Published in final edited form as:
Anal Bioanal Chem. 2010 June ; 397(3): 967–972. doi:10.1007/s00216-010-3556-4.

Hydrogen exchange mass spectrometry: what is it and what can it tell us?

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Abstract

Proteins are undoubtedly some of the most essential molecules of life. While much is known about many proteins, some aspects still remain mysterious. One particularly important aspect of understanding proteins is determining how structure helps dictate function. Continued development and implementation of biophysical techniques that provide information about protein conformation and dynamics is essential. In this review, we discuss hydrogen exchange mass spectrometry and how this method can be used to learn about protein conformation and dynamics. The basic concepts of the method are described, the workflow illustrated, and a few examples of its application are provided.

Keywords

Deuterium; Protein mass spectrometry; Protein dynamics; Protein conformation

Introduction

Proteins play a pivotal role in most biological processes, including DNA replication, cell division, cell death, immune response, and cellular signal transduction. Many of these processes are thought to be carried out by protein machines containing at least 10 or more proteins [1]. To fully understand how proteins drive and contribute to basic biological and biochemical events, techniques that probe the fundamental properties of proteins are necessary. Not only must these methods provide information about protein function, they must also help reveal how function is tied to protein conformation and dynamics. In the past 60 years or so, there have been profound advances in the techniques for protein analysis, including such Nobel Prize-winning methods as nuclear magnetic resonance (NMR), X-ray crystallography, small angle X-ray scattering, and cryo-electron microscopy. As with everything, each of these techniques has advantages and disadvantages for studying protein conformation and dynamics, and often a combination of methods is required for full protein understanding. An orthogonal biophysical technique that has also seen profound advances in the last 20 or so years is *H*ydrogen *E*xchange monitored by *M*ass *S*pectrometry (HX MS). HX MS probes solution conformation so crystallization is not required, it requires very little sample (500–1,000 picomoles for an entire experiment), it is amenable to studying proteins that are hard to purify or that can only be handled at low concentrations (as low as 0.1 μM), and it can reveal changes

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to conformation and dynamics on a wide timescale (for a recent review and perspective, see Ref. [2]).

Topics

Overview of hydrogen exchange: what is it?

Hydrogen exchange mass spectrometry exploits a fundamental chemical reaction unique to hydrogens found in proteins (for a historical perspective, see Ref. [3]). Certain hydrogens in proteins are in continuous exchange with the hydrogens in solution. If an aqueous, all-H_2O solvent is replaced with an isotope of hydrogen that has distinctive spectroscopic properties, then one can follow this exchange process. For most modern HX experiments, deuterated or "heavy" water (D_2O) is used. In particular, the hydrogen bonded to the backbone nitrogen (also referred to as the backbone amide hydrogen) is useful for probing protein conformation. Figure 1a shows the location of the backbone amide hydrogens relative to other chemical groups within the protein. Note that every amino acid except for proline has a backbone amide hydrogen, meaning that there is a sensor at every amino acid along the length of the protein chain. While other hydrogens on side chains can also undergo exchange, the rate of side chain exchange is generally much faster than that of the backbone positions and any deuterium that is incorporated in non-backbone positions reverts back to hydrogen later in the experiment (described below).

There are various factors that affect hydrogen exchange in proteins [4]. The four primary factors are pH, temperature, solvent accessibility, and hydrogen bonding. Temperature and pH can be controlled experimentally meaning that the rate and location of exchange then becomes a function of hydrogen bonding and solvent accessibility. While amide hydrogens of fully solvent-exposed peptides at pH 7 exchange very rapidly with rates of 10–1,000 s^{-1} (depending on solvent conditions, etc.) [5], in folded proteins amide hydrogens display a variety of exchange rates depending on their position within the protein and whether they are involved in intramolecular hydrogen bonding. Figure 1b illustrates how solvent accessibility and hydrogen bonding relate to exchange in proteins. Regions that are highly dynamic and solvent-exposed (like the loops connecting the alpha helices) will exchange rapidly while regions that are less dynamic (i.e., "rigid") and/or involved in hydrogen bonding networks or buried within the interior of the protein (such as β-sheets or α-helices) will exchange slower. In folded proteins some amide hydrogens exchange quickly while others exchange much slower on timescales from minutes to months [4,6]. Note also that the backbone amide hydrogens are the ones participating in the hydrogen bonds that hold α-helices and β-sheets together. If there is a change to the solvent accessibility or the hydrogen bonding network of a protein, the rate and location of deuterium incorporation can be altered.

