A Mass Spectrometry-Based Probe of Equilibrium Intermediates in Protein-Folding Reactions†

Susie Y. Dai‡ and Michael C. Fitzgerald*

Department of Chemistry, Duke University, Durham, North Carolina 27708

Received June 28, 2006; Revised Manuscript Received August 23, 2006

ABSTRACT: Described here is a mass spectrometry- and H/D exchange-based approach for the detection of equilibrium intermediate state(s) in protein-folding reactions. The approach utilizes the stability of unpurified proteins from rates of H/D exchange (SUPREX) technique to measure the *m* value (i.e., $\delta\Delta G/\delta$[denaturant] value) associated with the folding reaction of a protein. Such SUPREX *m*-value analyses can be made over a wide range of denaturant concentrations. Thus, the described approach is well-suited for the detection of high-energy intermediates that might be populated at low denaturant concentrations and hard to detect in conventional chemical denaturation experiments using spectroscopic probes. The approach is demonstrated on four known non-two-state folding proteins, including α-lactalbumin, cytochrome *c*, intestinal fatty acid binding protein (IFABP), and myoglobin. The non-two-state folding behavior of each model protein system was detected by the described method. The cytochrome *c*, myoglobin, and IFABP systems each had high-energy intermediate states that were undetected in conventional optical spectroscopy-based studies and previously required other more specialized biophysical approaches (e.g., nuclear magnetic resonance spectroscopy-based methods and protease protection assays) for their detection. The SUPREX-based approach outlined here offers an attractive alternative to these other approaches, because it has the advantage of speed and the ability to analyze both purified and unpurified protein samples in either concentrated or dilute solution.

A full understanding of the processes by which proteins fold into their native three-dimensional structures requires the biophysical characterization of not only the native and denatured state(s) but also the partially folded intermediate state(s) that may be populated in their folding/unfolding reaction. In many cases, the detection of protein-folding intermediate state(s) can be challenging because they are often poorly populated and thus escape detection by many biophysical techniques. The apparent absence of partially folded equilibrium states in the protein-folding reactions of many small, single-domain proteins has led to the development of two-state thermodynamic models for their folding and unfolding reactions, in which only the native and denatured states are assumed to be populated. However, the appropriateness of such two-state models can sometimes come into question, because a protein system that appears to be a two-state folder may populate partially folded intermediate states that have escaped detection.

The most common experimental approaches for differentiating two-state and non-two-state folding behavior have involved measuring the cooperativity of chemical denaturant-induced equilibrium unfolding transitions and/or measuring the coincidence of such unfolding transitions generated using multiple spectroscopic probes [e.g., fluorescence and circular dichroism (CD)[1]] (*1*). The coincidence of unfolding transitions monitored by different structural probes is often used to support two-state folding models, and the non-coincidence of such transitions is often used to support non-two-state or multistate models of protein folding. One problem with such conventional spectroscopy-based approaches is that the unfolding transition often occurs at a high denaturant concentration, where high-energy, partially folded intermediate states are not often heavily populated. Thus, a protein that appears to have a cooperative, two-state unfolding transition at high denaturant concentrations may not display such behavior at low denaturant concentrations or in the absence of the denaturant. More recently, several new biophysical approaches have been explored for the detection of partially folded equilibrium unfolding intermediates (*2*−*5*).

Here, we report on a new experimental approach for the assessment of two-state folding. The approach utilizes the stability of unpurified proteins from rates of H/D exchange (SUPREX) technique (*6*−*10*) to probe the chemical denaturant-induced equilibrium unfolding properties of proteins at low denaturant concentrations. The approach outlined here is analogous to the native-state nuclear magnetic resonance (NMR) approach that has been described by Englander and co-workers (*4*). However, SUPREX relies on a matrix-

† This work was supported in part with funds from a PECASE Award (NSF-CHE-00-94224) to M.C.F.

* To whom correspondence should be addressed: Department of Chemistry, Box 90346, Duke University, Durham, NC 27708-0346. Telephone: 919-660-1547. Fax: 919-660-1605. E-mail: michael.c.fitzgerald@duke.edu.

‡Present address: The Scripps Research Institute, Jupiter, FL 33458.

[1] Abbreviations: MALDI, matrix-assisted laser desorption ionization; SUPREX, stability of unpurified proteins from rates of H/D exchange; IFABP, intestinal fatty acid binding protein; GdmCl, guanidinium chloride; CD, circular dichroism.

10.1021/bi061295h CCC: $33.50
Published on Web 10/03/2006

assisted laser desorption ionization mass spectrometry (MALDI−MS) readout; therefore, unlike the native-state NMR method, the methodology described here is not limited to the analysis of protein-folding reactions in highly concentrated and purified protein samples. Rather, it can be applied to dilute protein samples containing multiple protein components (*11*). This facilitates biophysical studies of protein-folding reactions under more biologically relevant solution conditions.

Described here is the application of SUPREX to the detection of partially folded intermediate states of four model protein systems, including α-lactalbumin, cytochrome *c*, intestinal fatty acid binding protein (IFABP), and myoglobin. Three of the four model protein systems in this study (i.e., cytochrome *c*, IFABP, and myoglobin) display two-state folding behavior in conventional chemical denaturant-induced equilibrium unfolding experiments (*12*−*14*), but the population of partially folded intermediate states has been observed in other biophysical studies of these three proteins at low denaturant concentrations (*15*−*20*). In the case of the fourth protein, α-lactalbumin, there is biophysical evidence for non-two-state folding behavior at a wide range of denaturant concentrations (*21*).

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (99.9 atom %D), sodium deuteroxide, and deuterium chloride were purchased from Aldrich (Milwaukee, WI). Deuterated phosphoric acid was obtained from Cambridge Isotope Laboratories (Andover, MA), and guanidinium chloride (GdmCl) (OmniPur) was from EM Science (Gibbstown, NJ). Sinapinic acid (SA) was either from Acros Organics (Pittsburgh, PA) or from Aldrich. Trifluoroacetic acid (TFA) was from Halocarbon (River Edge, NJ), and acetonitrile (MeCN) was from Fisher (Pittsburgh, PA). Trizma base and the protein samples (bovine ubiquitin, bovine α-lactalbumin, horse heart cytochrome *c*, and horse skeletal muscle myoglobin) were purchased from Sigma (St. Louis, MO) and used without further purification. The IFABP was kindly provided by Professor Carl Frieden (Washington University).

