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Conformational insight into multi-protein signaling assemblies by hydrogen-deuterium exchange mass spectrometry

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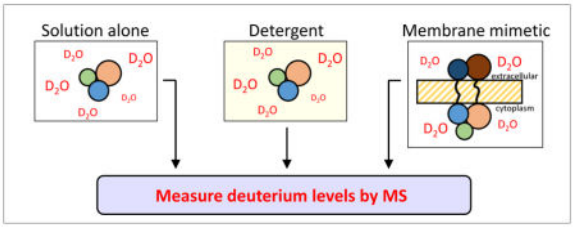
Abstract

Hydrogen-deuterium exchange (HDX) mass spectrometry (MS) can provide information about proteins that can be challenging to obtain by other means. Structure/function relationships, binding interactions, and the effects of modification have all been measured with HDX MS for a diverse and growing array of signaling proteins and multiprotein signaling complexes. As a result of hardware and software improvements, receptors and complexes involved in cellular signaling – including those associated with membranes – can now be studied. The growing body of HDX MS studies of signaling complexes at membranes is particularly exciting. Recent examples are presented to illustrate what can be learned about signaling proteins with this technique.

Graphical Abstract



Introduction

Few would debate that at present it can be challenging to determine the structure of multi-protein signaling assemblies, let alone to determine conformational changes important for function. Size, complexity, and/or proximity to membranes of many signaling assemblies can often complicate or completely inhibit analysis. Mass spectrometry (MS) methods have a role to play in conformational studies and can be used not only to characterize the quaternary structure of assemblies, but also to obtain details about conformation and conformational changes [1–3]. Selective protein labeling using covalent labels or hydrogen-deuterium exchange (HDX), with subsequent MS detection, may provide insight into

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conformational features, including for many proteins intractable by other methods. This review will cover the use of HDX MS to study signaling complexes, especially including the analysis of assemblies in detergent and in the presence of membranes (FIGURES 1,2).

The theory and practice of HDX MS have been reviewed extensively (recent examples: [4, 5••, 6]). In short, backbone amide hydrogens in the protein exchange with deuterons in solution. The rate of this exchange is dependent on pH, temperature, hydrogen bonding, and solvent accessibility. With proper control of pH and temperature, deuteration reports on conformation and conformational changes affected by hydrogen bonding and solvent accessibility. Generally, poorly hydrogen bonded and/or solvent exposed regions will incorporate deuterium more quickly than those that are strongly hydrogen bonded and/or solvent protected. A common approach is to compare two (or more) different conformational states of the same proteins(s), and report where differences in exchange, and hence conformation, exist between the states (FIGURE 3). In a typical implementation, a protein-containing solution (solution A) of a given composition at physiological pH (in H_2O) is diluted with a solution (solution B) of the same composition and pH (labeling pH) made with all D_2O. The only difference between solution A and B is the presence of deuterium. Once the deuterated solution is added, the labeling reaction proceeds for various amounts of time before being slowed 4–5 orders of magnitude by reduction of the pH to 2.5 and the temperature to 0 °C (quench conditions). Labeled protein in quench conditions can be digested into smaller fragments with an aspartic protease, such as pepsin, and the resulting peptide fragments – which bear a signature of deuterium that reports on the conformation at the labeling pH – are then separated by liquid chromatography (LC). The LC is coupled to a mass spectrometer and the mass of each labeled peptide is measured. HDX MS as a technique is 25 years old and has become much more accessible in recent years due to advances in methodology, hardware and data processing software [7,8]. Single proteins involved in signal transduction have been studied by HDX MS for many years, while analyses of multi-protein complexes and assemblies have recently become technically feasible and more common. HDX MS has become more amenable for the analysis of large proteins and complexes due largely to advances in chromatographic peak capacity by utilization of UPLC rather than HPLC, more sensitive mass spectrometers, and the development of powerful data processing software (described in more detail in [4,7,8]).

