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Comparison of two ESI-MS based H/D exchange methods for extracting protein folding energies

Rohana Liyanage, Nagarjuna Devarapalli, Latisha M. Puckett, N.H. Phan, Jennifer Gidden, Wesley E. Stites, Jackson O. Lay Jr.*

Department of Chemistry and Biochemistry University of Arkansas, Fayetteville, AR 72701, United States

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ABSTRACT

In this report, the model proteins staphylococcal nuclease and ubiquitin were used to test the applicability of two new hydrogen/deuterium exchange (HX) electrospray ionization mass spectrometry (ESI-MS) methods for estimating protein folding energies. Both methods use the H/D exchange of globally protected amide protons (amide protons which are buried in the hydrophobic core) to elucidate protein folding energies. One method is a kinetic-based method and the other is equilibrium-based. The first method, the HX ESI-MS kinetic-based approach is conceptually identical to SUPREX (stability of unpurified proteins from rates of H/D exchange) method but is based on ESI-MS rather than MALDI-MS (matrix assisted laser desorption mass spectrometry). This method employs the time-dependence of H/D exchange using various denaturant concentrations to extract folding energies. Like SUPREX, this approach requires the assumption of EX2 exchange kinetics. The second method, which we call a protein equilibrium population snapshot (PEPS) by HX ESI-MS uses data collected only for a single time point (usually the shortest possible) to obtain a snapshot of the open and closed populations of the protein. The PEPS approach requires few assumptions in the derivation of the equations used for calculation of the folding energies. The extraction of folding energies from mass spectral data is simple and straightforward. The PEPS method is applicable for proteins that follow either EX1 or EX2 HX mechanisms. In our experiments the kinetic-based method produced less accurate ΔG_{H_2O} and m_{GdHCl} values for wild-type staphylococcal nuclease and mutants undergoing H/D exchange by EX1, as would be expected. Better results were obtained for ubiquitin which undergoes HX by an EX2 mechanism. Using the PEPS method we obtained ΔG_{H_2O} and m_{GdHCl} values that were in good agreement with literature values for both staphylococcal nuclease (EX1) and ubiquitin (EX2). We also show that the observation of straight lines in linear extrapolation method (LEM) plots is not a reliable indicator of the validity of the data obtained using the LEM approach.

1. Introduction

Typically a specific conformation of a folded protein is biologically active and an unfolded or misfolded state is less so. Unfolding or misfolding may thus be associated with disease when biologically important proteins have less than the necessary function needed for full health [1]. Protein folding is significantly influenced by non-covalent interactions such as hydrogen bonding, ionic interactions, van der Waals' forces and hydrophobic effects [2,3]. While the protein is constantly folding and unfolding, bonding forces generally ensure that the native state is more strongly populated than any other conformation. Any changes in the protein's environment that disrupt these forces and increase the extent of unfolding may also denature the protein. Agents such as urea or guanidine hydrochloride (GdHCl), as well as pH and temperature changes have been used to deliberately denature proteins [4–8]. Such deliberate modifications to the protein's environment have been used to determine protein folding energies [4]. In the literature, most protein denaturation studies have been monitored using spectroscopic probes. Typically these are based on tryptophan or tyrosine fluorescence, or circular dichorism (CD) [4–6,9].

While these approaches are widely used, they have some disadvantages. Tryptophan residues are relatively rare in protein sequences and the resulting fluorescence changes only convey information about local changes. Tyrosines are more common, but also only report local structure. Tyrosine fluorescence is difficult to follow if tryptophan is also present. Circular dichroism signals from helices and sheets are more global in nature with respect to protein

* Corresponding author at: Department of Chemistry and Biochemistry, University of Arkansas, CHEM 119, 345 N. Campus Drive, Fayetteville, AR 72701, United States. Tel.: +1 479 575 5480; fax: +1 479 575 04049.
E-mail address: jlay@uark.edu (J.O. Lay Jr.).

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structure, but measuring the comparatively weak CD signal requires a large amount of protein present in high concentration. Thus, these techniques are not ideally suited for measurement of folding energies in complex environments where other proteins or compounds contribute similar and overlapping spectroscopic signals [10–16].

Mass spectrometry, on the other hand, is well suited for studying proteins at low levels and in complex mixtures. Most often MALDI and ESI are the approaches of choice. Examples from recent literature include MS-based studies of protein folding, protein structure, mapping changes associated with mutations, protein–ligand interactions and protein–protein interactions. H/D exchange [17,18], chemical modification, and limited proteolysis [19] are among the techniques most frequently coupled with MS for this purpose. Of these, H/D exchange has been the most popular and when carefully applied, can accurately reflect biologically relevant solution-phase properties including binding and folding [20–32]. Most of these methods are kinetics-based. The widely used SUPREX [25,27] and related technique SPROX [32] (stability of proteins from rates of oxidation) extract folding and binding properties for proteins and protein–ligand complexes. More recently, methods have been developed specifically for probing complex bimolecular interactions involving proteins. For example PLIMSTEX (protein–ligand interactions by mass spectrometry, titration, and H/D exchange) [31] can be used to measure protein–ligand and protein–protein interactions. A modification of PLIMSTEX called SIMSTEX (self-association interactions using mass spectrometry, self-titration and H/D exchange) [30] can also be used to measure self-association of proteins associated with protein aggregation.

The two primary MS-based methods which have been used to extract protein folding energies are SUPREX and SPROX. SUPREX involves the study of H/D exchange of the globally protected amide protons using MALDI HX-MS [25,27]. SPROX is based on chemical oxidation of globally protected methionines using H_2O_2 [32]. Both MALDI and ESI have been used with SPROX [32]. The SUPREX and SPROX approaches are conceptually similar and can be utilized to measure protein stability in complex environments. The application of both of these methods requires a number of assumptions. SUPREX relies on the assumption of EX2-HX kinetics while SPROX assumes that the protein folding equilibrium is not affected by time dependent protein oxidation. In this work we show that SUPREX may be extended from MALDI to ESI. Our ESI method is sufficiently different from SUPREX that we refer to it herein as the kinetic-based method rather than as ESI/SUPREX. By comparison, we also demonstrate the advantages of an equilibrium-based approach that samples the equilibrium population of the folded and unfolded states using H/D exchange. We call this approach a protein equilibrium population snapshot or PEPS. This PEPS approach requires fewer assumptions and can be used to extract protein folding energies and m_{GdHCl} values. Using several examples from two types of proteins the ΔG_{H_2O} and m_{GdHCl} values obtained using both ESI-based approaches (the kinetic-based method and PEPS) are compared to each other and with previously reported values.

2. Experimental and theory

2.1. Chemicals

The construction of the mutants of staphylococcal nuclease used in this study has been described elsewhere [3,5]. Bovine ubiquitin, sodium hydrogen phosphate, and acetonitrile (ACN) were purchased from Sigma–Aldrich. Trifluoroacetic acid (TFA) was purchased from Halocarbon. Guanidine hydrochloride, omni pure, was purchased from VWR. Sodium phosphate and sodium chloride were purchased from Mallinckrodt. Deuterium oxide (D_2O; 99.8% atom D) was purchased from Cambridge Isotope Laboratories. The GdHCl solution (6 M, 100 mM NaCl, 25 mM sodium phosphate, pH 7.0) used in these experiments was prepared carefully to maintain the correct concentration and pH as described previously [5,6]. H_2O and D_2O solutions were also prepared using the same buffers described above. All protein samples were dissolved in the H_2O buffer to achieve a final protein concentration of about 2 mg/ml. The pH measurements were not adjusted for deuterium. The HPLC mobile phases were 0.1% TFA (A) and 0.1% TFA in ACN (B).