Monitoring hydrogen exchange

The different chemical properties of hydrogen and deuterium allow several biophysical techniques to distinguish between the two isotopes. If deuterium incorporation into proteins is monitored with NMR, for example, the amide proton peaks (from hydrogen) disappear as the protein becomes deuterated because deuterium is NMR silent. As the mass of hydrogen is 1.0078 Da and the mass of deuterium is 2.0141 Da, deuterated proteins will have a larger mass than non-deuterated proteins [7]. All that remains to convert deuteration information into conformational information is to determine how fast a protein is deuterated and where the deuterium goes.

A general workflow for a hydrogen exchange experiment monitored with mass spectrometry is shown in Fig. 2. This figure shows the continuous labeling experiment, which is the most common [8]. Protein solutions are initially equilibrated at room temperature, physiological pH, and in all-H_2O buffer. A dilution of 10- to 20-fold into the identical buffer except with all

D_2O initiates the labeling process. The excess deuterium insures the exchange kinetics favor labeling from hydrogen to deuterium [9]. The labeling is allowed to proceed for various amounts of time (e.g., 10 s, 1 min, 20 min, 8 h) and then the labeling is quenched by adjusting the pH of the sample to 2.5 and lowering the temperature to 0 °C. These quench conditions ensure retention of the deuterium label for MS analysis by decreasing the rate of amide exchange approximately five orders of magnitude [6,10]. The quenched protein sample can then be sprayed directly into a mass spectrometer (using liquid chromatography as the interface) to determine the mass of the intact protein. Measuring the mass of the whole protein gives global information about the protein(s) being studied but does not locate where the deuterium is within the protein. Proteins can also be digested post-quench but prior to chromatography. This digestion must be conducted under quench conditions to ensure retention of the deuterium label; therefore, acid proteases such as pepsin must be used [10,11]. Note that all peptic peptides must also be identified, typically done in separate MS/MS experiments. With a digestion experiment, deuterium can be localized within the short peptides produced by the digestion. The location cannot be refined to single amino acids without the use of more sophisticated mass spectrometry methods [12]

What can hydrogen exchange tell us?

HX MS can provide very useful information, including: how conformation changes upon binding, protein folding and unfolding pathways, glimpses into the structure of proteins that will not crystallize or are not amenable to NMR, which regions of a protein are solvent-exposed and dynamic, location and properties of binding sites and surfaces, dissociation constants and measures of protein stability under various conditions. The raw mass spectra of the mass of the protein, or each peptide from the digestion, are converted into a deuterium uptake plot where the level of deuterium is plotted versus the labeling time. Deuterium uptake plots for both intact analysis and peptide analysis contain information about how solvent-exposed and dynamic a protein is in solution. For a detailed explanation of all the information that can be derived from deuterium uptake plots see Ref. [13].

