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REVIEW ARTICLE

Unraveling the dynamics of protein interactions with quantitative mass spectrometry

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Abstract

Knowledge of structure and dynamics of proteins and protein complexes is important to unveil the molecular basis and mechanisms involved in most biological processes. Protein complex dynamics can be defined as the changes in the composition of a protein complex during a cellular process. Protein dynamics can be defined as conformational changes in a protein during enzyme activation, for example, when a protein binds to a ligand or when a protein binds to another protein. Mass spectrometry (MS) combined with affinity purification has become the analytical tool of choice for mapping protein–protein interaction networks and the recent developments in the quantitative proteomics field has made it possible to identify dynamically interacting proteins. Furthermore, hydrogen/deuterium exchange MS is emerging as a powerful technique to study structure and conformational dynamics of proteins or protein assemblies in solution. Methods have been developed and applied for the identification of transient and/or weak dynamic interaction partners and for the analysis of conformational dynamics of proteins or protein complexes. This review is an overview of existing and recent developments in studying the overall dynamics of *in vivo* protein interaction networks and protein complexes using MS-based methods.

Keywords: Quantitative proteomics, protein interaction networks, metabolic labeling, label-free, spectral counting, H/D exchange

Introduction

Most of the key biological processes such as DNA replication, transcription, and translation are controlled by the assembly of protein molecules through coordinated mechanisms of action (Alberts, 1998). Proteins in these multiprotein complexes involve either transient and/or weak and stable interactions, which form a network of coordinated interactions for communicating different cellular signals. To understand these cellular processes, there is a need to study the composition, stoichiometry, posttranslational modifications (PTMs), and dynamic changes in protein complexes. The term dynamics has a broad meaning that includes changes in the composition and conformation of protein complexes due to stable or strong, transient, and/or weak interactions with proteins, metal ions, and ligands. Conformational changes are also observed because of PTMs of amino acids in protein complexes. Furthermore, metal ions or cofactors have important roles in a variety of processes including protein folding, conformational change, stability, assembly, and catalysis. In fact, the conformation and activity of one-third of all proteins is controlled by metal ions (Tainer et al., 1992). Transient and/or weak interacting proteins undergo rapid association and dissociation with various protein complexes and might be important in controlling critical aspects of a biological process. The main technology used to study protein interaction dynamics is quantitative proteomics, where mass spectrometry (MS) is used to determine changes in protein abundances. To fully understand the importance of these interaction partners in biological context, there is a need to study the dynamically interacting proteins *in vivo* and the changes in protein conformation in response to a range of interactors.

There are various established methods for studying protein complex composition and conformational changes. Existing approaches for studying protein–protein

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interactions in different protein complexes are yeast two-hybrid assays (Fields and Sternglanz, 1994; Suter et al., 2008; Williamson and Sutcliffe, 2010), fluorescence imaging (Zal, 2008), protein microarray (Spisák and Guttman, 2009), and affinity purification–MS. Among all of these, the combination of affinity purification and MS (AP-MS) has been widely used to decipher the composition of protein complexes due to its sensitivity, specificity, and accuracy. One challenge is to determine the bona fide protein interactions with bait proteins and protein complexes, and there are several methods to accomplish this (Tackett et al., 2005; Selbach and Mann, 2006; Sardiu et al., 2008; Hubner et al., 2010). In addition, high-throughput analysis of biological samples is also possible because of automation of the technique and advancements in bioinformatics for data evaluation. The existing MS methods are useful not only for studying protein sequences, stable interaction partners, and PTMs, but also for investigating dynamics of protein complexes (Hernández and Robinson, 2001; Gingras, 2005). Current MS methods for studying conformational dynamics are hydrogen/deuterium (H/D) exchange, hydroxyl radical labeling, and ion charge state distribution (Konermann et al., 2008). This review will focus on various studies and developments aimed toward looking at the dynamics of protein complexes using quantitative MS. The first part of this review will focus on studying dynamics of protein complex composition due to the transient and/or weak interacting proteins using proteomic approaches. The second part of the review will discuss various methods available for investigating conformational dynamics of protein complexes in association with various binding partners (proteins, metal ions, and ligands) as well as changes due to PTMs.

Quantitative proteomics approaches

Quantitative proteomics approaches can be broadly divided into label and label-free methods. The available labeling approaches are further divided into chemical (ICAT, ICPL, and iTRAQ) and metabolic labeling (SILAC) groups (reviewed in Kline et al., 2009). Metabolic labeling is carried out *in vivo* at the protein level, whereas the chemical labeling is an *in vitro* approach that can be carried out at either the peptide or protein level (reviewed in Kline et al., 2009) (Figure 1). Metabolic labeling methods rely on *in vivo* incorporation of isotope-labeled amino acids into the cellular proteome of an entire organism during protein biosynthesis (reviewed in Kline et al., 2009). Labeled quantitative proteomics approaches primarily obtain quantitative data from peptide intensities in parent ion scans. The label-free method emerged as an alternative to labeling methods because it is cost-effective and especially useful when a large number of samples have to be run for a comparative analysis. Analysis of proteins by label-free quantitative proteomics does not involve alterations of proteins or peptides in contrast to labeling methods. The most commonly used approaches for label-free quantification are spectral counting (reviewed in Lundgren et al., 2010) and total ion current (TIC) (Asara et al., 2008) (Figure 1).

Application of a particular labeling method to a study depends on the biological system under investigation. Chemical methods (ICAT, ICPL, and iTRAQ) are used for labeling proteins or peptides after purification of the sample; thus, the sample is completely independent of the source and preparation (Gouw et al., 2010). Chemical labeling methods virtually can be used for any type of biological sample and the time needed for this method is much shorter than the metabolic labeling and can be performed at a lower cost. The chemical methods further divided into two subcategories based on their level of labeling. The ICAT (isotope-coded affinity tag) (Gygi et al., 1999) and ICPL (isotope-coded protein labeling) (Kellermann, 2008) are useful for incorporation of isotopes at the protein level and iTRAQ (isobaric tag for relative and absolute quantitation) at peptide level (Aggarwal

