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A General Mass Spectrometry-Based Assay for the Quantitation of Protein−Ligand Binding Interactions in Solution

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Conventional methods for the quantitative analysis of protein−ligand binding interactions have typically employed either titrations and direct binding detection[1] or measurements of protein stability changes upon ligand binding.[2] However, the calorimetric[3] and spectroscopic[4] techniques traditionally used in these methods have several experimental limitations. Stability measurements require relatively large amounts of pure protein and are not inherently high-throughput, while direct binding detection methods require labeled ligand and selective detection. Here we describe a novel strategy for characterizing the thermodynamic properties of protein−ligand interactions that is not subject to the above limitations. The strategy relies on a new H/D exchange- and mass spectrometry-based approach, termed SUPREX (stability of unpurified proteins from rates of H/D exchange), to measure protein stability changes upon ligand binding. We have recently shown that SUPREX can be used to measure the thermodynamic stability of proteins both in vitro and in vivo with good accuracy and high precision.[5] This report describes the use of SUPREX to measure dissociation constants (K_d values) of protein−ligand complexes in solution.

The four model protein−ligand complexes studied in this work include: the protein−protein complex formed between the B1 domain of protein G and the Fc portion of a mammalian IgG antibody;[6] the protein-peptide complex formed between the S-protein and S-peptide;[7] the ternary protein−DNA complex composed of Trp repressor (TrpR), two molecules of L-tryptophan (W), and a 25-base pair duplex of DNA containing TrpR's cognate DNA sequence;[8] and the protease−inhibitor complex formed between the feline immunodeficiency virus protease (FIV Pr) and a small molecule inhibitor, TL3.[9] The reported dissociation constants for these protein−ligand systems measured by traditional methods range from 10^{-5} to 10^{-10} M.

Each protein and protein−ligand complex was subjected to H/D exchange by dilution into a series of deuterated exchange buffers containing different concentrations of a chemical denaturant, either urea or guanidinium chloride (GdmCl). After a specific exchange time, the deuterium content of each protein sample was determined by measuring the mass increase using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The change in mass relative to the fully protonated sample (Δmass) was plotted as a function of [denaturant], and the data were fit to a sigmoidal function to extract $C_{SUPREX}^{1/2}$ values (i.e., the [denaturant] at the transition midpoint). Shown in Figure 1 are typical SUPREX curves (Δmass versus [denaturant] plots) obtained in our analysis of the TrpR system. It is noteworthy that the SUPREX technique allowed us to use TrpR samples taken directly from lysates of recombinant *Escherichia coli* cultures overexpressing the protein without any purification. As expected, the $C_{SUPREX}^{1/2}$ values determined from the TrpR data in Figure 1 were higher in the presence of the W and DNA ligands, indicative of binding-induced stabilization.

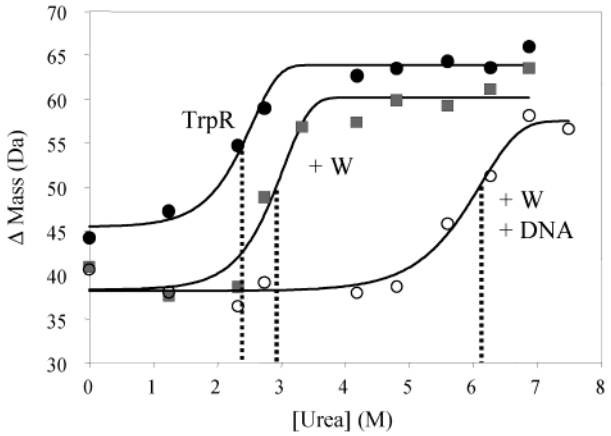

Figure 1. SUPREX data obtained on TrpR in the absence of ligand (●), in the presence of L-tryptophan (W) (■), and in the presence of W and cognate DNA (○). The dotted lines indicate the transition midpoints of each curve.

SUPREX curves such as those depicted in Figure 1 were generated for each model protein system in this study. The $C_{SUPREX}^{1/2}$ values we determined are summarized in Table 1. Ultimately, these values were used in eq 1 to determine folding free energies (i.e., ΔG_f° values) for each protein in the presence and in the absence of ligand (see Table 1).[5d]

$$-\Delta G_f^0 = mC_{SUPREX}^{1/2} + RT\left[\ln\frac{\left(\frac{\langle k_{int}\rangle t}{0.693} - 1\right)}{\frac{n^n}{2^{n-1}}[P]^{n-1}}\right] \quad (1)$$

In eq 1, m is defined as $\delta\Delta G_f^\circ/\delta$[denaturant], $C_{SUPREX}^{1/2}$ is the [denaturant] at the SUPREX transition midpoint, R is the gas constant, T is the temperature in Kelvin, $\langle k_{int}\rangle$ is the average intrinsic exchange rate of an amide proton, t is the H/D exchange time, n is the number of subunits in the protein, and [P] is the protein concentration expressed in n-mer equivalents. We have previously shown that eq 1 can be used to calculate accurate folding free energies from SUPREX data, provided that the protein under study is a two-state folder and under so-called EX2 H/D exchange conditions.[5d,10] These criteria were satisfied for all the protein systems in this study.

