# 28

# Hydrogen/Deuterium Exchange Mass Spectrometry: Potential for Investigating Innate Immunity Proteins

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## 1 Introduction

Innate immunity has evolved to provide a rapid, non-specific defensive response to invading pathogens (Kenzel and Henneke 2006). As central participants in the innate immune response, proteins mediate both the initiation and propagation of the defense reaction through their interactions with pathogen-derived macromolecules and other host proteins. Although the complement system constitutes an important line of defense, unregulated or misdirected activation of the components of innate immunity is detrimental and is associated with disease states, including autoimmunity and immune-mediated tissue injury. (Carroll and Holers 2005). Therefore, great effort has been expended to structurally characterize the molecular determinants of proteins of the innate immunity system that dictate their functions. Understanding protein structure and intermolecular interactions are key steps not only in delineating the mechanisms of innate immunity but also in the development of therapeutics that can modulate these interactions. Despite active investigation, a precise structural characterization of many such proteins remains elusive.

The gold standard for describing the static structure of macromolecules is X-ray crystallography. Crystallographic models are capable of providing precise descriptions of protein structure and, in the case of macromolecular complexes, intermolecular interfaces in the solid state. Unfortunately, this technique is often hindered by the inability to generate well-diffracting crystals, particularly in the case of heavily glycosylated proteins, flexible modular proteins or protein complexes. Once obtained, these solid-state models are snapshots of the single protein conformation and they do not necessarily provide insight into the range of structural fluctuations that proteins may adopt in solution. Filling this void, hydrogen/deuterium exchange has emerged as a complementary and powerful technique. This approach is capable of providing

not only insight into the structure of proteins in solution but also information regarding intermolecular interactions in the liquid phase (Busenlehner and Armstrong 2005; Hoofnagle, Resing, and Ahn 2003; Lanman and Prevelige 2004; Wales and Engen 2006).

The amide backbone of proteins provides a unique tool for investigating protein structure. The backbone hydrogen atoms, bound to the amide bond nitrogen atoms, are capable of exchanging with water-derived hydrogens in solution at neutral pH. Hydrogen/deuterium exchange is a method for measuring this exchange process and is accomplished by substituting deuterium oxide ($D_2O$) for water in the buffer of the protein. Amide hydrogen atoms exchange with solvent deuterium atoms in a time-dependent fashion, the rate of which is influenced by their local environment. Solvent-exposed amide hydrogens undergo exchange with an average rate of $k_i \approx 10\ s^{-1}$ at neutral pH, while amide hydrogens that are buried in the protein interior and/or participating in hydrogen bonds have a significantly slower exchange rate ($k_i \approx 10\ s^{-9}$ at neutral pH), or do not exchange at all (Busenlehner et al. 2005; Zhang and Smith 1993). Thus, the rate of exchange for backbone amide hydrogens provides information about the solvent accessibility of these amides, and thus insight into the structure, together with the dynamics of the protein.

Measuring the kinetics of hydrogen/deuterium exchange requires an analytical technique capable of providing exchange information about individual, or small stretches of, amide hydrogens on the protein backbone. Traditionally, nuclear magnetic resonance (NMR) spectroscopy was the technique of choice for following the exchange process (Englander and Kallenbach 1983; Englander, Mayne, Bai, and Sosnick 1997). However, NMR spectroscopy requires a relatively high concentration of protein, and assignments are difficult when protein masses exceed 50 kDa. With the recent advances in mass spectrometry, electrospray ionization (ESI) mass spectrometry and matrix-assisted laser desorptive-ionization (MALDI) mass spectrometry have emerged as alternate methods for monitoring hydrogen/deuterium exchange rates (Eyles and Kaltashov 2004). These techniques are capable of providing similar resolution of the exchange reaction and do not suffer from the protein size limits that are applicable to NMR spectroscopy.