Instrumentation. All MALDI spectra were collected on a Voyager DE biospectrometry workstation (Perspective Biosystems, Inc., Framingham, MA) using a nitrogen laser (337 nm). SA was the matrix in all experiments. All spectra were obtained in positive-ion mode using an acceleration voltage of 25 kV, a grid voltage of 23 kV, a guide-wire voltage of 75 V, a delay time of 225 ns, and 25−32 laser shots.

GdmCl concentrations were determined with a Bausch and Lomb refractometer as described previously (*1*). Measurements of pH were performed with a Jenco 6072 pH-meter equipped with a Futura calomel pH electrode from Beckman Instruments. To correct for isotope effects, the measured pH of each D_2O solution was converted to pD by adding 0.4 to the pH value.

Conventional Equilibrium Unfolding Studies. The unfolding of myoglobin was monitored by absorbance using a Hewlett-Packard 8452A diode array UV/vis spectrophotometer. The GdmCl denaturation solutions contained 20 mM sodium acetate and 20 mM potassium chloride at pH 5.8. This pH condition was used because it matched that used in ref *14*. The myoglobin-containing denaturation solutions containing the different GdmCl concentrations were incubated at least 15 min before the spectroscopic measurements were taken. Results from an earlier study (*22*) show that this 15 min equilibration time is more than adequate for myoglobin to reach equilibrium. A quartz cuvette with a light path length of 1.0 cm was used. The difference spectral measurements of myoglobin were monitored at a wavelength of 408 nm.

The conventional equilibrium unfolding studies of α-lactalbumin, cytochrome *c*, and IFABP were carried out on an Applied Photophysics π*-180 spectrometer (Applied Photophysics Ltd., Leatherhead, Surrey, U.K.). The unfolding reactions of α-lactalbumin and cytochrome *c* were monitored by CD, and the unfolding of IFABP was monitored by fluorescence. For α-lactalbumin and cytochrome *c*, the CD denaturation experiments were performed by using an automated titrator system. The CD signal was monitored at 220 nm. Titrations were set up by mixing 0 and 6 M GdmCl solutions containing the protein in 20 mM Tris buffer (pH 7.4). The mixing duration time was 60 s. After a delay of 5 s, 5000 optical signal data points were recorded over a time course of 30 s and the signals were averaged. Results from earlier biophysical studies on α-lactalbumin and cytochrome *c* suggest that the above mixing and delay times were sufficient for the protein samples to equilibrate (*23*−*25*). The fluorescence denaturation of IFABP was also performed with the titrator system. The total fluorescence was monitored using a filter with an optical cutoff of 305 nm. Excitation was at 280 nm. The titration was performed by mixing 0 and 10 M urea solutions containing 20 mM sodium phosphate, 100 mM potassium chloride (pH 7.5), and the protein. The mixing duration time was 60 s. After a delay of 5 s, the fluorescence signals were recorded over a time course of 30 s and the signals were averaged. Results from earlier biophysical studies on IFABP suggest that the above mixing and delay times were sufficient for the protein to equilibrate (*12*).

The final protein concentrations in all of the spectroscopy-based denaturation experiments were in the 10−20 μM range.

The calculation of *m* values in the spectroscopy-based denaturation experiments was based on the linear extrapolation method (LEM) (*1*) that exploits the well-documented linear relationship between the apparent unfolding free energy of a protein and the denaturant concentration described in eq 1

$$\Delta G_{u,app} = \Delta G_{H_2O} - m[\text{denaturant}] \quad (1)$$

In eq 1, $\Delta G_{u,app}$ is the apparent unfolding free energy at a given denaturant concentration. It is calculated from the fraction of unfolded protein (F_{app}) using eq 2

$$\Delta G_{u,app} = -RT \ln[F_{app}/(1 - F_{app})] \quad (2)$$

Values for F_{app} were determined according to eq 3

$$F_{app} = (y - y_f)/(y_u - y_f) \quad (3)$$

where *y* is the optical signal when the protein is equilibrated at a given denaturant concentration and y_f and y_u are the optical signals of the folded and unfolded forms, respectively, at each denaturant concentration in the unfolding transition.

Ultimately, a $\Delta G_{u,app}$ versus [denaturant] plot was constructed for the data in the unfolding transition of each protein and a linear least-squares analysis of the data was used to extract slope and *y*-intercept values, which were taken as the *m* and ΔG_u values, respectively.

SUPREX Analyses. The basic protocol used for the SUPREX analysis in this work was essentially identical to that described previously (*8*), except that a 12-channel pipettor was used to reduce the time for pipetting by transferring solutions in a batch instead of one by one. Also, to minimize the back-exchange of deuterons to protons, the quenched samples were loaded on a cold stainless-steel MALDI sample stage and incubated in a vacuum desiccator at 0 °C for 1−2 min before going into the MALDI mass spectrometer for analysis. No attempt was made to correct for the back-exchange of deuterons to protons during the MALDI sample preparation and analysis. Back-exchange (∼40%) did occur. However, it was relatively constant within a given SUPREX experiment (i.e., for all of the data points in a SUPREX curve). Thus, the back-exchange reaction only affected the amplitude of our SUPREX curves. It did not impact our ability to determine the transition midpoint of a curve (see below).

The GdmCl buffers (pD 7.4) used for α-lactalbumin and cytochrome *c* contained 20 mM sodium phosphate. The GdmCl buffers (pD 5.8) used for myoglobin contained 20 mM sodium acetate and 20 mM potassium chloride. The urea buffers (pD 7.5) used for IFABP contained 20 mM sodium phosphate and 100 mM potassium chloride. The working concentrations of each protein in our SUPREX analyses were similar to those used in our optical experiments (i.e., in the 10−20 μM range).