2.2. Instrumentation

The mass spectrometer was a Bruker Esquire 2000 (Billerica, MA) LC ion trap equipped with an electrospray ionization source. It was operated in positive ion mode with a nebulizing gas pressure (N_2) of 30 psi and a drying gas flow of 12 ml/min maintained at 250 °C. The mass spectrometer was optimized at m/z 1000 with low skimmer voltage (instrument default for this mass). The HPLC was a Hewlett Packard (Palo Alto, CA) 1100 series instrument equipped with an autosampler. HPLC (desalting) was accomplished using a Supelco C_{18} column (4.5 mm × 50 mm, 5 μm) at a flow rate of 0.8 ml/min using a rapid gradient from 5%B to 100%B over 3 min.

2.3. Kinetic-based H/D exchange method

The ESI kinetic-based experiments, very similar to MALDI based SUPREX, were carried out at room temperature, ∼25 °C. H/D exchange (wild-type staphylococcal nuclease, a mutant, or ubquitin) was initiated by 10-fold dilution of protein stock solution into a solution made from the D_2O buffer, H_2O buffer and the 6 M GdHCl stock solution. For staphylococcal nuclease this solution (100 μl) had the desired GdHCl concentration of 0 –1.5 M and was always 70% D_2O, v/v. For ubiquitin, D_2O had to be adjusted to 60%, v/v to achieve higher concentrations of 0–2.2 M GdHCl. Mixing was accomplished by vortexing the sample for about 5 s. Exchange times (1–120 min) were controlled using the HPLC autosampler which was started immediately on mixing. Samples were loaded into the autosampler tray after mixing, thus slight differences in the time taken to place the vial into the tray did not affect the timing sequence, initiated before vortex mixing. We assume H/D exchange is quenched on injection of 5–10 μl of sample into the acidic mobile phase (with 0.1% TFA) where both the mobile phase and column were kept at 0° C. A 3-min RP-HPLC separation was used for desalting to improve ESI spectra for the H/D exchanged samples. The resulting spectra of samples corresponding to specific H/D exchange times and GdHCl concentrations were used to obtain folding energies as described in section 2.5 below.

2.4. PEPS HX ESI-MS method

These experiments were similar to the kinetic-based experiments described above with the following differences. The equilibrium protein folded and unfold populations were first established by mixing 5–10 μl of protein stock solution with specific volumes of 6 M GdHCl stock and H_2O buffer to achieve final concentrations of between 0 and 2.5 M for staphylococcal nuclease and 0–4.2 M for ubiquitin in 100 μl total volume. Sufficient time, on the order of 5–10 min, was allowed to insure proper equilibration of the native and denatured states after denaturant addition and before the H/D exchange. Then, in contrast to the ESI SUPREX method, only a single (shortest possible) time of H/D exchange was employed to minimize the number of folding/unfolding cycles. In this study we report data for PEPS in separate experiments using the shortest (17 ± 3 s) and longer (45 s, and 60 ± 3 s) times for comparison. H/D exchange was again accomplished by a 10-fold dilution of the protein (5 μl) into an H/D exchange buffer (45 μl) identical to the

one described in Section 2.3. In this experiment the GdHCl was also present in the exchange buffer, in the same concentration as in the protein sample, to avoid dilution and thus maintain constant ionic strength. Because of the higher concentration of GdHCl used with ubiquitin the range of volumes possible for mixing needed to be adjusted, and in this case the v/v ratio for the percentage D_2O was maintained at 30%. The total time for exchange was measured in seconds rather than 1–120 min. Because of the very short time frame, the autosampler was not used for MS analysis. Instead 25 μl of sample was injected manually using a Rheodyne injector for HPLC/MS analysis.

2.5. Spectral data processing

For the kinetic-based method ΔG_{H_2O} and m_{GdHCl} values are derived from $C_{1/2}$ and $t_{1/2}$ values. These are the concentration and time where 50% of the maximum number of globally protected amide protons that can be detected in the experiment have exchanged. The PEPS method uses the ratio of closed and open state populations for the calculations. All of these parameters are derived from mass values, changes in mass, or peak intensity differences resulting from controlled H/D exchange. The accuracy of these measurements is dependent on the control of the experimental conditions as well as the ability to obtain accurate mass assignments and or intensity values. Mass values for the protein, denatured protein and H/D exchange products (including both folded and unfolded forms if differentiable) were obtained by deconvolution of ESI spectra using the deconvolution function in the Bruker Data Analysis 3.0 software. The spectra were typically smoothed one time using a 2 point (0.2 m/z) smooth to improve the deconvolution. In the special case of EX2, or in the absence of a denaturant where a single peak results from the deconvolution, the maximum intensity of that peak was taken as the average mass resulting from overall H/D exchange. If two peaks resulted from the deconvolution (EX1, two components resolved) the weighted average of the two peaks was calculated, when needed, using the equation below. This represents the weighted average H/D exchange for the protein overall.

$$\langle M \rangle = \frac{\sum M_i I_i}{\sum I_i} \quad (1)$$

In Eq. (1), I_i is the intensity detected at m/z value M_i. For abundance values the reported peak intensities resulting from deconvolution were used. This avoids uncertainties in the peak areas (widths) resulting from deconvolution of the spectra.

2.6. Theory, plotting and calculation of folding energies

There are two widely accepted general mechanisms for H/D exchange called EX1 and EX2 [33]. Both of these generally refer only to exchange of the globally protected amide hydrogens. These exchanges can be detected in the time scale of a typical laboratory experiments. EX1 is defined as the circumstance where a single unfolding event results in simultaneous exchange of most or all of the exchangeable protons within the molecule whereas EX2 exchange occurs when a single folding event leads to very few if any exchanges in the same single unfolding/folding event. Of these two types of exchange, EX1 is more often associated with a higher level of denaturation and EX2 with typical physiological behavior of proteins. EX2 with gradual H/D exchange over many unfolding events results in the observation of a single "population". This population has a distribution of isotopes and typically a Guassian-like appearance. In an MS experiment, the center of this distribution increases slowly in mass over time. On the other hand, EX1 exchange can lead to two different populations (one exchanged and one not) because each folding event results in a large and relatively well defined mass change. These two populations can be distinguished in a mass spectrometry experiment when the mass change is large enough to resolve and the half-life of the protein folding event is sufficient to allow both populations to be sampled. This explanation is simplified by the assumption of a two state model. More complicated behavior likely occurs in many biological systems. In mass spectrometry experiments the detection of two states would define the minimum but not necessarily the maximum number of states present during protein folding. Most methods that have been developed to access protein folding energies are kinetic-based and assume EX2 kinetics.