HDX MS of complexes that do not involve a membrane

Some of the earlier examples of how HDX MS can be used to study multi-protein signaling assemblies are also the simplest – they were performed in solution alone without any detergents or membrane mimetics (FIGURE 1A). In 2012, Choi et al. [9•] used HDX MS to investigate signaling by notch receptors, type I transmembrane proteins that communicate signals in response to transmembrane ligands on neighboring cells and regulate a variety of cellular events during development and in normal tissue homeostasis. Changes in protein dynamics and interactions were assessed upon stepwise assembly of a complex comprised of a transcriptional co-activator of the Mastermind family (MAML1), the Notch intracellular domain (NICD), and DNA-binding factor CSL. MAML stabilized the ankyrin (ANK) domain facilitating a stronger interaction with CSL, explaining why MAML is essential for

the cooperative dimerization of NTCs on paired-site DNA. At the time, this was a rather large multiprotein complex for HDX MS.

While there are numerous examples using HDX MS to follow signaling pathways in animals, West et al. [10••] used HDX MS to study the abscisic acid (ABA) receptor in plant biochemistry. The means by which plants regulate growth and respond to environmental cues can involve ABA pathways, initiated by the PYR/PYL/RCAR class of receptors, which promote signaling by disrupting phosphatase inhibition of Snf1-related kinases (SnRKs). The abscisic acid (ABA) receptor PYL2, HAB1 phosphatase, and SnRK2.3 and 2.6 were probed by HDX MS and the results revealed that in the presence of binding partners, the phosphatase adopts receptor-specific conformations involving a Trp385 “lock” required for signaling, and showed that kinase activity is linked to a more stable conformation.

The most studied signaling pathways by HDX MS are those including G-proteins and G-protein coupled receptors (GPCRs). Exchange has been monitored for G-proteins and GPCRs in all types of conditions, including alone in solution, in detergents, and with membrane mimetics (FIGURE 1). A recent illustrative example [11••] of HDX MS in solution (FIGURE 1A) involved a regulator of G protein signaling (RGS) which binds both active and inactive Gα proteins using two separate binding motifs. The N-terminal RGS motif binds to active Gα-GTP, and the C-terminal G protein regulatory (GPR) motif binds inactive Gα-GDP. Communication between these two G protein binding motifs was probed by HDX MS to determine whether regulator of G protein signaling 14 (RGS14) could interact with both forms of Gα at the same time. The results showed that RGS14 was a dynamic protein that underwent allosteric conformational changes when bound to Gα-GDP. When RGS14 formed a complex with Gα-GDP, there was stimulation of GTPase activity in Gα-GTP, meaning that RGS14 does interact with both Gα subunits simultaneously, thereby clarifying our understanding of how RGS14 integrates signaling by G protein subunits.

HDX MS in the presence of detergents

Although performing HDX MS experiments in solution alone can be valuable, many proteins, particularly those involved in signaling, may contain hydrophobic regions meant to interact with membranes and therefore may require a detergent to improve solubility. Several detergents [e.g., n-Dodecyl β-D-maltoside (DDM) [14•,15], lauryl maltose neopentyl glycol (LMNG) [16•], octyl-β-D-glucopyranoside (OG) [20••]] are compatible with MS and can be used for HDX MS studies (FIGURE 1B). There are multiple examples of HDX MS being used to investigate GPCR signaling pathways in the presence of detergents, e.g. [12,13]. While it is known that GPCRs initiate signaling through nucleotide exchange on heterotrimeric G proteins, the mechanism(s) of G protein activation is less clear. HDX MS was used to identify structural changes in the heterotrimeric G protein Gs upon formation of a complex with agonist-bound human β_2 adrenergic receptor (β_2AR) [14•]. This study showed that interactions between the receptor and the amino-terminal region of the α-subunit of Gs alter the phosphate-binding P-loop. As P-loop stabilization and β-phosphate coordination are critical to GDP and GTP binding affinity, destabilizing this region should facilitate nucleotide exchange and promote signaling.

A classic example [15•] of GPCR HDX MS in detergent and detergent:lipid micelles involved rhodopsin (Rho). Rho contains a specific binding site for rod G protein G_t that is exposed upon photoactivation. Both HDX MS and hydroxyl radical labeling were used to elucidate the structural changes in Rho that occur upon photoactivation. When dimeric, photoactivated Rho (Rho*) formed a complex with heterotrimeric G_t. Photoactivation resulted in structural relaxation of Rho, allowing the protein to adopt a conformation that favored binding with G_t. Upon binding Rho*, nucleotide-free G_t became far more dynamic, suggesting that it can more readily accept GTP and subsequently dissociate from the complex.