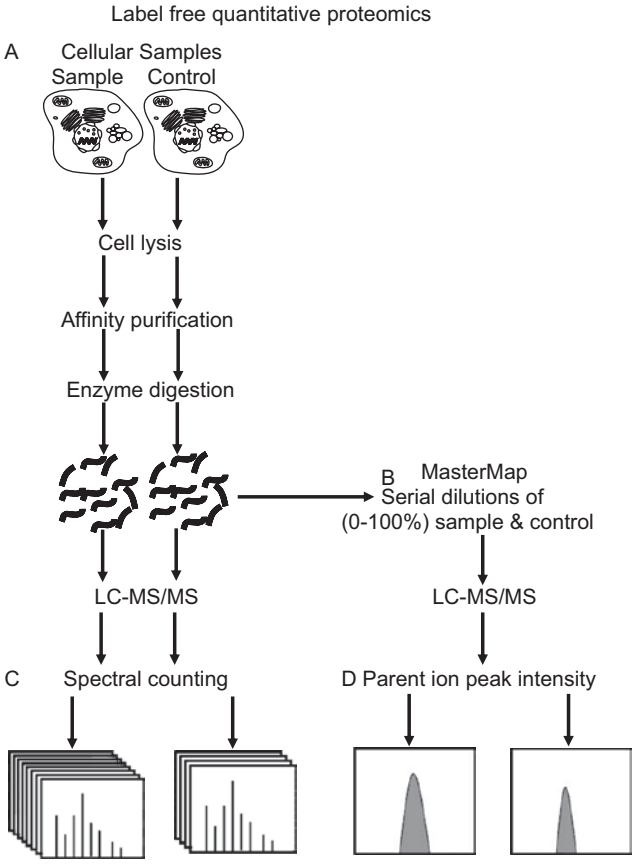

Figure 1. Label-free quantitative proteomics of protein complexes. (A) A sample and a control protein complex are studied separately by LC-MS/MS analysis. (B) The MasterMap approach was developed to eliminate background noise from the purification and samples are analyzed by an algorithm that performs multiple alignments and clustering of precursor ions peak intensity (Rinner et al., 2007). Commonly used quantification methods are spectral counting, where the total number of peptides that identify a protein are used for quantitative analysis (C) and parent ion peak intensity, where the MS scan peak intensity of individual peptides that identify a protein are used for quantitative analysis (D). (See colour version of this figure online at www.informahealthcare.com/bmg)

et al., 2006) (Figure 2). These approaches are particularly useful for studying dynamic changes of protein complexes isolated from tissues or organisms that cannot be metabolically labeled (for more information see Gygi et al., 1999; Ross et al., 2004; Schmidt et al., 2005). ICAT and ICPL approaches are comprised of an affinity handle (often biotin), an isotope linker (cleavable), and a protein reactive group (a thiol or amine reactive). Cysteine-containing peptides are used for quantification in ICAT approach, whereas in ICPL approach lysine-containing peptides and N-termini of proteins are used (Bantscheff et al., 2007). Trypsin digestion of ICPL-labeled samples does not allow cleavage at lysine residues, resulting in formation of longer peptides, which are often difficult to identify. iTRAQ is the only labeling method that relies on fragmentation data of peptides similar to label-free approaches. Therefore, the quantitative information is dependent on MS/MS spectra obtained only from the fragmented peptides. By labeling peptides with isobaric tags that are different in reporter and balancer groups, this method can be used simultaneously to analyze up to eight biological samples in contrast to other labeling methods that can compare only two or three samples in one experiment (Ross et al., 2004; Choe et al., 2007). These reagents are amine reactive, which are linked to the N-terminus of every peptide and lysine residue. In this method, the isobaric reagents are not quantified in the MS scan; instead, they undergo fragmentation during the MS/MS step yielding single low mass reporter ions (113, 114, 115, 116, 117, 118, 119, and 121 m/z are used for the eight-plexed reagent set) (Choe et al., 2007). Intensities of these reporter ions are used to calculate the relative abundance of peptides in each sample.

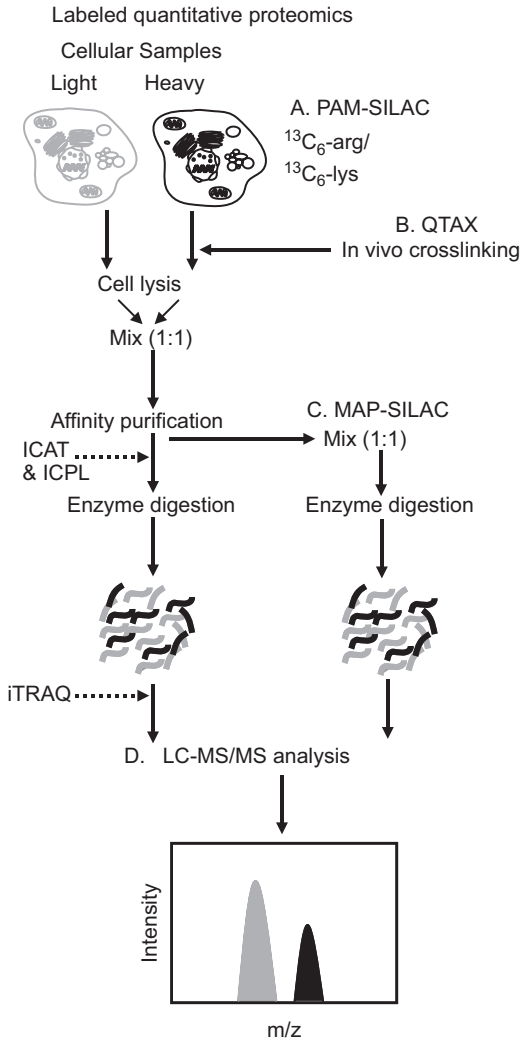

Figure 2. Labeled quantitative proteomics approaches. A sample and a control are labeled differently by heavy and light stable isotopes. The combined samples are analyzed by LC-MS/MS and quantified using relative intensity ratios of isotope-labeled peptides. Chemical labeling of samples can be done at protein level by ICAT or ICPL and at peptide level by iTRAQ as indicated by blue dotted line A. PAM-SILAC is the general SILAC approach where sample and control are combined before affinity purification. (B) In QTAX, the *in vivo* cross-linking is combined with MAP-SILAC and purification is done under denaturing conditions. (C) In MAP-SILAC, the sample and control are mixed after affinity purification. (D) In LC-MS/MS analysis, the same peptide but from heavy and light samples will co-elute and the peak areas are used to determine the relative abundance of one peptide over another. (See colour version of this figure online at www.informahealthcare.com/bmg)

