and the prostatic acidic phosphatase fragment of 39 residues (PAPf39). $^{22}$ The regions and rates of protection during fibril formation were interrogated in each case.

Chaperones. Cells are preprogrammed to deal with many types of protein folding and misfolding problems through the use of molecular chaperones. HDX MS is highly useful for studying not only how protein folding is altered by chaperones but also how molecular chaperones function. Many of the HDX MS reports on chaperones included in our analysis were aimed at understanding how the chaperones themselves function, e.g., in response to binding or ATP. A number of elegant studies were reported, including those on small heat shock proteins, $^{23}$ Hsp90, $^{16-20}$ the Hsp90 cochaperone Sti1, $^{21}$ Hsp70, $^{22}$ Hsp104/ClpB, $^{23,24}$ and GroEL/ES. $^{25}$ Following the actual folding of a protein substrate in the presence of a chaperone is a much more technically challenging HDX MS experiment. A study of the effects of GroEL/ES on a TIM-barrel substrate was reported by the laboratory of Hartl and colleagues. $^{31}$ The largely 2-state, cooperative folding of the substrate in the absence of GroEL/ES (Figure 3A) was changed by the chaperonin to be more than 30-fold faster and stepwise (Figure 3B). The spatial resolution from HDX MS allowed delineation of what regions of the substrate protein folded at what rates. Comparison of folding for a related substrate from a species with no GroEL/ES demonstrated that the pathway GroEL/ES enforced is correlated to folding in conditions of chaperonin independence. More HDX MS studies of protein folding and its machinery are certain to come in the future.

PROTEIN STRUCTURAL CHARACTERIZATION

Understanding the structure of a protein is an important contributor in understanding its function. HDX MS has an important role to play in this endeavor, as illustrated by this section with nearly 23% of the articles published during the 30
month period in question. Most experiments of this type compare at least two conformational forms and look for differences between the forms, interpreting the data on the crystal structure of one (or both) of the forms. The perturbations that may create multiple forms (not including interactions, discussed in the next section) include things such as outside forces (e.g., light, temperature, pressure), mutation, post-translational modifications (e.g., phosphorylation), multimerization, and intramolecular interactions. Experiments involving HDX MS data combined with other structural information from X-ray crystallography, NMR, cryo-EM, small-angle X-ray scattering (SAXS), and molecular dynamics simulations can draw the most informed conclusions about the structure of the protein(s). To explore the many examples of how HDX MS can be used for structural characterization, this section has been divided into two broad categories of proteins that are related to viruses and those that are not. Structural characterization is also found in articles that were classified in other sections because they contain multiple topics or because structural characterization was performed during protein interaction(s), performed on biopharmaceuticals, or performed on membrane proteins.

Nonviral, Nonmembrane Proteins. Protein structural characterization has been an increased focus of HDX MS in the past few years. An excellent example of HDX MS analysis of protein conformation was provided by Lee et al. where they describe the conformational changes of the LOV (light–oxygen–voltage) domain protein VIVID (VVD) in the absence and presence of the light. VVD adopts a more protected conformation in the light (Figure 4). The mass spectra (Figure 4A) of an intact protein continuous labeling experiment of the dark state (VVD) and the light state (VVD) revealed very different time scales of unfolding between the two states. Comparisons of deuteration at the peptide level (Figure 4B) showed the regions that incorporated more deuterium upon dimer formation in the dark state (Figure 4C). Light-induced conformational changes are only one variable that could be studied: reports of HDX MS analysis of the effects of temperature, wherein a study of the effects of cold on structure were examined or the effects of being in the solid state also appeared.

HDX MS is most informative when used in conjunction with information generated by other methods such as functional data, binding information, modeling, activity assays, or other structural tools. The combination of HDX MS with structural analysis using other tools is incredibly valuable, as shown by a number of recent examples. Noble et al. studied COPII proteins, which participate in transporting proteins from the ER to the Golgi apparatus, with cryo-EM, HDX MS, and modeling. Residues that mediate the COPII cage assembly were identified: HDX data identified four unique contact regions that interface two proteins Sec13 and Sec31, each of which is involved in the assembly and flexibility of the COPII cage. One of the peptides in the hinge

Figure 3. HDX MS in the study of chaperone-assisted protein folding. The TIM-barrel protein DapA was unfolded with denaturant and, upon dilution of the denaturant, allowed to fold and assemble into its native tetramer spontaneously (A) or in the presence of GroEL/ES (B). After various periods of refolding, pulse labeling, pepsin digestion, and mass analysis were performed. The protection half-times (colored by the categories shown: red, yellow, blue) for segments of the protein (left) were greatly accelerated by the chaperones compared to the spontaneous folding. The chaperones also changed the order of folding. The locations of each protection category are shown at the right on the crystal structure of the assembled tetramer. Reprinted with permission from ref 31. Copyright 2014 Elsevier.
region was found to exhibit bimodal isotope distributions. It was shown that cage assembly leads to a large conformational change at the interface between Sec13 and Sec31 and that Sec13 plays an important part in rigidifying the hinge by providing structural integrity. In another study, a combination of cross-linking and HDX MS was used to map the homodimer interface of the human −3 protein. Willander et al. used a crystal structure, molecular dynamics, and HDX MS on the BRICHOS domain from lung surfactant protein C to study the conformation of the wild-type and a BRICHOS mutant D105N. The dynamics of Family 1 glycoside transferase were studied by HDX MS in combination with molecular dynamics simulations, and the data revealed which parts of the enzyme become more solvent exposed despite the fact that most regions are buried in the crystal structure. Using HDX MS, X-ray structures, and SAXS modeling, detailed studies of heterodimers of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR) were completed. Solution NMR and HDX MS were combined to study the Cyclin-depdent kinase 2-associated protein 1 (CDK2AP1) and showed a region of intrinsic disorder and a 4-helix bundle.

HDX MS has seen multiple uses for analysis of multidomain proteins, quaternary interactions, and evolutionary relationships in proteins. For multidomain proteins, two studies we wish to highlight exemplify the possibilities. In the first study, Tsukamoto et al. used HDX MS of the four domain protein PDZK1, a regulator of an HDL receptor, to show that the PDZ domain does not exert its regulatory influence by modulating the conformation of the other PDZ domains. In the second study, a detailed analysis of the interactions that bridge the nitric oxide receptor and the catalytic domains of soluble guanylate cyclase was made to reveal the interdomain interactions that communicate nitric oxide occupancy from one region of the complex to the active site. Several other studies of interest include analysis of a two-domain protein with HDX MS to characterize the domain interface and analysis of ATP-induced dimerization.

The subdomains within a large prodomain protein, proenkephalin, were probed. Oligomerization of the RAGE receptor, which has been difficult to investigate with crystallography, was studied with HDX MS, molecular modeling, and molecular dynamics. The conformation of fibrinogen was probed with HDX MS to gain insights into regions that were not resolved in the crystal structure, such as the αC region which belongs to the Aα chain. In addition, the fibrinogen variant Bβ235 Pro/Leu, which is responsible for abnormal fibrin structure, was investigated by HDX MS. This variant showed enhanced deuteration in one region of the protein, immediately preceding the Bβ 235 mutation.

There were several reports of the HDX MS analysis of orthologs, proteins with the same function but from different species. Fang et al. studied the conformation of sliding clamps which function as DNA polymerase processivity factors. HDX MS data for a series of clamps from eight different species indicated that, despite their highly similar three-dimensional
structures, clamp proteins show a large range of dynamic behavior. A conserved pattern of alternating dynamics was observed in specific regions of the proteins. In a study of the orthologs of the receptor for activated kinase (RACK1) from human, yeast, and plant, rigid and dynamic regions in the orthologs were identified and compared to conclusions based on the crystal structure.

Recent studies of tRNA-synthetases exemplify how HDX MS analysis of the effects of mutation is powerful. Analysis of the Y341A mutant of human tyrosyl-tRNA synthetase, a functional mutant that would not crystallize, with HDX MS and SAXS revealed conformational changes essential for activation via uncovering of an internal tripeptide sequence.

In the seryl-tRNA synthetase, HDX MS was used to understand why a mutation (F383V) located ∼100 amino acids upstream of the nuclear localization signal (NLS) somehow abolished nuclear localization. The NLS was not resolved in a crystal of the protein, but HDX MS showed that the NLS was protected from exchange in the F383 V mutant versus the wild-type. In other mutagenesis analyses, HDX MS was used to show that mutation E208Q in the SecA translocase, a mutation that alters the active site, changes the global conformational state. Further HDX MS analysis of SecA focused on dimerization and how mutation would disrupt formation of the dimer. Mutagenesis in exchange of a protein activated by cAMP (EPAC) showed that a single mutation can shift the conformational dynamics toward the extended active conformation.

Changes to protein conformation as a result of post-translational modifications are quite tractable by HDX MS, including analysis of the effects of S-nitrosation, oxidation, addition of glutathione, modification to metal atoms such as in aquomet-hemoglobin, or binding to metal atoms (e.g., Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, or Ca\textsuperscript{2+}). Phosphorylation, one of the more recognizable post-translational modifications, is catalyzed by kinases while the reverse reaction is carried out by phosphatases. The effects of phosphorylation/dephosphorylation were studied in many proteins by HDX MS. Examples included interrogation of structural changes as a result of mutations that mimic

Figure 5. Application of HDX MS to the study of viruses and virus-related proteins. (A) Analysis of the binding to peripentonal hexons of the 33-residue N-terminal fragment of the precursor VI protein (pVIn) from human adenovirus. Exchange into purified hexons was compared with and without pVIn and the affected regions were localized. Modified with permission from ref 72. Copyright 2014 Elsevier. (B) Binding-induced changes in HIV Env trimers were determined when complexed with two different small molecule HIV entry inhibitors (NBD-556 or BMS-806) that block the CD4 binding site. Changes in exchange were compared to those seen when Env trimmers were bound to CD4 alone (not shown here). Modified with permission from ref 81. Copyright 2014 Elsevier.
phosphorylation (phosphomimetics S770D/S771D in the plasma membrane pH regulatory protein NHE1), the effects of serine phosphorylation in phenylalanine hydroxylase, mutation that alters the activity of the phosphatase SHP2, and conformational changes in an enzyme due to dephosphorylation. In addition, a number of studies that included HDX MS was performed on the enzymes that carry out phosphorylation, the kinases.

Knowledge of kinase structure and kinase regulation is important because kinases are tied closely to diseases such as cancer through signaling pathways. Therefore, many groups have studied kinases in an effort to understand how their conformation might affect their function. Both receptor and nonreceptor kinases have been studied by HDX MS. Itk and Btk are nonreceptor tyrosine kinases that belong to the Tec family kinases. HDX MS analysis of Itk and Btk showed that these two kinases have different dynamic behavior and that the activation loop of Btk is more solvent accessible than that of Itk thereby affecting activity. Panjarian et al. studied the Abl kinase in the context of SH3-linker mutations and showed that there is a remarkable allosteric network linking the SH3 domain, the myristic acid binding pocket, and the active site of c-Abl core. Activation of ErK2 occurs by enhancing hinge flexibility, shown by HDX MS analysis revealing that hinge mutations increase the flexibility and induce changes in the nucleotide binding mode. For the receptor-tyrosine kinases category, the kinase domain of epidermal growth factor receptor (EGFR) was recently studied by HDX. Wild-type EGFR kinase was compared to an L834R mutation that causes abnormally high activity by promoting EGFR dimerization. The HDX data indicated that the monomeric EGFR kinase has higher HDX rates and is dynamic especially in the C-terminal portion of the αC helix. In the L834R mutant, HDX was not significantly altered showing that the disordered state remains predominant. West et al. studied Snf1-related kinases (SnRKs) and regulation via abscisic acid (ABA), a plant hormone important for controlling growth and stress responses. The structural mechanism of proteins in the ABA signaling pathway (the ABA receptor PYL2, HAB1, phosphatase, and two kinases, SnRK2.3 and 2.6) were investigated with HDX MS. Among other methods, HDX MS has proven very effective in probing allostery. An allosteric effect may result from any type of ligand binding, post-translational modifications, or other conformational changes. Some of the articles already discussed above, and others not in this section but covered below, include an allosteric component in addition to their other findings. In a recent book chapter, Beckett describes the application of HDX to investigate an allosteric system focusing on the E. coli biotin repressor/BirA. The effects of phosphorylation and mutagenesis on the RegA response regulator were studied, and an allosteric coupling between the site of phosphorylation and the activating mutation was described. Winkler et al. studied the elements involved in allosteric regulation of phosphodiesterase activity by comparing different states of the blue-light-regulated phosphodiesterase 1.