In hydrogen/deuterium exchange mass spectrometry (HDXMS), proteins are exposed to $D_2O$ for varying amounts of time; aliquots are removed and quenched at pre-defined time points and then analyzed by mass spectrometry (Fig. 1). In the quench step, the pH of the aliquot is adjusted to pH ≈ 2.4, and the temperature is reduced, both serving to slow down non-specific exchange and “lock on” the deuterium atoms already incorporated. To achieve spatial resolution during the mass spectrometry step, proteins are subjected to rapid proteolysis, often with an acid-stable enzyme such as pepsin, and then analyzed (Cravello, Lascoux, and Forest 2003). In MALDI mass spectroscopy, aliquots are typically analyzed directly after proteolysis, without further chromatography or purification. When ESI mass spectroscopy is utilized, the incorporation of a brief liquid chromatography step prior to mass spectrometric analysis can further improve the resolution of the analysis. The data obtained through this analysis allows one to measure the change in m/z

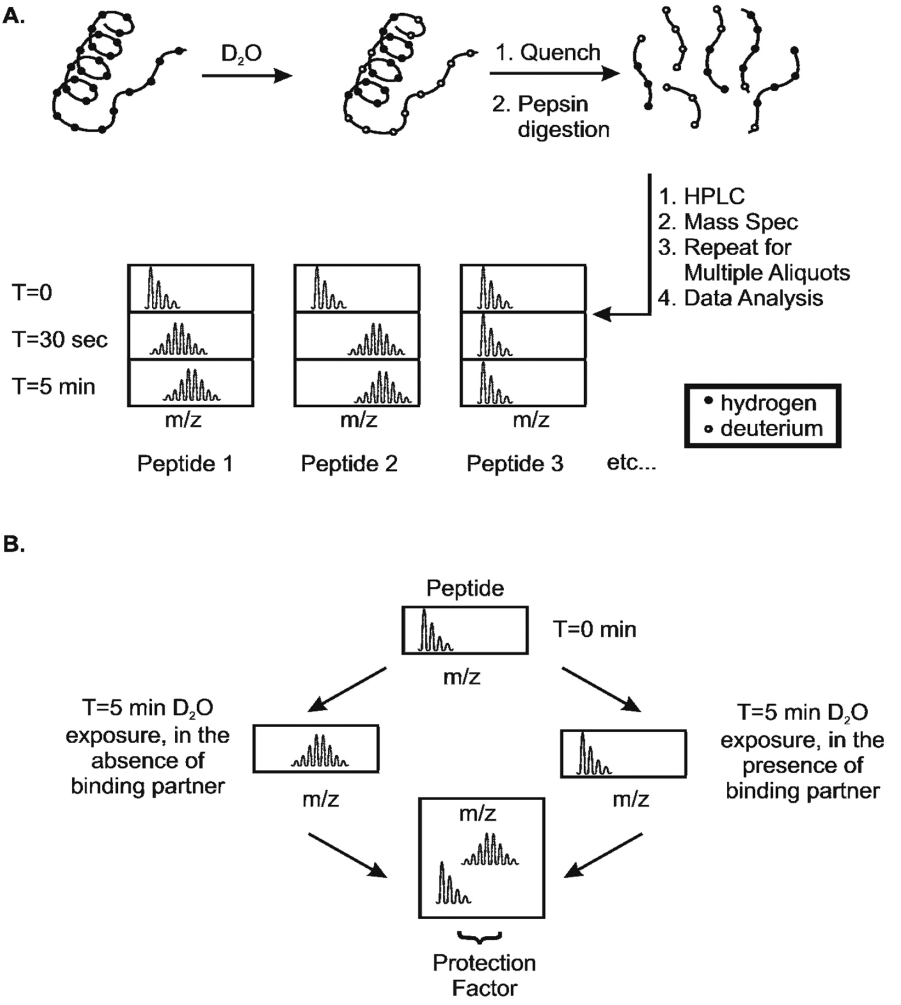


**Fig. 1A.** General overview of the hydrogen/deuterium exchange mass spectrometry method revealing expected mass envelope shifts for moderate (peptide 1), fast (peptide 2) and slow (peptide 3) exchanging peptides from a protein. **B.** Mass envelope shift for a single peptide derived from a protein involved in ligand binding. When the protein is incubated with D2O in the absence of a known ligand, there is an envelope shift for the peptide, suggesting deuterium exchange. When the protein is incubated with D2O in the presence of a known ligand, the same peptide is protected from deuterium exchange and does not readily undergo mass shift, suggesting that the peptide is shielded from solvent exposure as a result of ligand binding. This difference in mass shift between the bound and unbound states provides a quantitative measure of solvent protection, sometimes referred to as the protection factor.