HDX MS played an important role in studies by Shukla et al. [16•] of the interactions between β_2V_2R (a chimera of β_2AR and arginine vasopressin type 2 receptor) and β-arrestin 1. GPCR signaling can be attenuated by β-arrestins (proteins that desensitize G-protein signaling and instigate G-protein-independent signaling), which have themselves been studied by HDX MS [17–19]. The HDX MS studies of Shukla et al. demonstrated engagement of the finger loop of β-arrestin with the seven-transmembrane core of the receptor. Conformational changes in β-arrestin were found in both the N and C domains when in complex with β_2V_2R.

Quite recently, the T-cell receptor (TCR) associated kinase Zap70 was studied [20••] to determine how kinase conformation dictates its activity and ability to associate with the TCR. This example of HDX MS on a multiprotein signaling complex highlights how the method can prove invaluable for studying kinase recruitment to membrane receptors, a process known to be essential for signal transduction in cellular immune response pathways. Zap70 is inactive in the cytosol until recruited to the doubly phosphorylated immunoreceptor tyrosine-based activation motifs (p-ITAMS) of the TCR. HDX MS showed that TCR binding or phosphorylation of Zap70 shifts the protein from a closed, autoinhibited conformation to an open conformation, and kinase activity is controlled by TCR dwell time. The results suggested that kinase recruitment to the plasma membrane, and the associated changes in protein conformation and dynamics, can regulate the activity and function of receptors lacking inherent kinase activity.

HDX MS in the presence of membrane mimetics

Although detergents are useful for solubilizing proteins and complexes that associate with membranes, questions may linger about the biological relevance of results. Rather than detergents, and whenever possible, alternatives that more closely resemble an actual cell membrane are preferred. HDX MS can be performed in the presence of membrane mimetics (FIGURE 1C) and a number of studies have been performed on signaling complexes using membrane mimetics. The four major mimetics that have been implemented in HDX MS studies are bicelles, monolayers, liposomes, and nanodiscs (FIGURE 2). The composition of the membrane mimetic can be made to match the particular membrane of interest, be it the plasma membrane, the inner leaflet of the endoplasmic reticulum, etc. HDX MS of signaling protein complexes with membrane mimetics, while technically challenging, is particularly exciting as it probes contributions made to conformation and dynamics by the membrane itself.

There are advantages and disadvantages to each of the various mimetics (FIGURE 2). Bicelles represent an intermediate between detergents and lipid vesicles (liposomes) and are fairly easy to create and manipulate. Comparably less lipid is introduced into the MS when using bicelles (e.g., versus liposomes) but bicelles suffer the problem that they may be too small and too simplistic for many transmembrane proteins or large complexes. Duc et al. [21•] recently presented an example of using bicelles for HDX MS of three different GPCRs. Our group developed both nanodisc [22, 23, 24•] and monolayer [25] HDX MS methods, and we have worked with liposomes as well [26,27]. Nanodiscs offer the advantage that transmembrane proteins can be embedded into them without concerns about protein directionality as both faces of the nanodiscs are exposed to solvent. Disadvantages of nanodiscs are that assembly requires that the correct protein:lipid ratio be found, often empirically. In addition, because the protein:lipid ratio in the assembled nanodisc is fixed, should a protein need to move or change conformation or the packing of the lipid layers, the constraining effects of the membrane stabilizing protein (MSP) often prohibit this. Insertion of peripheral membrane proteins into nanodiscs after nanodisc assembly is not particularly efficient for this reason. Monolayers and liposomes do not suffer from such restrictions in lipid movement or packing. A major advantage of monolayers is that the lipid packing density can be precisely and reproducibly controlled [25] thereby allowing analysis of conformational features related to lipid packing and fluidity. Monolayers, however, only represent half of a traditional bilayer and are therefore mostly useful for the analysis of peripheral membrane proteins. Special apparatus must be used to create and maintain monolayers [25]. Liposomes can be easily and reproducibly created using straightforward extrusion methods. Both peripheral and transmembrane proteins can be analyzed with liposomes, although in the case of liposomes directionality of the proteins must be considered. Oscar Vadas and John Burke recently published an excellent review [5••] on the use of HDX MS to probe interactions of peripheral membrane proteins with lipids. Their review covers both theory and application, with a particular focus on the regulation of phospholipase A2 and phosphoinositide 3-kinases (PI3Ks). With the exception of nanodiscs where the membrane stabilizing protein is best removed prior to LC/MS analysis, lipid mimetics can be directly injected onto the LC traps/columns used for HDX MS and the lipids generally do not interfere with MS analysis provided that trapping columns are kept clean.