Virus-Related Proteins. The structural characterization of viruses is an ongoing topic of interest for the HDX MS community, with 12 papers appearing during January 2012 through June 2014. HDX MS can be used to understand whole viruses, assembled capsids, or just a single protein encoded by a viral genome. In an excellent example of the analysis of viral capsids, Snijder et al. characterized the N-terminal fragment of human adenovirus pV1 protein (termed pVIn). As shown in Figure 5A, when pVIn was incubated with purified hexons, there was protection from exchange in the region of mature capsids, or just a single protein encoded by a viral genome. In another example, the procapsid (prohead-1) of bacteriophage HK97 was analyzed by cryo-EM and HDX MS to understand how the protease cleavage events of maturation occur. Given the recent outbreaks of Ebola in Africa, two very timely papers described the role of HDX MS in studying Ebola virus (a negative-strand RNA virus). The conformation of the Ebola virus matrix protein VP40 and the role of the N- and C-terminal tails in assembly were investigated. Earlier HDX MS results localizing conformational differences between the only protein on the surface of the Ebola virus, the membrane-attached glycoprotein GP1,2 protein, and GI,2 from the related Sudan virus Gulu variant (SUDV-Gul) were discussed in light of structural data for complexes of GR1,2 with neutralizing antibodies. HDX MS was applied to another negative-strand RNA virus, Toscana virus which causes pediatric meningitis, and revealed which regions of the hexameric form of nucleoprotein N were dynamic. Hepatitis viruses were studied with HDX MS: hepatitis B capsid protein conformation was probed as were complexes with antibodies; a crystal structure of the hepatitis C envelope glycoprotein 2 was obtained with the aid of HDX MS identification of disordered regions. There were many HDX MS analyses of HIV proteins, including the very detailed analyses of the envelope glycoprotein (Env, various portions of the entire protein, gp 160, are called gp41 or gp120) by the Lee laboratory at the University of Washington as well as analysis of HIV viral accessory factors such as Nef. One example of the analysis of the Env protein is shown in Figure 5B where, in the presence of HIV entry inhibitors (NBD-556 or BMS-806), exchange into trimeric gp120 was altered in the variable regions V1 and V2 for NBD-556 but in quite different regions for BMS-806. Interactions between gp41 and gp120 were observed for monomers but not for trimers, and key differences in conformational stability between the gp120 proteins from different viral isolates were revealed.

INTERACTIONS

The activity and function of proteins is often regulated via binding to other molecules; understanding these events, therefore, can give critical insight into protein function and disease related states. In this section, we will focus on how HDX MS has been utilized (28% of the articles published during the 30 month period in question) to study protein interactions. We note that protein interaction studies are also present in articles that have fallen into other categories, again because classification is inevitably an imperfect process when articles cover multiple topics. The applications we will highlight in this section include antigen/antibody interactions (i.e., epitope mapping) and will include work on vaccine design, clotting factors, and allergens. Studies involving proteins in complex with large molecules, which we define as greater than molecular weight of 800 Da, will be discussed and include examples involving the effects of binding to protein subdomains and nucleic acids. Lastly, protein interactions with small molecules, defined here as smaller than molecular weight of 800 Da, will be reviewed and will cover topics involving nucleotide binding proteins and receptor/drug binding as well as the effects of anticancer drugs on protein targets.

Epitope Mapping. Epitope mapping is a crucial step in designing therapeutic antibodies and vaccines. HDX MS has been a useful, some would argue indispensable, technique for this...
Malito et al. probed the binding between a monoclonal antibody against factor H binding protein (fHbp), the vaccine antigen of *Neisseria meningitidis*. The epitope was probed with peptide arrays, phage display, X-ray crystallography, and HDX-MS; the agreement of the methods is shown in Figure 6. As the authors point out, HDX MS was "the most effective method to rapidly supply near-complete information about epitope structure". Some other methods for epitope mapping, while suitable for linear epitopes, struggle with providing full pictures of interactions that cover discontinuous conformational epitopes, a deficiency apparent in the missing regions in panels B and C of Figure 6. A second study by the same group analyzed two broadly cross reactive antibodies to fHbp with HDX MS to very rapidly characterize the complex, identify the epitopes, and compare the antibodies. These studies highlight how HDX MS is becoming an essential methodology for epitope characterization. While crystal structures still remain the gold-standard for complex characterization, HDX MS provides a rapid means, either in the absence of or in conjunction with an X-ray structure.

Figure 6. HDX MS for epitope mapping of a monoclonal antibody (mAb) against factor H binding protein (fHbp), a virulence factor, and vaccine antigen of the causative agent of bacterial meningitis, *Neisseria meningitidis*. Comparisons were made against (A) the known interface between factor H (fH) and fHbp, interface residues colored yellow in the structure at the left, and (B–E) epitope mapping data, colored red on the structure and linear representation, for mAb 12C1 and fHbp by various methods. HDX MS mapping (panel D) identified all regions but not with the resolution of the cocrystal structure. Reprinted with permission from ref 86. Copyright 2013 National Academy of Sciences of the United States of America.
structure, to focus attention on regions that are involved in interactions and inform other experiments that test such regions.

HDX MS can be used not only to characterize therapeutic antibodies destined to combat diseases but also to study antibodies that appear as the result of intervention with other therapeutic proteins. As an example, consider hemophilia A, a disorder characterized by the functional absence of a critical blood clotting protein, factor VIII (FVIII). Treatment with recombinant FVIII is hampered by the patient’s immune system, wherein intravenous infusion of recombinant FVIII causes many patients to develop antibodies against the clotting factor. HDX MS was used to characterize antibody binding to FVIII. Bloem et al. used HDX to identify epitopes in two C1 domain spikes on FVIII when in complex with a human mAb, KM33, while Sevy et al. identified epitopes of four anti-FVIII mAbs using HDX MS. Other interesting examples of HDX MS in epitope mapping studies include: analysis of allergens from almond nuts (pru du 6) or cashew nuts (ana o 2), studies of innate immunity where binding to IgG to ficolin complexes were characterized by HDX MS, generation of an algorithm for predicting B cell epitopes using as a model system the D8 protein of the vaccinia virus, a major target of the small pox vaccine. The prediction algorithm was validated using a combination of X-ray crystallography, ELISA, site-directed mutagenesis, and HDX MS. Lu et al. generated and characterized 13 mAbs against GroEL found in Francisella tularensis (FtGroEL) and used HDX to map the target epitopes and compare the antibodies. The HDX data suggested that the protective effects of the mAbs are due to stabilization of a structural rearrangement in FtGroEL. In the final example covered in this section, protein stabilization by antibody binding was described by Tiyanont et al. who monitored protein dynamics during activating- or inactivating-antibody binding to the regulatory region of human Notch3. Notch3 in complex with either EDTA or an activating antibody resulted in increased deuteration, signifying increased dynamics whereas binding of an inhibitory antibody reduced deuteration. This study points out that, although binding of an antibody to an antigen can be strong, the biological effects are not always inhibition of the function of the antigen.

**Protein/Large Molecule Interactions.** The previous section on epitope mapping covered the special case of protein/protein interactions where one of the partners is an antibody. Many other protein/protein interactions exist where no antibodies are involved. A multitude of proteins realize their biological function by interacting with other molecules. HDX MS can be used to map these interactions, and to address questions posed by the protein structure and function.

Figure 7. Example of mapping protein/protein interactions by HDX MS. (A) The effects of binding the nucleosome assembly protein 1 (Nap1) to the histone H2A−H2B heterodimer were shown by comparing exchange into the H2B portion of H2A−H2B alone (left) to exchange into H2B when bound to Nap1 (middle). The regions most affected by binding were mapped to the crystal structure of H2B (colored blue, right panel). (B) Analysis by HDX MS suggested regions where mutations might be made and tested in other assays; mutants 3 and 4 (residues changed indicated in yellow and red, respectively) later showed reduced binding to Nap1 in FRET assays. Modified with permission from ref 97. Copyright 2013 Elsevier.
functional roles through binding with other large biomolecules, including nucleic acids, peptides, or other proteins. In recent years, HDX MS has been applied to investigate protein/large molecule complexes, and here, we define a large molecule as something with molecular weight in excess of 800 Da, in order to further understand many biological systems. In general, the dissociation constant of the complex is known, and this simplifies the experiment because the mixing ratio of the component proteins can be calculated to maximize the amount of proteins that are bound. In cases where the dissociation constant is not known, 5-, 10-, or 20-fold excess of one protein can be added and the results compared, for example. Complications can arise in these types of HDX MS experiments when the binding constant is weak because signals from peptides of a protein that is not of interest can interfere with the signals of peptides from proteins that are of interest.

A good example that characterizes a protein/large molecule HDX MS is shown in Figure 7. Here, D’Arcy et al. investigated interactions between the histone H2A-H2B heterodimer and the nucleosome assembly protein Nap1. The authors report that the helices in the H2A-H2B sample partially disordered conformations at low ionic strength. Binding of Nap1 reduces these sampling events and competes for histone-DNA and interhistone interactions within the nucleosome. On the basis of the results of HDX MS, the authors proposed other mutants that should block interactions, which were then verified by non-MS methods. Such experiments highlight the importance of combining HDX MS measurements of protein/protein interactions with other types of assays.

There were many examples of how HDX MS can be used to study protein/protein interactions, ranging in application from analysis of identifying contact surfaces to monitoring structural changes in one or more members of the complex upon binding. Studies were performed on complexes with only two members as well as larger systems with multiple proteins; the proteins studied came from all types of organisms including human, yeast, E. coli, cyanobacteria, or other microorganisms. HDX MS data were often combined with other techniques, including methods such as circular dichroism, homology modeling, X-ray crystallography, and NMR.

Several reports focused on protein binding to peptides or small polypeptides including: analysis of how the conformation of the αβ T cell receptor (TCR) is influenced by binding to peptides presented by the class I major histocompatibility complex (MHC-I), a study of the protection of the plasminogen activator inhibitor-1 (PAI-1) by binding to the small 39-residue somatomedin B domain of the plasma glycoprotein vitronect-
The effects of cyclic AMP, a regulatory molecule for a number of proteins, on protein conformation and dynamics were explored in several publications. Underbake et al. showed that, in the GMP binding protein guanylate cyclase, nitric oxide binding produced profound changes in domains responsible for heterodimerization and signal transmission. HDX MS was used to study oxygen binding to myoglobin and hemoglobin, to understand the binding of severalaza-sugar molecules to a glycosylase, and to probe progesterone and propanolol binding to α1-acid glycoprotein (AGP), a protein in plasma that can bind to a variety of molecules. Hemychova et al. studied the interactions between a small molecule, Nutlin-3, and the protein MDM2 and showed that Nutlin-3 binding caused reduced HDX kinetics in regions surrounding the binding site and the hydrophobic pocket of the protein. Brier et al. investigated the regulatory effects of 4-hydrophenylacetic acid (4-HPA) on the DNA-binding protein neisserial adhesion (NadR). The HDX data show that 4-HPA binds between the two DNA-binding lobes of NadR and stabilizes this state with little conformational perturbation. These data suggest that 4-HPA regulates NadR by stabilizing a conformation unable to bind to DNA. Ghose et al. studied spore product hyase (SPL), which repairs UV lesion spore photoproducts in a S-adenosyl-l-methionine (SAM) dependent manner. SPL in complex with SAM and a synthetic nucleotide results in a significant decrease in deuterium incorporation, suggesting stabilization. However, SPL in complex with only the nucleotide produces no change in deuterium exchange, indicating that SAM is a required binding partner.