In contrast to chemical labeling, metabolic labeling is carried out *in vivo* at the protein level by growing cells in growth medium containing stable isotopes (^{13}C, ^{15}N, or ^{18}O in salts or amino acids) (Beynon and Pratt, 2005). SILAC is the most widely used metabolic labeling technique and involves the replacement of naturally occurring essential amino acids with heavy isotope-labeled amino acids during protein synthesis in the cell (Ong et al., 2002; Gouw et al., 2010) (Figure 2). This leads to a difference in mass for chemically identified peptides compared with the control sample, which can be detected by MS. The relative intensities of MS peaks are proportional to the abundance of peptides in the two samples. Labeled arginine and lysine amino acids are generally used because of the advantage that most tryptic peptides can be used for quantification (Gouw et al., 2010). Depending on the amino acid chosen for labeling, the SILAC approach can also be used for multiplexing experiments such as the use of multiplex isobaric tags in iTRAQ (Molina et al., 2009). For example, arginine is available in five different isotopic forms (Molina et al., 2009). SILAC has a major advantage over all the other available methods because it has the ability to incorporate stable isotopes before the purification of the protein complex, which may reduce the errors due to sample handling. However, it is very expensive and time-consuming because cells should be grown for several generations for complete labeling and cannot be applied to the analysis of tissue samples from patients. Despite these drawbacks, this is a widely used approach in the quantitative proteomics field. In summary, there are a variety of useful quantitative proteomics methods, and the majority of these methods have been used to study dynamics of protein complexes (reviewed in Oeljeklaus et al., 2009; Kaake et al., 2010b).

Studying dynamics of protein interactions by MS

Dynamics of protein interactions by label-free approaches

The label-free quantitative proteomics approaches are easy to perform, less expensive, and informative. However, each sample for comparison is processed separately during the analysis and errors might be introduced due to differential sample handling. One major challenge with the study of protein complexes and protein interaction networks is to distinguish bona fide interactors from false positive interactions. In one approach named the MasterMap, the human forkhead transcription factor FoxO3A (hemagglutinin-tagged) complex was co-immunoprecipitated and sequentially diluted into a control sample (Figure 1) to circumvent the problem of distinguishing the specific interacting partners from the background. Multiple MS1 signals from all the LC-MS runs were integrated into a MasterMap, which distinguishes the contaminant proteins from those that interact with the bait (Kühner and Gavin, 2007; Rinner et al., 2007). Contaminants were present at identical levels in both samples and in all dilutions, whereas the levels of proteins that interact specifically with the bait are gradually enriched in those dilutions that have more bait Co-IP samples (Rinner et al., 2007). This method can also be applied to detect changes in composition of protein complexes in different cellular states or types. In another example of a label-free analysis of protein complexes, Yang et al. used label-free quantitative method based on MS/MS average TIC to study the dynamics of insulin signaling. They used 15 bait proteins and total proteins were grouped across 31 biological samples (basal, stimulated, and one TAP control) by BLAST (Yang et al., 2009). The differences in protein levels were quantified by calculating the average TIC values from MS/MS spectra of identified peptides/proteins and the data displayed graphically using Cytoscape (Yang et al., 2009).

Spectral counting, where the total number of spectra that identifies a protein is used for quantitative analysis, has become an important and routine approach for analyzing protein complexes and protein interaction networks. One example of a spectral counting-based approach is the normalized spectral abundance factor (Zybailov et al., 2006), which has been used to analyze different forms of the transcriptional regulatory complex Mediator (Paoletti et al., 2006), a network of chromatin remodeling and nutrient sensing complexes (Sardiu et al., 2008, 2009a), and a deletion network of the Rpd3 histone deacetylase complex (Sardiu et al., 2009b). Other spectral counting approaches have been used to analyze PP2A phosphatases (Goudreault et al., 2009), a kinase and phosphatase protein interaction network (Breitkreutz et al., 2010), Mediator and HDAC1 and HDAC2 interactions (Malovannaya et al., 2010), human deubiquitylating enzymes (Sowa et al., 2009), and the human autophagy system (Behrends et al., 2010). The ease of use of spectral counting has likely played an important role in many of these studies since dozens to hundreds of samples are typically included in each analysis. Furthermore, spectral counting is a relatively straightforward approach when it comes to data analysis, resulting in its increased adoption. That being said, sophisticated statistical models have been developed, for example, for the general use of spectral counting in quantitative proteomics (Choi et al., 2008; Pavelka et al., 2008) and for the specific analysis of protein interaction networks (Sardiu et al., 2008; Choi et al., 2011). The use of metabolic labeling or chemical labeling in such studies would increase the cost dramatically and limit the ability of many researchers to carry out medium to large-scale protein interaction network analyses.

Dynamics of protein interactions by labeling approaches

Labeling approaches have also been used for the quantitative proteomic analysis of protein complexes. To begin, chemical labeling strategies have been used in these types of experiments. For example, the ICAT approach was used to find a 10th subunit of TFIIH transcription complex (Ranish et al., 2004), as well as to asses dynamic changes in transcription factor complexes during erythroid cell differentiation (Brand et al., 2004). The iTRAQ method has been used to analyze dynamics of Snf1 kinase complex in *Candida albicans* (Corvey et al., 2005) and to characterize protein–protein interactions as well as tyrosine phosphorylation dynamics (Pflieger et al., 2008). On the other hand, metabolic labeling approaches such as SILAC are more widely used compared with other methods in the field of quantitative proteomics. SILAC has been used for studying several applications including protein–protein interactions applied to EGF signaling (Blagoev et al., 2003) growth factor effects in mesenchymal stem cells (Kratchmarova et al., 2005), the identification of β-secretase substrates (Hemming et al., 2009), and kinetochore subunits (Akiyoshi et al., 2009). Refinements to the SILAC approach include the method called mixing after purification (MAP)-SILAC (Figure 2) is used to study the dynamic components of a protein complex (Wang and Huang, 2008). The standard SILAC method can also be represented as purification after mixing (PAM)-SILAC to distinguish it from the MAP approach (Wang and Huang, 2008). In the SILAC approach, generally the abundance of genuine interaction partners with the bait is always high compared with control sample and the nonspecific background proteins are close to 1 (Wang and Huang, 2008). In addition, scientists showed that this principle applies only to stable interactions and not necessarily to dynamic interactions. A standard SILAC approach involves purification of sample after mixing of two cell lysates that are differentially labeled. The dynamic interaction partners based on their on/off rates will reach to equilibrium between the two forms of proteins that are bound to the bait. Based on kinetic parameters of interacting proteins, the relative

abundance ratios of dynamic interactors will be equal to background proteins after a certain interval of time (Wang and Huang, 2008). Therefore, the standard SILAC approach cannot distinguish the specific dynamic interacting partners from background proteins based on the relative abundance ratios. This is problematic because most of the proteins involved in signaling events are dynamic and transient by nature (Kaake et al., 2010a).