(mass-to-charge ratio) for individual peptides as a function of time. Peptides that have a greater increase in m/z as a function of time, representing peptides with greater deuterium incorporation, are presumed to originate from less ordered and more solvent exposed environments. Although individual peaks yield the *average* deuterium uptake for a given peptide, the simultaneous analysis of multiple overlapping peptides provides resolution at nearly the amino acid level. {Peaks in the mass spectra are correlated with specific peptide stretches in the protein by performing MS/MS analysis of enzymatically cleaved proteins in their fully protonated state, using proteomic methods that are now routine (Domon and Aebersold 2006).}

HDXMS is a powerful technique that has been used to study a wide variety of proteins and macromolecules of varied sizes. Surprisingly, it has not found significant appreciation in the investigation of mechanisms of innate immunity, although it is capable of providing the precise structural information that is lacking for many of the proteins and protein complexes in this field. In this short review, we will highlight recent applications of HDXMS for the study of protein structure, protein-ligand interactions and protein-protein interactions in non-innate immunity systems. We will conclude by discussing our experience in using this technique to investigate the structure of complement protein C3 and its hydrolytic product C3($H_2O$).

## 2 Applications of HDXMS

### 2.1 Protein Structure: HIV and SIV Nef Proteins

The Nef protein of the human (HIV-1 and HIV-2) and simian (SIV) immunodeficiency viruses is an important factor in the pathogenesis of infections caused by these viruses. By enhancing viral replication, downregulating receptors on immune cells and activating specific host signaling pathways, Nef promotes high viral loads and is necessary for progression to AIDS (Arold and Bauer 2001; Renkema and Saksela 2000). Although HIV Nef and SIV Nef both bind to the Hck tyrosine kinase, the mechanism of these interactions varies; HIV Nef interacts with the SH3 domain, whereas SIV Nef interacts with the SH2 domain of this tyrosine kinase. How the Nef proteins from these two viruses differ in structure is unknown, as no structural information is available for SIV Nef. Although HIV Nef has been investigated with a combination of techniques, including X-ray crystallography (Arold, Franken, Strub, Hoh, Benichou, Benarous, and Dumas 1997; Lee, Saksela, Mirza, Chait, and Kuriyan 1996) and NMR spectroscopy (Grzesiek, Bax, Clore, Gronenborn, Hu, Kaufman, Palmer, Stahl, and Wingfield 1996; Grzesiek, Bax, Hu, Kaufman, Palmer, Stahl, Tjandra, and Wingfield 1997), these studies have been hampered by the idiosyncrasies of the protein. Full-length HIV Nef could not be crystallized, and crystallographic data are available only for deletion mutants containing residues 54-205 and 58-206. Even then, residues 54-69 and 149-178 are disordered in both crystal structure models. Similarly, because Nef forms multimers at the concentrations required for NMR studies, NMR spectroscopy has been accomplished only for a

deletion mutant of HIV Nef that is missing residues 2-39 and 159-173. Despite these limitations, a structural model of HIV Nef was developed from the available information (Geyer, Fackler, and Peterlin 2001). To better characterize the structure of HIV Nef and to gain insight into the structure of SIV Nef, Hochrein et al. took advantage of HDXMS to study both full-length proteins (Hochrein, Wales, Lerner, Schiavone, Smithgall, and Engen 2006).

Using an ESI-based HDXMS method with inline pepsin digestion, Hochrein and coworkers obtained deuterium exchange data for more than 85% of the primary sequence of both the HIV and SIV Nef proteins. Given the sensitivity of the instrumentation, they could utilize low concentrations of proteins, avoiding the aggregation that had prohibited NMR analysis of the full-length protein. With regard to HIV Nef, their results were in complete agreement with the pre-existing structure model; exchange was slow in areas shielded from the solvent and rapid in areas of predicted solvent exposure. It is particularly interesting that the exchange data from a central loop of the protein, obscured in the crystallographic map and deleted in the NMR experiments, suggested that this region was protected from solvent and likely possessed structure. When compared to HIV Nef, SIV Nef had a similar deuterium incorporation profile but appeared to have greater dynamics in solution.