To illustrate how HX MS data can be revealing, consider Fig. 3 which shows examples of deuterium uptake plots for two proteins under different hypothetical situations. In Fig. 3a, the use of HX MS to probe structural effects as a result of small molecule/inhibitor binding is represented; Fig. 3b illustrates results for protein–protein interactions in a hexameric protein assembly. In the intact analysis in Fig. 3a(i), when the protein is incubated with an inhibitor, less deuterium is incorporated over the course of the labeling experiment. The differences in deuteration appear at later times in the exchange process, which indicates changes in solution dynamics as a result of inhibitor binding [14]. The overall motions of the protein were decreased, that is its structure was rigidified and it could not flex as well in solution, hence less deuterium was incorporated. In order to identify which specific parts of the protein were changed upon binding, the pepsin digestion experiment can be performed prior to MS analysis. HX MS data from two peptides are shown in Fig. 3a(ii and iii). The deuterium incorporation for the peptide in Fig. 3a(ii) (residues 20–35) shows a similar shape to that of the intact protein in Fig. 3a(i), meaning this is a region that has become less dynamic as a result of inhibitor binding. As the location of the residues is known from the crystal structure of the protein, blue in the structure in Fig. 3a(ii), an interpretation of the meaning of less deuteration in this region of this protein can be made. In other regions (Fig. 3a(iii)), no changes are observed. Deuterium uptake curves that differ in the level of deuterium incorporated but remain parallel are more indicative of a change of solvent accessibility whereas those that have different slopes usually report on changes in protein dynamics and breathing (reviewed in Ref. [13]).

In addition to probing structural changes in proteins as a result of ligand/inhibitor binding, HX MS can be used to study many other types of protein interactions, including protein–protein

interactions. Since the majority of cellular processes are orchestrated by multiple proteins and large macromolecular protein complexes, being able to study the structural consequences of these interactions is extremely important. Figure 3b illustrates an example of how HX MS could be used to study protein–protein interactions. In this hexameric protein, exchange can be compared in the isolated monomer versus the monomer in the assembled hexamer (Fig. 3b (i)). Then, with the use of pepsin digestion, the specific regions affected by complex formation can be determined (residues 10–28 are involved in hexamer formation, residues 50–68 are not; Fig. 3b(ii and iii)).

One could imagine many protein–protein interactions in which determining the effects and location(s) of changes in HX upon binding would be useful. For example, HX MS has been used to study the structural changes induced by pH changes in the capsid protein of the brome mosaic virus [15] and it has been used to investigate conformational changes in the HIV-1 capsid protein as a result of HIV assembly and maturation [16]. The large size and complexity of viral capsids make them challenging to study by many structural means, particularly when detailed information about conformational changes is desired. HX MS, however, can allow access to such information and has therefore become important as a tool for probing large proteins and protein complexes.

HX MS is also sensitive to populations of molecules in solution. If multiple populations exist that are different structurally, different amounts of deuterium will be incorporated into each population and this can be observed in the raw mass spectra [17], as illustrated in Fig. 4. If the refolding rate (k_{-1}) of unfolded and exposed regions of a protein is slower than the deuterium labeling rate (Fig. 4a), the unfolded species will have sufficient time to get highly deuterated and therefore have a higher mass than the folded species [4,18,19]. D_2O is present in vast excess such that once an individual protein molecule has transitioned to the unfolded form and become deuterated, it is a permanent member of the unfolded species peak (red, bottom of Fig. 4a). The conversion of the folded to the unfolded species occurs at a rate indicative of the rate of protein unfolding in solution. This type of exchange behavior is rare for proteins, but when observed, can serve as an extremely powerful tool for studying protein–protein interactions. A good example comes from the SH3 domains of the Src family of tyrosine kinases [20]. Data from the HX of the SH3 domain of the Lyn kinase alone (Fig. 4b) and in complex with the HIV accessory protein Nef (Fig. 4c) indicate multiple populations existing in solution. The Nef protein has been shown to interact with different members of the Src family of tyrosine kinases, including the Lyn kinase, and to disrupt cellular signaling to enhance viral infection [21]. In HX MS of Lyn SH3 alone, the unfolding rate occurs with a half-life of approximately 12 min but when the Lyn SH3 domain is bound to Nef, the unfolding dynamics of Lyn SH3 are slowed to a half-life of longer than 30 min. From this type of data, one can extract relative binding affinities [22], or determine when and if certain sequences are able to associate intramolecularly [23,24].