Many small molecule therapeutics that combat cancer have been discovered. HDX MS has been applied to better characterize the effects of such molecules on the target protein. The effects of Taxol on microtubule associated protein 4 (MAP4) were studied. Yu et al. investigated the antimetabolites and the kinase domain of the PPAR γ subunit of AMPK with other small changes in the α and β subunits. The synthetic inhibitor exhibited large changes in the β subunit and the kinase domain of the α subunit. Other Griffin publications further demonstrate analyses of this type for the estrogen receptor α (ERα), the vitamin D receptor, peroxisome proliferator-activated receptor gamma (PPARγ), and the retinoid X receptor α (RXRa).

The analysis of protein binding to nucleic acids, or binding between proteins involved with nucleic acids, was described in a number of articles. Choi et al. used HDX MS to study the Notch transcription complex formed between the Notch intracellular domain (NICD), the DNA-binding factor CSL, and Mastermind Family 1 protein (MAML1). The role of each member of cooperative assembly of the complex was ascertained. Winkler et al. studied the interaction between the light-sensing antirepressor AppA and the transcriptional repressor PpsR. The HDX data for the complex showed how light activation of AppA−PpsR influences the PpsR effector region, altering how the complex would interact with DNA. A study of the TATA box-binding protein (TBP) and its interactions with domains of the progesterone receptor (PR) revealed conformational changes in both AF1 and AF2 domains of PR upon binding, as well as changes in flexibility within TBP. Binding between the processivity factor proliferating cell nuclear antigen (PCNA) in complex with an inhibitor called TIP identified regions involved in binding between TIP and PCNA, and the authors concluded that TIP binding disrupts the PCNA trimer formation and decreases its activity. In a study focusing solely on the effects of DNA binding to a protein, Roberts et al. utilized HDX MS and computational docking to investigate uracil−DNA glycosylase UNG, which is responsible for identifying and cleaving uracil from DNA, as it binds to a 30 bp DNA sequence. They reported that the UNG−DNA complex showed increased protection at the enzyme active site and two areas surrounding the active site. The effects of protein binding to sequences of RNA, in the form of aptamers, were investigated for both the serpin plasminogen activator inhibitor 1 (PAI-1), RNA aptamer binding resulted in major reduced deuterium incorporation within PAI-1, and the areas of reduction were mapped.

Protein/Small Molecule Interactions. We have just discussed where HDX MS has been used to characterize protein interactions with large biomolecules. Small molecule (defined here as smaller than a molecular weight of 800 Da) binding to proteins has also been investigated extensively with HDX MS. The small molecules in question are often therapeutic drugs but can also be natural substrates, regulatory molecules, metal ions, or antibiotics. Other possibilities include lipids, glycans, aromatic pollutants, metabolites, and the list goes on. One surprising finding from many HDX MS studies of small molecule binding is how influential these interactions are on proteins. Given the size differences between most small molecules and peptide peptides, even high concentrations of small molecules (i.e., weak dissociation constants) do not generally interfere with the LC/ MS steps or lead to suppression of peptide signals of interest.

Publications from Patrick Griffin’s laboratory epitomize analyses of protein/small molecule binding by HDX MS. An example is shown in Figure 8 where structural changes in AMP-activated protein kinase (AMPK) were monitored upon binding of nucleotides, cyclodextrin, and a small molecule activator. AMP binding resulted in conformational changes principally in the γ subunit of AMPK with other small changes in the α and β subunits. The synthetic inhibitor exhibited large changes in the β subunit and the kinase domain of the α subunit. Other Griffin publications further demonstrate analyses of this type for the estrogen receptor α (ERα), the vitamin D receptor, peroxisome proliferator-activated receptor gamma (PPARγ), and the retinoid X receptor α (RXRa). dx.doi.org/10.1021/ac5040242 | Anal. Chem. 2015, 87, 99−118
ClpP revealed, among other things, that ADEP1 destabilizes the N-terminal regions of ClpP subunits while stabilizing the equatorial belt, highlighting the allosteric nature of inhibition.

**BIOPHARMACEUTICALS**

Biopharmaceuticals are proteins that are used as drugs. A very important part of the development of such drugs is characterization of the protein, which occurs at many stages (e.g., research, development, processing, formulation, etc.). There are tremendous challenges to manufacturers during characterization due to the size of proteins, their complexity, and the necessity of analyzing higher-order structure. This section reviews the published work in the period that involved biopharmaceutical characterization by HDX MS. As mentioned near the beginning of this article, it is almost certain that a large body of HDX MS work on biopharmaceuticals goes unpublished; as a result, in this category, we are only discussing a fraction of the applications in this field. We will cover 17 articles in this section, half of which are related to antibodies. Several papers related to biopharmaceuticals have been covered in other sections of the review (e.g., epitope mapping) although we have tried to consolidate known biopharmaceutical products to the following section. Three subcategories addressed here are (1) general antibody characterization with a focus on the effects of post-translational modifications on antibody conformation and dynamics; (2) antibody aggregation; (3) studies that describe the conformation and dynamic behavior of nonantibody biopharmaceuticals.

**Antibodies.** Monoclonal antibodies primarily of the IgG isotype make up the majority of biopharmaceutical proteins currently employed for the treatment of diseases. Due to their large size and complexity (e.g., disulfide bonding, glycosylation, etc.), their structural characterization has been challenging. HDX MS is playing an important role in understanding the conformation and dynamics of such complex molecules. Work has been done to understand the effects of post-translational modifications, such as oxidation and glycosylation, on antibody structure. For example, a recent paper described how a single mutation in the C<sub>ε3</sub>-Fc domain of an antibody dramatically altered the conformation of the antibody by altering its glycosylation pattern. Antibody-drug conjugates have also been characterized by HDX MS, and the data indicate that, for the IgG in question, no major structural changes occurred in the molecule upon addition of the drug conjugates. A comparison between an originator antibody (Rituximab) and a biosimilar was published, highlighting how HDX MS can be used in comparability analyses of antibodies.

Proteins in vitro may be susceptible to aggregation induced by the environment. Biopharmaceuticals undergo many events in their lifetime, from expression and purification to formulation and storage. There can be a tendency to form aggregates which are obviously detrimental to the manufacturing process and most importantly can have a toxic effect on the patients. There are ongoing efforts to catalog, characterize, and ultimately understand aggregation, especially what might trigger it and what parts of the molecule are more prone to aggregation. HDX MS is a valuable method in the quest to understand aggregation, and several articles on aggregation recently appeared. Zhang et al. investigated the effects of thermal stress on bevacizumab (Avastin), a monoclonal antibody that targets the vascular endothelial growth factor A (VEGFA-A) and is used as an anticancer drug. The HDX MS data indicated that some peptides in the Fab and the C<sub>ε2</sub>-Fc regions incorporate more deuterium upon aggregation, and others, in the variable region, incorporate less deuterium upon thermally induced aggregation. Manikwar et al. explored how excipients influence aggregation, using HDX MS as the readout. Iacob et al. detailed how HDX MS and other methods in the biopharmaceutical industry can be used in combination to gain a more complete picture of aggregation for three monoclonal antibodies that have a propensity for forming dimers.

Other/Nonantibody Biopharm Proteins. In addition to antibodies, there were other biopharmaceuticals characterized by HDX. In an excellent example of the use of HDX MS for comparability studies, Houde and Berkowitz characterized recombinant coagulation factor IX (rFIX), a fusion protein version in which an Fc homodimer domain was connected C-terminally. Figure 9 shows the difference index plot for rFIX in the absence and presence of Ca<sup>2+</sup> (calcium binding is required for activation). Conformational changes are apparent upon Ca<sup>2+</sup> binding but expected due to the influence of calcium on the structure of rFIX. The same conformational changes were observed for the rFIX-Fc fusion protein, indicating that it too responds to activation in the same way as the nonfusion version. Other HDX MS data in the same study also show that, by all measures, the conformation of rFIX is not altered by the presence of the Fc domain. Experiments of this type clearly have implications not only for creation of new entities but also for demonstrating comparability during process change, upon modification, or for biosimilars.

The conformation and dynamics of insulin were characterized by HDX MS, including analyses of various forms/versions of insulin with different therapeutic properties. The effects of Endo H trimming of high mannose glycans from α-amylase were studied. Several forms of recombinant glucocerebrosidase, sold as Cerezyme, a treatment for Gaucher’s disease, were investigated by HDX MS, including mutants and ligand-bound forms. The effects of pegylation on the conformation and dynamics of granulocyte colony stimulating factor (G-CSF) were studied. Exchange into a new class of therapeutic agents, “stapled peptides,” was characterized by HDX MS, and the authors found that the location of the staple is important and is linked to both the deuterium kinetics and the rates of proteolysis of the stapled peptide. Finally, HDX of lyophilized protein was studied with the goal of understanding what may happen to protein structure during dehydration. The results of this work have important implications in formulation of biopharmaceuticals, including on the conditions during the freeze-drying process and for reconstitution. HDX MS can be used to probe all such events and inform each level of processing.

**MEMBRANE PROTEINS**

The application of HDX MS to the investigation of membrane proteins is a growing area of application. Membrane proteins are inherently difficult to deal with by many biophysical methods. HDX MS has the advantage of only needing small amounts of material and the ability to differentiate protein from lipid. Exchange can be performed in detergent or in membrane mimetics, provided the lipid component of the membrane mimetic can be removed sufficiently so as to not interfere with peptide ionization and detection. A great deal of the methodological challenges of analyzing membrane proteins by HDX MS have been met, leading to a number of publications in this area and increased understanding of these proteins. Topics in this section are divided into peripheral membrane proteins, which are less challenging to study, and transmembrane proteins.
Peripheral Membrane Proteins. Peripheral membrane proteins are not completely embedded but rather anchored to the membrane in some way. Protein conformation and dynamics can be influenced by the membrane itself, through electrostatic and hydrophobic interactions. It is therefore important to study peripheral membrane proteins in the presence of an actual membrane in order to obtain meaningful data. Several excellent, recent examples of the application of HDX MS to the analysis of peripheral membrane proteins come from Roger Williams’ lab. They have been investigating phosphoinositide 3-kinases (PI3Ks) which act just downstream of membrane receptors and are in close proximity to the membrane. There are two classes of PI3Ks; Class 1A includes isoforms p110α, p110β, and p110δ, which are regulated primarily by p85 and are activated by receptor tyrosine kinases; Class 1B contains isoform p110γ, is regulated by p101, and is activated by G-protein coupled receptors (GPCRs). HDX MS was used to study p110α and showed that p110α in complex with a regulatory subunit (p85γ) constitutes a complex catalytic cycle characterized by distinct conformational steps. There was disruption of the SH2 domain of p85γ with interfacial regions of p110γ, movement of the adapter binding domain in p101γ, and interaction of the kinase domain with lipids. Oncogenic mutants of p110α were then probed. All mutations changed the way the protein interacted with the lipid membrane, and selected mutants induced a variety of conformational changes in both p110α and its interaction p85γ. Some mutants mimicked conformational changes observed in natural activation. The other isoforms (p110β and p110δ) of Class 1A PI3K were also investigated by HDX MS, and results for all isoforms were compared. The binding of the regulatory subunit p85 to each isoform was mapped, and it was shown that each isoform had unique relationships with p85. Figure 10A summarizes some of the most recent findings wherein HDX MS probed the class 1B isoform PI3Kγ catalytic subunit (p110γ) as it interacts with its regulatory subunit (p101), lipid membranes, and G-protein Gβγ heterodimers. HDX MS analyses, which involved multiple proteins and liposomes and are a technical challenge in themselves, showed that the helical domain of p110γ is substantially protected when in complex with p101, exposed when bound to lipids, and protected once more when binding to G-protein Gβγ heterodimers. Then, Walser et al. used HDX MS to investigate how the p110γ catalytic subunit interacted with the adaptor subunit p84. Not only do all these studies with PI3K provide essential new information that is very difficult to obtain by other methods, but also they highlight the extreme utility of HDX MS for studying membrane proteins in lipid environments.

