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PLIMSTEX: a novel mass spectrometric method for the quantification of protein–ligand interactions in solution

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Abstract

Protein–ligand interactions by mass spectrometry, titration, and H/D exchange (PLIMSTEX) is a new mass spectrometric method for determining association constants and binding stoichiometry for interactions of proteins with various ligands, as well as for quantifying the conformational changes associated with ligand binding to proteins. The association constants determined with PLIMSTEX agree with literature values within a factor of six, establishing its validity for protein interactions involving metal ions, small organic molecules, peptides, and proteins. PLIMSTEX provides solution, not gas-phase, properties by taking advantage of ESI and MALDI mass spectrometry to measure accurately the mass of a protein as it undergoes amide H/D exchange. The approach sidesteps the problem of relating gas-phase abundances of the protein or protein–ligand complex ions to their solution concentrations. With on-column concentration and desalting, high picomole quantities of proteins are sufficient for reproducible mass detection, and the concentration of the protein can be as low as 10^{-8} M. It is amenable to different protein/ligand systems in physiologically relevant media. No specially labeled protein or ligand is needed. PLIMSTEX offers minimal perturbation of the binding equilibrium because it uses no denaturants, no additional spectroscopy or reaction probes, and no physical separation of ligand and protein during binding.

Keywords: Protein–ligand interaction; H/D exchange; ESI-MS; Association constant; Conformational change

1. Introduction

The interaction of ligands with proteins and the concomitant conformational change in the protein are of crucial importance in biophysics and drug design [1–3]. Although computer modeling has been used to predict binding affinities [2,4,5], the strengths of these interactions are normally determined by various experimental assays [6–9]. The experimental approaches for quantification of protein–ligand binding are (1) equilibrium titrations, in which the equilibrium concentrations of the ligand and protein are measured or deduced; (2) kinetic measurements, in which the on and off rate constants for ligand association are measured at binding equilibrium and the ratio gives the equilibrium constant; and (3) stability measurements, in which the changes in protein stability are followed during ligand binding, and the free energy difference between an apo protein and a ligand-bound protein is measured. Although these measurements enjoy good success, limitations do exist for some traditional methods such as calorimetry, radiolabeling, and spectroscopy because they may require large amounts or specifically labeled ligand or protein. Some methods require additional spectroscopic or reaction probes, denaturants, or measurements of equilibrium concentrations following a separation, which may

Abbreviations: H/D, hydrogen/deuterium; PLIMSTEX, protein–ligand interaction using mass spectrometry, titration and H/D exchange; ESI-MS, electrospray ionization-mass spectrometry; MALDI, matrix-assisted laser desorption ionization; CaM, calmodulin; IFABP, intestinal fatty acid binding protein; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]

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perturb the equilibrium. It is still of interest for biochemists and biophysicists to seek new methods for quantification of protein–ligand binding that have general applicability, high accuracy, relative simplicity, and high throughput.

Recently we developed a method [10] to quantify *P*rotein–*L*igand *I*nteractions in solution by *M*ass *S*pectrometry, *T*itration and H/D *Ex*change (PLIMSTEX). This strategy, which is not subject to many of the limitations discussed above, can determine the conformational change, binding stoichiometry, and affinity for a variety of protein–ligand interactions including those involving small molecules, metal ions, and peptides [10]. We also recently described the modeling procedures for PLIMSTEX and the effect of model modifications on precision and accuracy [11]. This modeling applies not only to PLIMSTEX but also to titration modeling, in general. Combined with kinetic measurements of H/D exchange, PLIMSTEX can provide insights on protein structure and protein–ligand interactions and reveal effects of media and ionic strength [12], species specificity, mutations on protein–ligand binding, and systematic changes in ligands [13]. The purpose of this account is to describe PLIMSTEX, provide perspective, and discuss its advantages with respect to conventional methods and to other mass spectrometry-based methods that can be used to study protein/ligand equilibrium.