The above problem was overcome by using a combination of PAM-SILAC and MAP-SILAC methods. Initially, the time controlled (Tc)-PAM-SILAC approach has been introduced to effectively distinguish the dynamically interacting specific proteins from the background proteins (Wang and Huang, 2008). This was developed based on two assumptions. First, the exchange of dynamic interacting proteins take place between the light and heavy forms during purification when both are present; second, the interaction swapping depends on interaction dynamics and incubation time. This approach was first used by Wang et al. to decipher 26S proteasome interacting proteins (Wang and Huang, 2008). They performed experiments with incubation times 20 min, 1 h, and 2 h, and found that for stable interactors the relative abundance ratio is time-independent, but for two proteins ADRM1 and UCH37 the SILAC ratios increased dramatically with shorter incubation times from 1.7 to 6.6 and 1.3 to 7.2, respectively. This strategy was able to identify the dynamic interactors with low on/off rates but still proteins with high on/off rates remained indistinguishable from the background proteins (Wang and Huang, 2008).

The MAP-SILAC has several advantages compared with conventional PAM-SILAC and was used to identify high on/off rate dynamic interactors. The specificity of interacting proteins will be preserved in MAP-SILAC because the mixing of differently labeled proteins occurs after purification and there is no chance for exchange of light/heavy proteins during purification (Wang and Huang, 2008). The use of specific incubation times as in Tc-PAM-SILAC decreases efficiency of protein purification but in MAP-SILAC purifications can be carried out under optimal incubation time for better purification efficiency. In MAP-SILAC, the relative abundance ratios for specific dynamically interacting proteins are always higher than the PAM-SILAC ratios because there is no exchange of labeled proteins in the MAP-SILAC method. All these features made MAP-SILAC a convenient method for identifying dynamically interacting proteins (Kaake et al., 2010a). The MAP strategy is more flexible and can be combined with any other quantitative method when using labeling at the peptide or protein level. The MAP approach combined with Tc-PAM-SILAC was used to completely decipher most of the dynamically interacting proteins of the 26S proteasome complex (Wang and Huang, 2008). In one study, 67 putative human proteasome interacting proteins were identified, among them 14 were misidentified as background proteins by the standard PAM-SILAC approach and 57 of them were not reported previously (Wang and Huang, 2008). In addition, the method was also able to distinguish 35 stable interactors and 16 dynamically interacting proteins from nonspecific background proteins (Wang and Huang, 2008).

The dynamics of various complexes including human TATA-binding protein transcription complexes (Mousson et al., 2008), human COP9 signalosome complex (Fang et al., 2008), mouse embryonic fibroblasts mitogen-activated protein kinase kinase 2 (MEKK2) (Xu et al., 2009), yeast elF2B-elF2, and cyclin–Cdc28 complexes (Kito et al., 2008) have been studied using combination of PAM- and MAP-SILAC approaches. In the study of associated proteins of TATA-binding protein complex, the BTAF1 is the only identified dynamically interacting protein (Mousson et al., 2008). Interestingly, it was found that the interaction BTAF1 with the TATA-binding complex is cell cycle-dependent, providing basis for further exploration into the mechanism of its control (Mousson et al., 2008). All these results showed that the combined MAP- and PAM-SILAC approaches is useful for studying dynamically interacting proteins in various complexes. However, the drawback with MAP-SILAC is that the purification of samples is to be performed separately, which could introduce some variability during sample handling (Kaake et al., 2010a). Due to this, caution needs to be taken to minimize the experimental errors while doing biological replicates and subsequent validation is necessary. In summary, the MAP-SILAC has a potential application in combination with PAM-SILAC for identifying dynamic interaction partners of various protein complexes. Although SILAC-based approaches have been widely used for the analysis of protein complexes, the computational methods used for determining SILAC ratios have not been extensively described in the literature with the exception of Census (Park et al., 2008; Park and Yates, 2010) and MaxQuant (Cox and Mann, 2008; Cox et al., 2009). In addition, both of these software packages are able to analyze more than just SILAC data (Cox and Mann, 2008; Park et al., 2008; Cox et al., 2009; Park and Yates, 2010).

Dynamics of protein interactions by in vivo cross-linking

Even though the SILAC approach has many applications, it cannot identify very unstable transient/weak interactors and other proteins that are not amenable to cell lysis and purification conditions. This has begun to be addressed by *in vivo* cross-linking technology and scientists later used a combination of SILAC with *in vivo* cross-linking to improve the identification of dynamically interacting proteins. *In vivo* cross-linking involves freezing the transient or labile interactions using various chemical cross-linking agents, thereby generating exact *in vivo* protein interaction network maps. In cross-linking approaches, the reactive groups present at the end of cross-linker form covalent bonds with target proteins and the spacer length provides some degree of specificity for the cross-linking process. The functional groups that are commonly used at the end of cross-linking reagents

are amine reactive *N*-hydroxy succinimide (NHS) esters (refer reviews for more details about chemical cross-linking reagents in Sinz, 2006, 2010). Some of the successful cross-linkers used for *in vivo* cross-linking are formaldehyde (Schmitt-Ulms et al., 2004; Vasilescu et al., 2004) and homo-bifunctional, thiol cleavable, and amine reactive di-thiobis-succinimidyl propionate (DSP) (Zlatic et al., 2010), BS3 (bis[sulfosuccinimidyl] suberate) (Chen et al., 2010). These chemical cross-linkers possess at least two reactive groups that are separated by a spacer length of 5–15 A°. Formaldehyde is the most widely used because of its unique physicochemical properties such as water solubility, cell membrane permeability, reversible short cross-linked bonds (2.3–2.7 A°), and also does not affect protein arrangements in the network. The cross-linking approach is highly beneficial for studying membrane protein interactions, because the purification conditions used for effective solubilization of proteins could destabilize the existing protein–protein interactions in the cell (Puts et al., 2010). Cross-linking approaches have been coupled to quantitative proteomics analysis for protein complex analysis.