There were a number of papers in the last 30 months that described investigations of human apolipoprotein A-1 (ApoA1), a protein essential for solubilizing and transporting cholesterol in the blood. ApoA1 is the primary component of high density lipoproteins (HDLs) and plays a major role in the structure of most HDL particles. HDL particles start out as discs of lipid molecules with two copies of ApoA1 circling the hydrophobic lipid tails at the equator. Rearrangements of the ApoA1 structure and addition of cholesterol can then transform the disc into spheres. HDX MS was used to obtain conformational information for ApoA1 in discoidal HDL particles of two sizes, and to probe the effects of point mutations in ApoA1 that lead to reduced HDL levels and to compare the conformation of ApoA1 in discs versus in spherical HDL particles. The HDX MS data indicated that several regions of lipid-free ApoA1 become significantly protected when incorporated into HDL particles with a 9.6 nm diameter whereas, for particles with a diameter of 7.6 nm, approximately 20% more ApoA1 residues are forced out of contact with lipids. EX1 kinetics were observed for ApoA1 indicating coexisting helical and nonhelical populations. The Iowa (G26R) and Milano (R173C) mutations of ApoA1 change helix packing, albeit in different ways and magnitudes,

Figure 9. Characterization of Fc fusion proteins and how their parts relate to naturally occurring versions. HDX MS of recombinant factor IX (rFIX) was compared to a fusion of rFIX with an Fc of an antibody (termed rFIX-Fc). The pattern of differences in deuterium levels of rFIX as a result of calcium binding was the same as that observed when the fusion form, rFIX-Fc, bound to calcium meaning that the Fc portion was not impaired in its calcium binding activity by being attached to the Fc. There were essentially no differences between exchange into rFIX and rFIX-Fc with calcium or without calcium. Reprinted with permission from ref 158. Copyright 2012 Wiley.
which contributes to proteolysis and the potential to form amyloids. A combination of methods including small angle neutron scattering, cross-linking MS, and HDX MS were used to construct a model of HDLs, including full length ApoA1. Both HDX MS and lysine acetylation MS were implemented to study conformational changes in ApoA1 caused by Apo A-II in discoidal HDL. When both ApoA1 and ApoA-II were incorporated in the same HDL particle, there were differences in deuterium incorporation in ApoA1 helices 3−4 and 7−9.

Phospholipase A₂ (PLA₂) was also a topic of interest. PLA₂ is a large protein superfamily, members of which catalyze the hydrolysis of phospholipids to produce fatty acids. They are involved in signaling, inflammation, and lipid membrane maintenance. Group VIIA lipoprotein associated (Lp-PLA₂) and its interactions with ApoA1 and HDLs were studied by HDX MS. The identity of three specific regions in PLA₂ with decreased deuteration were found. The authors note that the same regions in PLA₂ do not exhibit the same protection when associated with ApoA-II. Group VIA Ca²⁺ independent (iPLA₂) was also studied as was an inhibitor binding to iPLA₂ using a combination of MD simulations and HDX MS. It was shown that inhibitor binding resulted in protection in loop regions surrounding the active site, with some minor changes in regions distant from the active site, indicating that the whole enzyme is affected.

Monoacylglycerol lipase (Mgl) was studied by HDX MS. Mgl is a serine hydrolase that associates with lipid membranes and deactivates cannabinergic signaling in the central nervous system by hydrolyzing the lipid signaling molecule 2-arachidonylglycerol. Karageorgos et al. used HDX MS to monitor changes to Mgl when bound to small several molecule inhibitors. Reaction with a covalent inhibitor AM6580 that carbamylates Ser122 in Mgl resulted in HDX protection of helices 6α and 8α. Decreases in deuterium incorporation were also noted for another inhibitor AM6701 that was reversible; however, the decreased protection was not as pronounced. In a second publication, Nasr et al. used HDX MS to compare the conformation of Mgl free in solution versus bound to phospholipid nanodiscs to delineate the region of lipid association. Membrane anchoring was found to proceed through helix 8α in the lid domain and neighboring helix 9β, and this positions Mgl into an open conformation to facilitate ligand binding. Using the AM6701 inhibitor, it was shown that Mgl remains membrane bound, even when inactivated.

Figure 10. Membrane protein investigations with HDX MS. (A) Exchange into phosphoinositide 3-kinase γ (also termed p110γ) was compared to exchange during interactions with its regulatory/adaptor subunit p101 (left). The complex of p110γ/p101 was then labeled with and without empty liposomes (middle) or liposomes containing G-proteins (Gβγ) (right) to ascertain the role of the membrane and effects of binding. See ref 229 for full details. Reprinted with permission from ref 229. Copyright 2013 National Academy of Sciences of the United States of America. (B) HDX MS of BmrA, a bacterial multidrug ABC transporter, was performed while the protein was in n-dodecylβ-D-maltoside (DDM) detergent. HDX results for analysis of an apo form were superimposed on the 3D model of the open conformation (left), and analysis of a closed form ATP-Mg-bound mutant were superimposed on a 3D model of the closed conformation (right). Coloring is based on the percentage of deuteration after 1 h of labeling, according to the color scale at the right. Reprinted with permission from ref 181. Copyright 2012 National Academy of Sciences of the United States of America.
Other peripheral membrane proteins were investigated. HDX MS aided in understanding how an enzyme required for synthesis of phosphatidylcholine is activated via a lipid-induced amphipathic helix. Koshy et al. studied the cytoplasmic fragment of CheW and CheA proteins, which are bacterial chemotaxis receptors. Glabal HDX data show that CF exhibits much slower exchange when in complex with CheW and CheA compared to when in solution. Lee et al. monitored the structural transitions of α-synuclein as it bound to phospholipid vesicles.

Transmembrane Proteins. Transmembrane proteins, or those that span the membrane, are even more challenging than peripheral membrane proteins. The highly hydrophobic regions that span the membrane can be difficult to digest and separate from the lipid component. Nevertheless, some success has been realized, including the example of this type of HDX MS shown in Figure 10B. In the work shown in Figure 10B, the bacterial ABC transporter, BmrA, was investigated. BmrA is a multidrug transporter that normally protects healthy cells by binding and expelling foreign organic compounds but must shift between open and closed conformations for this function. HDX MS revealed regions located in the intercellular domain with very different exchange kinetics depending on the conformational state. Another carrier, the bovine mitochondrial ADP/ATP carrier, has also been studied by HDX MS. Similar to BmrA, mitochondrial ADP/ATP shifts between two distinct conformations to function and HDX revealed differences between these states were localized to the loop regions within the mitochondrial matrix. The glycero facilitator (GF), a transmembrane protein responsible for transporting water and glycerol, was probed with HDX MS. One of the transmembrane helices, TM7, displayed much faster exchange kinetics relative to the other helices suggesting that TM7 is less stable than the other helices in order to aid transport of molecules across the membrane.

Methodological developments are key to the analysis of transmembrane proteins. The proteins must be in the lipid to be in their native state for deuteriation, but then the lipid must be removed. Various methods are described in these articles for accomplishing this. The folding of membrane proteins and ways to study it are also interesting. Bacteriorhodopsin, the prototypical GPCR, was studied by pulse-labeling HDX MS to monitor refolding induced by dilution from sodium dodecyl sulfate (SDS). The protein obtained structure rather slowly as it refolded from SDS. The methodology explored could be applied to other membrane proteins. The transmembrane enzyme gamma-glutamyl carboxylase (GGCX), for which there is no solved structure, was studied with HDX MS. GGCX converts select glutamic acid to γ-carboxy glutamic acid in vitamin K-dependent (VKD) proteins, and GGCX mutations have been linked to clotting disorders. GGCX in nanodiscs was labeled when free or bound to an 18 residue consensus propeptide from VKD substrates. Major differences in hydrogen exchange were observed for sites involved in propeptide and glutamate binding. The authors also noted other regions within GGCX exhibiting minor differences when in complex with the propeptide. Overall, HDX MS work on transmembrane proteins is progressing slowly but is expected to increase once the methods become more tractable.

**METHODS**

In the course of applying a method to problems, better ways of addressing the problems invariably appear. It is through a wide variety of applications that methods refinement and development can truly become efficient. During the past 30 months of applications, there have been a number of developments in the methodology that have made the applications possible. Nineteen percent of the articles surveyed had something to do with methods development. We have divided their classification into pre-LC, LC-MS, software, and other.

Improvements to the Pre-LC steps were reported and include work describing the enzymatic digestion step, and di sulfide bond reduction using electrochemical cells, affinity capture, methodological consideration for reducing back-exchange in MALDI- and electrospray, and offline fast mixing for HDX labeling. Reports of methods improvements in the LC-MS portions of the experiment included subzero cooling to minimize back exchange during LC/MS, descriptions of top-down HDX MS studies using ECD or ETD fragmentation where the goal is the elimination of proteolytic digestion and/or improvement to the spatial resolution of the exchange information, microfluidic systems for exchange, LC and MS, work related to improving separation and understanding LC column parameters, comparison of various platforms for HDX MS, and description of fine isotope structure in HDX MS. A number of software improvements was made including new software packages or updates to existing software. Other interesting studies, including methods descriptions, histidine exchange, and useful tools, were published.

**CONCLUDING REMARKS**

Methods developments and refinements in HDX MS technology have placed this tool in the hands of nonspecialists. There will no doubt be additional future improvements in the methodology, but even at the current stage, a great deal of studies are now possible by more people than ever before. The applications of the method are therefore expected to continue to be strong and even increase in coming years. When one considers the vast number of possible forms of proteins, some known but many unknown, perhaps HDX MS will never run out of proteins to study. It is imperative that those active in the field continue to expand the reach of the method, apply the method to more and more types of proteins, systems, and purposes, train new investigators, and push forward with more improvements. There are still many things that remain to be studied.

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Appendix III

Publication

Hydrogen Exchange Mass Spectrometry of Proteins at Langmuir Monolayers

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Supporting Information

ABSTRACT: Hydrogen exchange (HX) mass spectrometry (MS) is valuable for providing conformational information for proteins/peptides that are very difficult to analyze with other methods such as peripheral membrane proteins and peptides that interact with membranes. We developed a new type of HX MS measurement that integrates Langmuir monolayers. A lipid monolayer was generated, a peptide or protein associated with it, and then the monolayer-associated peptide or protein was exposed to deuterium. The deuterated species was recovered from the monolayer, digested, and deuterium incorporation monitored by MS. Test peptides showed that deuterium recovery in an optimized protocol was equivalent to deuterium recovery in conventional solution HX MS. The reproducibility of the measurements was high, despite the requirement of generating a new monolayer for each deuterium labeling time. We validated that known conformational changes in the presence of a monolayer/membrane could be observed with the peptide melittin and the myristoylated protein Aef-1. Results in an accompanying paper show that the method can reveal details of conformational changes in a protein (HIV-1 Nef), which adopts a different conformation, depending on whether or not it is able to insert into the lipid layer. Overall, the HX MS Langmuir monolayer method provided new and meaningful conformational information for proteins that associate with lipid layers. The combination of HX MS results with neutron or X-ray reflection of the same proteins in Langmuir monolayers can be more informative than the isolated use of either method.

