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Quantification of Protein−Ligand Interactions by Mass Spectrometry, Titration, and H/D Exchange: PLIMSTEX

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Protein−ligand binding is of crucial importance in biophysics and drug design because binding controls regulatory proteins. Changes in protein stability upon ligand binding,[1,2] measurements of on and off-rate constants,[3] or equilibrium titrations[4] are commonly used to quantify binding. Calorimetry,[5,6] radio-counting,[7] and spectroscopy[8,9] have limitations because they may require large amounts of or specifically labeled materials. Often needed are additional spectroscopic or reaction probes, denaturants, or measurements of equilibrium concentrations following a separation, which may perturb the equilibrium.

The sensitivity and specificity of mass spectrometry (MS) have recently made it important in proteomics and protein research.[10,11] One example is a new MALDI-MS-based stability determination method (SUPREX) that measures K_d values of protein−ligand complexes.[12] Here we describe a complementary means to quantify protein−ligand interactions in solution by MS, titration, and H/D exchange (PLIMSTEX). The strategy is less subject to the limitations described above, and no denaturant is needed, unlike SUPREX. Moreover, binding and intermediate binding states can be monitored, and variations of protein, ligand, buffer, salt concentrations, pH, and temperature can be accommodated.

To quantify affinity, PLIMSTEX requires that a change occur in H/D exchange during a titration. The method has its basis in reactivity, similar to footprinting,[13] but it is analogous to titration monitoring by spectroscopic methods. PLIMSTEX relies on spectral position shifts (ΔD_i) rather than spectral amplitudes (e.g., absorbance). The approach, although indirect, overcomes a major difficulty of modern MS to measure, without discrimination, solution concentrations at any point, including at equilibrium.

We chose four model protein−ligand interactions to illustrate the method: the 1:1 interaction between rat intestinal fatty acid binding protein (I-FABP) and potassium oleate;[14,15] the 1:1 interaction between GDP-bound human $p21^{H-ras}$ protein (Ras-GDP) and Mg^{2+};[16] the 1:1 interaction between Ca-saturated porcine calmodulin (holo-CaM) and melittin;[17,18] and the 1:4 interaction between apo-CaM and Ca^{2+}.[19,20] These are widely studied systems, and their K values range from 10^4 to 10^8 M^{-1}.

The protein is first equilibrated with different concentrations of ligand in aqueous buffer, and H/D exchange is then initiated by adding D_2O, which has buffer and salt concentrations as in the starting solution. The protocol utilizes a high D/H ratio in the forward and a high H/D in the back-exchange, and allows in-situ desalting. When the exchange reaches a near steady state (e.g., 1−3 h, based on a kinetic study conducted previously), the exchange is quenched by lowering the pH to 2.5 with cold, 1 M HCl. The solution is loaded on a C18 guard column (at 0 °C), and the solution is desalted with ice-cold, aqueous formic acid (pH 2.5), back exchanging the labile, non-amide sites of the immobilized protein. A molecular-weight measurement reveals the extent of D uptake by amide linkages, reflecting the protein's state prior to quench. Rapid elution (by isocratic flow of solvent with high organic content or with a fast, pH-2.5 gradient) delivers the protein to an electrospray ionization (ESI) source. (We conducted mass analysis with either a Finnigan LCQ or a Micromass Q-TOF working in the positive-ion mode.)

Quenching and desalting cause the ligand(s) to dissociate, liberating the protein for which the number of deuteriums on solvent-accessible amides can be measured by MS. A plot of the mass difference between the deuterated and nondeuterated protein (deuterium uptake) versus the total ligand concentration gives the PLIMSTEX curve. This is fit by using a 1:n (n is the number of binding sites) protein:ligand sequential binding model (see Supporting Information). A search gives the overall equilibrium binding constants (β_i, $i = 1$ to n) and deuterium shift (difference between the average deuterium level of each binding species and that of the apo-form, ΔD_i, $i = 1$ to n). A positive ΔD_i indicates that binding of i ligand(s) to the protein leads to more protection and less uptake of D as compared to the apo-form. A negative ΔD_i points to the formation of a more open structure relative to its apo form. When $\Delta D_i \approx 0$, little change occurs upon binding. At least two runs were performed for each PLIMSTEX titration to give an average for the parameters (β_i, ΔD_i, and D_0). Macroscopic K_i's were calculated from β_i's.

The PLIMSTEX curve from the titration of wild-type rat I-FABP with K^+-oleate was fit using a 1:1 binding model (Figure 1), giving K_1, ΔD_1, and D_0 for apo-I-FABP as $(2.6 \pm 0.2) \times 10^6$ M^{-1}, 13.8 ± 0.7, and 95.4 ± 0.5, respectively. The results indicate a relatively strong interaction between oleate and I-FABP, causing 14 backbone amide protons of the apo-form to become protected with binding to oleate. The curves for the other binding systems are available as Supporting Information.

Modeling the titration results for the four systems (Table 1) gives the binding stoichiometry from the best fit. The positive ΔD_i values quantify the protection against H/D exchange due to a ligand interaction or to a ligand-induced conformational change that makes the protein less solvent accessible. The K_i values derived from PLIMSTEX agree reasonably well (within a factor of 4) with literature values except for that for the holo-CaM/melittin system. The binding constant for this interaction is ~6-fold lower than the literature value (3.3×10^8 M^{-1}). The latter binding constant, which is commonly cited, was determined using an affinity column to separate free [^{3}H] mono-acetyl-melittin from CaM-bound melittin and quantify it by liquid-scintillation counting.[18] If the high affinity is correct, then the most appropriate protein concentration for the titration would not be 150 nM, but 3 nM, a concentration that challenges current MS.