2. Experimental

2.1. Protocol for H/D exchange and LC/MS analysis

The general protocol for PLIMSTEX is illustrated in Fig. 1. The experiment was begun by allowing the protein to equilibrate with different concentrations of ligand in aqueous buffer solutions. D_2O containing the same concentrations of buffer and salts as in the starting solution was added to initiate H/D exchange. The protocol utilized a high D/H ratio in the forward and a high H/D ratio in the back-exchange, and carried the added advantage of in situ desalting. When the system reached a near steady state (1–3 h of exchange) where the fast exchangeable hydrogens had reached equilibrium while the slow exchangers had not (as established by a kinetic study conducted previously), the exchange was quenched by adding cold 1 M HCl to decrease the pH to 2.5. The solution was then loaded on a small C18 column (or C4 column for large protein), cooled to 0 °C, and the labile, non-amide sites of the immobilized protein were back-exchanged to the H form. The solution was desalted by washing with ice-cold, aqueous formic acid (pH 2.5). The protein, which now bears an isotopic-exchange "signature" in its amide linkages, reflecting its state in the initial solution, was then introduced into a mass spectrometer, and its molecular weight was determined. Rapid elution (by an isocratic flow of solvent at 30–35 μL/min with high organic composition or with a fast, pH 2.5 gradient) delivered the protein to an electrospray ionization (ESI) source. We conducted mass analysis with either a Finnigan LCQ ion trap or a Micromass Q-TOF working in the positive-ion mode although MALDI should also be an appropriate method.

Insulin, a 51-amino acid protein known to self-associate in solution can serve as an example of protein–protein interactions. To obtain data similar to PLIMSTEX, the concentration of protein in solution was varied, and amide exchange was initiated, followed by quenching of the exchange, and injection of the ice-cold solution into the Q-TOF mass spectrometer. After the quench, the oligomers dissociated into monomers, but the increase in mass of the monomer (compared to the control) was measured to give a weighted average of the increase in mass of the various oligomers. These data were used to trace the path back to obtain species-specific deuterium number for each oligomer, and to calculate the association constants for the oligomerization [14].

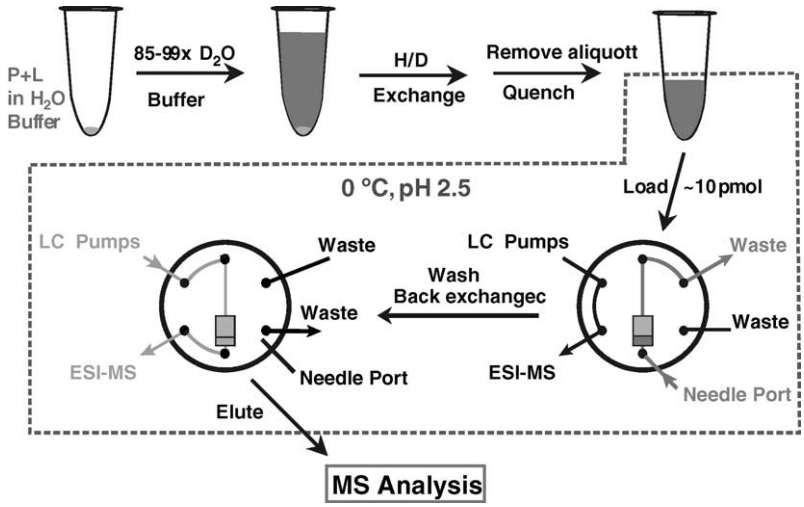

Fig. 1. A general H/D exchange and LC/MS protocol for PLIMSTEX. (P is protein and L is ligand.)

2.2. Modeling titration curves

A detailed modeling procedure for analyzing PLIMSTEX data was described previously and will not be repeated here [11]. For fitting the insulin self-association data, the modeling was modified to acknowledge that both ligand and protein were the same, and the modifications will be described elsewhere [14]. The self-association modeling, as that for PLIMSTEX, was executed in Mathcad 2001 (MathSoft, Inc., Cambridge, MA). The remaining constants and variables were the same as for modeling of titrations with small ligands [11].