QTAX (quantitative analysis of tandem affinity purified *in vivo* cross-linked protein complexes) is an alternative approach to combined PAM- and MAP-SILAC, which combines *in vivo* cross-linking with tandem affinity purification and SILAC for studying dynamic interaction partners (Guerrero et al., 2006) (Figure 2). Here, formaldehyde was used for freezing stable and transient interactions of the cell (Guerrero et al., 2006). The combination of this strategy with a modified tandem affinity purification protocol and an isotope labeling method was used to identify novel proteasome interactors (Guerrero et al., 2006). The 26S proteasome subunits were tagged with a HBH (histidine–biotin–histidine) tag, grown in light medium, and the untagged strain was grown in heavy medium containing ^{13}C- and ^{15}N-labeled arginine and lysine (Guerrero et al., 2006). Finally, MAP-SILAC approach was used for quantification of peptides and SILAC ratios were used to differentiate background proteins from proteasome interaction partners (Guerrero et al., 2006). This approach was able to detect the proteasome interaction network containing 471 proteins, which is higher than the previously reported interactors by other methods (Guerrero et al., 2008; Kaake et al., 2010a). Although this method is effective in identifying a wide range of interactors, the efficiency depends on the cross-linker characteristics such as cell permeability, spacer arm length, the structure of interacting proteins, and the accessibility of cross-linkers to the amino acids of protein complexes.

The main drawback for the wide application of cross-linking is the lack of conventional database searching algorithms to identify cross-linked peptides and efficient cross-linking reagents. The widely used formaldehyde cross-linker results in cross-linking proteins to nucleic acids and formation of a mixture of heterogeneous products, which are difficult to analyze by MS. The concentration of formaldehyde and incubation times used for cross-linking should be considered because 90% protein loss occurs at a concentration of 1% v/v applied to cells for 30 min (Sutherland et al., 2008). In an attempt to overcome some of these issues, the protein interaction reporter (PIR) technology was developed which involves introduction of labile bonds in a cross-linker that can be cleaved directly in the ion source of mass spectrometer and releases two intact peptide chains (Hoopmann et al., 2010; Tang and Bruce, 2010). These peptides are identified by MS/MS fragmentation and the peptide precursor mass is used for identification, which reduces the complexity of the sample and simplifies data interpretation (Hoopmann et al., 2010; Tang and Bruce, 2010). Custom algorithm X-links was also developed for the identification of PIR-labeled products and to assign cross-link types (Hoopmann et al., 2010; Tang and Bruce, 2010). These cross-linkers can be useful in structural studies and in stabilizing transient interaction partners.

Taken together, the quantitative methods presented above are useful to study large-scale protein interaction network data, to distinguish nonspecifically interacting contaminants from the specific partners, as well as to identify transient/weak interacting partners. Among all these approaches, the choice of the method depends on the question to be answered and the type of system. Label-free methods are highly preferred for studying large-scale protein interaction networks and improvements are needed in quantification methods for better accuracy. The labeling methods are a common choice for studying dynamics of protein complexes and SILAC has the advantage of likely introducing fewer errors over chemical labeling. Unlike in SILAC, chemical labeling can be used to label at the protein or peptide level; however, the labeling reaction could be challenging. SILAC has been shown to be very successful for studying transient interactors in combination with MAP-SILAC or *in vivo* cross-linking (Kaake et al., 2010a). All these methods are useful to unravel protein–protein interaction networks and dynamic interactors in large protein complexes but not helpful for studying dynamics in individual proteins and protein binding to a ligand or another protein. Approaches that can be used to study dynamics in individual proteins, protein binding to ligands, or proteins binding to other proteins include H/D exchange and hydroxyl radical probing coupled to MS.

MS methods for studying conformational dynamics of protein interactions

The structural and conformational changes in protein complexes happen due to interactions with proteins, metal ions, and ligands (substrate, inhibitor, and cofactor) or due to PTMs. Understanding conformational changes is important, since dynamics of proteins play a critical role in protein function and even some of the proteins become inactive when folded incorrectly. For example, most of the enzymes are active when they are properly

folded and in addition, their function varies based upon the conformation (Bobst et al., 2008). This also conveys the importance of protein conformation in designing ligands to treat various diseases since the design of drug molecules is based on the active conformation of proteins involved in diseases. H/D exchange MS is useful for studying proteins at very low concentrations (500–1000 picomoles), can probe conformational states in solution, and can reveal conformational dynamics (reviewed in Engen, 2009; Marcsisin and Engen, 2010). Another great advantage of H/D MS is that it can analyze multiple proteins and ligands because the LC (liquid chromatography) can separate mixtures. Other MS approaches used for the study of protein structure and dynamics are hydroxyl radical labeling (Xu and Chance, 2007) and ion charge state distribution (Konermann et al., 2008).

An H/D exchange experiment requires the replacement of regular water with deuterated water (D_2O). H/D exchange is a chemical reaction in which the covalently bonded hydrogen atom (O–H, N–H, and S–H groups) from the protein is exchanged with the deuterium present in the water solvent. Hydrogens, which are not involved in hydrogen bonding and exposed to solvent, can exchange with deuterium, whereas the hydrogen involved in stable intramolecular hydrogen bonding and/or the permanently buried inside the protein will be protected from H/D exchange (reviewed in Wales and Engen, 2006; Marcsisin and Engen, 2010). Exchange of hydrogen attached to backbone amide group is the only one quantifiable as the hydrogen attached to polar groups is in fast exchange that is difficult to quantify. The rate of amide hydrogen exchange is proportional to fluctuations in protein structure; therefore, the H/D exchange rate is a good indicator of regional flexibility within a protein (Marcsisin and Engen, 2010). The general workflow and experimental conditions used for the H/D exchange MS was shown in Figure 3. The measurement of H/D exchange over a time course can give information about the conformation of a protein as well as the dynamic changes in conformation due to various factors like binding to proteins or drugs, or PTMs (Busenlehner and Armstrong, 2005; Wales and Engen, 2006; Tsutsui and Wintrode, 2007).