Membrane proteins are involved in many cellular processes ranging from regulation, recognition, metabolism, transport, and signaling. Recent accounts indicate that ~58% of utilized drug targets were membrane proteins. Despite much effort focused on both transmembrane and peripheral membrane proteins, it has been difficult to obtain high-resolution structural information for many membrane proteins. Membrane protein structures account for ~2.5% of all coordinate files deposited in the Protein Data Bank (www.rcsb.org). The major obstacle to structural characterization of membrane proteins is often the membrane itself, which is generally not compatible with structural studies and many biophysical measurements. Solubilizing membrane proteins with detergents to make them compatible with aqueous buffers and methodologies is one alternative, but this is not always successful, and even when successful, questions can linger as to how detergents may alter protein structure. Analytical methods that make use of membrane mimetics (i.e., artificial membranes) are attractive alternatives, because the structure of the membrane protein is more likely to be preserved in the mimetic.

We have previously applied hydrogen exchange (HX) mass spectrometry (MS) to membrane proteins, primarily using liposomes or nanodiscs as the membrane mimetic. Other groups have also used HX MS for membrane proteins, utilizing detergents [see, e.g., refs 15−17], and liposomes [see, e.g., refs 18−20]. Each membrane mimetic has advantages and disadvantages. Detergents may force the protein into a non-native conformation and can suppress peptide ionization if not properly removed prior to electrospray. Creation of both liposomes and nanodiscs can be challenging. For liposomes, there can be issues with reproducibility; lipid membrane curvature effects (especially in vesicles, see refs 22 and 23 for review), and protein directionality, while nanodiscs have a background, undesirable protein component (the membrane stabilizing protein) and the lipid packing density is not easily modified.

The lipid packing density, the number of lipid molecules per unit area, is fluid in cellular membranes with some regions packed more tightly than other regions, often dependent on the lipid composition, degree of hydrocarbon chain saturation, percentage of cholesterol, and other factors. Some proteins may alter the packing density of surrounding lipids, perhaps as a result of conformational changes during function.
Langmuir monolayers are formed from amphiphilic molecules spread at an air/liquid interface. Monolayers comprised of lipids are analogous to one leaflet of a lipid bilayer membrane; that is, two monolayers make a membrane. When these monolayers are generated and maintained within a Langmuir trough, the user is able to finely control and change the lipid packing density throughout the experiment with a high degree of reproducibility. The advantage of fine control over the monolayer was recently utilized with neutron reflectometry (NR) to investigate the conformational changes in HIV-1 Nef upon association with monolayers of different lipid packing density. NR is capable of resolving and modeling an overall, monolayer-associated shape profile, but is silent to the finer details of dynamics and local conformation. The opportunity to combine local information provided by HX with global structural information by neutron or X-ray reflectivity is a great advantage. However, a disadvantage of Langmuir monolayers is that they are only half of a biological membrane and therefore applicable only for peripheral membrane proteins or peptides that interact with one leaflet of the membrane bilayer.

In the current work, we report on development of a new method for studying peripheral membrane proteins and peptides that interact with lipid layers using HX MS and Langmuir monolayers. We show that this method is capable of investigating protein/peptide conformations over a range of lipid packing density and compositions, and that it can be applied to proteins/peptides that anchor or interact with one face of a lipid bilayer. The method was validated by comparing solution HX and monolayer HX of membrane-binding peptides and proteins known to exhibit structural changes when associated with lipid layers. In an accompanying paper, we have used the HX MS Langmuir monolayer method to monitor the conformational changes of the HIV-1 Nef protein upon lipid interaction. Previous NR experiments identified a conformational change in HIV-1 Nef that is dependent on lipid packing density and the ability of the Nef protein to insert into the lipid layer. The Langmuir HX MS method proved to be particularly valuable for characterizing this lipid insertion event. Overall, we expect Langmuir monolayer HX MS methodology will be applicable to a large number of interesting proteins and systems.

### EXPERIMENTAL PROCEDURES

**Chemicals and Materials.** Melittin (≥98%, Catalog No. M2272), pepsin (porcine gastric mucosa, Catalog No. P6887), aspergillosepsin (Protease from Aspergillus saitoi, Catalog No. P2143), angiotensin I (≥90%, Catalog No. A9650), bradykinin (≥98%, Catalog No. B3259) and leucine enkephalin (≥95%, Catalog No. L9133) were purchased from Sigma–Aldrich (St. Louis, MO). Cathepsin B and cathepsin L were purchased from CalBioChem (La Jolla, CA). Protease inhibitors were purchased from Roche (Indianapolis, IN). Dithiothreitol and sodium dodecyl sulfate were purchased from Sigma (St. Louis, MO). Thiophenylacetyl-AMC, fluorescein isothiocyanate (FITC)-labeled casein, FITC-labeled BSA, FITC-labeled transferrin, and succinimidyl ester were purchased from Invitrogen (Carlsbad, CA). 

**Methods.**
Myristoylated Arf-1 Expression and Purification. Myristoylated human Arf-1 (myrArf-1)39 expression and purification was similar to that described previously for myristoylated HIV-1 Nef.39 The Arf-1 gene was purchased from Addgene (Plasmid No. 28168) and subcloned into the pET-Duet vector. The pET-Duet vector contained human N-myristoyltransferase 1 (hNMT1) in the first multiple cloning site and myrArf-1 with a C-terminal polyhistidine tag in the second multiple cloning site. Protein was isolated using Ni-NTA agarose (QIAGEN, Valencia, CA), washed and eluted as described for HIV-1 Nef.39 Purity and proper myristylation were confirmed by polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray mass spectrometry.

Solution Hydrogen Exchange. Solution hydrogen exchange experiments were carried out at room temperature (22 °C) by diluting peptide or protein stock solutions in equilibration buffer (50 mM citric acid, 50 mM sodium phosphate, 150 mM NaCl, pH 6.0, H2O) 15-fold with labeling buffer (50 mM citric acid, 50 mM sodium phosphate, 150 mM NaCl, pH 6.0, 99.8% D2O). Melittin buffers (both H2O and D2O) also contained 5 mM EDTA. Both Arf-1 buffers were adjusted to pH 7.0 and 2 mM magnesium chloride was added. Following dilution into D2O, samples were continuously labeled for predetermined times ranging from 10 s to 4 h before being quenched to pH 2.6 using a 0 °C solution of quench buffer (0.8% formic acid and 0.8 M guanidine hydrochloride in H2O). Quenched samples were digested on ice for 5 min by adding pepsin and aspergillopepsin (60 and 70 μg, respectively, dissolved in water). We found this digestion strategy and enzyme combination maximized the digestion of protein in the presence of lipid (further described below), so it was also used for solution digestion. Digested samples were injected into a sample tube at 0 °C using a Waters nanoAcquity UPLC with HX technology40 (Milford, MA) for desalting, separation, and mass analysis (details below).

Monolayer Hydrogen Exchange Experiments. Monolayer HX experiments utilized a modified Langmuir trough system (Figure 1) and the previously described protocol for generating the monolayer.79,80 The total volume of the trough (blue features in Figure 1) was 18 mL. All experiments were performed at room temperature (22 °C). For melittin experiments, 1,2-dipalmitoyl-sn-glycero-3-phosphoserine (DPPS) was used for the monolayer; for all other experiments, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) was used. Lipid was spread from a mixture of chloroform/methanol (70/30 by volume) onto the surface of aqueous subphase (equilibration buffer for each type of peptide/protein, as described for solution experiments) that had been placed in the trough. Once the chloroform and methanol had evaporated, the deposited lipids were compressed to a set pressure using a motorized, computer-controlled movable barrier. By controlling the position of the barrier via computer, the monolayer pressure could be fixed at any value. After the pressure had stabilized, peptide/protein was injected into the subphase beneath the lipid monolayer (to a final concentration of ~1 μM) using gel-loading pipet tips. If peptide/protein interacted with the lipids in the monolayer and inserted hydrophobic residues into the lipid tails, thereby crowding the monolayer and increasing the molecular packing density, the barrier position automatically adjusted to maintain constant pressure (see Figure S1 in the Supporting Information). The resulting barrier movement increased the surface area of the monolayer. Interaction of the protein with the Langmuir monolayer was allowed to proceed until the relative barrier position was <5 mm from the back edge of the trough. Then, 100 mL of labeling buffer were rapidly circulated through the trough using a peristaltic pump (Figure 1). The subphase exchange process took 10 s, after which the timing for continuous labeling experiments began. Adsorbed samples were deuterated for predetermined times ranging from 10 s to 4 h. After the labeling time, ~300 μL of monolayer, subphase buffer and the monolayer-associated protein were quickly vacuum-aspirated into a sample tube at 0 °C (Figure 1). Quench buffer (as used in solution labeling above) was quickly added (within <5 s) to drop the pH of the aspirated sample to 2.5−2.6. The quenched sample was digested for 5 min at 0 °C (all parameters identical to solution HX experiments) before injection into the UPLC-MS system.

Mass Analyses and Data Processing. Peptide separation was performed at 0 °C using a Waters nanoAcquity UPLC with HX technology40 (Milford, MA). Peptides were trapped on a Waters UPLC BEH C18 1.7 μm VanGuard BEH column and desalted with 0.1% formic acid in water for 3 min at 100 μL/min. To reduce the amount of lipids entering the mass spectrometer, an additional Vanguard BEH column was placed directly in line with the analytical column (Waters HSS T3 1.8 μm C18, 1.0 mm × 50 mm). This first trap captures the majority of lipids present in the sample and is replaced frequently or washed with chloroform overnight40 to remove lipids. Peptides were eluted over 6 min using a 5−35% gradient of water/acetonitrile with 0.1% formic acid flowing at a rate of 60 μL/min. Lipids that did elute only appeared at high acetonitrile concentrations when, for most samples, the LC system was disconnected from the mass spectrometer. Mass measurements were performed with a Waters Synapt G2 HDMS equipped with a standard ESI source and lock-mass correction using (+2) Glu-fibrinogen peptide. All mass spectra were acquired in MS2 mode39 and spanned a range of 50−2000 m/z. The peptic peptides from myrArf-1 were identified (Figure S2 in the Supporting Information) with a combination of exact mass measurements and MS/MS. Data processing to extract deuterium levels for each peptide was performed with DynamX software (Waters). All deuterium levels are reported as relative,40 and there were no corrections for back-exchange. Relative deuterium incorporation curves for identified peptides of myrArf-1 are provided in Figure S3 in the Supporting Information.

■ RESULTS AND DISCUSSION

Langmuir Trough Design and Circulation Testing. To perform HX MS with a Langmuir monolayer, the Langmuir trough used previously for NR studies66 was adapted to facilitate HX labeling experiments. The major modification allowed for efficient and quick exchange of the aqueous subphase (the buffer under the monolayer) for a deuterated labeling buffer. Several ports were machined into opposite sides of the trough and additional Teflon tubing was connected to a high-speed peristaltic pump to rapidly exchange the subphase
with fresh buffer (as shown in Figure 1). Circulation tests were performed to determine the smallest volume of subphase buffer required for complete subphase exchange. For these tests, buffer-containing dye was used and absorbance measurements of the subphase buffer were taken before and after circulation. The efficiency of subphase exchange was calculated using a ratio of the two absorbance measurements. Subphase exchange with 50 mL of buffer (~2 times the total volume of the trough and tubing) produced 83.8% exchange of the subphase. Circulating 100 mL of fresh buffer (4 times the volume of the trough and tubing) yielded 96.3% exchange of the subphase. With the peristaltic pump used, the minimum time required to circulate 100 mL of buffer without disturbing the monolayer was 10 s. For each labeling experiment (deuteration time point), a new monolayer was generated and 100 mL of new labeling buffer were circulated for 10 s. Thus, every data point was a biological replicate with a new lipid monolayer. This strategy required experiments to test the reproducibility of monolayer creation, lipid interaction, deuterium labeling, and quenching of the exchange reaction, as outlined below.