3. Results and discussion

3.1. PLIMSTEX determines K_i, stoichiometry, and protection (ΔD_i)

If a non-covalent protein–ligand complex can be introduced into the gas phase, the molecular mass reveals its stoichiometry. This measurement can be obfuscated by non-specific binding. An alternate route to stoichiometry and to affinity is PLIMSTEX. PLIMSTEX generates a plot of the mass difference between a deuterated and non-deuterated protein (deuterium uptake) versus the total ligand concentration (example in Fig. 2). To determine stoichiometry PLIMSTEX requires that the titration be done at high protein concentration (Fig. 3). To quantify affinity, PLIMSTEX requires that a change occur in the extent of H/D exchange during a titration. The change may be a conformational change and/or stability difference between the apo- and ligand-bound protein.

Quenching and desalting cause the ligand(s) to dissociate, liberating the protein for measurement by mass spectrometry to give the number of deuteriums taken up by solvent-accessible amides. Typically, the deuterium uptake values decrease with increasing ligand concentration, which reflects an increased protection (overall deuterium shift ΔD) of the backbone amide protons on formation of the protein–ligand complex. Intermediate states for multiple ligand binding can also be monitored when a specific deuterium shift (ΔD_i) can be related to a specific binding species.

We applied PLIMSTEX to examine affinity constants (K_i) and stoichiometry, and to assign protection against H/D exchange in interactions involving small organic molecules [fatty-acid carboxylates binding to intestinal fatty-acid-binding protein (I-FABP)], metal ions [Mg^{2+} binding to GDP-bound human ras protein, or Ca^{2+} binding to apo calmodulin (CaM)], and peptides [melittin binding to Ca^{2+}-saturated calmodulin (holo CaM)]. Recently, we extended PLIMSTEX to protein–protein interactions, using the self-association of various insulins as models [14]. The insulin amide exchange during the self-association showed that the number of exchangeable deuteriums decreased with increasing concentration of insulin, demonstrating that association occurs and more amide hydrogens become protected as a result.

The affinity constants determined by PLIMSTEX for the test system are within a factor of six of those previously determined using conventional methods (Table 1). The positive ΔD_i values (Table 1) give a quantitative measure of the *increased* protection for the protein from H/D exchange. The protection arises from either direct ligand interaction or ligand-induced conformational change that makes the protein less solvent accessible (shown schematically in Fig. 2). Negative ΔD_i values, on the other hand, indicate decreased protection and an opening of structure with ligand binding. The ΔD values in the case of insulin represent changes in the protection in the oligomer compared to that in monomer.

PLIMSTEX curves are sensitive to the total protein concentration and do not yield reliable K values when the protein is titrated at high concentrations (~100 times the $1/K$ or K_d). Nevertheless, when the concentration is too high, "sharp-break" curves (Fig. 3) are obtained and can be used for stoichiometry determination. These curves may also be

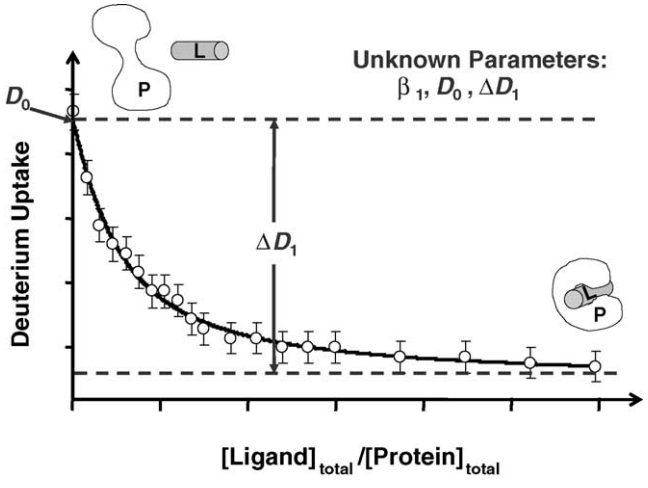

Fig. 2. Schematic illustration of a PLIMSTEX curve for 1 to 1 protein–ligand binding. (P is protein and L is ligand.)