The kinetic-based method employed herein is similar to SUPREX [25,27] which measures the hydrogen/deuterium (H/D) exchange reaction of globally protected amide protons in proteins. For proteins with two state equilibrium unfolding behavior, the H/D exchange reaction can be described by Eq. (2):

$$(\text{closed})^{H} \underset{k_{cl}}{\overset{k_{op}}{\rightleftarrows}} (\text{open})^{H} \xrightarrow{k_{int}} (\text{open})^{D} \quad (2)$$

where (closed^{H}) is a closed native protein with no deuterium. This state of the protein is H/D exchange incompetent in the sense that only exterior (surface) amide protons can be exchanged. If this state is subjected to a quick H/D exchange, almost no globally protected amide protons get exchanged. On the other hand, the open form of the undeuterated protein $(\text{open})^{H}$ is denatured and more sites are accessible to exchange. This form is H/D exchange competent in the sense that the amide protons which were globally protected (interior) are now exposed. After H/D exchange has occurred there also exists a population which is denatured and deutarated $(\text{open})^{D}$. Under these circumstances the values k_{op} and k_{cl} represent the rate constants for the opening and closing reactions of the protein (respectively), and k_{int} is the intrinsic exchange rate of unprotected amide protons. Note that there is no reverse arrow between $(\text{open})^{H}$ and $(\text{open})^{D}$ in Eq. (1) because the reaction is performed in an environment that is predominantly D_2O. Using this two state protein folding mechanism, the observed H/D exchange rate constant, k_{ex}, can be written as

$$k_{ex} = \frac{k_{op} k_{int}}{(k_{op} + k_{cl} + k_{int})} \quad (3)$$

If $k_{cl} \gg k_{op}$ and k_{int}, Eq. (3) reduces to Eq. (4) when 50% of the globally protected amides protons have exchanged. This circumstance where $k_{cl} \gg k_{op}$ and k_{int} is the definition of EX2 kinetics [33]. A detailed discussion of the derivation of Eq. (4) from Eq. (3) can be found elsewhere [25,27].

$$K_f^{app} = \frac{\langle k_{int} \rangle t_{1/2}}{0.693} - 1 \quad (4)$$

K_f^{app} is the apparent equilibrium folding constant ($K_f^{app} = k_{cl}/k_{op}$), $t_{1/2}$ is the H/D exchange time (for 50% exchange) in minutes and $\langle k_{int} \rangle$ is the average intrinsic exchange rate of unprotected amide protons and assumed to be a constant. $\langle k_{int} \rangle$ is pH dependent and approximated to be $\sim 10^{(pH-5)}$ protons per minute [25,27,34].

One of the most common methods for the analysis of traditional solvent-induced equilibrium denaturation curves is the linear extrapolation method (LEM) [7,8,35] given by Eq. (5).

$$\Delta G_{app} = \Delta G_{H_2O} + m_{GdHCl}[\text{GdHCl}] \quad (5)$$

where ΔG_{app} is $-\text{RT ln } K_f^{app}$, [GdHCl] is the molar GdHCl concentration, m_{GdHCl} is $\frac{\delta \Delta G_{H_2O}}{\delta C}$ and ΔG_{H_2O} is the folding free energy of the protein in the absence of denaturant. When 50% of the globally protected amide protons have exchanged, Eq. (5) can be re-written

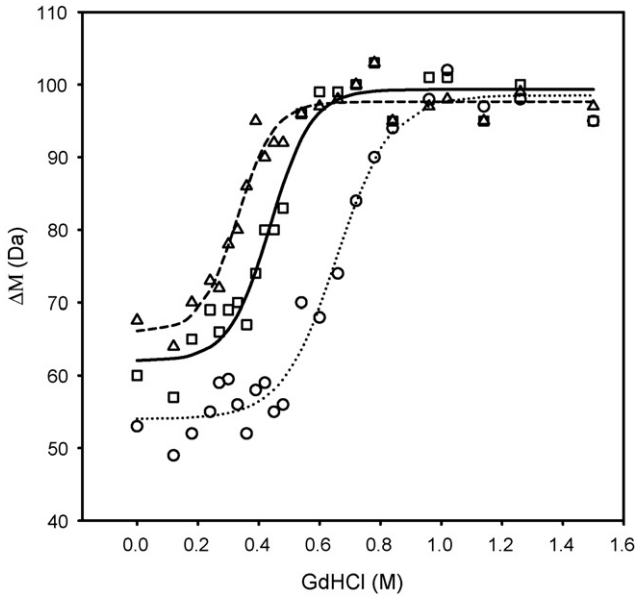

Fig. 1. Average mass shift (ΔM) upon H/D exchange as a function of [GdHCl] for wild-type staphylococcal nuclease. Three curves corresponding to exchange times of 1.5 min (○), 11 min (□), and 19.5 min (△). The average uncertainty associated with the mass shift estimations at different GdHCl concentrations is about ±2 Da.

(using Eq. (4) as

$$\Delta G_{app} = -RT \ln[(< k_{int} > t_{1/2}/0.693) - 1]$$
$$= \Delta G_{H_2O} + m_{GdHCl}[C_{1/2}]. \quad (6)$$

Values of $C_{1/2}$ for given exchange times ($t_{1/2}$) can be obtained by utilizing plots of the sort displayed in Figs. 1 and 2. In Fig. 1, the average mass shift (ΔM) from H/D exchange is plotted as a function of [GdHCl] for a fixed exchange time. $C_{1/2}$ is determined from the transition mid point by fitting the plots to a sigmoidal curve using regression analysis tools as described elsewhere [25,27]. In Fig. 2, ΔM is plotted as a function of time instead of [GdHCl]. Values for $C_{1/2}$, and $t_{1/2}$ from these plots can be obtained simply by drawing a horizontal line across the 50% deuteration level as shown in Fig. 2a. Here the minimum and maximum values for ΔM are defined as the average value for ΔM with no denaturant (minimum) and the average value for the maximum ΔM change (maximum). The *x*-axis value at the intersection of individual data curves with the horizontal 50% ΔM line gives the $t_{1/2}$ value for each concentration. $C_{1/2}$ and $t_{1/2}$ values produced by either method can be used with Eq. (6) to generate standard LEM plots as shown in Fig. 3. In the plot, the *y*-axis intercept yields ΔG_{H_2O} at zero denaturant concentration and the gradient gives the corresponding m_{GdHCl} values.

The PEPS HX ESI-MS method is different from the kinetic-based method. A partially denatured and briefly exchanged protein is probed by ESI. Ideally two different and distinguishable peaks result from the different extents of H/D exchange of the folded and unfolded states. The intensity values from the discrete peaks in the deconvoluted ESI spectrum are presumed representative of the equilibrium populations of native and denatured states. Similar to the kinetic-based method, PEPS also assumes a two state folding mechanism, as depicted in Eq. (7).

$$\text{Native state (closed)} \rightleftharpoons \text{Denatured state (open)} \quad (7)$$

H/D exchange is used to obtain the population ratio of the two states at equilibrium as a function of GdHCl concentration and K_{app} can then be determined by Eq. (8) for different denaturant concentrations if it is assumed that the peak intensities correspond