**Reproducibility and Deuterium Recovery of the Trough-Labeling Protocol.** After buffer exchange, the labeled species (peptide or protein) was aspirated directly from the trough into a cold sample tube and deuterium labeling was quenched. Some lipid molecules from the monolayer and subphase buffer were also present in each aspirated sample. The variables associated with aspiration were tested to determine the deuterium recovery, as a function of protocol, including D,O circulation, time, temperature, and quench efficiency. For this purpose, we utilized a solution of fully deuterated peptides to monitor deuterium loss in a manner similar to that commonly used to monitor deuterium loss in HX MS protocols (e.g., during sample handling, LC separation or mass analysis). A monolayer was prepared on equilibrium buffer and 100 mL of a mixture of deuterated leucine enkephalin, angiotensin I, and bradykinin (in labeling buffer) were circulated under the monolayer. The peptide mixture/monolayer was aspirated into the collection tube, quench buffer was added, and then the solution was immediately injected into the UPLC system (termed trough labeling). In parallel, the same deuterated peptide mixture was added to quench buffer directly without trough circulation or aspiration (termed solution labeling), or directly injected into the UPLC without quenching (termed direct injection). Note that, because of differences in timing, species in the trough could have been exposed for deuterium for up to 20 s at what we called a 10 s labeling-time point (up to 10 s of exposure during the 100 mL deuterated buffer circulation + 10 s of exchange before aspiration and quenching), while exchange in solution for 10 s was, in fact, exposure to deuterium for 10 s before quenching. We did not make a correction for this timing difference in any of the experiments. Using the trough protocol or a conventional solution protocol (Figure 2), there was little difference in the measured deuterium levels in leucine enkephalin, angiotensin I, or bradykinin, indicating that the additional sample handling steps in the trough protocol did not lead to more back-exchange of deuterium than a conventional solution labeling protocol. The error of triplicate measurements of deuterium uptake for the trough protocol was also very low (less than ±1%), indicating that reproducible monolayer creation (recall that each experiment was a new, unique monolayer), buffer exchange/circulation, aspiration, and quenching can all be achieved. These results show that the Langmuir monolayer trough system and associated protocol can provide both reproducible and reliable measurements of HX for peptides/proteins in the presence of a Langmuir monolayer.

**Validation with an Amphiphatic Peptide.** A major incentive for developing this Langmuir monolayer method for HX MS was to investigate membrane-related conformational changes in proteins and peptides. To test the experimental system with a simple model compound, we measured HX in the peptide melittin in the presence of a monolayer (trough labeling) and compared with HX in the absence of lipids (solution labeling). Melittin is a well-characterized, 26-residue peptide that is known to interact with lipid membranes. The amphipathic nature of melittin allows it to associate with lipid membranes, despite being soluble in water.

At higher concentrations, melittin can form pores in bilayers and also in monolayers. Because of the conformational changes melittin undergoes in the presence of lipids, which include creation of hydrogen bonds and secondary structure, it was an ideal candidate to test lipid-associated protection from HX.

We compared the deuterium incorporation for melittin in solution and associated with a monolayer of DPPS (historically, DPPS has been used for monolayer/membrane studies of melittin and, therefore, we used it rather than DPPG for all of the other studies reported here). First, melittin was labeled in solution for 10 and 30 s and the relative deuterium levels measured. Then, following these solution HX measurements, melittin was injected beneath a Langmuir monolayer at a pressure of 20 mN/m and allowed to adsorb for ~15 min. Melittin insertion into the monolayer was evidenced by the backward movement of the barrier, indicating an increase in surface area of the monolayer (data not shown). Once the barrier had migrated ~25 mm, deuterium was circulated,
Validation with a Protein. After validating that our Langmuir monolayer HX MS method could be used to monitor conformational changes and lipid-induced protection from exchange in a peptide, we sought to monitor a protein known to undergo significant conformational changes in the presence of membranes. We elected to use ADP-ribosylation factor 1 (Arf-1), a 20 kDa guanine-nucleotide-binding protein that is involved in vesicle formation and trafficking.\textsuperscript{37,44} Proper functioning of Arf-1 requires lipid association via membrane binding and nucleotide exchange. In addition to membrane anchoring via an N-terminal amphipathic helix, Arf-1 is myristoylated on the glycine residue at position 2, and this modification is crucial for membrane association.\textsuperscript{45} MyrArf-1 is a prototypical protein for a myristoyl switch mechanism, where the transition between two different conformations moves the myristoyl group from a position hidden within a hydrophobic pocket of Arf-1, when the protein is cytosolic and GDP-bound, to a position where the myristoyl group is inserted into the membrane when Arf-1 is GTP-bound and membrane-anchored.\textsuperscript{45} Nucleotide exchange in Arf-1 by a guanine exchange factor accompanies the switch mechanism.\textsuperscript{46,47} In the GDP-bound form, myrArf-1 associates with the membrane\textsuperscript{48} with the N-terminal portion (residues 1–16) interacting directly with the membrane and residues 17–177 facing the solvent.\textsuperscript{33,48,49} Other conformational changes can occur upon the switch from GDP-bound to GTP bound, as high-resolution structures of the GDP-bound soluble and GTP-bound membrane-associated forms show. The well-defined membrane association, clear conformational changes and available structural details for myrArf-1 made it a good model to validate our Langmuir monolayer HX system for studies of protein conformational changes upon membrane association.

HX of monolayer-bound myristoylated Arf-1 bound to GDP was performed in the trough system with a lipid packing pressure of 20 mN/m and deuteration incorporation was compared to HX of myrArf-1 in solution. Digestion of labeled and quenched myrArf-1 produced 32 peptides, resulting in 92\% coverage of the protein backbone (see Figure S2 in the Supporting Information). Overlapping peptides were found in almost all areas of the protein, and similar HX trends were observed in redundant peptides (a full dataset is shown in Figure S3 in the Supporting Information). All deuteration incorporation measurements were made in triplicate, again with a new monolayer (DPPG) spread for every data point. There were distinct differences in exchange, as a result of monolayer association (Figure 4a). In solution, residues 1–21 incorporated a high amount of deuterium at 10 s and deuteration remained high for all time points (Figure 4b(i)), indicative of a lack of structure in this region of myrArf-1. In the presence of the DPPG monolayer, this region of myrArf-1 showed a reduction of >5 Da in relative deuterium incorporation at 10 s, which is consistent with the stabilization or creation of structure. However, this region remained dynamic and incorporated as much deuterium as the solution state after 10 min (Figure 4b(i)). Similar HX results (early protection that changed to high deuteration) were observed in the amphi-α-helical sequences through stabilization with hydrocarbon staples,\textsuperscript{52} suggesting that the N-terminus of myrArf-1 is unstructured and highly dynamic in solution, but becomes partially ordered as a helix upon monolayer interaction, yet still remaining somewhat dynamic. This...
The hypothesis is consistent with published myrArf-1 data describing the formation and insertion of an N-terminal α-helix.\textsuperscript{33,51,52}

The myrArf-1 used here was bound to GDP, and we did not investigate the additional conformational changes that occur as a result of nucleotide exchange to GTP after membrane interaction by the myristoyl group.\textsuperscript{33} Nevertheless, we did observe that peptides in the switch 1 region of myrArf-1, residues 41–50 (Figure 4b(i)), and peptides encompassing the switch 2 region, residues 63–89 (Figure 4b(iii)), incorporated less deuterium at early labeling times in the monolayer-associated form. Peptides in areas involved in GTP binding showed increases in deuterium uptake after 4 h (see Figures 4a and 4b(iv)). In solution, these regions were more protected, exchanging, at most, one or two amide hydrogens for peptides between residues 121–135 and less than one amide hydrogen for residues 166–170 at 10 s (also see Figure S3 in the Supporting Information). Our results are in agreement with prior studies, where it has been shown that much of the GTP binding residues both switch regions and the C-terminal portion of myrArf-1 are exposed when membrane-bound, in order to interact with exchange factors and complete nucleotide exchange necessary for myrArf-1 function.\textsuperscript{33,51,53,54}

Overall, myrArf-1 provided good validation that changes in protein conformation upon membrane association could be measured and localized within the protein using our Langmuir monolayer HX MS system.

**CONCLUSIONS**

We have developed a new method for analyzing conformational features of membrane-associated peptides and peripheral membrane proteins by combining HX MS and Langmuir monolayers. We believe this to be the first description of using HX MS and Langmuir monolayers to study conformation during membrane association. A Langmuir trough setup that...
had been originally designed for neutron reflection studies was modified to rapidly introduce deuterated buffer under a monolayer where peptide/protein had associated. The strength of the interaction of the peptide/protein with the monolayer was sufficient enough that peptide/protein was not washed away during exchange of the subphase, giving us confidence that nonspecific or casual binding events were likely not responsible for the HX differences that were observed. The method had a deuterium recovery equivalent to that of solution HX experiments; the additional steps associated with the monolayer did not accelerate back-exchange. With this method, conformational changes could be monitored in peptides (melittin) and proteins (myrArf-1). The reported lipid-induced structural changes observed in these systems are consistent with what is predicted or known from other published data. An important advantage of using the Langmuir monolayer system is that the lipid packing density can be controlled and reproduced from monolayer to monolayer. For proteins that undergo conformational changes as a result of lipid packing density, this is a very valuable feature. In an accompanying paper, we show that when the packing density is altered, different conformations of the myrNef protein result.

Of course, monolayers are not membranes and so the question remains as to how well results obtained for monolayer interaction of proteins that interact with membranes in cells represent reality. An answer may be dependent on the particular protein. At one extreme, transmembrane proteins are likely not suited for analysis in this Langmuir monolayer system. At the other extreme, those peripheral membrane proteins that enter only one monolayer of a membrane may behave in very similar manners in Langmuir monolayers, as they do in the presence of a lipid bilayer. The behavior of (and validity of Langmuir monolayers for) proteins falling between the extremes is less certain, and more measurements are needed. It may be possible to perform HX with tethered lipid bilayers, rather than just monolayers, thereby allowing a much wider range of inserted or membrane-associated proteins to be investigated.

The aspiration step to remove sample from the trough requires some practice to reliably obtain only 300 μL and capture mostly monolayer and peptide/protein with minimal excess of subphase buffer. With practice, one can become quite good at the entire protocol, including aspiration, as demonstrated by the reproducibility of the measurements. During the course of the work reported here and in the accompanying paper, 48 independent Langmuir monolayers were prepared and analyzed (peptide standards (Figure 2) in triplicate; melittin (Figure 3) in triplicate for 2 time points, equaling a total of 6; myrArf-1 (Figure 4) in triplicate for 5 time points, equaling a total of 15; myrNef (accompanying paper) in triplicate, 4 time points at 2 pressures, for a total of 24). The deuterium measurement reproducibility from monolayer to monolayer was remarkably high and gave us high confidence in the measurements (triplicate results would not be as meaningful were the three MS measurements of deuterium obtained from one monolayer). As we have advocated, replication that recapitulates as much biology (in this case, fresh monolayers and protein binding) as possible results in the most meaningful results. One downside to the method is that 100 mL of 99.9% deuterium buffer were required per analysis (we consumed >5 L in all the work here); modifications to the trough and associated pump tubing could reduce the required volume (and cost) substantially. While lipids were present in each aspirate and were injected into the LC/MS system with every run, they were mostly captured by the inline traps and did not interfere with the signals of nearly all peptides. The exception to this were highly hydrophobic peptides that eluted very late in the gradient, when lipids were also eluting.