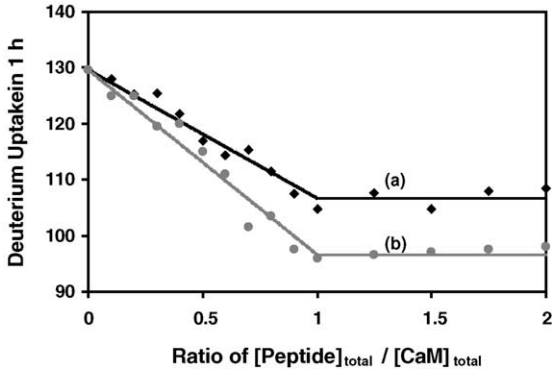

Fig. 3. Sharp-break PLIMSTEX curves at high protein concentration. (a) Melittin (a 26-amino acid peptide) titration and (b) mastoparan (a 14-amino acid peptide) titration of 15 μM Ca^{2+}-saturated porcine calmodulin (CaM–4Ca) in 50 mM HEPES, 100 mM KCl, 0.49 mM Ca^{2+}, 99% D_2O, apparent pH 7.4. Data points are based on the average of two runs for each titration system, and the break points clearly indicate 1 to 1 protein–ligand binding stochiometry.

Table 1
Titration parameters obtained by PLIMSTEX

Protein (C_{total}) + ligand (1 to n)	ΔD_i[a]	PLIMSTEX[a] K_i (M^{-1})	K_i (Literature)/ K_i (PLIMSTEX)[b]
Rat I-FABP (0.3 μM) + oleate (1 to 1)	13.8 ± 0.7[c]	K_1: $(2.6 \pm 0.6) \times 10^6$	1.2[d]
Human ras-GDP (1.5 μM) + Mg^{2+} (1 to 1)	25.6 ± 0.6[c]	K_1: $(4.1 \pm 0.2) \times 10^4$	1.7[e]
Porcine apo-CaM (15 μM) + Ca^{2+} (1 to 4)	12.6 ± 0.3[f]	K_3: $(7 \pm 2) \times 10^4$, K_4: $(1.1 \pm 0.4) \times 10^5$, K_3K_4: $(9 \pm 1) \times 10^9$ M^{-2}	K_3: 0.6[g], K_4: 2.8[g], K_3K_4: 1.4[g]
Porcine holo-CaM (0.15 μM) + melittin (1 to 1)	29.3 ± 0.8[c]	K_1: $(5.4 \pm 0.9) \times 10^7$	6.1[h] or 0.2[i]
r-Human insulin + r-human insulin (mono- to di- to hexamer)	14 ± 2[j], 23 ± 3[k]	K_{12}: $(7 \pm 1.2) \times 10^5$, K_{26}: $(2 \pm 0.7) \times 10^9$	K_{12}: 0.2[l], K_{26}: 0.2[l]

[a] Each protein–ligand titration was done in duplicate. Values were determined by fitting the average data at similar conditions. A sub-sampling method was used to evaluate the second order statistics of the parameters.
[b] K_i (Literature) was determined under comparable experimental conditions (e.g., similar pH, ionic strength, if available) are selected.
[c] ΔD_1.
[d] From Ref [42].
[e] From Ref [64].
[f] ΔD_4.
[g] From Ref [65].
[h] From Ref [66] for CaM from bovine brain.
[i] From Ref [67] for CaM from wheat germ.
[j] ΔD_{12}.
[k] ΔD_{26}.
[l] From Ref [68].

useful in purity determinations of a protein if a pure ligand were available as a titrant.