All of the H/D exchange times used in our SUPREX analyses on α-lactalbumin, cytochrome *c*, and myoglobin were long compared to reported unfolding and refolding times of these proteins in denaturant-containing buffers (*22*−*25*). However, this was not the case for IFABP, which has been reported to take 10−20 s to unfold and refold in denaturant-containing buffers (*12*). Thus, it was necessary to use the "pre-equilibrium" SUPREX protocol that we recently reported on to collect the IFABP SUPREX curves at short H/D exchange times (i.e., those between 15 and 120 s) (*10*). The protonated denaturation buffers (pH 7.5) used in conjunction with the pre-equilibrium protocol contained 20 mM sodium phosphate and 100 mM potassium chloride. The urea concentrations in these protonated buffers also matched those in the deuterated H/D exchange buffers. The IFABP samples were pre-equilibrated in the protonated denaturation buffers for 30 min prior to their dilution into the deuterated H/D exchange buffers.

m-Value Determination by SUPREX. The *m*-value determinations in this work were made by recording a series of different SUPREX curves at different H/D exchange times. The Δmass values used to construct each SUPREX curve (i.e., the Δmass versus [denaturant] plot) were the average values determined from 5 to 10 MALDI mass spectra. The data points in each SUPREX were fit using a nonlinear regression routine in Sigma Plot to the following sigmoidal equation:

$$\Delta\text{mass} = \Delta M_0 + \frac{a}{1 + e^{-\left(\frac{[\text{denaturant}] - C^{1/2}_{\text{SUPREX}}}{b}\right)}} \quad (4)$$

In eq 4, ΔM_0 is the Δmass before global exchange, *a* is the amplitude of the curve in Da, [denaturant] is the denaturant concentration, $C^{1/2}_{\text{SUPREX}}$ is the [denaturant] at the transition midpoint of the curve, and *b* is a parameter that describe the steepness of the transition. Equation 4 was adapted from the generic four-parameter sigmiodal equation in Sigma Plot.

The $C^{1/2}_{\text{SUPREX}}$ values obtained from eq 4 were then used in eq 5 to determine the *m* value and the unfolding free energy

$$RT \ln\left(\frac{\langle k_{\text{int}}\rangle t}{0.693} - 1\right) = -mC^{1/2}_{\text{SUPREX}} + \Delta G^0 \quad (5)$$

In eq 5, *m* is defined as $-\delta\Delta G/\delta$[denaturant] in kcal mol^{-1} M^{-1}, ΔG^0 is the free energy of unfolding in the absence of the denaturant in kcal mol^{-1}, *R* is the gas constant in kcal mol^{-1} K^{-1}, *T* is the temperature in Kelvin, $\langle k_{\text{int}}\rangle$ is the average intrinsic H/D exchange rate for an amide proton in s^{-1}, and *t* is the H/D exchange time in seconds. This equation is valid for finite values of *t*, such that the $C^{1/2}_{\text{SUPREX}}$ value is greater than or equal to 0 M GdmCl. The derivation of eq 5 can be found in ref *9*. Values for $\langle k_{\text{int}}\rangle$ were estimated using the relationship $10^{\text{pH}-5}$ min^{-1} (*6*), and they were assumed to be independent of the denaturant concentration (*26*).

The $C^{1/2}_{\text{SUPREX}}$ values used to generate the $RT\ln(\langle k_{\text{int}}\rangle t/0.693 - 1)$ versus $C^{1/2}_{\text{SUPREX}}$ value plots in this work were the average of 2−4 determinations (i.e., 2−4 SUPREX curves were recorded at each H/D exchange time (see Table 1). Ultimately, linear least-squares analyses of the resulting $RT\ln(\langle k_{\text{int}}\rangle t/0.693 - 1)$ versus $C^{1/2}_{\text{SUPREX}}$ value plots yielded a slope and *y* intercept. The slope was taken as the *m* value. According to eq 5, the *y* intercept corresponds to the ΔG_u value of the protein when it is a two-state folder. Such ΔG_u values are not reported here because they are not meaningful for the non-two-state folding proteins.

RESULTS

SUPREX Analyses. The four model proteins in this study (α-lactalbumin, cytochrome *c*, IFABP, and myoglobin) were each subject to a SUPREX analysis that involved the acquisition of SUPREX curves using a series of different exchange times that ranged from 0.25 to 1325 min depending upon the protein (see Table 1). Shown in Figure 1 are representative SUPREX curves obtained for each protein using short and long exchange times. Equation 4 (see the Experimental Procedures) was employed to extract a $C^{1/2}_{\text{SUPREX}}$ value from each SUPREX curve, and the values extracted from each SUPREX curve recorded in this study are summarized in Table 1.

One general observation that can be made regarding the SUPREX data in Figure 1 is that the amplitudes of the curves obtained using the longer exchange time were smaller than the amplitudes of the curves obtained using the shorter exchange time. This is a result of the pretransition baseline of the curves collected at the longer exchange times being shifted to higher Δmass values. Such shifts are observed because H/D exchange reactions resulting from local unfolding reactions in the protein (i.e., so-called local H/D exchange

Table 1: SUPREX Exchange Times and Values $C_{SUPREX}^{1/2}$ of Model Protein Systems in This Work

	exchange time (min)	$C_{SUPREX}^{1/2}$ [a] (M)
α-lactalbumin	15	1.7
	30	1.3
	60	1.2
	90	0.9
	180	0.8
	240	0.6
cytochrome *c*	3	2.1
	10	1.9
	30	1.7
	45	1.5
	60	1.5
	90	1.4
	120	1.3
	150	1.1
	180	1.0
IFABP	0.25	3.5
	0.5	3.4
	1	2.9
	2	2.3
	3	2.1
	10	1.9
	30	1.6
	60	1.5
	100	1.3
	140	1.1
	200	0.8
myoglobin	15	0.8
	45	0.6
	60	0.5
	105	0.4
	195	0.4
	420	0.4
	1325	0.4

[a] Each $C_{SUPREX}^{1/2}$ value is the average value obtained from 2−4 SUPREX curves. The standard deviation associated with each value was typically ±0.1 M.

reactions) largely define the Δmass value in the pretransition baseline. As the H/D exchange time is increased in the SUPREX experiment, the local H/D exchange reactions proceed further toward completion; therefore, Δmass values in the pretransition region also increase. It is important to note, however, that the local H/D exchange reactions defining the pretransition baselines of SUPREX curves do not have a significant denaturant dependence. Thus, the Δmass values in the pretransition baseline of a specific SUPREX curve are generally constant. In contrast, the globally protected protons generally have a strong denaturant dependence to their H/D exchange reactions. Ultimately, this strong denaturant dependence creates the transition region of a SUPREX curve, and it is the transition region that defines the most important parameter of a SUPREX curve, the $C_{SUPREX}^{1/2}$ value.