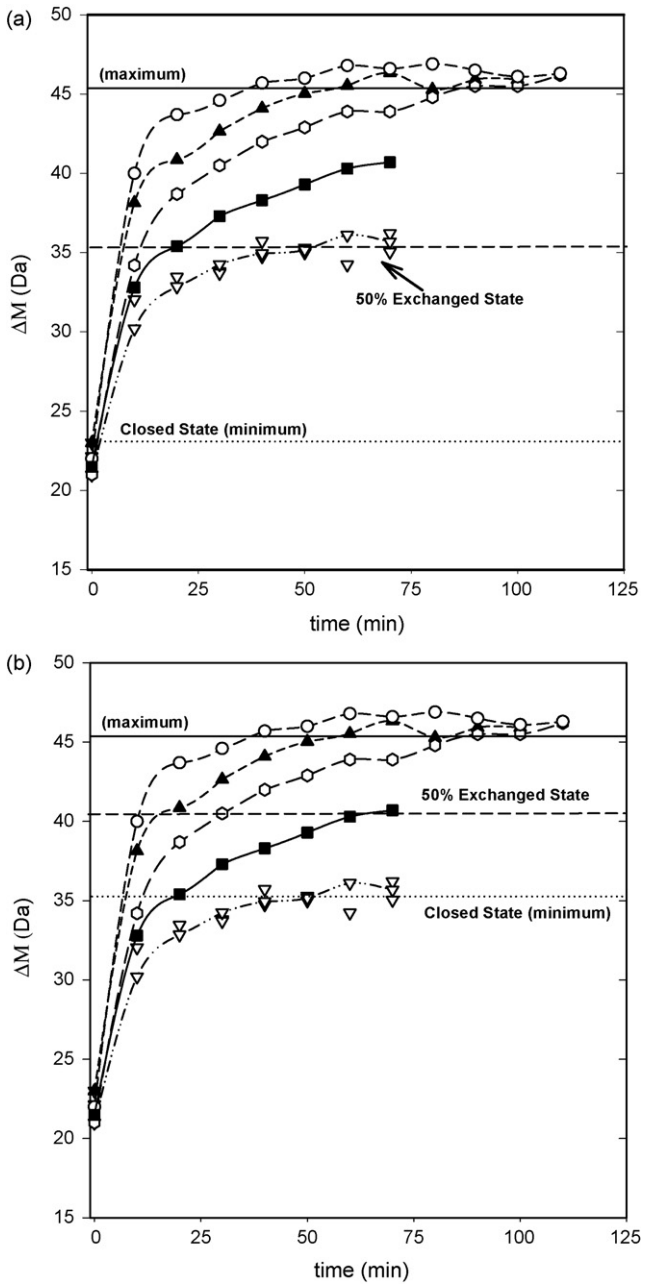

Fig. 2. Average mass shift (ΔM) upon H/D exchange as a function of exchange time for ubiquitin. Seven curves correspond to different GdHCl concentrations: 0.00, 0.48, 0.84 and 1.26 M (▽), 1.56 M (■), 1.76 M (□), 1.92 M (▲) and 2.16 M (○). The bottom (dotted) line corresponds to a closed state (minimum exchange); the middle (dashed) line corresponds to 50% exchange and the top (solid) line corresponds to the maximum exchange. In (b) these lines have been adjusted to encompass only denaturation concentration dependent H/D exchange to facilitate assessment of 50% exchange in the denatured (unfolded) protein. The average uncertainty associated with the mass shift estimations at different GdHCl concentrations is about ±2 Da.

approximately to the population values for the two states they are presumed to represent.

$$K_{app} = \frac{I_n}{I_d} \quad (8)$$

In Eq. (8), I_n and I_d are the intensities of the exchanged native (closed) and exchanged denatured (open) states, respectively, for specific denaturant concentrations which may be directly detected by mass spectrometry if the protein undergoes HX by an EX1 mechanism.

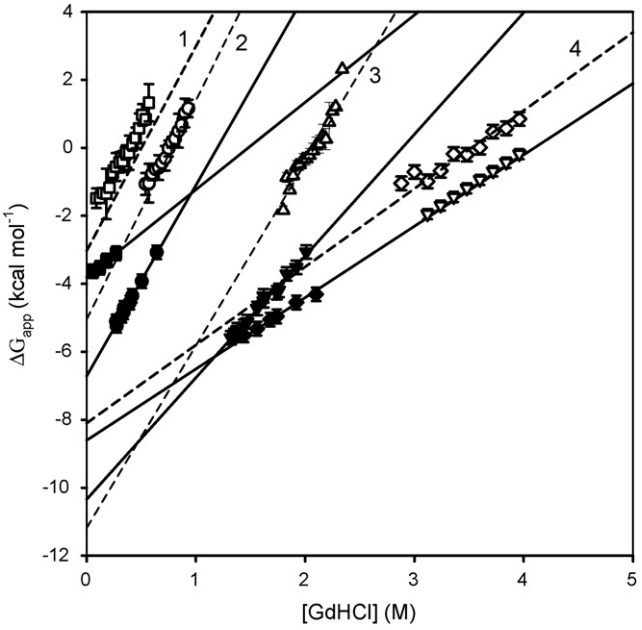

Fig. 3. Solvent denaturation plots using the linear extrapolation method (LEM) for three staphylococcal nucleases and ubiquitin obtained from HX ESI-MS kinetic-based method and PEPS HX ESI-MS method compared to literature fluorescence and CD data. For staphylococcal nuclease: open symbols represent the PEPS method and solid symbols represents the kinetic-based HX-MS methods; 23I/25I/66L/72L (△); wild type (□), 117G/124L/128A/41I/59A/21K/21N (○); dashed lines (1)–(3) show fluorescence data [5,6,36]. For ubiquitin (◊) dashed line (4) represents literature NMR data [39] and (▽) literature CD data [29].

Alternatively mass differences can also be used. For a given [GdHCl] the minimum exchange [ΔM_n, no denaturant], maximum exchange [ΔM_d, denaturant no longer increases exchange] and the specific exchange for a given concentration [ΔM] gives K_{app} values for each denaturant concentration. Here [ΔM] could be the mass change from the single peak resulting when the folded and unfolded states do not give two discrete masses, or the weighted average if they do.

$$K_{app} = \frac{\Delta M_d - \Delta M}{\Delta M - \Delta M_n} \quad (9)$$

Having both options is important. Ideally, the magnitude of the relative k values for folding and unfolding should not matter. If the HX mechanism is EX2 or the peaks cannot be resolved then a single peak is observed in the mass spectrum and mass differences must be used. For EX1 mechanisms if two states can be observed then the intensity values can be taken directly from the spectra to obtain K_{app}. In this case the process is greatly simplified as illustrated using PEPS HX of a staphylococcus nuclease. Low resolution ESI-MS detected two states (folded and unfolded) after H/D exchange as shown in the deconvoluted spectrum. Observation of two discrete peaks resulting from H/D exchange of a single protein is indicative of EX1 conditions. The different populations of the two states are clearly evident in Fig. 4a for processed deconvoluted data and unprocessed raw data in Fig. 4c for different denaturant concentrations. In this example, K_{app} for a given [GdHCl] is simply taken as the ratio of I_n and I_d. The percentage of folded protein calculated this way is compared to the percentage of folded protein under similar conditions sample using fluorescence in Fig. 4b.