Outlook

Because the function of proteins is dictated by their structure and movements in solution, any biophysical technique which allows for the molecular investigation of proteins will be invaluable to aiding in our understanding of these molecular machines. Amide hydrogen exchange monitored by mass spectrometry is just one such technique for studying proteins. To become robust and high throughput, HX MS faces some challenges. Development of robotics and total automation of sample handling and data processing will make the method more amenable to high-throughput types of studies. Advances in liquid chromatography and mass spectrometry will also continue to improve the HX MS experiment. A major limitation in terms of chromatography during HX MS experiments is that separation must be done at 0 °C where chromatographic efficiency in traditional HPLC is relatively poor. New separation media and

the use of UPLC [25] are addressing this issue and also allowing for analysis of bigger and bigger proteins and protein systems [26]. The large amount of data produced during hydrogen exchange experiments have historically made data processing time-consuming. Reducing the data to a form that is easily understood in terms of conformation for a given protein can be arduous. Recent software developments (e.g., HYDRA [27], HD Desktop [28], and TOF2H [29]) significantly reduce the burden of HX MS data analysis although much more work needs to be done in this area. More sensitive mass spectrometers will also improve HX MS. One important parameter of an HX MS experiment is obtaining the protein to be studied. If proteins are rare, difficult to overexpress or obtain in the concentrations needed for some biophysical methods, or just otherwise uncooperative, there may be significant problems obtaining suitable material for analysis. Luckily, HX MS has some of the lowest requirements of any of the biophysical techniques that can provide conformational data, thereby providing access to some of these proteins that are difficult to deal with, particularly the estimated 30% of eukaryotic proteins that contain unstructured regions [30]. We envision that conformational data on many proteins that were considered impossible to analyze will be provided by HX MS in the future.

Acknowledgments

We would like to thank Dr. Thomas Wales and Dr. Thomas Smithgall for the Lyn SH3:Nef binding data. This work was supported in part by the National Institutes of Health (R01-GM070590 and R01-GM086507). This is contribution 959 from the Barnett Institute.

References

1. Alberts B. Cell 1998;92:291–294. [PubMed: 9476889]
2. Engen JR. Anal Chem 2009;81:7870–7875. [PubMed: 19788312]
3. Englander SW, Mayne L, Bai Y, Sosnick TR. Protein Sci 1997;6:1101–1109. [PubMed: 9144782]
4. Englander SW, Kallenbach NR. Q Rev Biophys 1983;16:521–655. [PubMed: 6204354]
5. Bai Y, Milne JS, Mayne L, Englander SW. Proteins 1993;17:75–86. [PubMed: 8234246]
6. Smith DL, Deng Y, Zhang Z. J Mass Spectrom 1997;32:135–146. [PubMed: 9102198]
7. Katta V, Chait BT. Rapid Commun Mass Spectrom 1991;5:214–217. [PubMed: 1666528]
8. Wales TE, Engen JR. Mass Spectrom Rev 2006;25:158–170. [PubMed: 16208684]
9. Englander JJ, Rogero JR, Englander SW. J Mol Biol 1983;169:325–344. [PubMed: 6312054]
10. Zhang Z, Smith DL. Protein Sci 1993;2:522–531. [PubMed: 8390883]
11. Cravello L, Lascoux D, Forest E. Rapid Commun Mass Spectrom 2003;17:2387–2393. [PubMed: 14587084]
12. Rand KD, Zehl M, Jensen ON, Jorgensen TJ. Anal Chem 2009;81:5577–5584. [PubMed: 19601649]
13. Morgan CR, Engen JR. Curr Protoc Protein Sci 2009;58(17.16):11–17.10.1002/0471140864.ps1706s58
14. Maier CS, Deinzer ML. Methods Enzymol 2005;402:312–360. [PubMed: 16401514]
15. Wang L, Lane LC, Smith DL. Protein Sci 2001;10:1234–1243. [PubMed: 11369862]
16. Lanman J, Lam TT, Emmett MR, Marshall AG, Sakalian M, Prevelige PE Jr. Nat Struct Mol Biol 2004;11:676–677. [PubMed: 15208693]
17. Miranker A, Robinson CV, Radford SE, Aplin RT, Dobson CM. Science 1993;262:896–900. [PubMed: 8235611]
18. Weis DD, Wales TE, Engen JR, Hotchko M, Ten Eyck LF. J Am Soc Mass Spectrom 2006;17:1498–1509. [PubMed: 16875839]
19. Engen JR, Smith DL. Methods Mol Biol 2000;146:95–112. [PubMed: 10948498]
20. Wales TE, Engen JR. J Mol Biol 2006;357:1592–1604. [PubMed: 16487539]
21. Trible RP, Emert-Sedlak L, Smithgall TE. J Biol Chem 2006;281:27029–27038. [PubMed: 16849330]
22. Trible RP, Emert-Sedlak L, Wales TE, Ayyavoo V, Engen JR, Smithgall TE. J Mol Biol 2007;374:121–129. [PubMed: 17920628]