Burke, Vadas, and others in Roger William's lab at the Medical Research Council in Cambridge have performed some very noteworthy and demonstrative HDX MS studies on p110β in the presence of lipid vesicles. p110β is a unique PI3K in that it can be activated by both receptor tyrosine kinases (RTKs) and GPCRs, and it has been specifically implicated in the growth of tumors deficient in phosphatase and tensin homolog deleted from chromosome 10 (PTEN). The first study using HDX MS monitored activation of p110β through direct binding to the Gβγ subunit of GPCRs in order to determine which functions of p110β could be attributed to GPCR signaling [28••]. The results identified the p110β binding site, and mutations at this site prevented Gβγ from binding and activating PI3Kβ (a dimer of p110β and the p85 regulatory subunit) independent of basal or RTK-mediated activity. Disruption of this important binding interface had profound anti-cancer effects on PTEN-null tumor cells in culture, indicating that targeting GPCR signaling could be a viable therapeutic

approach for p110β-dependent tumors. A later study [29••] investigated the regulation of PI3Kγ, which plays roles in multiple areas of therapeutic interest including inflammation, cardiac function, and tumor progression. HDX MS helped map the interactions between this enzyme and its regulatory partners, including lipid membranes, and showed that lipid interaction induces a conformational change that results in exposure in a key helical domain responsible for diverse regulatory interactions in class I PI3Ks. Finally, the same lab investigated the distinct roles of the intrinsically disordered tail regions of PTEN in regulating substrate specificity and membrane activity [30].

Vacuolar protein sorting 34 (Vps34) is a class III PI3K that phosphorylates phosphatidylinositol (PI) to generate phosphatidylinositol 3-phosphate (PI3P) and fulfills an important role in intracellular membrane trafficking. In a recent study [31••], HDX MS was used to study Vps34 in endosomal complex II. Endosomal complex II is involved in endocytic sorting, autophagy, and cytokinesis, and includes proteins Vps34, Vps15, Vps30/Atg6, and Vps38. It was hoped that HDX MS would be able to characterize any changes in the complex due to lipid binding, and also to provide a rationale for differential treatment of lipid vesicles depending on their size. The HDX MS data revealed conformational changes in the complex upon binding to the membrane, and identified a distinct “aromatic finger” motif in Vps30 that could explain the ability of complex II to phosphorylate giant liposomes that complex I cannot.

Relaying signals across membranes can involve situations where interactions on one side of a membrane elicit changes on the other side – how that happens exactly is unclear for most systems but conformational changes are a reasonable explanation. HDX MS proved valuable in an analysis of this type in bacterial chemotaxis receptors, proteins that enable bacteria to respond to environmental cues and trigger cell motility. Ligand binding to the extracellular portion of the receptor initiates a conformational change in the periplasmic and transmembrane domains, but how that signal is relayed through the cytoplasmic domain to regulate the activity of intracellular kinases was unknown. Initial global HDX MS (where there is no protease digestion after quenching) of complexes associated with lipid vesicles was used to measure the overall dynamics of a receptor cytoplasmic fragment (CF), complexed with histidine kinase CheA, and scaffolding protein CheW [32]. It was found that CF incorporates significantly less deuterium when bound in the complex compared to alone in solution, suggesting that it becomes highly-structured upon complex formation. The same group later reported the local HDX MS (where there is protease digestion after quenching, FIGURE 3, and therefore improved spatial resolution of where the deuteration had occurred) analysis of complexes containing CF, CheA, and CheW [33••]. The authors found that functionally-important regions of the receptor fragment known to be involved in chemotaxis exhibited significant signaling-associated changes in exchange rates. The results also suggested a more significant interaction between the receptor signaling subdomain and CheA and CheW in the kinase-activating state, as evidenced by significantly reduced exchange rates in this region. Surprisingly, both increased and decreased exchange rates were observed in the methylation subdomain. This result is partially consistent with proposals that the domain is stabilized by the complex, but also suggests a heterogeneous behavior worthy of further examination.