3. Results and discussion

Data obtained from two HX-ESI-MS based techniques, a kinetic-based approach and PEPS method, are presented. The ΔG_{H_2O} and m_{GdHCl} values are compared to the previously reported fluorescence [3,5,6,36], and NMR/CD [29] values for staphylococcal nuclease and ubiquitin. These two proteins have been extensively studied as models in protein folding studies [5,37–39]. Moreover these two proteins follow different HX mechanisms. Staphylococcal nuclease clearly follows EX1 HX kinetics [40] as shown in Fig. 4 (distinct populations) whereas ubiquitin reportedly follows EX2 HX kinetics even at high denaturant concentrations [29]. Most other HX methods require assumptions or prior knowledge of the proteins folding characteristics [25,27], including exchange mechanisms. The PEPS approach presented herein is different in that regard, and should provide reasonably accurate folding energies for proteins exhibiting either EX1 or EX2 mechanisms.

3.1. Kinetic-based method

Fig. 1 shows three stability curves for a staphylococcal nuclease wild type obtained at different times (1.5, 11 and 19.5 min). The individual points on these plots correspond to the weighted average mass shift (ΔM) of the protein upon H/D exchange for each given time and denaturant concentration. A value referred to as the $C_{1/2}$ for each curve can be obtained by finding the concentration of GdHCl at which the mass change is half way between the minimum and maximum values for each curve. This can be accomplished either by estimation or curve fitting. Typically sigmoid curves are fitted. The mass increase resulting from H/D exchange reflects the time spent in the open configuration. For longer H/D exchange times and the increasing numbers of open/closed cycles, the probability of any given globally protected amide proton being exchanged increases. Likewise increases in the denaturant concentration increase the equilibrium population of the open form and thereby also increase the accessibility of protected amide protons. This is evident from Fig. 1, where the $C_{1/2}$ value at which 50% of the globally protected amide protons were exchanged decreased with increasing H/D exchange time. Eq. (6) relates the constants (T, k_{int} and R) and variables ($t_{1/2}$ and $C_{1/2}$) to folding energies. Thus the $C_{1/2}$ values were used to prepare ΔG_{app} vs [GdHCl] plots for 14 staphylococcal nuclease mutants giving rise to ΔG_{H_2O} at zero denaturant concentration and m_{GdHCl} values listed in Table 1.

Three examples are also plotted in Fig. 3 along with literature data from fluorescence studies of the same mutants [5,6,36]. Overall the estimated ΔG_{H_2O} values for folding energies of the staphylococcal nucleases obtained using the kinetic-based method showed a clear correlation with the values obtained from tryptophan fluorescence studies, even though the absolute values were consistently high (Fig. 5a). It should be noted that there are minor differences in the temperatures for the MS-based data and the fluorescence data, but these temperature differences are not large enough to account for the observed differences in ΔG_{H_2O}. Similarly, m_{GdHCl} values derived from mass changes are significantly lower than those from fluorescence but seem to agree better for more stable proteins as clearly seen when the difference between fluorescence and kinetic-based m_{GdHCl} values data (Δm) is plotted as a function of protein folding energies in Fig. 5b.

A ΔG_{app} vs [GdHCl] plot for ubiquitin, also generated accordingly using the kinetic-based method, is presented in Fig. 3 along with a plot of literature (NMR and CD) values [29,39]. A different plotting approach is shown in Fig. 2. The advantage of using the method in Fig. 2 is that some specific behaviors (Fig. 2b) that affect the derived ΔG_{H_2O} and m_{GdHCl} values and are indicative of the kinetics of the experiment are readily detected although they are not otherwise obvious. For example, Fig. 2 shows nearly identical behavior for no denaturant added and the three lowest concentrations of denaturant added (up to 1.2 M) in the H/D exchange for ubiquitin. This holds true for times up to 120 min. The lack of

denaturant concentration dependence suggests that this plateau corresponds to a closed state where exchange occurs but the denaturant has had no effect on protein folding. Thus in Fig. 2b, we have redefined the closed state "minimum" exchange by moving the dotted horizontal line labeled "minimum" vertically to match the beginning of measurable denaturant concentration dependent H/D exchange. By chance this state would correspond to about 50% of the overall H/D exchange observed in this experiment as noted in Fig. 2a, but is about the same exchange that would have occurred without any denaturant at all. Only addition of GdHCl above 1.2 M causes proportional increases in H/D exchange until a maximum value is reached (with no subsequent change). This is the same maximum value defined in Fig. 2a. This state at the maximum concentration corresponds to the state where all the globally protected amide protons are fully exchanged. We propose that the correct closed state which has minimum H/D exchange capacity should be taken as the lower (dotted) line in Fig. 2b rather than the arbitrary level selected (dotted line) in Fig. 2a. Using the approach depicted in Fig. 2b the ΔG_{H_2O} and m_{GdHCl} from HX-ESI-MS kinetic-based method for ubiquitin agree with the CD and the SUPREX values [29], whereas the other plotting approach resulted in 2.0 kcal mol^{-1} lower values (Table 3, Fig. 3).

3.2. PEPS HX-ESI-MS method

The main differences between these kinetic-based and PEPS methods are that (a) the denaturant is mixed with, and allowed to equilibrate with the protein before H/D exchange rather than added at the same time as the H/D exchange mixture, and (b) the protein H/D exchange is quenched more rapidly (seconds rather then minutes) after exposure to D_2O. This brief exchange is done at different denaturant concentrations to reproducibly sample different concentrations of the open and closed forms of the protein. A brief exposure limits the number of cycles of protein folding and unfolding and thereby should label primarily the amide hydrogens which are highly solvent accessible. If this can be achieved then, at