Notably, the $C_{SUPREX}^{1/2}$ values obtained for each protein were shifted to lower denaturant concentrations when longer exchange times were used in the SUPREX experiment. This is predicted by eq 5, which has been previously described for the analysis of SUPREX data (*8*, *9*). Equation 5 also predicts that a plot of $RT\ \ln(\langle k_{int}\rangle t/0.693 - 1)$ versus the observed $C_{SUPREX}^{1/2}$ values will be linear for proteins that meet the requirements of so-called "ideal proteins". Ideal proteins for SURPEX analyses include those that exhibit reversible, two-state unfolding and EX2 exchange behavior. Plots of $RT\ \ln(\langle k_{int}\rangle t/0.693 - 1)$ (i.e., $\Delta G_{u,app}$) versus

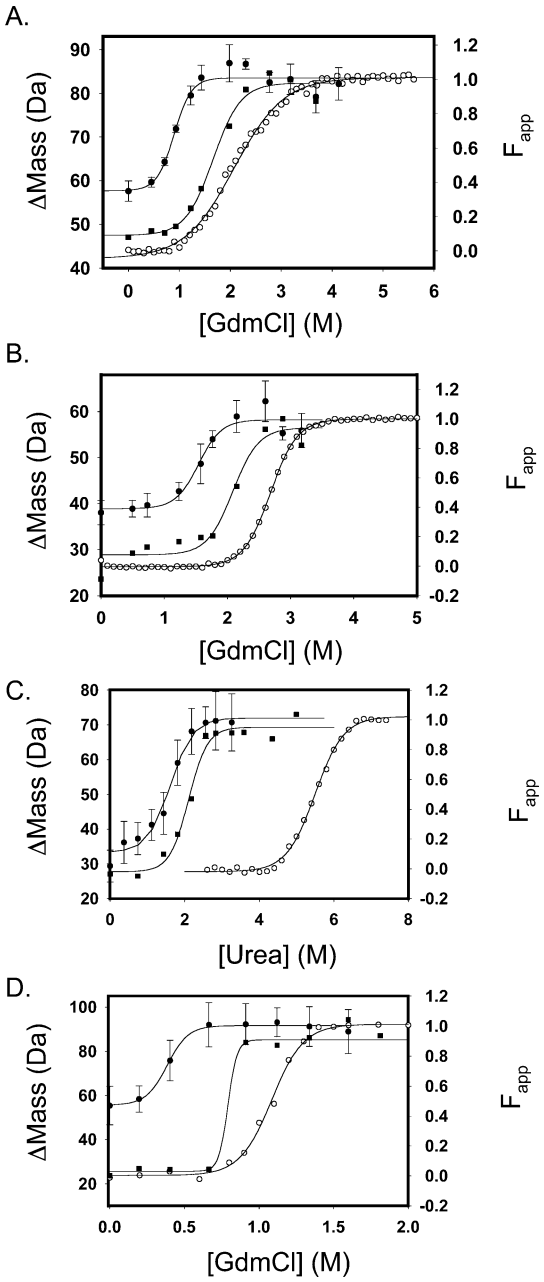

FIGURE 1: Chemical denaturant-induced equilibrium unfolding curves obtained for (A) α-lactalbumin, (B) cytochrome *c*, (C) IFABP, and (D) myoglobin using SUPREX (● and ■ and left *y* axes) and conventional spectroscopy-based techniques (○ and right *y* axes). The SUPREX curves for α-lactalbumin were collected with 90 min (●) and 15 min (■) H/D exchange times; the SUPREX curves for cytochrome *c* were collected with 45 min (●) and 3 min (■) H/D exchange times; the SUPREX curves for IFABP were collected with 30 min (●) and 2 min (■) H/D exchange times; and the SUPREX curves for myoglobin were collected with 420 min (●) and 15 min (■) H/D exchange times, respectively. The conventional CD denaturation data on α-lactalbumin and cytochrome *c* were collected using CD spectroscopy as a structural probe, and the denaturation data for IFABP and myoglobin were collected using fluorescence and UV−vis absorbance spectroscopy, respectively, as a structural probe. Error bars representing ±1 standard deviation are included for the first SUPREX curve in each case. The error bars are typical of those obtained in all of our SUPREX experiments. Error bars for the optical experiments are not shown, because they were less than the size of the symbol.

$C_{SUPREX}^{1/2}$ values were constructed for each protein in this study (see Figure 2) using the data in Table 1. Interestingly,

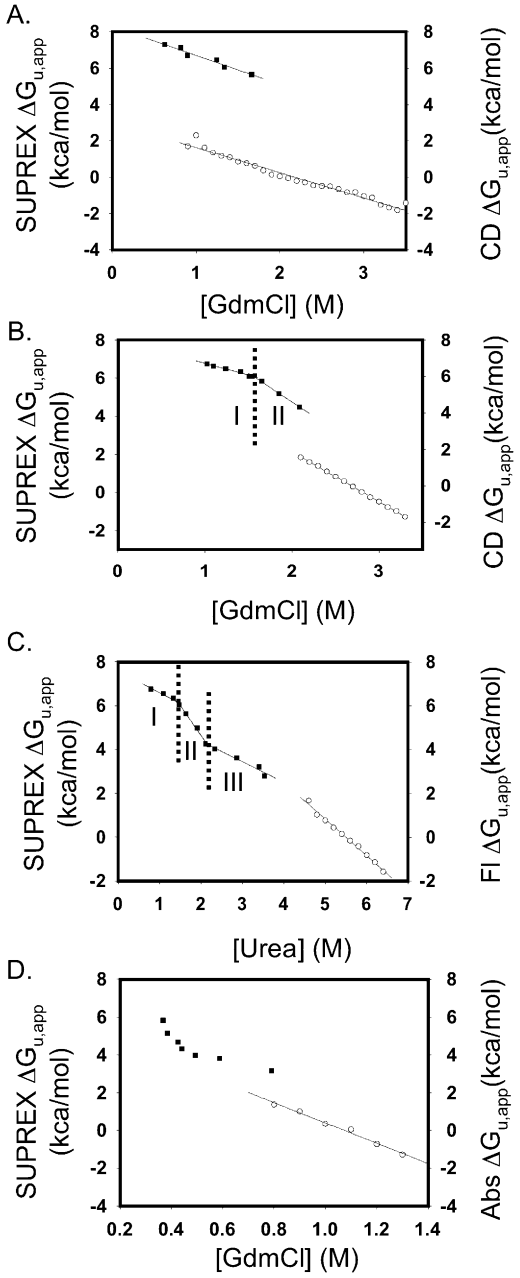

FIGURE 2: Apparent free energy versus denaturant concentration plots for (A) α-lactalbumin, (B) cytochrome *c*, (C) IFABP, and (D) myoglobin derived from SUPREX data (■ and left *y* axes) and conventional spectroscopy data (○ and right *y* axes). The solid lines represent the best linear least-square fitting of the data to either eq 1 for the spectroscopy data or eq 5 for the SUPREX data. Note that $\Delta G_{u,app}$ corresponds to the $RT \ln(\langle k_{int}\rangle t/0.693 - 1)$ term in eq 5. In B and C, the SUPREX data in the specified regions (i.e., I, II, and III) were independently fit to eq 5.

only the α-lactalbumin data were well-fit to eq 5. The *m* value extracted from the fitting is given in Table 2.