The complimentary approach to H/D exchange for the analysis of protein conformation by MS is hydroxyl radical labeling (reviewed in Xu and Chance, 2007). This involves a covalent modification of solvent-exposed amino acid side chains by hydroxyl radicals and the reactivity depends on the chemical nature of the side chain (reviewed in Xu and Chance, 2007). This leads to either an increase or decrease in the mass of amino acids due to change in the chemical structure of side chains, which can be identified by LC-MS/MS (Takamoto and Chance, 2006). For example, formation of a hydroxyl group on the side chain of aliphatic and aromatic acids increases their mass by 16 Da and acidic residues loose CO_2, which reduces their mass by 32 Da. The aromatic amino acids can even form +32 and +48 adducts with subsequent interaction with the hydroxyl radical (Takamoto and Chance, 2006). The sulfur-containing amino acids cysteine and methionine are highly reactive followed by Trp, Tyr, and Phe, and overall 14 of 20 amino acids represent potential modification sites (Takamoto and Chance, 2006). Hydroxy radicals used for experiments are generated by Fenton chemistry (Sharp et al., 2003, 2004) or homolytic cleavage of hydrogen peroxide by UV light (Hambly and Gross, 2005). There are some drawbacks for this method compared with H/D exchange. This is more nonselective because each amino acid reacts differently with hydroxyl radicals and highly reactive methionine residues can undergo oxidation even in the absence of reagent. This leads to the formation of a mixture of reaction products. Unlike in H/D exchange, the long contact with hydroxyl radicals causes structural damage to proteins (see reviews in Takamoto and Chance, 2006; Xu and Chance, 2007; Konermann et al., 2008). H/D exchange is a well-established technique for various biophysical studies, although hydroxyl radical labeling is in the relatively early stages of development and has excellent potential as a bioanalytical method.

Conformational dynamics due to protein–protein interactions

H/D exchange is useful to determine local and distal dynamic changes involved in activation of an enzyme. Protein kinase A is involved in cAMP signal transduction and its activation is a good example for subunit–subunit interaction in which catalytic regulatory subunit interactions affect distal substrate binding (Anand et al., 2002). In another study, Horn et al. studied the role of dynamics in protein–protein binding of wild-type growth hormone (wt-hGH) and human growth hormone variant (hGHv) to the extracellular domain of their receptor (hGHbp). The engineered helix-1 of hGHv of unbound protein undergoes significant fluctuations compared with wild type and it binds better to hGHbp receptor (Horn et al., 2006). These studies imply that one way to increase protein–protein binding is destabilization of an unbound protein by mutagenesis that results in improved binding with other partners in a protein complex (Horn et al., 2006). These results support the importance of dynamics in molecular recognition and protein–protein interactions.

H/D exchange has been used to study conformational dynamics of regular protein complexes such as heat shock proteins (Wintrode et al., 2003) and also to study the assembly of viral proteins and membrane proteins (Lanman et al., 2003). Human immunodeficiency virus (HIV) has a high mutation rate, which makes it resistant to available drugs (Adamson and Jones, 2004). One of the present available strategies is to target viral capsid protein (CA) of HIV (Adamson and Jones, 2004). CA is a 25.6 kDa protein composed of an N-terminal domain (NTD) and C-terminal dimerization domain (CTD) connected through a flexible linker (Adamson and Jones, 2004). Lanman et al. (2003) investigated the dynamics of the monomeric and assembled capsid protein by H/D

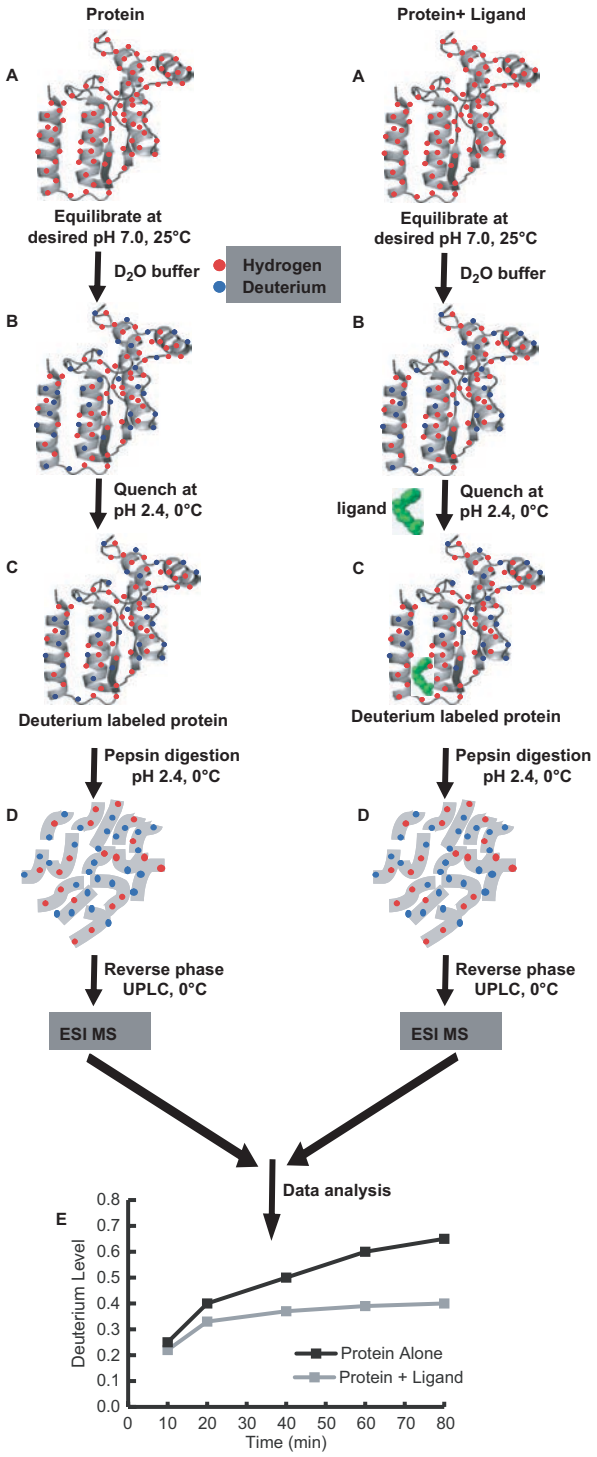

Figure 3. General scheme for the H/D exchange mass spectrometry analysis of protein alone and protein + ligand binding. (A) Protein equilibrated at pH 7.0 and 25°C is diluted into the same buffer in which water is replaced by D_2O. (B) The exchange reaction is stopped at various time intervals by decreasing pH to 2.4 and the temperature to 0°C. The same procedure will be followed to the protein and ligand solution (right side). (C) The samples are digested by enzyme pepsin (5 min), which is active at lower pH. (D) The digested samples are separated by chromatography column and eluted into mass spectrometer. (E) The levels of deuterium uptake are analyzed and plotted against time for the protein alone and protein + ligand comparison.

exchange MS and identified previously unrecognized inter subunit interactions between NTD and CTD. This technique has also been used for studying other viral capsid proteins by different groups (Wang et al., 2001b). The study of membrane protein structure and dynamics is difficult by most physical techniques because of solubility issues and size of proteins. Recently, H/D exchange has been used in integration with nanodiscs for emulating a native environment of membrane proteins (Hebling et al., 2010). This technique was applied to study the native conformation of a 94-kDa γ-glutamyl carboxylase (GGCX) transmembrane protein (Hebling et al., 2010).