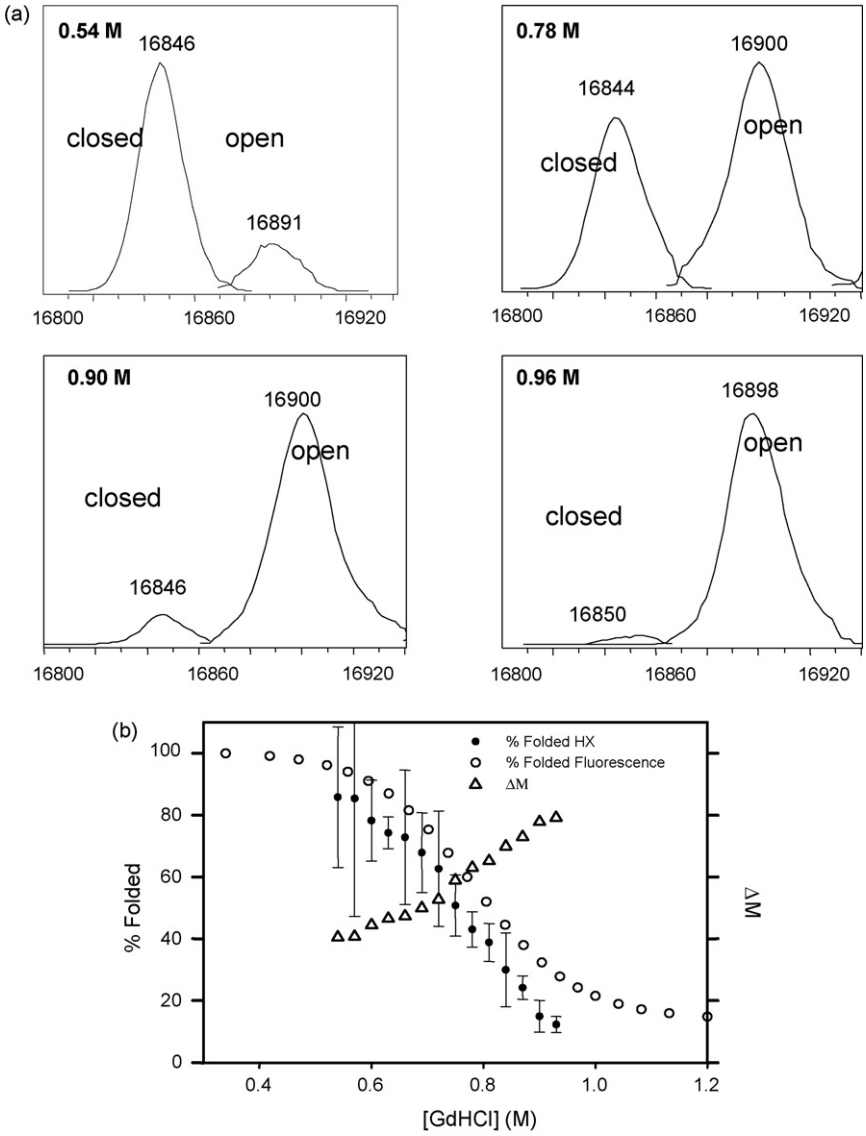

Fig. 4. Staphylococcal nuclease wild type by PEPS method. The top panel (a) shows variations in peak intensities corresponding to the deuterated open and closed forms as a function of GdHCl concentration. The middle panel (b) shows ΔM (Δ) {right side y-axis}, the percent of folded protein (●) calculated using ΔM, and the percent of folded protein from fluorescence data (○) [5,6,36]. All are plotted as a function of GdHCl concentration. The bottom panel (c) shows the ESI mass spectra which were deconvoluted to produce the data in panel (a).

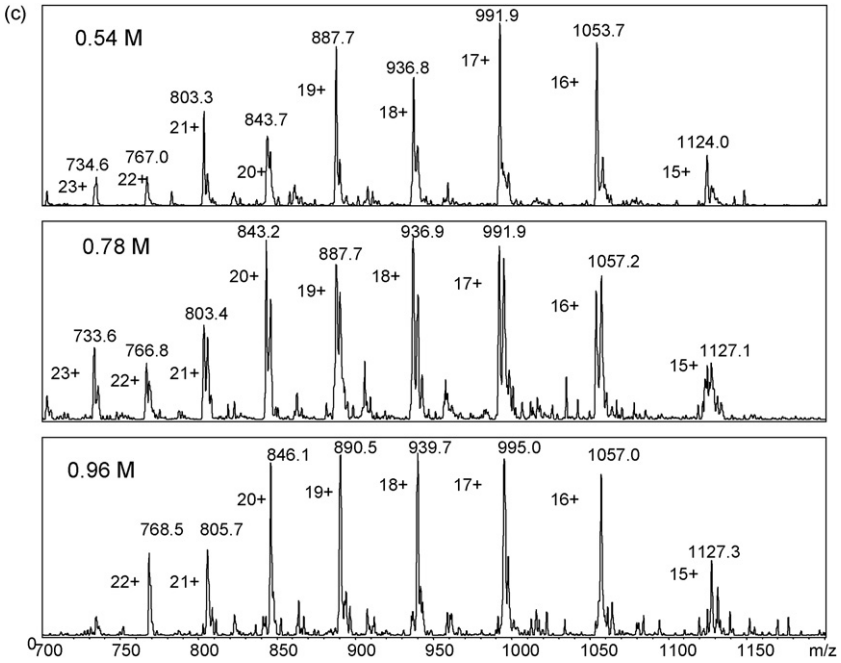

Fig. 4. (*Continued*).

the limit of zero time the extent of H/D exchange (if it could occur instantaneously) could allow the open and closed populations to be monitored. For short exchange times and only weakly denatured states, increased GdHCl concentration results in a greater extent of unfolded protein compared to folded protein. While about the same number of amide protons are exchangeable in the unfolded state (the mass difference between the folded and unfolded state changes very little in Fig. 4a) the number of these accessible protons that exchange increases as shown in Fig. 4a. For exchange times of 45 and 60 s similar results were observed. The calculated values deviated more from the reported values at longer times, as expected. However, at the longer exchange times the ΔG_{H_2O} values decreased by about 10% compared to the values for 17 s (data not shown). Thus this approach might not work for exchange times longer than 60 s.

Thus, irrespective of the HX behavior (EX1 or EX2), the percentage of folded protein, ΔG_{H_2O} and m_{GdHCl} can be calculated. For EX1 using Eq. (8) (I_n/I_d) and $\Delta G_{app} = -RT \ln K_f^{app}$ are used. For EX2 only one m/z value will be detected for all forms (closed and open) and ΔMs must be used rather than I_n/I_d and Eq. (8). For EX2 Eqs. (9) and (5) are used with graphical plotting. Within experimental error (ΔG_{H_2O} is ~0.3 kcal mol^{-1} and for m_{GdHCl} it is 0.5 kcal mol^{-1} M^{-1}) either approach gives the same value (data not shown). The ΔG_{app} values thus obtained were plotted as a function of [GdHCl] and the results for the three staphylococcal nuclease proteins and ubiquitin are shown in the Fig. 3.

Using the PEPS approach the ΔG_{H_2O} and m_{GdHCl} values for the three staphylococcal nuclease proteins for which data are currently available (the wild type, 23I/25 V/66L/72L, and 117G/124L/128A/41I/59A/21K/21N) are in much better agreement with the fluorescence values than values derived from the kinetic-based method, as seen in Fig. 3 and Table 2. This is because this protein exchanges by an EX1 mechanism [18,40]. The experimental data (open symbols) almost overlay the literature values (dash

Table 1
ΔG_{H_2O} and m_{GdHCl} values obtained for staphylococcal nucleases at room temperature ~25 °C using the HX ESI-MS kinetic-based method. ΔG_{H_2O} and m_{GdHCl} values from fluorescence studies [5,6,36] are at 20 °C unless otherwise stated. ΔG and m units are kcal mol^{-1} and kcal mol^{-1} M^{-1}. Estimated average uncertainty for the ΔG is ~0.3 kcal mol^{-1} and for m is 0.1 kcal mol^{-1} M^{-1}.

Staphylococcal nuclease	ΔG_{H_2O} (MS)	ΔG_{H_2O} (fluoresence)	m_{GdHCl} (MS)	m_{GdHCl} (fluoresence)
Wild type	−6.7	−5.0[a]	5.6	6.5[a]
23L/25I/66L/72V	−3.8	−2.8	3.0	6.4
23I/25I/66L/72L	−4.1	−2.7	3.9	6.9
23I/25V/66L/72L	−3.8	−2.8	2.6	6.2
23I/25V/66I/72V	−3.5	−2.4	1.9	6.4
23L/25V/66L/72V	−3.9	−3.0	2.2	6.9
23L/25I/66L/72L	−4.1	−3.0	2.5	5.9
23I/25I/66I/72V	−3.7	−2.3	3.1	6.2
23I/25I/66L/72V	−4.0	−2.8	2.4	6.0
66I/72V/92V/99L	−4.5	−3.7	3.2	6.9
66I/72L/92V/99I	−4.6	−4.0	3.0	6.7
66I/72V/92L/99L	−4.4	−2.8	2.9	6.7
66I/72V/92L/99I	−4.5	−3.4	2.5	6.7
D21N	−7.0	−6.6	4.8	6.3
117G/124L/128A/41I/59A/21K/21N	−10.4	−11.0	3.6	5.3

[a] Measured at 25 °C.