This example of an HDXMS application confirms several key features of the method. Most importantly, it allowed for the structural characterization of full-sequence HIV Nef, which was not amenable to investigation by X-ray crystallography or NMR spectroscopy. Conversely, the HDXMS technique was itself validated by its agreement with the available structural model. Finally, using this technique, the authors were able to gain insight into a related but previously uncharacterized protein, SIV Nef.

## 2.2 Protein-Protein Interactions: Anthrax Lethal Factor

*Bacillus anthracis*, the organism that causes anthrax, secretes three monomeric proteins that mediate its toxicity toward host cells. Interestingly, these monomeric proteins participate in a series of intermolecular protein-protein interactions with one another, which facilitates entry of the toxins into host cells (Bradley, Mogridge, Mourez, Collier, and Young 2001; Molloy, Bresnahan, Leppla, Klimpel, and Thomas 1992; Scobie, Rainey, Bradley, and Young 2003). Assembly of the toxin begins when one of these secreted proteins, called protective antigen (PA), binds to host-cell surface receptors and is proteolytically cleaved. The cleaved, receptor-bound PA fragment can then bind additional PA proteins and spontaneously form the circular, homo-heptameric pre-pore. The pre-pore is capable of binding up to three copies of either of the two remaining secreted proteins, edema factor (EF) or lethal factor (LF). It has been suggested that EF and LF both bind to the pre-pore via their N-terminal 250 residue domains, termed $EF_N$ and $LF_N$, in a competitive fashion (Elliott, Mogridge, and Collier 2000). The resulting pre-pore-EF or pre-pore-LF complexes are then internalized into the endosomal compartment, where the factors dissociate and the pre-pore forms a membrane-spanning pore. The factors EF and LF can then

translocate into the cytosol, where they exert their toxic effects (Zhang, Finkelstein, and Collier 2004).

Understanding the protein-protein interactions that mediate this process has the potential to contribute to the development of anthrax-directed therapeutics. Previous work in this area, using mutagenesis, has indicated that a small patch of seven residues on $EF_N/LF_N$ is responsible for binding to the pre-pore (Lacy, Mourez, Fouassier, and Collier 2002). However, complementary studies with the pre-pore revealed a much larger footprint on the pre-pore that was binding to $EF_N/LF_N$ (Cunningham, Lacy, Mogridge, and Collier 2002). Melnyk et al. utilized HDXMS to better define the interacting surface between PA and $LF_N$, and with this information, they performed directed mutagenesis to identify critical residues in the pre-pore-$LF_N$ interaction (Melnyk, Hewitt, Lacy, Lin, Gessner, Li, Woods, and Collier 2006).

Utilizing an ESI-based HDXMS technique with inline pepsin digestion, Melnyk and colleagues studied the termolecular complex of $LF_N$ with two molar equivalents of trypsin-activated PA. Under optimized conditions, they obtained deuterium exchange data for over 75% of the primary sequence of $LF_N$. In the absence of PA, HDXMS analysis revealed rapid deuterium uptake in an area of $LF_N$ that is known from the X-ray crystal structure to be surface-exposed. In the presence of two equivalents of activated PA, four discrete sites on $LF_N$ (residues 95-120, 137-147, 177-189, and 225-235) were protected from the solvent. These residues formed one continuous surface on $LF_N$, which was larger than the protein-protein interface surface previously identified through mutagenesis studies. To better define the residues that are critical for binding, they prepared 27 mutants of $LF_N$ by incorporating single amino acid substitutions into the observed interface region. When assayed for their ability to bind cells in the presence of PA, binding-defective mutants revealed the importance of multiple amino acids, including Asp-182 and Glu-135, which were separated by a larger-than-expected ~40 Å.