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Table 1. Thermodynamic Parameters Obtained by SUPREX

model system	$C_{SUPREX}^{1/2}$ [den] (M) [a]	ΔG_f° [d] (kcal/mol)	SUPREX K_d [e]	reported K_d
B1 domain	0.9 ± 0.1 [b]	-5.1 ± 0.4		
+ Fc frag	1.7 ± 0.1 [b]	-6.3 ± 0.7	0.52 ± 0.14 μM	0.24 μM [f]
S-protein	0.6 ± 0.2 [c]	-3.7 ± 0.2		
+S-peptide	4.5 ± 0.3 [c]	-9.7 ± 0.7	2.4 ± 0.6 nM	1.1 nM [g]
TrpR	2.4 ± 0.2 [c]	-20.2 ± 0.4		
+ W	2.9 ± 0.3 [c]	-21.7 ± 0.7	130 ± 20 μM	42 μM [h]
+ W+DNA	6.1 ± 0.2 [c]	-29.1 ± 0.5	0.16 ± 0.09 nM	0.25 nM [i]
FIV Pr	0.5 ± 0.1 [b]	-14.3 ± 0.6		
+ TL3	1.1 ± 0.1 [b]	-18.2 ± 0.7	520 ± 330 nM	41 nM [j]

[a] Standard errors of curve fitting are reported. [b] The denaturant was GdmCl. [c] The denaturant was urea. [d] Calculated using eq 1; reported with standard error (see Supporting Information for exact assay conditions). [e] Calculated using eq 2; the mean and average deviation of at least three trials are reported. [f] From ref 6c. [g] From refs 7b, c. [h] From ref 8a. [i] From ref 8b. [j] K_I value from ref 9.

In our calculations of ΔG_f° values using eq 1 values for $\langle k_{int} \rangle$ were estimated using the program SPHERE[11] or using the relationship $\langle k_{int} \rangle = 10^{pH-5}$ which holds at room temperature and at pH > 4, and n was defined by the multimeric state of the protein under study. The m values used in eq 1 to calculate ΔG_f° values for each protein in the absence of ligand were estimated, as the data in Myers et al. suggests, using a value of either 0.026 or 0.013 kcal mol^{-1} M^{-1} per amino acid residue (for GdmCl or urea, respectively) and a correction term for the number of disulfide bonds. The m values used in eq 1 to calculate ΔG_f° values for each protein in the presence of ligand were the sum of two components, m_p and m_b. The m_p component was equivalent to the m value associated with the protein's folding/unfolding reaction (i.e., $\delta \Delta G_f^\circ / \delta$[denaturant]), and it was estimated from the data in Myers et al. as described above. The m_b component was the "m value" associated with the binding reaction (i.e., $\delta \Delta \Delta G_f^\circ / \delta$[denaturant]). This m_b component includes the denaturant dependence due to burial of surface area in the binding site (on both protein and ligand) and the denaturant dependence due to any conformational changes in the protein or ligand upon binding. Values for m_b were experimentally determined, as described below.

A unique characteristic of SUPREX curves is that $C_{SUPREX}^{1/2}$ values can be shifted to lower concentrations of denaturant by using longer exchange times or using experimental conditions that increase $\langle k_{int} \rangle$ values (i.e., elevated temperatures, and high pHs). This makes it possible to define the denaturant dependence of a given binding reaction by measuring ligand-induced stability changes at different $C_{SUPREX}^{1/2}$ values. In the work described here, the ligand-induced stability changes ($\Delta \Delta G_f^\circ$ values) that we determined for the B1 domain, the FIV Pr, and the TrpR protein were independent of the $C_{SUPREX}^{1/2}$ values used to calculate them (i.e., $m_b = 0$). However, in the case of the S-protein/S-peptide complex, the $\Delta \Delta G_f^\circ$ values that we measured by SUPREX were linearly dependent on the $C_{SUPREX}^{1/2}$ values used to calculate the ΔG_f° value for the complex. This linear relationship was used to define an m_b value of 0.33 kcal mol^{-1} M^{-1} for the S-protein/S-peptide binding reaction (see Supporting Information for details on the calculation of this m_b value).

Dissociation constants, K_d values, were calculated from $\Delta \Delta G_f^\circ$ values using eq 2.[2]

$$\Delta \Delta G_f^\circ = -nRT \ln[1 + ([L]/K_d)] \qquad (2)$$

In eq 2, n is the number of independent binding sites (see Supporting Information for n value assignments), R is the gas constant, T is the temperature in Kelvin, and [L] is the molar concentration of free ligand. The SUPREX-derived K_d values obtained for the protein−ligand complexes in this study are summarized in Table 1. They are all in reasonable agreement (i.e., within a factor of 3) with previously established K_d values with the exception of the FIV Pr system in which the SUPREX K_d is ~10-fold larger than the K_I measured in a fluorescence-based assay. The D_2O and/or GdmCl in our SUPREX buffers appears to alter the binding affinity of TL3. For example, there may be a nonlinear GdmCl dependence to the FIV Pr/TL3 binding reaction at low concentrations of GdmCl (i.e., <0.5 M) (see m_b value discussion in Supporting Information).

Our results demonstrate that the SUPREX technique is generally applicable to the thermodynamic analysis of a variety of different protein−ligand complexes including those that involve small molecules, nucleic acids, peptides, and other proteins. In most cases, the SUPREX technique can be used to accurately predict the K_d value of a protein−ligand complex to within a factor of 2 to 3. Important experimental advantages of the SUPREX technique over conventional methods include: the ability to make measurements in a high-throughput and automated fashion, the ability to analyze picomole quantities of material, and the ability to analyze either purified or unpurified protein−ligand complexes. These advantages make SUPREX an attractive technique for studying the thermodynamic properties of protein−ligand complexes in vivo, in vitro, and on a proteomic scale.

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Supporting Information Available: Assay conditions, m_b value calculations, and n value assignments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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