Conformational dynamics due to PTMs

PTMs of specific amino acids of a protein potentially affect the structure and conformational dynamics of protein complexes, and are typically important for controlling protein function. These modifications even alter conformational mobility of regions far from the site of covalent modification. Non-enzymatically controlled modifications such as deamidation, oxidation, isomerization, and disulfide scrambling have little or no effect on the conformational changes of protein complexes and are often involved in protein degradation, protein aging, and so on (Hipkiss, 2006). For example, conformational dynamic studies focused on phosphorylation of methylesterase CheB showed that the phosphorylated state is more solvent accessible compared with non-phosphorylated state (Hughes et al., 2001), which would likely not be possible to detect using X-ray crystallography due to the two second lifespan of the transiently active phosphorylation state of the enzyme. These studies have been extended to investigate the impact of other modifications (oxidation, fucosylation, and galactosylation) on the conformational dynamics (Houde et al., 2010). CheB is a bacterial chemotaxis receptor modification enzyme involved in a wide variety of phosphotransfer-dependent signal transduction pathways by specifically demethylating methylglutamate residues of other proteins (Simms et al., 1985). The crystal structure of the non-phosphorylated form was shown to act as an inhibitor because the regulatory domain of the protein obstructs substrate access to the active site located in the C-terminal domain (Djordjevic et al., 1998). Later, the changes in the transiently active phosphorylation state were probed using H/D exchange where greater solvent accessibility of the catalytic domain was observed (Hughes et al., 2001). Furthermore, deletion studies of the regulatory domain demonstrated decreased solvent accessibility to the catalytic domain (Hughes et al., 2001). These studies are an excellent example demonstrating the affect of phosphorylation on the conformation of a protein.

Recently, Houde et al. (2010) studied the affects of methionine oxidation (heavy-chain residues Met 253 and Met 429), fucosylation, and galactosylation on the conformational dynamics of IgG1 antibody and its binding to the $Fc_\gamma RIIIa$ receptor. All these modifications change the local environment of CH_2 domain residues (243–247).

The galactosylation modification of IgG1 increases the structural rigidity of the polypeptide backbone at Lys 247 residue, and this leads to increased receptor binding. The fucosylated–hypergalactosylated IgG1 binding to $Fc_{\gamma}RIIIa$ affects residues 243–247 and 279–301 of the CH_2 domain (Houde et al., 2010). These changes were not seen in binding of unmodified IgG1 with the receptor, which suggested a different mode of binding due to changes in conformation or conformational dynamics (Houde et al., 2009, 2010). Additional examples of the use of H/D exchange to study the effects of PTMs include an analysis of the C-terminal of the Src kinase (Hamuro et al., 2002) and extracellular signal-regulated protein kinase 2 (Hoofnagle et al., 2001).

Ligand binding induced changes in protein structure and dynamics

H/D exchange is one of the most commonly used techniques to probe ligand (substrate, inhibitor, and cofactor)-binding sites and to correlate observed changes in protein dynamics to biological functions (Sinz, 2007). Structure-based drug design mostly involves the use of static protein structures determined by X-ray crystallography for docking ligands, but these results can be improved by incorporating flexibility in the protein structure during the docking process. The dynamic nature of proteins in solution can be studied by H/D exchange and can be implemented in structure-based drug design (Sinz, 2007). The amide hydrogens of ligand-bound proteins are generally in less exchange with the deuterium compared with unbound form because of the shielding by the ligand (Sinz, 2007). Two methods developed to study association constants, stoichiometry of protein–ligand interactions, and quantifying the conformational changes associated with ligand binding to proteins are: protein–ligand interactions in solution by MS, titration, and H/D exchange (PLIMSTEX) (Zhu et al., 2003a, 2005) and stability of unpurified proteins from rates of H/D exchange (SUPREX) (Ghaemmaghami et al., 2000). The basic principle in PLIMSTEX (Zhu et al., 2003a, 2005) and SUPREX (Ghaemmaghami et al., 2000) is to measure the uptake of deuterium as a function of ligand and denaturant concentration, respectively.

An example of the use of H/D exchange to probe the structure and dynamics of protein complexes because of binding to various ligands includes an analysis of the peroxisome proliferator-activated receptor γ ($PPAR_{\gamma}$). $PPAR_{\gamma}$ is a transcription factor involved in regulating glucose homeostasis and adipocyte differentiation (Hamuro et al., 2006). Many studies suggest that ligand binding induces changes in the conformation and dynamics of the ligand-binding domain (LBD) of $PPAR_{\gamma}$ that leads to alterations in the gene expression profile (Berger et al., 2003; KallenBerger et al., 2003). The conformational dynamics of the LBD of $PPAR_{\gamma}$ are well-studied in the presence and absence of various drugs (agonists, partial agonist, and antagonist) using H/D exchange coupled with proteolysis and MS. The LBD of $PPAR_{\gamma}$ is more stabilized in binding to the agonist than the partial agonist or antagonist (Hamuro et al., 2006). They also observed that the helices 11 and 12 of LBD are as dynamic as in unbound form except for binding to agonist. The stabilization of helix 12 by the agonist supports the previous data showing the importance of conformation of helix 12 for proper gene activation. These results demonstrate the importance of changes in dynamics of ligand-bound proteins to biological function and the utility of H/D exchange technology to study them (Hamuro et al., 2006; Chandra et al., 2008). Similar types of studies have been applied to probe dynamic and conformational changes relevant to enzyme catalysis of hypoxanthine–guanine phosphoribosyltransferase (HGPRT).