Referring to Fig. 3, we see that the binding of mastoparan, which is a 14-amino acid (aa) residue peptide from the wasp and is approximately half the size of melittin (a 26 aa residue peptide from bee venom), causes more CaM protection than that of melittin. The number of amide hydrogens that are protected is greater for the smaller mastoparan than for melittin, ruling out a direct block of the surface amides, and indicating significant conformational change with the binding. The PLIMSTEX result is in accord with the proposed structure of the holo-CaM–melittin complex [15] for which the holo-CaM changes from an open dumbbell shape to a closed globular shape with both domains interacting with the peptide. The conformational change induced by mastoparan binding may involve that small peptide being surrounded by the two domains of CaM, whereas this full interaction may not be possible for the longer peptide melittin. These two examples demonstrate a potential for PLIMSTEX to quantify the conformational changes associated with protein–ligand binding.

3.2. PLIMSTEX relies only on measurement of m/z not concentration

One asset of modern mass spectrometry in protein science is that ESI and MALDI [16,17] can introduce non-covalent complexes into the gas phase [18–20]. Using these complexes allows the relative and absolute binding affinities to be deduced when one assumes that the gas-phase ion abundances (peak intensities) for the complex, apo protein, and ligand are directly related to their equilibrium concentrations in solution [21–24]. Other cases make use of the intensity of the complex and the protein at high ligand concentration to calculate the binding constants. The advantage of all these approaches [21,25–27] is that they are rapid, but their validity requires that the complex and the protein are put in the gas phase with equal efficiency.

Unfortunately, the nature of ESI causes it to be discriminatory in terms of ion abundances and the resulting peak intensities especially when measuring a system at equilibrium [28,29]. Electrostatic forces in complexes are strengthened in a solvent-less environment, and electrostatically bound protein–ligand complexes may be more stable in the gas phase than in solution. Binding that is largely governed by hydrophobic interactions in solution, however, weakens in the vacuum of a mass spectrometer, and complexes bound by hydrophobic forces break apart to an unpredictable extent, leading to incorrect affinities [19,30,31]. One may correct for fragmentation of a non-covalent complex in the gas phase by using response factors that relate the mass spectrometer signal to the concentration of the complex in solution and ultimately give the correct stability of the complex. A recently announced method [32] cleverly uses only the signal intensity of the complex and follows it in a titration, much the same way as PLIMSTEX uses only the changing mass of the protein during a titration. Modeling of the changing intensity as ligand is added gives the response. Although use of response factors may avoid some of the problems of direct measurements, the ionization process must still bring detectable amounts of protein–ligand complex into gas phase, and this remains problematic for weak binding systems. Furthermore, for systems having a low K_a, the titration must be performed at high concentration of ligand and protein, regions where the response of ESI may be nonlinear [33–37].

An additional problem for all direct methods is that they cannot use high ionic strength and nonvolatile buffers, which

are needed to simulate physiological conditions. ESI does not work under these conditions, and non-specific adducts may be produced, confusing the stoichiometry and affinity determinations. Furthermore, if the affinity is to be measured in water, then ESI must be done with solutions that have high contents of water, but this requirement is often incompatible with successful ESI. Another problem arises because different source configurations (e.g., normal versus nano ESI) and desolvation conditions may give different results in affinity determination [38].

PLIMSTEX avoids these problems by following changes in H/D exchange by using the shifts in the mass spectrum. As such, it takes advantage of the increasing ability of mass spectrometers to measure accurately m/z. The signal intensities for the complex are not required. The measurement of mass is not compromised by the nature of ESI to discriminate ion abundances. The basis for PLIMSTEX is reactivity, similar to footprinting [39], but there is a strong analogy to titration monitoring by spectroscopic methods (e.g., absorbance or fluorescence). SUPREX, another recent method for measuring the free energies of binding from H/D exchange rates during unfolding (for some examples of the method, see [40,41]), also takes only a single parameter from the mass spectrum (i.e., the m/z) and also avoids the complications of relying on ESI signal intensities.

3.3. PLIMSTEX requires low quantities of protein

NMR, X-ray crystallography, and calorimetry-based approaches typically require millimolar concentrations and milliliter volumes. This hinders their use for proteins that are available only in low quantities and/or are difficult to purify. Furthermore, measuring affinity may require a concentration regime that is too low for determining the enthalpy of binding, and these regimes may be experimentally inaccessible to conventional methods such as isothermal titration calorimetry [42]. Spectroscopy-based approaches such as fluorescence or circular dichroism generally require less sample, but when the binding is weak, these methods also require more sample [43].