As in the case of the previously described study, the work by Melnyk et al. validates the HDXMS technique in terms of the crystal structure model of $LF_N$ and the pattern of deuterium uptake. In addition, by comparing the deuterium uptake profile of the protein in the absence and then the presence of its binding partner, the authors were better able to describe the interaction surface between $LF_N$ and its binding partner. Additional use of site-directed mutagenesis allowed them to define which residues that are important in binding and to obtain valuable structural information regarding this critical interaction.

## 2.3 Protein-Ligand Interactions: PPARγ

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-dependant transcription factor whose actions influence glucose homeostasis and adipocyte differentiation (Gampe, Montana, Lambert, Miller, Bledsoe, Milburn, Kliewer, Willson, and Xu 2000; Yang, Rachez, and Freedman 2000). Because nuclear receptors such as PPARγ modulate fundamental biological processes such as metabolism and cellular differentiation, they are natural targets for drug development

(Olefsky and Saltiel 2000). PPARγ itself is a key regulator of glucose and lipid homeostasis and is a target of the anti-diabetes drugs rosiglitazone and pioglitazone. In addition, several agonists, partial agonists and antagonists of PPARγ have been synthesized (Berger et al. 2003; Brown et al. 1999; Leesnitzer et al. 2002).

It is known that nuclear receptors such as PPARγ undergo conformational changes upon ligand binding (Kallenberger, Love, Chatterjee, and Schwabe 2003). While the PPARγ full agonists rosiglitazone and pioglitazone are effective drugs in the treatment of diabetes mellitus, they cause undesirable side effects, such as edema and weight gain. Interestingly, a partial-agonist of PPARγ, nTZDpa, induces fewer side effects. While an X-ray crystal structure model of the ligand binding domain (LBD) of PPARγ with a partial agonist exists (Oberfield, Collins, Holmes, Goreham, Cooper, Cobb, Lenhard, Hull-Ryde, Mohr, Blanchard, Parks, Moore, Lehmann, Plunket, Miller, Milburn, Kliewer, and Willson 1999), such structural information for the LBD of PPARγ with agonists and antagonists is not available. Knowledge of the extent to which full agonists, partial agonists and antagonists influence PPARγ structure, and thus function, could be useful in the development and screening of new medications targeted toward nuclear receptors.

Hamuro and coworkers (Hamuro, Coales, Morrow, Molnar, Tuske, Southern, and Griffin 2006) have characterized the effects of agonists, partial agonists and antagonists on PPARγ structure using HDXMS. Utilizing an ESI-based approach with inline pepsin digestion, they characterized the deuterium exchange profile of the LBD of PPARγ alone and also in the presence of two full agonists (including rosiglitazone), the partial agonist nTZDpa, and a covalently binding antagonist. They identified 49 peptides for which they could monitor the deuterium uptake, spanning 97% of the PPARγ LBD sequence.

In the absence of ligand, the deuterium uptake profile of the PPARγ LBD was consistent with its known structure. When the PPARγ LBD was analyzed in the presence of either of the two full agonists tested, the deuterium uptake was remarkably different. Specifically, the exchange profile was dramatically slowed in nine segments, suggesting that multiple areas of the protein became less flexible/solvent-exposed, including the entire binding cavity, after binding the agonist. In contrast, data obtained with the PPARγ LBD in the presence of an antagonist revealed a slowing of exchange in only five segments. The partial agonist had the smallest structural perturbation, decreasing the exchange in only three segments. All four ligands slowed the exchange at residues 281-287; however, only the two agonists slowed the exchange at residues 445-451 and 472-477. From this information, the authors concluded that the full agonists stabilize the PPARγ LBD more fully than do the partial agonist or antagonist. In addition, given the unique ability of the full agonists to stabilize the two stretches (445-451 and 472-477) in the LBD, these data provide the foundation for future structure-function research with this important class of nuclear receptors.