HGPRT is a key enzyme in the salvage pathway of purine nucleotide formation, which catalyzes the reversible Mg^{2+}-dependent 5-phosphoribosyl group from α-D-5,5-phosphoribosyl 1-pyrophosphate (PRPP) to N9 of hypoxanthine or guanine to form nucleotide IMP or GMP, respectively. The immucillin phosphates ImmGP and ImmHP, which are transition state analogs of HGPRT, act as inhibitors (Li et al., 1999; Shi et al., 1999). The differences in solvent accessibility of HGPRT upon binding to nucleotides (GMP·Mg) (binary), nucleotide and substrate (Michaelis complex of $IMP{\cdot}MgPP_i$), or transition state inhibitors ($ImmGP{\cdot}MgPP_i$) (ternary equilibrium complex) were studied by H/D exchange technique. The exchange rates of peptides in catalytic site loops, a loop buried in catalytic site, and a connected peptide of the subunit tetramer were studied. The stronger protection of the catalytic loop and the nucleotide phosphate-binding loop was observed in HGPRT binding to an inhibitor, possibly due to formation of new hydrogen bonds. This provided new insights for the mechanism of H/D exchange into enzymatic reactions involving transition state analogs (Wang et al., 2001a).

There are additional examples of the use of H/D exchange for the study protein and ligand binding. Ceccarelli et al. (2004) used both the crystal structure and H/D exchange to study the binding of alcohol dehydrogenase enzyme to the cofactor NAD^+, and they suggested that the changes in protein structure and dynamics were due to transition from the open to closed conformation caused by cofactor binding. Wang et al. (1998) analyzed ligand binding-induced conformational changes in Yersinia protein tyrosine phosphatase by ESI-FT-ICR and found dramatic differences in solvent accessibility that are not apparent from X-ray crystal structures. Finally, analysis of ligand, Mg ATP, or protein, RNA poly(C) binding-induced conformational changes in *Escherichia coli* transcription termination factor Rho helicase elucidated the primary interaction site for binding to RNA (Stitt and Xiao, 2010). These studies further demonstrate the utility of H/D exchange coupled to MS for the analysis of binding event induced protein dynamics.

Metal ion binding (metalloproteins)

Metalloproteins are proteins with metal ions as cofactors involved in a wide range of biological functions.

H/D exchange MS and ion charge state distribution are used for studying conformational changes upon metal ion binding and its effect on the structure and dynamics of proteins (reviewed in Kaltashov et al., 2006). The compact native protein structure after transition into gas phase gives rise to a relatively small number of charges on the surface of protein compared with unfolded protein because the folded compact protein does not allow protons to be accommodated (Kaltashov et al., 2006). The ion charge state distribution of a protein also changes in the presence of various conditions such as use of denaturant, pH, and so on. This is used to evaluate protein compactness and also conformational heterogeneity under different conditions (Konermann and Douglas, 1997). The potential of ESI-MS to analyze mass shift and ion charge state distribution in a single experiment that links metal ion binding or dissociation to its conformational transitions has made this approach particularly useful for exploring the dynamics of metalloprotein complexes (Kaltashov et al., 2006). The downside of this method is that it can detect only conformational transitions that result in significant changes in the solvent-exposed surface area. For example, ion charge state distribution was not able to distinguish the two distinct conformations, apo- and holo-forms of (human serum transferring) hTf/2N (Jeffrey et al., 1998). The Ca^{2+}-induced conformational changes in porcine calmodulin (CaM) and the effect of cations and ionic strength were initially studied by H/D exchange and later using PLIMSTEX technology (Zhu et al., 2003b). CaM is a ubiquitous Ca^{2+}-binding protein present in all eukaryotic cells. Binding to Ca^{2+} leads to conformational changes in CaM, which allows it to bind and activate target proteins (James et al., 1995). Initial studies showed that the sequential binding of calcium ions to CaM (Kilhoffer et al., 1992) also decreases in binding affinity with increasing concentration of other cations (Linse et al., 1991). Later, PLIMSTEX technology showed that the CaM-4Ca species had the largest conformational change and the CaM-*x*Ca-binding events follow a three group (fast, intermediate, slow), pseudo first-order kinetic model (Zhu et al., 2003b).

In summary, H/D exchange is a powerful technique in probing local and distal structural and conformational dynamics of proteins in a complex as well as in observing interactions with various binding partners. Unlike in hydroxyl radical foot printing, H/D exchange is more selective and the nature of exchange is always the same regardless of amino acid chain attached to the amide group. Developments such as PLIMSTEX (Zhu et al., 2003a) and SUPREX (Ghaemmaghami et al., 2000) even quantify the conformational changes associated with ligand binding to proteins. Besides all these improvements, H/D exchange suffers some limitations and needs to be improved. A major development in HPLC separation of large size protein complexes at 0°C is needed because the time available for running chromatography allows only separation of peptides, and limits the size and complexity of proteins to be studied. The largest system that so far had been studied was viral capsid proteins containing smaller subunits of identical copies (Tsutsui and Wintrode, 2007). Other improvements include developing proteases, which are active at pH 2.5, and new gas phase fragmentation methods for preventing H/D scrambling (Tsutsui and Wintrode, 2007).

Concluding remarks

Most biological processes are dictated by dynamic changes in the composition, structure, and conformation of protein complexes. MS coupled with affinity purification is the only technique that can be used to investigate both the transient and/or weak interactors and conformational dynamics of protein complexes. Recent developments in identifying dynamic interaction partners coupled with studying structural and conformational dynamics of large protein complexes will likely continue to be beneficial to the structural biology research community. Label-free quantitative proteomics methods are particularly useful for evaluating large-scale protein interaction network mapping. Methods such as the QTAX strategy are able to identify highly transient interaction partners of protein–protein interaction networks (Guerrero et al., 2008). However, for widespread application of detection of transient interactions there is a need to develop cross-linking reagents that are very specific and can simplify the computational search for cross-linked peptides. H/D exchange MS has become an attractive technique for its ability to study both conformational dynamics and structural changes. Due to its ability to probe location of binding sites of ligands along with structural and conformational changes even at low concentrations of proteins, H/D exchange is emerging as a complement to other structural techniques such as NMR and X-ray crystallography (Wales and Engen, 2006). However, application of H/D exchange to large protein complexes and for H/D exchange to become a high-throughput technique requires improvements in chromatography separation and the software used for data analysis.

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Declaration of interest

The authors declare that they have no conflicts of interest.

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