The movement of $C_{SUPREX}^{1/2}$ values with the exchange time was not well-described by eq 5 for cytochrome *c*, IFABP, and myoglobin. However, in the case of cytochrome *c* and IFABP, it is noteworthy that specific regions of the $RT \ln(\langle k_{int}\rangle t/0.693 - 1)$ versus $C_{SUPREX}^{1/2}$ value plots could be well-fit to eq 5. These regions are depicted in Figure 2. The *m* values extracted from the fitting of these individual regions of the cytochrome *c* and IFABP SUPREX data in Figure 2 using eq 5 are summarized in Table 2. Note that a SUPREX derived *m* value is not reported for myoglobin because the $RT \ln(\langle k_{int}\rangle t/0.693 - 1)$ versus $C_{SUPREX}^{1/2}$ value plot of the SUPREX data obtained for this protein was nonlinear.

Table 2: Thermodynamic Parameters Obtained by SUPREX for the Model Proteins in This Study

	m value[a] (kcal mol^{-1} M^{-1})
α-lactalbumin	1.6 ± 0.2
cytochrome *c*	1.3 ± 0.1 (region I, <1.5 M GdmCl) 3.0 ± 0.1 (region II, >1.5 M GdmCl)
IFABP	1.0 ± 0.1 (region I, <1.5 M urea) 2.7 ± 0.1 (region II, 1.5−2.1 M urea) 0.9 ± 0.1 (region III, >2.1 M urea)
myoglobin	N/A

[a] Values calculated using SUPREX data in Figure 2 as described in the Experimental Procedures. Reported errors are the standard errors of fitting.

Table 3: Thermodynamic Parameters Obtained by a Conventional Spectroscopy-Based Method for Model Proteins in This Study

	m value[a] (kcal mol^{-1} M^{-1})	ΔG_u[a] (kcal mol^{-1})
α-lactalbumin	1.4 ± 0.1 (1.3[b])	3.0 ± 0.2 (3.4[b])
cytochrome *c*	2.8 ± 0.1 (3.0[c])	7.4 ± 0.2 (8.1[c])
IFABP	1.7 ± 0.1 (1.8[d])	9.2 ± 0.3 (10.0[d])
myoglobin	5.4 ± 0.2 (4.4[e])	5.8 ± 0.2 (5.3[e])

[a] Thermodynamic parameter determined from conventional denaturation data acquired in this work using LEM. Values are reported with standard error. Values in parentheses were previously reported by others. [b] Values were estimated from denaturation data in ref *21*. [c] Data are from ref *13*. [d] Data are from ref *12*. [e] Data are from ref *14*.

Conventional Equilibrium Unfolding Studies. Conventional chemical denaturation curves utilizing various spectroscopic probes were also used to characterize the denaturant-induced equilibrium unfolding properties of the four model proteins in this study. Shown in Figure 1 are the conventional chemical denaturation curves recorded for each protein in this study. Note that these denaturation curves were recorded under solution conditions that were similar (e.g., the same buffer composition, pH, and temperature) to those used to acquire the SUPREX curves in this work, with the exception that the SUPREX buffers were prepared in D_2O and the conventional denaturation buffers were prepared in H_2O.

The conventional denaturation curves in Figure 1 were used to evaluate ΔG_u and *m* values for each model protein according to the LEM (*1*). In this method, the data points in the transition region of each unfolding curve were used to calculate an apparent unfolding free energy, $\Delta G_{u,app}$ value, at each denaturant concentration in the transition region, and plots of $\Delta G_{u,app}$ versus [denaturant] (see Figure 2) were used to calculate ΔG_u and *m* values for each protein, as described in the Experimental Procedures. These values are summarized in Table 3. Note that the spectroscopy-derived ΔG_u and *m* values determined in this work were in reasonably good agreement with similar values previously reported in other denaturation experiments on these same proteins, albeit under slightly different solution conditions (see Table 3).

DISCUSSION

SUPREX and Conventional Spectroscopy-Based Data. There are important parallels between SUPREX and conventional spectroscopy-based approaches for the thermodynamic analysis of protein-folding reactions. Both approaches

utilize chemical denaturants and rely on a linear extrapolation method to evaluate the ΔG_u and *m* values associated with the folding/unfolding reaction of a protein in the absence of the denaturant. Both approaches also require that similar assumptions (e.g., reversible and two-state unfolding) be valid for the accurate determination of such values. In theory, the ΔG_u and *m* values derived by SUPREX and conventional spectroscopy-based approaches should be the same provided that the additional assumptions required by SUPREX (i.e., EX2 exchange and a random coil-like denatured state) are also met. This has been demonstrated for several two-state folding systems (*9*, *10*).

The proteins studied in this work were assumed to be in the EX2 exchange regime. Indeed, the results of the mass spectral analyses in this study were consistent with EX2 exchange behavior (i.e., only one protein ion signal was observed in each mass spectrum, and the double peaks indicative of EX1 exchange were not detected). Recently, we have also shown that problems associated with non-EX2 exchange behavior in SUPREX can generally be minimized if H/D exchange times are chosen so that $C_{SUPREX}^{1/2}$ values are $\leq \sim 2$ M (*27*). This was the case for all of the experiments in this work, with the exception of several IFABP SUPREX curves collected at short exchange times.

An important difference between SUPREX and conventional spectroscopy-based methods for the thermodynamic analysis of protein-folding/unfolding reactions is that a much longer linear extrapolation is required for the evaluation of ΔG_u and *m* values by conventional spectroscopy methods than by SUPREX (see Figure 2). This is because the $C_{SUPREX}^{1/2}$ value of a SUPREX curve is not only a function of the ΔG_u and *m* values of a protein, as in the case of the conventional methods, but it also depends upon $\langle k_{int} \rangle$ and the exchange time, *t* (*6*). The use of longer exchange times in the SUPREX experiment moves the $C_{SUPREX}^{1/2}$ value of a SUPREX curve to lower denaturant concentrations. It is this feature of SUPREX that enables the analysis of protein-folding reactions at low denaturant concentrations. For example, in our earlier work on ubiquitin (*10*), it was possible to generate SUPREX curves with $C_{SUPREX}^{1/2}$ values ranging from 1 to 3 M GdmCl, even though the unfolding transition of the protein occurs at GdmCl concentrations between 3 and 5 M. In the case of ubiquitin, the same linear dependence of the unfolding free energy on the denaturant concentration (i.e., *m* value) was found in the 1−3 M range by SUPREX as was found in the 2−5 M range by the conventional CD spectroscopy-based method. Such behavior is consistent with the well-established two-state folding behavior of ubiquitin in denaturant-induced equilibrium unfolding experiments, which was also detected in a comparable low GdmCl concentration range (1.5−1.8 M) probed by NMR (*28*).