## 3 HDXMS Utilized to Explore the Structures of C3 and C3($H_2O$)

The complement system is an important component of innate and acquired immunity and a primary contributor to the inflammatory response. It consists of approximately 30 proteins that generally circulate in the blood in inactive forms (zymogens). Upon activation by one of three known pathways, the classical, lectin or alternative pathways, the active complement proteins assume varied roles, functioning as fluid-phase or cell-bound enzymes, chemoattractants, opsonins and cytolytic factors (the membrane attack complex) (Walport 2001a, 2001b). Complement protein C3 plays a central role in complement activation and is capable of binding to over 20 different proteins (Lambris 1988; Sahu and Lambris 2001). As a result of enzymatic activity occurring within the complement cascade, C3 undergoes significant conformational changes as it is activated and then degraded (Becherer, Alsenz, and Lambris 1990).

In addition to the changes in C3 structure that are mediated by its interactions with other proteins, C3 undergoes conformational changes during its non-enzymatic conversion to C3($H_2O$). C3($H_2O$) is the product of spontaneous hydrolysis of the C3 thioester bond. C3($H_2O$), through the binding to factor B, forms the alternative complement pathway convertase C3($H_2O$)Bb (Fishelson, Pangburn, and Mullereberhard 1984; Xu, Narayana, and Volanakis 2001). Because the alternative pathway convertase is implicated in several autoimmune diseases, there is interest in characterizing the molecular determinants that lead to its formation (Thurman and Holers 2006). X-ray crystal structures have been reported for C3 and its breakdown products C3c and C3d, but no crystal structure has been published for C3($H_2O$) (Janssen, Huizinga, Raaijmakers, Roos, Daha, Nilsson-Ekdahl, Nilsson, and Gros 2005; Nagar, Jones, Diefenbach, Isenman, and Rini 1998).

To better characterize the structural changes that occur with the formation of C3($H_2O$), our laboratory utilized MALDI-based HDXMS to investigate the structural differences between C3 and C3($H_2O$) (Winters, Spellman, and Lambris 2005). To begin these studies, we utilized ESI mass spectrometry to identify 354 peptides produced from the pepsin-catalyzed digestion of C3. The identified peptides accounted for 80% of the total C3 primary sequence. Once this task was completed, deuteration studies and enzymatic digestions were performed *in silico*, and the resulting fragments were analyzed by MALDI-time of flight (MALDI-TOF) mass spectrometry. With this technique, we were able to monitor the deuterium incorporation for 31 peptides derived from the C3 and C3($H_2O$) sequences, with a significant difference in uptake observed between the two proteins in the case of 17 peptides.

Analysis of these data in the context of the known interactions in which C3 and C3($H_2O$) participate was provocative. As compared to C3, C3($H_2O$) exhibited increased deuterium uptake at one of its factor I cleavage sites, via the spanning peptide 944-967. Factor I is responsible for degrading C3($H_2O$) and C3b after their activation, thereby limiting excessive complement activation. Therefore, the data suggest that conformational changes in this region might predispose the molecule to digestion by factor I. With these data, 30% of the C3d region of C3 and C3($H_2O$) could also be analyzed. There was a difference in deuterium incorporation for 7 out of

10 peptides identified from this region after conversion from C3 to C3($H_2O$). This diffusely distributed and extensive change in deuterium incorporation suggested a significant alteration in the structure of this region of the protein. Interestingly, the recently published X-ray crystal structure of C3b, the protein with which C3($H_2O$) shares significant functional similarities, does indeed exhibit a significant change in the C3d region of the protein.

## 4 Conclusions

Hydrogen/deuterium exchange, coupled with mass spectrometry, has emerged as a powerful technique for the structural analysis of proteins. It is capable of providing structural information, including data concerning solvent-exposed surface areas of previously uncharacterized proteins. For proteins whose crystal structures have been determined, the information obtained from HDXMS is additive and can provide clues to the structural dynamics of the protein in solution. Finally, and perhaps most significantly, HDXMS has been shown to be a powerful tool in the mapping of protein-protein and protein-ligand binding sites. Our laboratory has initiated studies utilizing HDXMS to investigate the interactions of C3 with other complement components. These studies are directed toward precise characterization of the mechanisms through which C3 interacts with its molecular partners within the complement cascade.

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