Table 2
ΔG_{H_2O} and m_{GdHCl} values obtained for staphylococcal nucleases at room temperature ~25 °C using the PEPS HX ESI-MS method compared to fluorescence values [5,6,36]. Units of ΔG_{H_2O} and m_{GdHCl} units are kcal mol^{-1} and kcal mol^{-1} M^{-1} respectively. Estimated average uncertainty for the ΔG_{H_2O} is ~0.3 kcal/mol and for m_{GdHCl} it is 0.1 kcal mol^{-1} M^{-1}.

Staphylococcal nuclease	ΔG_{H_2O} (MS)	ΔG_{H_2O} (fluoresence)	m_{GdHCl} (MS)	m_{GdHCl} (fluoresence)
Wild type	−4.7	−5.0[a]	6.3	6.5[a]
23I/25I/66L/72L	−2.4	−2.7	6.5	6.9
117G/124L/128A/ 41I/59A/21K/21N	−11.1	−11.0	5.4	5.3

[a] Measured at 25 °C.

lines) in the Fig. 3. Small differences might be explained by different experimental conditions. For example, for 23I/25 V/66L/72L, the fluorescence values in the Fig. 3 and Table 2 were obtained at 20 °C, whereas for other two staphylococcal proteins fluorescence data was obtained at 25 °C. All MS experiments were done at room temperature.

Using the PEPS approach the ΔG_{H_2O} and m_{GdHCl} values for the ubiquitin are also in excellent agreement with the NMR [29,39] values as seen in Fig. 3 and Table 3. The experimental data (open diamond symbol) almost overlay with literature values (dash line 4) in the Fig. 3. Even though there are some differences among CD, NMR, SUPREX, SPROX-ESI and values obtained from the two new HX-ESI-MS methods, those differences are clearly within the experimental error, Table 3. In this case, with ubiquitin, where EX2 exchange conditions apply, the kinetic-based method, and PEPS give nearly the same value.

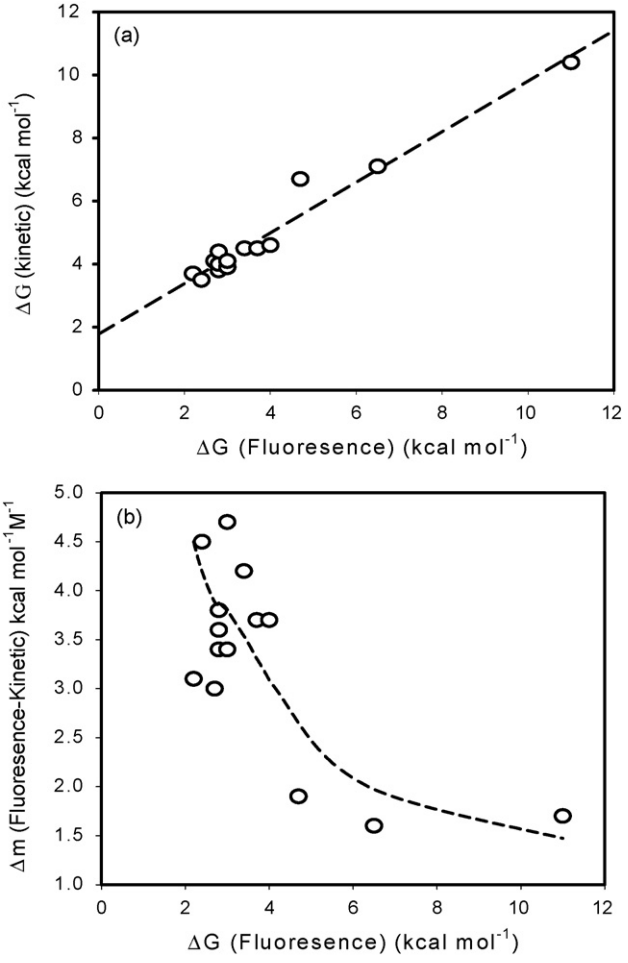

Fig. 5. ΔG_{H_2O} by fluorescence versus (a) ΔG_{H_2O} by H/D kinetic-based method or (b) Δm {m by flurorescence-m by H/D kinetic-based method} for 14 staphylococcal nucleases. x-axis values taken from references [5,6,36]. y-axis values derived from references (m by fluorescence) and this paper (m and ΔG_{H_2O} by H/D kinetic-based method). Average uncertainty associated with ΔG_{H_2O} is 0.3 kcal mol^{-1} and for (Δm) is 0.1 kcal mol^{-1} M^{-1}.

Table 3
ΔG_{H_2O} and m_{GdHCl} values obtained for ubiqutin at room temperature ~25° C using HX ESI-MS kinetic-based and PEPS methods compared to CD, NMR [39], SUPREX and SPROX [32] literature values. Units of ΔG_{H_2O} and m_{GdHCl} units are kcal mol^{-1} and kcal mol^{-1} M^{-1} respectively.

Method	ΔG_{H_2O}	m_{GdHCl}
NMR data	−8.1	2.3
SUPREX data	−8.7 ± 0.2	2.1 ± 0.2
SPROX-ESI	−8.0 ± 0.3	2.1 ± 0.1
SOROX-MALDI	−6.3 ± 1.0	1.6 ± 0.3
CD data	−8.5 ± 0.3	2.1 ± 0.2
HX-ESI-MS-kinetic-based[1]	−6.4 or −8.4 ± 0.2	2.0 ± 0.2
PEPS-HX-ESI-MS	−8.1 ± 0.3	2.3 ± 0.3

−6.4 for the plotting method shown in Fig. 2a, −8.4 using the improved plotting method shown in Fig. 2b.

4. Conclusions

Estimated ΔG_{H_2O} values for folding energies of 14 staphylococcal mutants obtained using an HX ESI-MS kinetic-based method showed a clear correlation with the values obtained from tryptophan fluorescence studies, even though the absolute values were consistently high. The difference is attributed to EX1 exchange where some of the assumptions underlying the kinetic-based method (and SUPREX) are not valid. Even though EX1 HX kinetics were in effect, we still produced linear LEM plots using Eq. (5) for all the staphylococcal nucleases. Thus, any notion that it is possible to judge the validity of the LEM approach based solely on the ability to obtain a straight line plot should be reconsidered.

Values obtained in kinetic-based experiments using ubiquitin showed better results and also that the results can be further improved by consideration of only the denaturant concentration dependent H/D exchange. The PEPS method produced ΔG_{H_2O} and m_{GdHCl} values that agreed well with fluorescence, NMR or CD values for three staphylococcal proteins and ubiquitin. The three staphylococcal proteins selected for study had low, medium and high folding energies and EX1 HX kinetics. All three gave results almost identical with literature values showing PEPS ability to measure folding energies from −2 to −11 kcal mol^{-1} and under typically intractable EX1 conditions. Ubiquitin, which undergoes EX2 HX kinetics, also gave PEPS derived values for folding energies that were in good agreement with literature values. Based on a subset of proteins investigated herein, the PEPS method appears to provide ΔG_{H_2O} and m_{GdHCl} values consistent with fluorescence, NMR, and CD studies and requires fewer assumptions. For EX2 exchange both the kinetic-based method and the PEPS method should be valid.