α-*Lactalbumin.* The *m* values determined for α-lactalbumin by SUPREX and the conventional CD denaturation method (1.6 and 1.4 kcal mol^{-1} M^{-1}, respectively) are very similar, and they are relatively small compared to that expected for a two-state folding protein the size of α-lactalbumin (i.e., 142 amino acids with 4 disulfide bonds). A two-state folding protein of such size would be expected to yield an *m* value of 2.4 kcal mol^{-1} M^{-1} according to the data presented by Myers and co-workers (*29*). The low *m* value measured for α-lactalbumin is consistent with the population of partially folded intermediate states in the denaturant-induced equilibrium unfolding reaction of the protein (*30*). Indeed, the population of at least three states (folded, unfolded, and partially folded) has been reported for the GdmCl-induced equilibrium unfolding reaction of α-lactalbumin (*21*). This earlier work utilized multiple structural probes (i.e., near-UV CD and far-UV CD spectroscopy) to detect the non-two-state folding behavior of α-lactalbumin (*21*). It is also noteworthy that this partially folded state of α-lactalbumin appears to be populated at a wide range of GdmCl concentrations.

The $RT \ln(\langle k_{int} \rangle t/0.693 - 1)$ values calculated for α-lactalbumin in the SUPREX experiment were consistently higher than the $\Delta G_{u,app}$ values calculated in the CD experiment (see Figure 2A). In theory, the SUPREX and CD denaturation data for α-lactalbumin shown in Figure 2A should coincide with each other at the denaturant concentrations in which they overlap. The presence of "super-protected" amide protons in the unfolded state of α-lactalbumin may be one explanation for the discrepancy between the two data sets. Super-protected amide protons (i.e., amide protons that appear to exchange significantly slower than the global stability of the protein predicts) have been observed in NMR-based H/D exchange studies of several other proteins that appear to have structured denatured states (*31*−*33*). Interestingly, the results of other biophysical studies have suggested that the denatured state of α-lactalbumin may not be a random-coil-like configuration but that some structure might persist (*34*).

Cytochrome c. Unlike α-lactalbumin, the equilibrium unfolding behavior of cytochrome *c* varied with the denaturant concentration. Similarly, large *m* values of 3.0 and 2.8 kcal mol^{-1} M^{-1} were determined by SUPREX and CD at high denaturant concentrations (i.e., >1.5 M). The magnitudes of both the CD- and SUPREX-derived *m* values in these high denaturant concentrations were similar to the value of 2.8 kcal mol^{-1} M^{-1} that is expected for a two-state folding protein the size of cytochrome *c* (*29*). Significantly, the SUPREX experiment enabled the unique ability to measure the *m* value of cytochrome *c* at low denaturant concentrations (i.e., <1.5 M). In this denaturant concentration range, a relatively small *m* value of 1.3 kcal mol^{-1} M^{-1} was recorded.

The small *m* value determined in our SUPREX experiments on cytochrome *c* at denaturant concentrations <1.5 M is indicative of non-two-state folding and the population of partially folded intermediate state(s) at such low denaturant concentrations (*30*). While such partially folded intermediate state(s) went undetected in the conventional CD denaturation experiments reported here and elsewhere (*13*, *35*), their presence has been noted in other studies (*18*−*20*). For example, there is electrochemical evidence of their existence, as well as data obtained using proteolysis and MALDI−MS that supports their existence (*18*, *19*). Native-state NMR studies performed on cytochrome *c* also revealed the presence of partially folded states (*20*) Significantly, the results of these earlier studies all indicated that the population of the equilibrium intermediate state(s) was strictly dependent upon the denaturant concentration, with the population of these state(s) being favored at lower denaturant concentrations. Our SUPREX data accurately detected such equilibrium unfolding behavior for cytochrome *c*.

As in the case with α-lactalbumin, there is a discrepancy between the SUPREX-derived $\Delta G_{u,app}$ values and the CD-derived $\Delta G_{u,app}$ values at overlapping denaturant concentrations (see Figure 2B). Again, this discrepancy is most likely related to the super-protection of amide protons in the unfolded state of cytochrome *c*. In the case of cytochrome *c*, such super-protection has been documented in native-state NMR experiments by Englander and co-workers (*20*). In their NMR and H/D exchange studies on cytochrome *c*, Englander and co-workers observed the super-protection of some amide protons to be on the order of 2 to >30-fold (*36*). It is noteworthy, that a $\langle k_{int} \rangle$ value reduction of 70-fold (i.e., using a value of 0.06 s^{-1} instead of 4.2 s^{-1}) would eliminate the discrepancy between our SUPREX- and spectroscopy-derived $\Delta G_{u,app}$ values. This degree of super-protection that is observed in our SUPREX experiments is within the same order of magnitude as that observed in the native-state NMR experiment (*20*). We note that there is also biological evidence for such residual structure in the acid denatured state of cytochrome *c* (*37*, *38*).

IFABP. Our SUPREX results with IFABP indicate that the equilibrium unfolding behavior of this protein also varied with the denaturant concentration. At low denaturant concentrations (<3.5 M) exclusively probed by SUPREX, three different regions with three different *m* values were observed (Figure 2C). The largest *m* value of 2.7 kcal mol^{-1} M^{-1} was observed at urea concentrations between 1.5 and 2.1 M (region II), and the two smaller *m* values of 1.0 and 0.9 kcal mol^{-1} M^{-1} were observed in the 0.9−1.5 M and 2.1 − 3.5 M ranges, regions I and III (respectively). None of the SUPREX derived *m* values were similar to the *m* value of 1.7 kcal mol^{-1} M^{-1} determined in our fluorescence denaturation experiments. However, it is important to note that the SUPREX and fluorescence data were not obtained at overlapping urea concentrations. In theory, SUPREX curves recorded at shorter exchange times could move IFABP $C_{SUPREX}^{1/2}$ values to the higher denaturant concentrations probed in the fluorescence denaturation experiment. In practice, it was not possible to use H/D exchange times less than 0.25 min^{-1} in the SUPREX experiment because the small uncertainties in the H/D exchange time (∼1−2 s) introduced during pipetting become significant. For example, a 3 s exchange time would be needed to move the IFABP SUPREX curve to 5 M urea. Such an exchange time is not practical because the pipet transfer of protein into and then out of the exchange buffer takes several seconds.