Owing to the high sensitivity of mass spectrometers and the chromatographic concentrating procedure in our protocol, we are able to measure a wide range of protein concentrations in PLIMSTEX by simply adjusting the injection for MS analysis. Small quantities (high picomole) and low concentration (nanomolar) of proteins are sufficient for mass measurement in each acquisition. For each PLIMSTEX curve consisting of more than 10 data points, nanomoles or less of protein are needed. The direct methods by mass spectrometry and SUPREX [44] also need only small amounts of protein.

3.4. PLIMSTEX works in biologically relevant media at high ionic strength

Taking the advantage of clean-up (desalting) and concentrating procedures, we are able to use various proteins, buffer systems, salts, and pH in the exchange protocol. These allow PLIMSTEX to measure protein–ligand binding in biologically relevant media at high ionic strength, which is not possible for direct ESI measurements.

High sensitivity is often achieved because the pH is decreased to quench the exchange, and metal cations and ligands normally dissociate and are removed by chromatography prior to MS analysis. Further, all forms of the protein revert back to the apo state, giving minimal signal dispersion and good signal-to-noise ratio. The clean-up improves the mass resolving power because metal-ion interference is removed. By maintaining a high D/H ratio in the forward exchange and a high H/D ratio in the back exchange, we find a narrow isotope distribution and concomitant improved mass resolving power. By rapid desalting on the guard column and eluting quickly with a high concentration of organic in the LC solvent, we normally maintain the time between quenching and analysis to be less than 1 min, minimizing back exchange. For example, when we applied PLIMSTEX to the binding of the small peptides, melittin and mastopran, to calmodulin (Fig. 3), we found that the peptide signals correspond to complete deuteration, indicating negligible back exchange.

SUPREX can also work at high ionic strengths [45] but requires that the ΔG of binding be obtained by using denaturants.

3.5. PLIMSTEX does not need specially labeled protein or ligand

Many conventional methods require that the protein be specially labeled so that it can generate the signals that are a measure of concentration. For example, ^{13}C and/or ^{15}N isotope-enriched proteins or special isotope-labeled ligands are commonly used in NMR. Radio-labeled materials are essential when counting is used. For protein–ligand systems that do not contain chromophores or fluorophores, additional labels must be included. Some affinity studies need special chemical reaction probes, and these probes may be expensive or difficult to obtain, thus hindering their application to a wide range of protein–ligand systems.

PLIMSTEX relies on the hydrogen/deuterium exchange of amide hydrogens that are present in all protein systems; therefore, no special labeling is necessary. Other mass spectrometry-based methods also do not need special labeling, but they suffer from discrimination effects of ESI or MALDI.

3.6. PLIMSTEX avoids perturbation of the binding equilibrium

The use of D_2O as an exchange reagent produces the least perturbation of any chemical method. No additional reagents are added when using PLIMSTEX. No physical separations of the free ligand or protein from the protein–ligand binding system are required as in affinity chromatography, size

exclusion chromatography, and ultra-filtration. Certain methods that track stability of protein–ligand interactions (e.g., circular dichroism and other spectroscopy methods [46–48] as well as SUPREX [40,49]) require denaturants, and they may perturb the original binding equilibrium. ESI or MALDI-based methods that attempt to measure directly the solution concentrations may also perturb the equilibrium during the ionization process. The perturbation causes additional formation or fragmentation of the complex depending on the mode of binding (electrostatic or hydrophobic) in the complex [19,30,31].

3.7. PLIMSTEX has potential for throughput in drug discovery and proteomics

With the introduction of combinatorial chemistry, many high throughput-screening technologies are being developed for discovering drugs, for screening small molecule-protein affinities, and for determining protein–protein binding interactions. Associated analytical measurements include NMR, X-ray crystallography, mass spectrometry, chemical microarrays [50,51] and protein microarrays [52,53]. An automated approach for the analysis of protein structure by H/D exchange and MS was reported recently [54]. Many techniques developed in high throughput screening methods use automated sample preparation with robot systems and parallel LC/MS with autosampling and online desalting. These can be adapted for PLIMSTEX.