The shape of any titration curve is very sensitive to the total concentration of protein, suggesting another application for PLIMSTEX. If we use a protein concentration of 100 times the $1/K$ (or K_d), the break in the titration curve should become sharp. The ratio of $[\text{ligand}]_{\text{total}}$ to $[\text{protein}]_{\text{total}}$ at the break would clearly indicate binding stoichiometry. An example (Figure 2) is the titration

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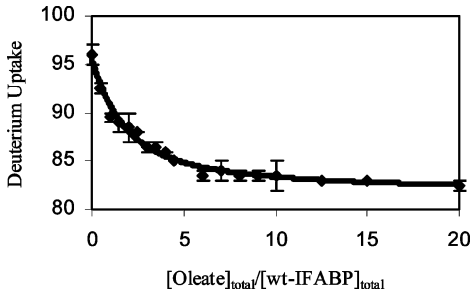

Figure 1. PLIMSTEX data for 0.3 μM wild-type I-FABP titrated with K^+-oleate in 95% D_2O, 20 mM pyrophosphate buffer, 135 mM KCl, and 10 mM NaCl (pH = 9.0), after 3 h of exchange. Error bars are from two independent runs. The solid line represents the fit by PLIMSTEX, using 1:1 binding and a three-parameter model (K_1, D_0, and ΔD_1).

Table 1. Titration Parameters Obtained by PLIMSTEX

protein (C_{total}) + ligand (1 to n)	ΔD_i	PLIMSTEX[a] K_i (M^{-1})	literature[b] K_i (M^{-1})
I-FABP (0.3 μM)	13.8	$(2.6 \pm 0.2) \times 10^6$	K_1: 3.0×10^6 [d]
+ oleate (1 to 1)	± 0.7[c]		(4.8[e] or 25[f]) $\times 10^6$
Ras-GDP (1.5 μM)	25.45	$(4.0 \pm 0.3) \times 10^4$	K_1: 6.9×10^4 [g]
+ Mg^{2+} (1 to 1)	± 0.07[c]		
Holo-CaM (0.15 μM)	29.3	$(5.44 \pm 0.03) \times 10^7$	K_1: 33×10^7 [h]
+ melittin (1 to 1)	± 0.2[c]		
Apo-CaM (15 μM)	14.1	$(5.4 \pm 0.5) \times 10^4$	K_3: 3.98×10^4 [j]
+ Ca^{2+} (1 to 4)	± 0.5[i]	$(0.9 \pm 0.1) \times 10^5$	K_4: 3.16×10^5 [j]
		$(5.0 \pm 0.4) \times 10^9$	K_3K_4: 12.6×10^9 [j]

[a] Average deviation from at least two independent titration curves. [b] K's determined under comparable experimental conditions (e.g., similar pH, ionic strength, if available) are selected. [c] ΔD_1. [d] From ref 15. [e] From kinetic analysis in ref 14. [f] From equilibrium analysis in ref 14. [g] From ref 16. [h] From ref 18. [i] ΔD_4. [j] From ref 19.

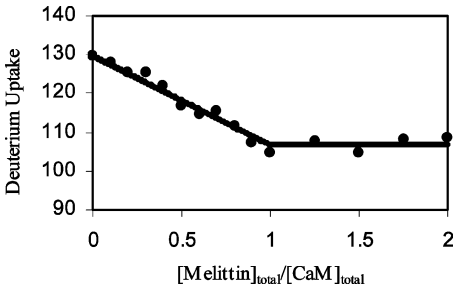

Figure 2. PLIMSTEX curve for titrating 15 μM CaM with melittin in 99% D_2O, 50 mM HEPES buffer, 0.1 M KCl (pH = 7.4), H/D exchange time = 1 h. The data points, which are the average of two independent runs, clearly indicate 1:1 binding stoichiometry.

curve for 15 μM holo-CaM with melittin. Although the conditions are not appropriate for determining K, the curve clearly shows 1:1 binding and increasing protection from exchange accompanying binding, in agreement with the proposed NMR structure for this ternary complex.[17] These "sharp-break" PLIMSTEX curves may also be useful in purity determinations if K is known (or estimated) and the concentration of one component, protein or ligand, is known.

In summary, PLIMSTEX can be applied to determine the conformational change, binding stoichiometry, and affinity associated with a wide range of protein−ligand interactions including those that involve small molecules, metal ions, and peptides. At concentrations too high for determining affinity, PLIMSTEX may be used to determine quickly binding stoichiometry and possibly the purity of proteins. Taking advantage of concentrating the protein on-column and desalting, we can use different concentrations of proteins, buffer systems, salts, and pH in the exchange protocol. High picomole quantities of proteins are sufficient, offering detection limits well below those of NMR and X-ray crystallography.

PLIMSTEX can evolve. For example, the information level may be increased to the peptide or even amino acid level by digesting the protein before analysis and using MS/MS.[21] More sophisticated modeling with statistical analysis and sub sampling will increase the reliability of the affinity determination and allow for fuller interpretation of the titration curve. Automation of sample handling could make PLIMSTEX a high throughput method for library screening and proteomics. High throughput will usually require a "rough" titration or some other method to give an estimate of K_i, to within a factor of 10 or 100, followed by a titration under conditions where the protein concentration is approximately $1/K_i$. The H/D exchange time would be approximately 1−3 h, although some fine-tuning may be needed for different classes of proteins or interactions.

We are now applying PLIMSTEX to determine effects of media, ionic strength, and species on CaM binding and the effects of mutation on FABP binding; these results will be reported soon.

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Supporting Information Available: Information on modeling, experiments, and other PLIMSTEX curves for the systems in Table 1 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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