23. Chen S, Dumitrescu TP, Smithgall TE, Engen JR. Biochemistry 2008;47:5795–5803. [PubMed: 18452309]
24. Hodkinson JP, Jahn TR, Radford SE, Ashcroft AE. J Am Soc Mass Spectrom 2009;20:278–286. [PubMed: 18996721]
25. Wales TE, Fadgen KE, Gerhardt GC, Engen JR. Anal Chem 2008;80:6815–6820. [PubMed: 18672890]
26. Houde D, Arndt J, Domeier W, Berkowitz S, Engen JR. Anal Chem 2009;81:5966. [PubMed: 19606834]
27. Slysz GW, Baker CA, Bozsa BM, Dang A, Percy AJ, Bennett M, Schriemer DC. BMC Bioinformatics 2009;10:162. [PubMed: 19473537]
28. Pascal BD, Chalmers MJ, Busby SA, Griffin PR. J Am Soc Mass Spectrom 2009;20:601–610. [PubMed: 19135386]
29. Nikamanon P, Pun E, Chou W, Koter MD, Gershon PD. BMC Bioinformatics 2008;9:387. [PubMed: 18803853]
30. Dyson HJ, Wright PE. Curr Opin Struct Biol 2002;12:54–60. [PubMed: 11839490]

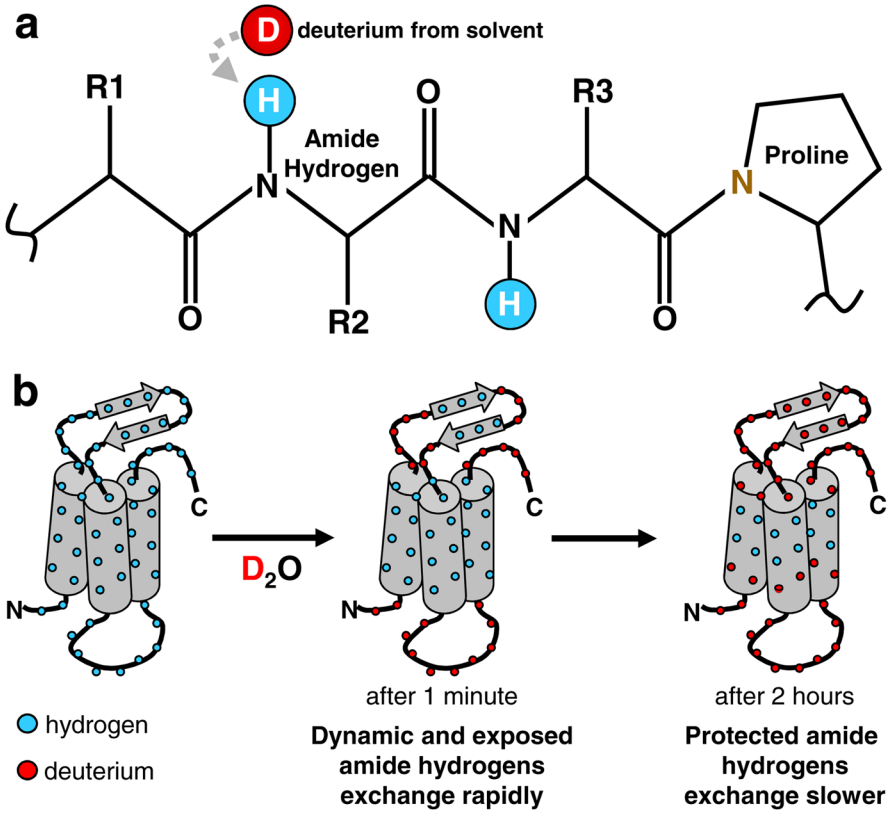

Fig. 1.

Overview of amide hydrogen exchange in proteins. **a** Part of an amino acid sequence is shown. All backbone nitrogens have an amide hydrogen (*blue*), except the nitrogen in proline (*brown*), thereby providing a sensor at each amino acid along the length of the protein chain. The backbone amide hydrogens (*blue*) are in continuous exchange with hydrogens in the solvent. Some hydrogen atoms located within the amino acid side chains (R1, R2, etc.) also become deuterated; however, these positions revert back to hydrogen during the liquid chromatography (LC) step of the experiment. Hydrogen