Closing thoughts

As instrumentation and software have continued to improve, HDX MS has found its way into the analysis of sophisticated signaling complexes. Larger and larger systems are becoming more tractable, and the inclusion of membrane mimetics makes it possible to determine the contribution of the membrane in signaling processes. In most experiments, conformational synchronization, meaning all the protein molecules must be in the same signaling state (e.g., all proteins phosphorylated, bound, etc.) remains important in order to interpret the data. This variable can be controlled and when HDX MS data are related to functional assays, the results can be exceedingly insightful.

Acknowledgments

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Highlights

- Hydrogen-deuterium exchange mass spectrometry (HDX MS) is now more routine
- Large signaling proteins can be studied with HDX MS, from kinases to GPCRs
- Membrane mimetics and HDX MS probe signaling complexes at the membrane
- HDX MS with membranes elucidates the role of membrane in conformation
- HDX MS can be leveraged to facilitate x-ray crystallography of large complexes

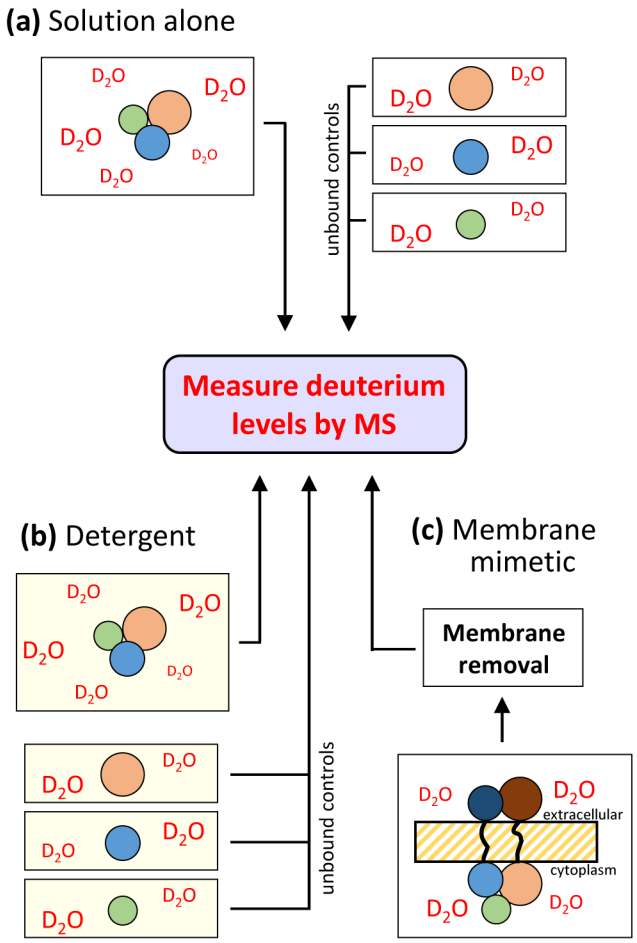

Figure 1.

Summary of the ways in which comparative HDX MS can be used to study multi-protein complexes. (a) Solution alone. Each protein (green, orange, blue circles) is labeled individually in an unbound state (right panel), and the results compared to labeling of each protein when part of a complex (left panel). (b) In detergent. Labeling and comparisons are similar to solution alone except detergent (yellow background) is present to aid in solubilizing the hydrophobic regions of certain proteins. (c) Membrane mimetics. Protein complexes are assembled in a membrane mimetic (e.g., bicelles, monolayers, lipid vesicles, nanodiscs). Deuterium labeling is measured after membrane component removal by chromatography and compared with the labeling of each component alone (not shown).

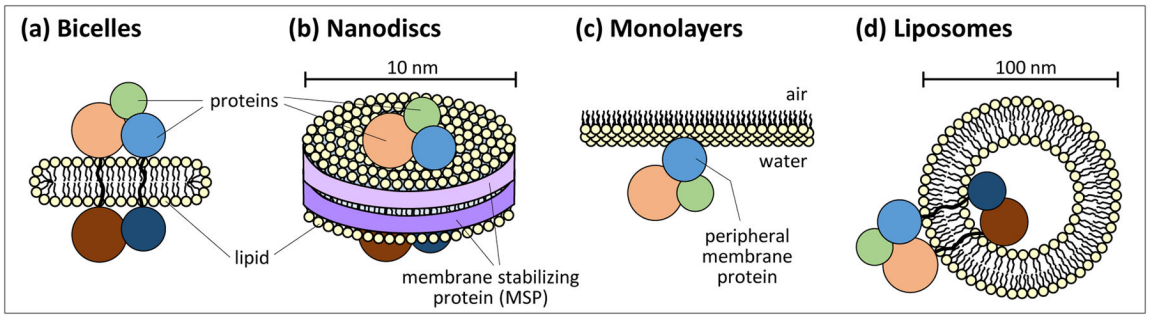

Figure 2.