Although PLIMSTEX was originally developed using LC/ESI-MS, it does not eliminate the possibility of using MALDI for the protein–ligand titration. A different desalting procedure is needed, and the conditions for quench and analysis would be controlled differently than when using LC/ESI-MS. If future studies show the MALDI-MS gives similar results in ligand titrations, many current automated procedures for MALDI-MS could also be immediately adopted for PLIMSTEX, making it a high throughput method for library screening, drug discovery, and proteomics. SUPREX and other MS methods also have the potential for high throughput [23,55].

3.8. PLIMSTEX has potential for peptide resolution

Current PLIMSTEX assays give H/D exchange profiles that provide a global view of the intact protein. One of the advantages of using MS to measure exchange is that the information can be extended to the peptide and even the amino acid level by enzyme digestion and/or by MS/MS analysis [56–61]. Once the binding affinity and protection in the intact protein are determined by the current PLIMSTEX strategy, increasing the resolution involves digesting the protein with pepsin after the exchange is quenched. Pepsin is used because it works under the low pH of the quench. The resulting peptides would be analyzed by MALDI-MS, or LC/ESI-MS and MS/MS. We recently implemented and compared different approaches for pepsin digestion [62] of IFABP, CaM, and ras protein. The on-line digestion on a custom-built immobilized pepsin column [62,63] followed by LC-MS and MS/MS gave the best sequence coverage and experimental control. Some advantages of the on-line compared to a solution approach are that there is less pepsin interference in the mass spectrum, more complete digestion, more reproducible cleavage sites, and less digestion time (leading to less back exchange). We applied this on-line digestion to ligand binding of IFABP [62].

Using mass spectrometry to measure directly the complex and estimate the affinity does not give the opportunity to resolve the binding at peptide levels because the information about binding is lost once the complex is broken apart in solution or by MS/MS.

3.9. Current challenges and future directions for PLIMSTEX

Present successes in PLIMSTEX rely on a measurable deuterium shift upon ligand binding. This normally requires a conformational change or a relatively large shielding in the ligand-binding region. We are considering modifications of the current PLIMSTEX procedures so that they are applicable for proteins that do not significantly change conformation during ligand binding. This may be achieved by using competition with a known protein that can serve as an indicator or by employing a pulsed-labeling strategy to shorten H/D exchange time and allow us to focus on the fast exchanging amide hydrogens that may be directly perturbed by ligand binding but would not show difference in longer H/D exchange time.

PLIMSTEX has been applied to protein interactions involving various ligands such as metal ions, peptides, small proteins, and small organic molecules. We wish to continue the validation of PLIMSTEX by applying it to more proteins with wide range of molecular weight and other ligands including nucleic acids and other proteins, and we encourage others to do so as well. One extension is to self-association, and we have preliminary data that the approach is valid for self-association of insulin [14]. The complementary approach of SUPREX can also be applied to protein multimers [44]. The preliminary data (Table 1) show that the binding constants agree with the literature value within a factor of 5.

The current modeling procedure was implemented using Mathcad, which may not be efficient for more complicated protein–ligand binding systems than tested thus far. Other programs (e.g., in C or C^{++}) should increase the calculation speed and be more user-friendly. A kinetics factor may be built into the model to accommodate different exchange times used for the titration and to assist the evaluation of a best time-to-quench for a titration study. An example of a more complex system is the binding of two different ligands to one protein or two proteins competing for a single ligand.

Automation of sample handling and the LC/MS process could make PLIMSTEX a high throughput method for library screening and proteomics. More method development is needed for automated PLIMSTEX experiments and data analyses. In addition, improvements are needed for experiments aimed at increasing the structural resolution by enzyme digestion and MS/MS.

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