bonded to carbon does not exchange. **b** Once placed in a solution of D_2O, backbone amide hydrogens will become deuterated. The exposed and “dynamic” regions of proteins will exchange rapidly while protected and “rigid” regions of proteins will exchange slower. The rate of exchange for any given amide hydrogen is dictated by pH, temperature, solvent accessibility, and hydrogen bonding. The temperature and pH are controlled during an HX MS experiment leaving the structurally related parameters of solvent accessibility and hydrogen bonding as the governing factors for deuteration

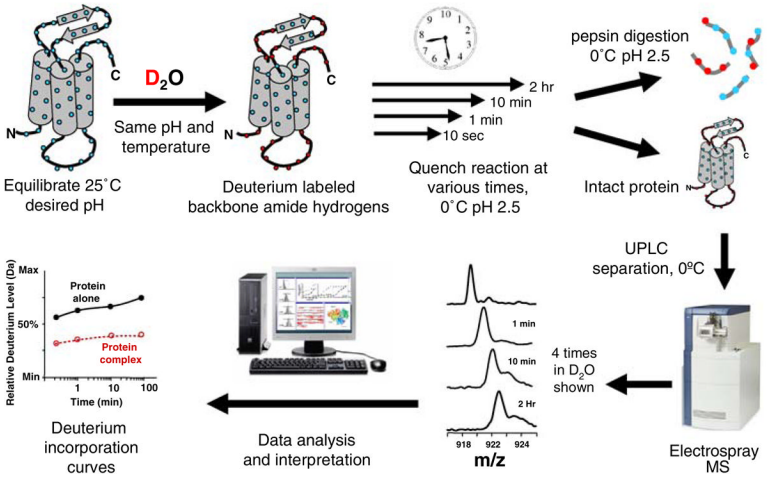

Fig. 2.

Workflow of a typical HX MS experiment. Protein samples are equilibrated at the desired temperature and pH, in a buffer compatible with the protein. Protein solutions are then diluted (typically 10-fold or more) with the identical buffer containing 99.9% D_2O instead of H_2O. The exchange reaction proceeds for various amounts of time and is quenched by lowering the pH to 2.5 and the temperature to 0 °C. The pH and temperature adjustment reduces the amide exchange rate to its minimum. Deuterated, quenched protein can then be either directly injected into a mass spectrometer for mass analysis or digested with an acid protease prior to liquid chromatography and mass analysis. The mass spectra are analyzed and the uptake of deuterium over time determined and plotted, either for the intact protein, or for each of the peptic peptides