The folding mechanism of IFABP has been well-studied (*12*, *16*, *17*). The equilibrium unfolding behavior determined by conventional spectroscopy-based approaches has been modeled by a two-state process, because the chemical denaturation curves recorded by fluorescence, CD, and absorbance were coincident and cooperative, each yielding an *m* value on the order of that expected for a two-state folding protein the size of IFABP (∼1.7 kcal mol^{-1} M^{-1}) (*12*, *29*). However, several intermediate states have been detected using more specialized NMR-based methods (*16*, *17*). For example, NMR studies utilizing two-dimensional ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra collected on IFABP revealed the presence of at least two partially folded equilibrium intermediate states of IFABP populated at low denaturant concentrations (i.e., in the 0−4.5 M range) (*17*). ^{19}F NMR was also used to detect an intermediate state that went undetected by fluorescence, CD, and absorption measurements (*16*). This intermediate state was found to be populated between 4−7 M urea and had very little secondary structure.

The three different linear phases of our SUPREX data are consistent with the H/D exchange of IFABP, being described by at least three different equilibria over the range of denaturant concentrations studied in our SUPREX experiments, 0−4 M. Because it was not possible to obtain SUPREX and fluorescence data in overlapping denaturant concentrations, it is not clear if the presence of phase III in our SUPREX data is an artifact of the IFAPB intermediate detected by ^{19}F NMR or if an additional intermediate state of IFABP has been detected in our SUPREX experiments. Detailed information about the states populated in each equilibria cannot be ascertained from our SUPREX data. However, the SUPREX data do clearly indicate that the equilibrium unfolding reaction of IFABP is not well-modeled by a simple, two-state process at low denaturant concentrations. This conclusion is consistent with those arrived at in the NMR studies noted above. However, unlike in the NMR studies (*16*, *17*), the SUPREX studies described here required no special covalent labeling of the protein and significantly less protein material was required for analysis.

Myoglobin. Our SUPREX results on myoglobin indicate that there is a nonlinear dependence of $\Delta G_{u,app}$ (i.e., the $RT \ln(\langle k_{int} \rangle t/0.693 - 1)$ values) on [GdmCl]. The nonlinearity is especially pronounced at low denaturant concentrations (<0.5 M). Similar nonlinearities have been previously observed in other chemical denaturation studies on myoglobin (*39*). In these earlier studies that employed conventional spectroscopic techniques, acidic conditions had to be used to determine apparent ΔG values in the low GdmCl concentration range (<0.5 M). These ΔG values obtained under acidic conditions were then employed to calculate corresponding ΔG_{app} values at neutral pH. The resulting plot of ΔG_{app} versus [GdmCl] looked very similar to that of our SUPREX data in Figure 2D (see Figure 3 in ref *39*). It has been suggested that such nonlinearities for myoglobin are due to the population of partially folded intermediate states at low denaturant concentrations (*15*). However, other explanations have also been proposed to explain increases in the *m* value of a protein at low denaturant concentrations. For example, in the case of barnase (*40*), potential interactions between the chemical denaturant and the native protein were thought to alter the *m* value. Regardless of the source of the nonlinearity, it is significant to this work that the nonlinear behavior of myoglobin was directly detected in our SUPREX analyses at pD 5.8. No extrapolation of low pH data was needed to arrive at such conclusions.

CONCLUSIONS

The SUPREX *m*-value analyses of the four model proteins described here validate the utility of SUPREX for probing equilibrium unfolding intermediate states in protein-folding reactions. The SUPREX-based approach outlined here successfully detected the non-two-state folding properties of all four model proteins chosen for this study, including the three proteins with high-energy equilibrium intermediate states that were undetected in conventional optical spectroscopy-based

studies. The detection of these states previously required other biophysical approaches such as NMR spectroscopy-based methods and protease protection assays. The SUPREX-based approach outlined here offers an attractive alternative to these other approaches, because it can be rapidly applied to small amounts of purified and/or unpurified protein.

ACKNOWLEDGMENT

The authors are grateful to Professor Carl Frieden for providing the purified IFABP sample in this work.