Another major advantage of this technique is the opportunity to combine global structural information from other techniques, such as neutron or X-ray reflection with local information from HX. Our Langmuir monolayer HX MS method was designed to be integrated with a neutron or X-ray reflection workflow. In such a scheme, protein association with monolayers can be monitored in the same trough described here using neutron or X-ray reflection, the profile of the protein, with respect to the monolayer obtained, packing density of the monolayer modulated (if desired), and the impact on conformation monitored. Once an interesting conformation is identified by neutron or X-ray reflection (or at any point, conformation or condition such as packing density), the protein could be labeled with deuterium right at the reflectometer and the sample passed to a UPLC-MS system for HX measurement. We believe there are significant benefits to such a strategy and the presented data provide strong justification for interrogating membrane protein association using both neutron reflection and HX MS, which we further describe and exemplify in the accompanying paper.

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Hydrogen exchange mass spectrometry of proteins at Langmuir monolayers

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SUPPORTING INFORMATION

Figures S1, S2, S3
Figure S1.
Change in surface pressure (black) and barrier position (red) in the trough during adsorption of myrArf-1 at low lipid packing monolayers. “I” represents when protein was injected underneath the monolayer, “D” represents the subphase exchange with D$_2$O, and “A” represents aspiration of the sample.

The surface pressure was held constant after addition of protein. The barrier gradually moved back as more protein associated and inserted residues into the monolayer. The barrier was stopped within a few millimeters before reaching the end of the trough. The surface pressure rose slightly after the barrier was stopped indicating that protein was still associating with the monolayer. Aspiration dropped the surface pressure quickly and the barrier rapidly moved forward in order to reestablish the preset surface pressure.
Figure S2.
Coverage maps of identified peptides (green bars) from myrArf-1 generated using a mixture of pepsin and aspergillopepsin, and followed during HX MS experiments. Reproducible peptides were found at least three times in quadruplicate runs. The sequence coverage and redundancy are indicated below the map.
Figure S3.
Deuterium incorporation curves for myrArf-1 comparing trough samples (red) and solution samples (blue). The average deuterium incorporation from at least three independent experiments is plotted with the error bars representing the spread of the measurements.
Appendix IV

Publication

Membrane-Associated Conformation of HIV-1 Nef Investigated with Hydrogen Exchange Mass Spectrometry at a Langmuir Monolayer

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4Supporting Information

ABSTRACT: In the companion paper to this work, we described development of a new type of hydrogen exchange (HX) mass spectrometry (MS) measurement that integrates Langmuir monolayers. With Langmuir monolayers, the lipid packing density can be reproducibly controlled and changed as desired. Analysis of HX in proteins that may undergo conformational changes as a function of lipid packing (for example, conformational rearrangements after insertion into a lipid layer) are then possible. We previously used neutron reflection to characterize just such a conformational change in the myristoylated HIV-1 Nef protein (myrNef): at high lipid packing density, myrNef could not insert into the lipids and maintained a compact conformation adjacent to the monolayer, whereas at lower lipid packing density, myrNef was able to insert. We now report that N-terminal arm residues, causing displacement of the core domain away from the monolayer. In order to locate where conformation may have been altered by lipid association, we applied the HX MS Langmuir monolayer method to myrNef associated with monolayers of packing densities identical to those used for the prior neutron reflection measurements. The results show that the N-terminal region and the C-terminal unstructured loop undergo conformational changes when associated with a low density lipid monolayer. The results are not consistent with the hypothesis of myrNef dimerization upon membrane association in the absence of other myrNef binding partners. The HX MS Langmuir monolayer method provides new and meaningful information for myrNef that helps explain necessary conformational changes required for function at the membrane.

Interrogating conformational changes in proteins when they interact with a lipid membrane is highly desirable. A well-known obstacle, however, to such structural characterization is the membrane itself, which is generally not compatible with structural studies and many biophysical measurements. In the highly likely event that high-resolution structural methods such as X-ray crystallography are unable to provide information, alternative methods have been utilized that are less susceptible to interference from the membrane. We have used neutron reflection (NR) methods‡§ to study peptides and proteins at lipid monolayers (one leaflet/half of a lipid membrane). While NR is capable of resolving and modeling an overall, lipid-associated shape profile, it is silent to the finer details of protein motion/dynamics and local conformation. The opportunity to combine the local information provided by other methods with global structural information by NR (or also X-ray reflection) is very attractive. To this end, we developed (described in an accompanying paper‡) hydrogen exchange (HX) mass spectrometry (MS) methods that use the same Langmuir monolayer trough system that is central to NR. This strategy was intended to combine information from NR and HX MS for a multifaceted and more-comprehensive characterization of protein conformation at membranes. Overall shape, distance from the monolayer, and nuclear density are given by the NR measurements, while protein dynamics, and location of protected/unprotected backbone amide hydrogen, are given by the HX MS measurements.

HX MS Langmuir monolayer data are obtained using the same Langmuir trough used to obtain NR data. This system allows for very fine and highly reproducible control over the packing density of the lipid layer. Unlike other parameters (e.g., lipid composition, headgroup charge, lipid tail chain length, etc.), lipid packing density is a parameter that is not easy to control in many membrane mimetics used for biophysical analyses. However, such control can be essential for monitoring conformational changes in proteins that are sensitive to lipid packing. In addition, it may also be possible to perform HX with tethered lipid bilayers, allowing a much wider range of inserted or membrane-associated proteins to be investigated.
In our recent analysis of the HIV-1 Nef protein, we observed a conformational transition that is sensitive to lipid packing. Biological evidence has shown that membrane association is important for the cellular functions of the 25 kDa myristoylated Nef protein from HIV-1 (myrNef), including interaction with various signaling molecules also localized to the cytoplasmic face of the plasma membrane and valuable information about myrNef at the membrane can rearrange its conformation but when lipid packing density was solution. The results reported here show that, indeed, new conformation at a monolayer with the conformation in transition that was dependent on lipid packing density. When possible for us to study the conformational transition in myrNef ∼70 Å away. Very importantly, however, was the discovery and characterization with NR of the conformational transition that was dependent on lipid packing density. When lipid packing density was low, myrNef was able to insert and rearrange its conformation but when lipid packing density was high, quite a different conformation was obtained. Development and validation of the Langmuir monolayer HX MS method, as reported in an accompanying paper, made it possible for us to study the conformational transition in myrNef in more detail and to compare the all-important functional conformation at a monolayer with the conformation in solution. The results reported here show that, indeed, new and valuable information about myrNef at the membrane can be obtained from HX MS in Langmuir monolayers.

■ EXPERIMENTAL PROCEDURES

Myristoylated Protein Expression and Purification. Myristoylated HIV-1 Nef (strain SF2) expression and purification were as described previously. Briefly, myristoylated Nef (myrNef) was expressed using a pET-DesT vector (obtained from the Wilboud laboratory) containing human N-myristoyltransferase 1 (hNMT1) in the first multiple cloning site and Nef with a C-terminal polyhistidine tag in the second multiple cloning site. Nef was isolated using Ni-NTA agarose (Qiagen, Valencia, CA), washed and eluted as described previously. Purity and proper myristoylation were confirmed by polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray mass spectrometry.

Solution Hydrogen Exchange. Solution hydrogen exchange experiments were carried out at room temperature (22 ºC) by diluting myrNef stock solutions in equilibration buffer (50 mM citric acid, 50 mM sodium phosphate, 150 mM NaCl, pH 6.0, H2O) 15-fold with labeling buffer (50 mM citric acid, 50 mM sodium phosphate, 150 mM NaCl, pH 6.0, 99.8% D2O). Both Nef buffers contained 1 mM dithiothreitol. Following dilution into D2O samples were continuously labeled for predetermined times ranging from 10 s to 1 h before being quenched to pH 2.6, using a 0 °C solution of quench buffer (0.8% formic acid and 0.8 M guanidine hydrochloride in H2O). Quenched samples were digested on ice for 5 min by adding pepsin and aspergillopepsin (60 μg and 70 μg, respectively, dissolved in water). Digested samples were injected into a Waters nanoAcquity UPLC with HX technology (Milford, MA) for desalting, separation, and subsequent mass analysis.

Monolayer Hydrogen Exchange Experiments. Monolayer HX experiments were performed as described in the accompanying paper. All myrNef experiments were performed at room temperature (22 ºC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) was used for the monolayer. MyrNef was injected under the monolayer to a final concentration of ∼1 μM and the barrier position and pressure were monitored (Figure 1). Interaction of the protein with the Langmuir monolayer was allowed to proceed until the relative barrier position was <5 mm from the back edge of the trough (for inserting species, see Figure 1a) or until it remained constant (for noninserting species, see Figure 1b). Then, 100 μL of labeling buffer were rapidly circulated, protein-isolated, and exchange-quenched, as described. The quenched sample was digested for 5 min at 0 °C (all parameters identical to solution HX experiments) before injection into the UPLC-MS system.

Mass Analyses and Data Processing. Peptide separation, mass analysis, and data processing were performed as described in the accompanying paper. The peptic peptides from myrNef are shown in the Supporting Information (Figure S1). Since all experiments were performed under very similar experimental conditions, all deuterium levels are reported as relative, and there were no corrections for back-exchange. The relative deuterium incorporation curves for the peptides of myrNef are provided in Figure S2 in the Supporting Information.

■ RESULTS AND DISCUSSION

Myristoylated Nef was subjected to HX both in the Langmuir trough system and in solution, and the results were compared. Nef was studied at two Langmuir monolayer pressures: 20 mN/m, where insertion occurs, and 35 mN/m, where insertion does not occur. As described in Akgun et al., the barrier position reports on how peptide/protein interacts with the lipids in the monolayer. If proteinaceous material inserts into the lipid layer, the monolayer becomes more crowded and increases the molecular packing density. To maintain constant pressure, the position of the barrier holding the monolayer in place must be adjusted (done automatically by computer). As expected, myrNef inserted into the monolayer at 20 mN/m (Figure 1a) but not at 35 mN/m (Figure 1b). These conditions are the same as those used for the previous NR experiments with myrNef. A total of 31 peptides were generated from digestion of myrNef that was aspirated from the Langmuir monolayer, resulting in 90% coverage of the protein backbone (see Figure S1 in the Supporting Information). Overlapping peptides were identified in almost all areas of the protein, and similar HX trends were observed in areas of redundancy (see the full dataset in Figure S2 in the Supporting Information). Deuterium incorporation was measured for at least triplicate biological replicates of myrNef with a fresh monolayer spread for every deuterium labeling time. While the reproducibility of HX MS measurements in Langmuir monolayers was discussed in detail.

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in the accompanying manuscript, the error bars in the deuteration incorporation graphs for each peptide (Figure S2 in the Supporting Information) demonstrate that reliable conclusions could be reached from the triplicate measurements. The exchange of myrNef was measured at pH 6.0, a pH at which the functional activity of Nef, as measured by Hck activation, was indistinguishable from that at pH 7.3 (Figure S3 in the Supporting Information) or higher (as shown in ref 22).