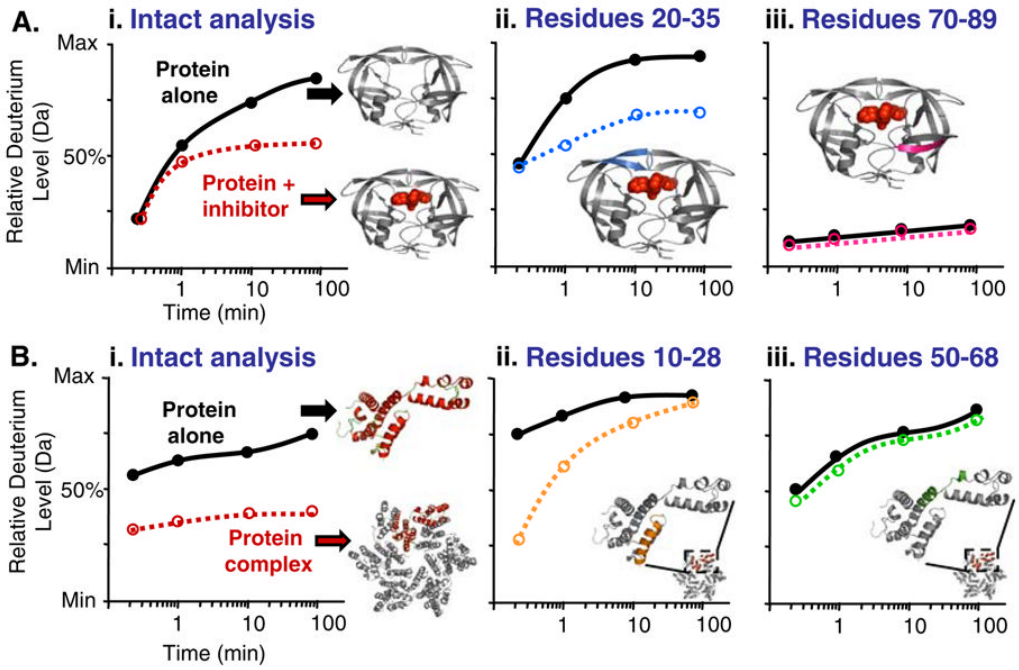

Fig. 3.

HX MS reports on the effects of protein binding: **a** protein plus small molecule inhibitor or **b** protein–protein interactions as a result of quaternary structure formation. In **a**, analysis of the intact, undigested protein (*i*) can reveal if there are any global conformation and/or dynamic changes as a result of inhibitor binding. In this example, the inhibitor causes a reduction in deuterium incorporation (*red curve*). By digesting the deuterated protein with a protease, one can observe the location of the differences (*ii* and *iii*). Residues 20–35 of this protein (*ii*) undergo a reduction in deuterium incorporation upon inhibitor binding while residues 70–89 (*iii*) do not. The location of each peptide is known (*structural insets*). In **b**, deuteration of the monomeric subunit of a hexameric protein was compared with deuteration of the same monomer when part of the hexameric assembly found in vivo. Intact protein analysis (*i*) indicates protection from exchange in the hexamer and the location was determined to be primarily residues 10–28 (*ii*) and not residues 50–68 (*iii*)