REFERENCES

1. Pace, C. N. (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves, *Methods Enzymol. 131*, 266−280.
2. Dobson, C. M. (2004) Experimental investigation of protein folding and misfolding, *Methods 34*, 4−14.
3. Eyles, S. J., and Kaltashov, I. A. (2004) Methods to study protein dynamics and folding by mass spectrometry, *Methods 34*, 88−99.
4. Krishna, M. M., Hoang, L., Lin, Y., and Englander, S. W. (2004) Hydrogen exchange methods to study protein folding, *Methods 34*, 51−64.
5. Rounsevell, R., Forman, J. R., and Clarke, J. (2004) Atomic force microscopy: Mechanical unfolding of proteins, *Methods 34*, 100−111.
6. Ghaemmaghami, S., Fitzgerald, M. C., and Oas, T. G. (2000) A quantitative, high-throughput screen for protein stability, *Proc. Natl. Acad. Sci. U.S.A. 97*, 8296−8301.
7. Powell, K. D., and Fitzgerald, M. C. (2001) Measurements of protein stability by H/D exchange and matrix-assisted laser desorption/ionization mass spectrometry using picomoles of material, *Anal. Chem. 73*, 3300−3304.
8. Powell, K. D., Wales, T. E., and Fitzgerald, M. C. (2002) Thermodynamic stability measurements on multimeric proteins using a new H/D exchange- and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry-based method, *Protein Sci. 11*, 841−851.
9. Powell, K. D., and Fitzgerald, M. C. (2003) Accuracy and precision of a new H/D exchange- and mass spectrometry-based technique for measuring the thermodynamic properties of protein−peptide complexes, *Biochemistry 42*, 4962−4970.
10. Dai, S. Y., Gardner, M. W., and Fitzgerald, M. C. (2005) Protocol for the thermodynamic analysis of some proteins using an H/D exchange- and mass spectrometry-based technique, *Anal. Chem. 77*, 693−697.
11. Wang, M. Z., Shetty, J. T., Howard, B. A., Campa, M. J., Patz, E. F., Jr., and Fitzgerald, M. C. (2004) Thermodynamic analysis of cyclosporin a binding to cyclophilin a in a lung tumor tissue lysate, *Anal. Chem. 76*, 4343−4348.
12. Ropson, I. J., Gordon, J. I., and Frieden, C. (1990) Folding of a predominantly β-structure protein: Rat intestinal fatty acid binding protein, *Biochemistry 29*, 9591−9599.
13. Hagihara, Y., Tan, M., and Goto, Y. (1994) Comparison of the Conformational stability of the molten globule and native states of horse cytochrome *c*. Effects of acetylation, heat, urea, and guanidine hydrochloride, *J. Mol. Biol. 237*, 336−348.
14. Ahmad, F., Yadav, S., and Taneja, S. (1992) Determining stability of proteins from guanidinium chloride transition curves, *Biochem. J. 287* (part 2), 481−485.
15. Yadav, S., Taneja, S., and Ahmad, F. (1992) Measuring the conformational stability of proteins, *Indian J. Chem. 31*, 859−864.
16. Ropson, I. J., and Frieden, C. (1992) Dynamic NMR spectral analysis and protein folding: Identification of a highly populated folding intermediate of rat intestinal fatty acid-binding protein by ^{19}F NMR, *Proc. Natl. Acad. Sci. U.S.A. 89*, 7222−7226.
17. Hodsdon, M. E., and Frieden, C. (2001) Intestinal fatty acid binding protein: The folding mechanism as determined by NMR studies, *Biochemistry 40*, 732−742.
18. Ferri, T., Poscia, A., Ascoli, F., and Santucci, R. (1996) Direct electrochemical evidence for an equilibrium intermediate in the guanidine-induced unfolding of cytochrome *c*, *Biochim. Biophys. Acta 1298*, 102−108.
19. Yang, H. H., Li, X. C., Amft, M., and Grotemeyer, J. (1998) Protein conformational changes determined by matrix-assisted laser desorption mass spectrometry, *Anal. Biochem. 258*, 118−126.
20. Bai, Y., Sosnick, T. R., Mayne, L., and Englander, S. W. (1995) Protein folding intermediates: Native-state hydrogen exchange, *Science 269*, 192−197.
21. Kuwajima, K., Nitta, K., Yoneyama, M., and Sugai, S. (1976) Three-state denaturation of α-lactalbumin by guanidine hydrochloride, *J. Mol. Biol. 106*, 359−373.
22. Sykes, P. A., Shiue, H., Walker, J. R., and Bateman, R. C. (1999) Determination of myoglobin stability by visible spectroscopy, *J. Chem. Ed. 76*, 1283−1284.
23. Ikeguchi, M., Kuwajima, K., Mitani, M., and Sugai, S. (1986) Evidence for identity between the equilibrium unfolding intermediate and a transient folding intermediate: A comparative study of the folding reactions of α-lactalbumin and lysozyme, *Biochemistry 25*, 6965−6972.
24. Kuwajima, K., Hiraoka, Y., Ikeguchi, M., and Sugai, S. (1985) Comparison of the transient folding intermediates in lysozyme and α-lactalbumin, *Biochemistry 24*, 874−881.
25. Bai, Y. (1999) Kinetic evidence for an on-pathway intermediate in the folding of cytochrome *c*, *Proc. Natl. Acad. Sci. U.S.A. 96*, 477−480.
26. Loftus, D., Gbenle, G. O., Kim, P. S., Baldwin, R. L. (1986) Effects of denaturants on amide proton exchange rates: A test for structure in protein fragments and folding intermediates, *Biochemistry 25*, 1428−1436.
27. Dai, S. Y., and Fitzgerald, M. C. (2006) Accuracy of SUPREX (stability of unpurified proteins from rates of H/D exchange) and MALDI mass spectrometry-derived protein unfolding free energies determined under non-EX2 exchange conditions, *J. Am. Soc. Mass Spectom.*, in press.
28. Sivaraman, T., Arrington, C. B., and Robertson, A. D. (2001) Kinetics of unfolding and folding from amide hydrogen exchange in native ubiquitin, *Nat. Struct. Biol. 8*, 331−333.
29. Myers, J. K., Pace, C. N., and Scholtz, J. M. (1995) Denaturant *m* values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding, *Protein Sci. 4*, 2138−2148.
30. Soulages, J. L. (1998) Chemical denaturation: Potential impact of undetected intermediates in the free energy of unfolding and *m*-values obtained from a two-state assumption, *Biophys. J. 75*, 484−492.
31. Nocholson, E. M., Mo, H., Prusiner, S. B., Cohen, F. E., and Marqusee, S. (2002) Differences between the prion protein and its homolog doppel: A partially structured state with implications for scrapie formation, *J. Mol. Biol. 316*, 807−815.
32. Llinas, M., Gillespie, B., Dahlquist, F. W., and Maqusee, S. (1999) The energetics of T4 lysozyme reveal a hierarchy of conformations, *Nat. Struct. Biol. 6*, 1072−1078.
33. Shortle, D. R. (1996) Structural analysis of non-native states of proteins by NMR methods, *Curr. Opin. Struct. Biol. 6*, 24−30.
34. Demarest, S. J., and Raleigh, D. P. (2000) Solution structure of a peptide model of a region important for the folding of α-lactalbumin provides evidence for the formation of nonnative structure in the denatured state, *Protein: Struct., Funct., Genet. 38*, 189−196.
35. Chan, C.-K., Hu, Y., Takahashi, S., Rousseau, D. L., and Eaton, W. A. (1997) Submillisecond protein folding kinetics studied by ultrarapid mixing, *Proc. Natl. Acad. Sci. U.S.A. 94*, 1779−1784.
36. Bai, Y., Milne, J. S., Mayne, L., and Englander, S. W. (1993) Primary structure effects on peptide group hydrogen exchange, *Proteins 17*, 75−86.
37. Jeng, M.-F., and Englander, W. (1991) Stable submolecular folding units in a non-compact form of cytochrome *c*, *J. Mol. Biol. 221*, 1045−1061.
38. Chalikian, T. V., Gindikin, V. S., and Breslauer, K. J. (1995) Volumetric characterization of the native, molten globule and unfolded states of cytochrome *c* at acidic pH, *J. Mol. Biol. 250*, 291−306.
39. Pace, C. N., and Vanderburg, K. E. (1979) Determining globular protein stability: Guanidine hydrochloride denaturation of myoglobin, *Biochemistry 18*, 288−292.
40. Johnson, C. M., and Fersht, A. R. (1995) Protein stability as a function of denaturant concentration: The thermal stability of Barnase in the presence of urea, *Biochemistry 34*, 6795−6804.

BI061295H