It is not necessary to speculate about whether or not exchange is EX1 or EX2. ESI-MS can readily detect EX1 exchange where both the open and closed exchanged states can be detected. The HX ESI-

MS kinetic-based method, like SUPREX relies on the assumption of a specific HX mechanism, namely EX2. Thus detection of EX1 kinetics is important for evaluation of H/D exchange data. Because of the ability to operate under both EX1 and EX2 conditions, the applicability of the PEPS method appears broader than the kinetic-based method. When two populations can be detected in spectra the procedure is extremely simple. Thus we also believe that this approach offers advantages in terms of speed and simplicity. We expect that this approach can be easily adopted to obtain protein–ligand and protein–protein interaction energies.

The results presented herein show that the two new techniques, especially PEPS, have advantages over other methods. However, it is equally clear that there is still room for improvement and additional studies are already planned. Two of the most obvious areas that should be fruitful are to gain better control over temperature and time. The best thermodynamic analysis is possible with accurate and precise control of temperature. We have done these experiments at room temperature with less than optimal control over temperature. Even if temperature was more precisely controlled, it would still be desirable to collect data at different temperatures, and such studies are underway. Second, the mixing and, in the equilibrium method, the injections are done manually. This obviously introduces a degree of variability in the timing of the experiments. Further, manual methods have a longer dead time than automated methods. Therefore our future efforts will also focus on automation of the mixing and injection process. Finally, we are well aware that the results for the equilibrium method are dependent upon the time that labeling is allowed to proceed and that the shortest time that allows labeling of the open configuration should give the most accurate results. Our results using PEPS at 17 s are already in very good agreement with a limited number of previously characterized proteins, but we believe additional studies, better temperature control and automation will improve the accuracy and precision of these measurements.

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References

[1] C.M. Dobson, Trends Biochem. Sci. 24 (1999) 329.
[2] F.H. Beijer, H. Kooijman, A.L. Spek, R.P. Sijbesma, E.W.M. Angew, Chem. Int. Ed. 37 (1998) 75.
[3] J. Chen, W.E. Stites, Biochemistry 40 (2001) 14012.
[4] K. Maki, H. Cheng, D.A. Dolgikh, H. Roder, J. Mol. Bio. 368 (2007) 244.
[5] M.P. Byrne, W.E. Stites, Biophys. Chem. 125 (2007) 490.
[6] J. Chen, W.E. Stites, Biochemistry 40 (2001) 14015.
[7] J.A. Schellman, Biopolymers 17 (1978) 1305.
[8] J.A. Schellman, Ann. Rev. Biophys. Chem. 16 (1987) 115.
[9] K. Maki, H. Cheng, D.A. Dolgikh, M.C.R. Shastry, H. Roder, J. Mol. Bio. 338 (2004) 383.
[10] J.F. Brandts, L.N. Lin, Biochemistry 29 (1990) 6927.
[11] J.J. Hill, C.A. Royer, Methods Enzymol. 278 (1997) 390.
[12] B.W. Sigurskjold, Anal. Biochem. 277 (2000) 260.
[13] M. Straume, E. Freire, Anal. Biochem. 203 (1992) 259.
[14] A. White, Biochem. J. 71 (1959) 217.
[15] E. Chen, M.J. Wood, A.L. Fink, D.S. Kliger, Biochemistry 37 (1998) 5589.
[16] Peter S. Kim, Robert L. Baldwin, Annu. Rev. Biochem. 51 (1982) 459.
[17] S.W. Englander, T.R. Sosnic, J.J. Englander, L. Mayne, Curr. Opin. Struct. Biol. 6 (1996) 18.
[18] W. Englander, J. Am. Soc. Mass Spectrom. 17 (2006) 1481.
[19] J.G. Williams, K.B. Tomer, C.E. Hioe, S. Zolla-Pazner, J. Am. Soc. Mass Spectrom. 17 (2006) 1560.
[20] P.L. Roulhac, K.D. Powell, S. Dhungana, K.D. Weaver, T.A. Mietzner, A.L. Crumbliss, M.C. Fitzgerald, Biochemistry 43 (2004) 15767.
[21] J.C. Williams, P.L. Roulhac, A.G. Roy, R.B. Vallee, M.C. Fitzgerald, W.A. Hendrickson, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 10028.
[22] L. Tang, E.D. Hopper, Y. Tong, J.D. Sadowsky, K.J. Peterson, S.H. Gellman, M.C. Fitzgerald, Anal. Chem. 79 (2007) 5869.
[23] H. Xiao, I.A. Kaltashov, S.J. Eyles, J. Soc. Am. Mass Spectrom. 14 (2003) 506.
[24] M.M. Zhu, D.L. Rempel, Z. Du, M.L. Gross, J. Am. Chem. Soc. 125 (2003) 5252.
[25] S. Ghaemmaghami, M.C. Fitzgerald, T.G. Oas, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 8296.
[26] K.D. Powell, S. Ghaemmaghami, M.Z. Wang, L. Ma, T.G. Oas, M.C. Fitzgerald, J. Am. Chem. Soc. 124 (2002) 10256.
[27] K.D. Powell, M.C. Fitzgerald, Biochemistry 42 (2003) 4962.
[28] A.H. Wani, J.B. Udgaonkar, Biochemistry 45 (2006) 11226.
[29] S.Y. Dai, M.C. Fitzgerald, J. Am. Soc. Mass Spectrom. 17 (2006) 1535.
[30] R.K. Chitta, D.L. Rempel, M.A. Grayson, E.E. Remsen, M.L. Gross, J. Am. Soc. Mass Spectrom. 17 (2006) 1526.
[31] J.B. Sperry, X. Shi, D.L. Rempel, Y. Nishimura, S. Akashi, M.L. Gross, Biochemistry 47 (2008) 1797.
[32] M.W. Graham, T. Liangjie, M.C. Fitzgerald, Anal. Chem. 80 (2008) 4175.
[33] D.D. Weis, T.E. Wales, J.R. Engen, M. Hotchko, L.F.T. Eyck, J. Am. Soc. Mass Spectrom. 17 (2006) 1498.
[34] Y. Bai, J.S. Miline, S.W. Englander, Proteins 17 (1993) 75.
[35] C.N. Pace, K.L. Shaw, Proteins Suppl. 4 (2000) 1.
[36] J.M. Chen, Z.Q. Lu, J. Sakon, W.E. Stites, J. Mol. Bio. 303 (2000) 125.
[37] H.M. Went, C.G. Benitez-Cardoza, S.E. Jackson, FEBS Lett. 567 (2004) 333.
[38] M.D. Crespo, E.R. Simpson, M.S. Searle, J. Mol. Biol. 360 (2006) 1053.
[39] T. Sivaraman, C.B. Arrington, A.D. Robertson, Nat. Struct. Biol. 8 (2001) 331.
[40] S. Bédard, L.C. Mayne, R.W. Peterson, A.J. Wand, S.W. Englander, J. Mol. Biol. 376 (2008) 1142.