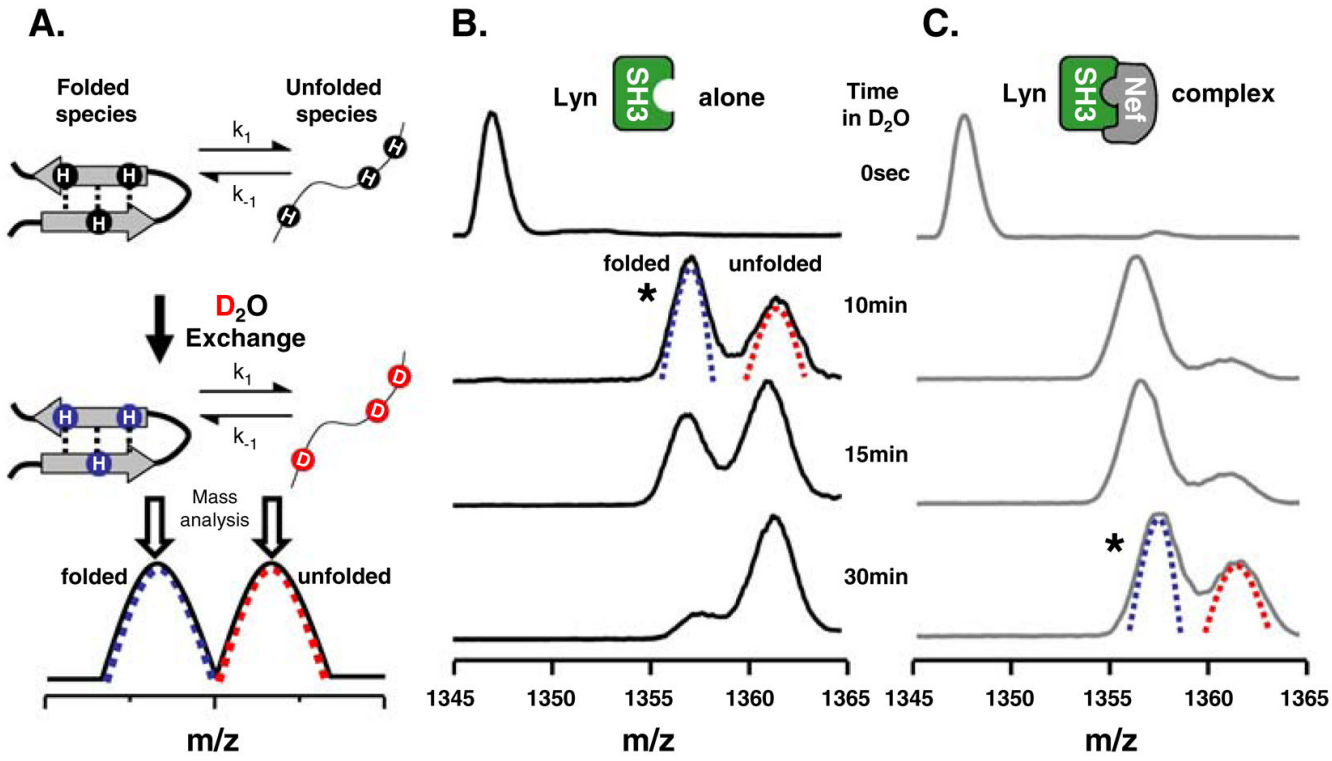

Fig. 4.

Using hydrogen exchange to monitor the HIV-1 Nef:Lyn SH3 complex. **a** An advantage of HX MS over some other biophysical techniques is the ability to detect different protein populations in solution. In this example, the two populations appear in the mass spectra: one representing the folded state (*blue distribution*) and the other representing the unfolded state (*red distribution*). The appearance of two distributions occurs when the rate of interconversion of the two populations (i.e., folded and unfolded) is slower than the amide exchange rate (recently discussed in Ref. [18]). If a molecule unfolds, it will become totally deuterated, hence the higher mass. **b**,**c** The *S*rc *H*omology *3* (SH3) domain of the Lyn kinase shows a bimodal pattern in intact protein HX MS (+6 charge state shown). This pattern indicates partial, cooperative unfolding as described in **a**. One of the labeling timepoints captures the populations at an approximate 60:40 ratio (folded to unfolded), indicated by the *asterisk*. The actual 50:50 population point (or $t_{1/2}$ for the unfolding reaction) is observed 12 min after deuterium labeling begins. In **c**, the unfolding is shown for the +6 charge state of intact Lyn SH3 bound to the HIV-1 accessory protein Nef [22]. Notice that while the pattern of the bimodal distribution of folded and unfolded species appears similar to that shown for Lyn SH3 (**b**), the time at which it appears during the deuterium labeling time course is much longer in the bound form (**c**). The 60:40 ratio is again marked with an *asterisk* and occurs at a much later time in the labeling timecourse (30 mins). Data such as these give insight into the molecular mechanisms which govern enhancement of HIV replication through the Nef:Lyn complex