For the case of association with monolayers at a pressure of 20 mN/m, HX in myrNef increased in some regions and decreased in others, relative to that measured for myrNef in solution (Figure 2; also see Figure S4 in the Supporting Information). In solution, the core of myrNef did not incorporate much deuterium (e.g., Figure 2b(ii)) and, after 1 h of labeling, remained fairly protected from labeling, which was consistent with a well-folded structure with little protein dynamics, as seen previously by HX MS. Increased deuterium incorporation upon lipid association was observed principally in the core domain and some parts of the C-terminal region (Figure 2a). Lipid association did not appear to change the dynamics of the core as the slopes of the deuterium incorporation graphs were similar to those of myrNef in solution (also see ref 23). Lipid association exposed several backbone amide hydrogens in the Nef core: peptide 89−100 showed an increase of several Daltons (Figure 2b(iii)), as well as other peptides (e.g., residues 116−142) from within the Nef core (see Figure 2a, as well as Figure S2 in the Supporting Information). In solution, these residues were less deuterated, perhaps because of interaction with residues of the N-terminal arm.

Both the N-terminal myristoylated region and the C-terminal disordered loop of myrNef showed significant reduction in deuterium incorporation when the protein was associated with the Langmuir monolayer at 20 mN/m (see Figures 2a, 2b(i), 2b(ii), and 2b(iv)). This protection from labeling suggests either contact with and burial into the lipid layer, structural creation and stabilization, or a combination of both. Protection from exchange in the first ~20 residues is consistent with the hypothesis that both the myristic acid and residues from the N-terminus including a positively charged region between residues 17−22 insert into the membrane. Insertion of arm residues, in addition to the myristate group, is indicated by the large movement of the barrier (Figure 1a). In solution, residues next to the lipid binding region (e.g., peptides 35−51 and 35−58) were deuterated rapidly at 10 s (see Figure S2 in the Supporting Information), suggesting high solvent accessibility or a lack of structure, but, upon lipid association, displayed reductions of 2−3 Da in deuterium incorporation at 10 s (see Figure 2a). An α-helical sequence (αH2) is located within that span of residues, and we speculate that this helical region may become stabilized when the N-terminal arm separates from the core upon membrane binding. Reduced deuteration of the C-terminal disordered loop of lipid-associated myrNef (e.g., residues 147−173) suggests that some backbone amide hydrogens in the C-terminal loop become protected, perhaps by hydrogen bonding with the core of Nef, or become more stabilized. Arolld and Baur predicted that, upon binding to the membrane, Nef would adopt a "signaling" conformation where the C-terminal loop wrapped around the core domain. Interactions with the core domain, they argued, would avoid rapid endocytosis and removal from the membrane, while other events such as phosphorylation or binding to another protein could trigger the loop to become exposed. The observed protection of the C-terminal loop in our HX MS data agrees with this earlier prediction.

Recently, a structure of HIV-1 Nef in complex with the AP-2 clathrin adaptor complex was reported that contained the loop region. In that structure, two helical regions within the loop are apparent: a helix from residues 150−157 (αH4) and another single turn helix from 167 to 170 (αH5). αH5, along with a series of turns at the C-terminal end of the loop (residues 171−179), are located between AP-2 α−α and the Nef core, with αH5 packing against the β-sheet of the core and the turn-rich section of the loop from 171 to 179 anchored by a hydrogen bond between Asp174 and Gln104 and by a salt bridge between Asp173 of the loop and Nef Arg134 of the core.
β-sheet. It was concluded that these interactions between loop and core play an important role in organizing this region of the loop. Our HX results are consistent with interactions between loop and core upon membrane binding when the N-terminal arm separates from the core.

In addition to providing new insight into the disposition of the C-terminal loop, these HX MS data strongly suggest that myrNef does not dimerize through its core upon membrane binding, since the residues on the core domain show more deuterium uptake relative to the solution form, rather than less deuterium, as would be expected for dimer-induced protection.

Dimerization of Nef has been identified in cells and shown to be critical for down-regulation of CD4 receptors. We have seen no evidence of dimer formation in previous solution HX MS measurements of either myrNef or nonmyristoylated Nef. Crystal structures of truncated Nef as a dimer in complex with SH3 and SH3–SH2 domains of Src family kinases have been reported. The dimerization interface between the two core domains is different in these two structures, which is consistent with the hypothesis that Nef dimerization is driven by interaction with the host protein and not by an inherent affinity of the core domain for itself. The present HX MS data, showing strong evidence that the core regions of Nef do not come together in a dimer when associated with the monolayer, support this host-protein-dependent dimerization hypothesis.

With the Langmuir trough system, the pressure of the monolayer is measured continuously (Figure 1) and can be controlled by manipulating the barrier position. Therefore, HX can be performed at various pressures that can be generated very reproducibly. We used this strategy to compare HX in myrNef when associated with a DPPG monolayer with a pressure of 20 mN/m (as above, Figure 2) versus a monolayer with a pressure of 35 mN/m. At higher lipid packing density, the arm of myrNef is unable to insert into the monolayer and...
myrNef remains in a closed conformation directly against the lipid headgroups, as shown previously by NR.5 A model of myrNef conformation with either low (20 mN/m) or high (35 mN/m) lipid packing density, based on the NR data, is shown in Figure 3a. At higher lipid packing density, residue insertion at the monolayer was prohibited and greatly altered the HX of myrNef; deuterium incorporation was significantly higher throughout the protein at 20 mN/m versus 35 mN/m (Figure 3b). In addition, the HX of myrNef at the 35 mN/m monolayer was not identical to that of myrNef in solution (full dataset shown in Figure S2 in the Supporting Information). There was less deuterium in residues 1−83 and 146−184 of myrNef associated with the 35 mN/m monolayer, compared with myrNef in solution, implying stabilization of these parts of the structure, protection by the lipid layer, conformational rearrangements, or all three. The deuteration levels of residues 84−145 and 185−210 were largely the same at the two monolayer pressures. MyrNef with the 20 mN/m monolayer had deuterium levels in residues 1−83 and 146−184 that were intermediate between the 35 mN/m monolayer and solution HX measurements. Our interpretation of these results is that, when myrNef associates with the monolayer at higher pressures (35 mN/m) through interaction of the positively charged residues (17−22) with the negatively charged headgroups, the arm partially dissociates from the core, allowing the C-terminal loop to associate with the core. Yet, because the lipid packing is too high for αH1 (residues 6−22) to insert, myrNef remains in a closed conformation directly against the lipid layer in an orientation such that the N-terminal portion (residues 30−84) and the C-terminal loop are occluded from the solvent by proximity to the monolayer. When the monolayer pressure is lower (20 mN/m) and αH1 can insert into the monolayer, the N-terminal arm releases completely from the core and the conformation becomes extended (as shown on the left of Figure 3a). In that case, more deuterium can be incorporated into the N-terminal region and the C-terminal loop (Figure 3b), although not as much as for the solution form, because of the interaction of the loop with the core in the monolayer-bound state.

**CONCLUSIONS**

The newly developed method for analyzing conformational features of membrane-associated peptides and peripheral membrane proteins combines HX MS and Langmuir monolayers. An advantage of using the Langmuir monolayer system is that the lipid packing density can be controlled and reproduced from monolayer to monolayer. For proteins that undergo conformational changes, as a result of lipid packing density, this is a very valuable feature. We showed how packing density could be altered by and result in different conformations of the myrNef protein. Our myrNef Langmuir monolayer HX exchange data support and expand upon previous conformational studies by neutron reflection.5 In particular, these data provide the first direct evidence that that
the C-terminal loop is occluded from solvent in the monolayer-associated and open conformation form (as predicted for the "signaling" form by Arold and Baur (10) and that myrNef does not dimerize upon associating with membranes. The latter suggests that dimerization of myrNef is driven by interaction with host proteins, something that could now be examined directly with the HS MS Langmuir system.

Another major advantage of using HS MS at Langmuir monolayers is the opportunity to combine global structural information from techniques such as neutron or X-ray reflection with more local information from HS. Our Langmuir monolayer HS MS method was designed to be integrated with a neutron or X-ray reflection workflow. In such a scheme, protein association with monolayers can first be investigated with the same trough described here using neutron or X-ray reflection, the profile of the protein (with respect to the lipid layer obtained), packing density of the monolayer modulated (if desired), and the impact on conformation monitored. Once an interesting conformation is identified by neutron or X-ray reflection (or at any point, conformation, or condition such as packing density), the protein could be labeled with deuterium right at the reflectometer and the sample passed to a nearby UPLC-MS system for HX measurement. We believe there are significant benefits to such a strategy and the presented data provide strong justification for interrogating membrane protein association using both neutron reflection and HS MS.

### ASSOCIATED CONTENT

#### Supporting Information

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**Notes**

The authors declare the following competing financial interest(s): J.R.E. is a consultant of the Waters Corporation.

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The membrane-associated conformation of HIV-1 Nef investigated with hydrogen exchange mass spectrometry at a Langmuir monolayer

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SUPPORTING INFORMATION

Figures S1, S2, S3 and S4
Figure S1.
Coverage map of identified peptides from myrNef generated using a mixture of pepsin and aspergillopepsin, and followed during HX MS experiments. Reproducible peptides (blue bars) were found at least three times in quadruplicate runs. The sequence coverage and redundancy are indicated below the map.
Figure S2.
Deuterium incorporation curves for myrNef comparing exchange in the trough systems with two lipid packing densities (red & green) versus exchange in solution (blue). All measurements shown were performed at pH 6.0. The average deuterium incorporation from three independent experiments is plotted with the error bars representing the spread of the measurements.
Figure S3.
Nef retains function at both pH 6.0 and pH 7.3. The activity of Hck-YEEI was measured in the Z’Lyte \textit{in vitro} kinase assay as a reporter for Nef functionality, as described in Emert-Sedlak et al. (2009). \textit{ACS Chem. Biol.} 4(11), 930-947. (a) Hck activation at pH 6.0. Hck-YEEI was titrated into the assay alone or with a ten-fold molar excess of Nef (myr or non-myr). Maximum activation was realized at 64 ng/well with both constructs. (b). The same assay was repeated at pH 7.3 to mimic the conditions used for some previous solution HX experiments. Maximum Hck-YEEI activation was also realized at 64 ng/well for both constructs.
a. Solution

Figure S4.

(a). The relative percent deuterium incorporation for myrNef in solution. Time in deuterium is shown at the top and % relative deuterium is colored on the structure according to the scale shown. These results are derived from the data shown in Figure S2.
Figure S4 (continued).
(b). The relative percent deuterium incorporation for myrNef with a Langmuir monolayer at 20 mN/m. Time in deuterium is shown at the top and % relative deuterium is colored on the structure according to the scale shown. These results are derived from the data shown in Figure S2.
(c) The effect of lipid association on myrNef deuteration. The average amount of deuterium (in Da) for HX in solution was subtracted from the average amount of deuterium after HX in the trough (monolayer associated 20 mN/m) and the value colored (positive values in reds, negative values in blues, as indicated – i.e. blue: less deuterium when with lipid, red: more deuterium when with lipid). Time in deuterium is shown at the top. These results are derived from the data shown in Figure S2 as summarized in Figure 2a.
**d. with monolayer, 35 mN/m**

![Diagram showing relative percent deuterium incorporation for myrNef with a Langmuir monolayer at 35 mN/m. Time in deuterium is shown at the top and % relative deuterium is colored on the structure according to the scale shown. These results are derived from the data shown in Figure S2.](image)

**Figure S4 (continued).**

(d). The relative percent deuterium incorporation for myrNef with a Langmuir monolayer at 35 mN/m. Time in deuterium is shown at the top and % relative deuterium is colored on the structure according to the scale shown. These results are derived from the data shown in Figure S2.
Figure S4 (continued).
(e). The effect of lipid packing density on myrNef deuteration. The average amount of deuterium (in Da) after HX using a monolayer pressure of 35 mN/m was subtracted from the average amount of deuterium after HX using a monolayer pressure of 20 mN/m and the value colored (positive values in reds, as indicated). Time in deuterium is shown at the top. These results are derived the data shown in Figure S2 as summarized in Figure 3b.