Membrane mimetics used for HDX MS. Lipid headgroups are shown in yellow; proteins are shown with colored circles (as in Fig. 1). These cartoons are not to scale. The typical size of nanodiscs (b) and liposomes (d) are indicated with scale bars. Bicelle (a) size depends on protein size and lipid choice, but is generally much smaller than nanodiscs. Monolayers (c) can be of any size, dependent on the apparatus. Nanodiscs (b) contain a membrane stabilizing protein (MSP), present in two copies (purple bands) that wrap the inner lipid disc and hold it together. While bicelles, nanodiscs and liposomes can be used for both transmembrane (as shown here, panel a, b, d) and peripheral membrane proteins, monolayers (c) are generally restricted to just peripheral membrane proteins.

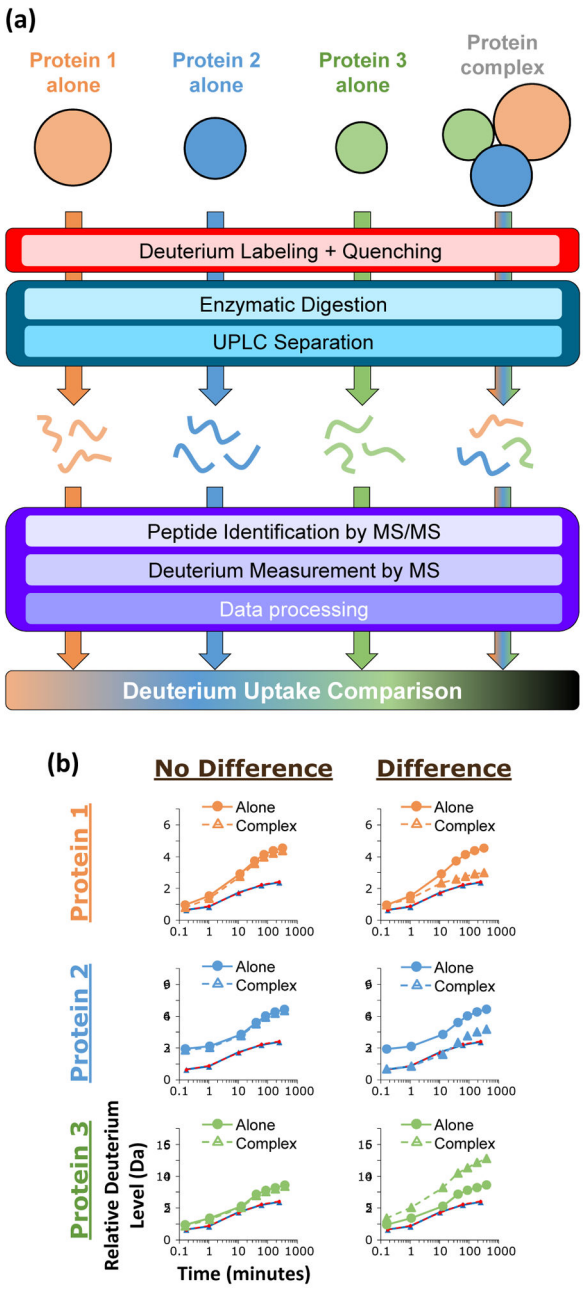

Figure 3.

A typical multi-protein HDX MS experiment, adapted from [34]. (a) Each protein, both alone and in complex, is subjected to the same workflow: labeling with deuterium at physiological pH, quenching of the labeling reaction, digestion, MS measurement of deuterium, and data processing. Note that the peptides in which deuterium is measured bear a signature of the deuterium incorporation into the folded, native protein/protein complex. (b) Deuterium incorporation is compared at the peptide level to identify regions of the protein(s) that incorporated a different (graphs on the right) or the same (graphs on the left)

amount of deuterium. Deuterium incorporation is shown for peptides derived from the protein complex (dotted lines) and from each protein alone (solid lines).