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

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Described here is the impact of so-called non-EX2 exchange behavior on the accuracy of protein unfolding free energies (i.e., ΔG_u values) and *m* values (i.e.,-$\delta\Delta G_u/\delta$[denaturant] values) determined by an H/D exchange and mass spectrometry-based technique termed stability of unpurified proteins from rates of H/D exchange (SUPREX). Both experimental and theoretical results on a model protein, ubiquitin, reveal that reasonably accurate thermodynamic parameters for its folding reaction can be determined by SUPREX even when H/D exchange data is collected in a non-EX2 regime. Not surprisingly, the theoretical results reported here on a series of hypothetical protein systems with a wide range of biophysical properties show that the accuracy of SUPREX-derived ΔG_u and *m* values is compromised for many proteins when analyses are performed at high pH (e.g., pH 9) and for selected proteins with specific biophysical parameters (e.g., slow folding rates) when analyses are performed at lower pH. Of more significance is that the experimental and theoretical results reveal a means by which problems with non-EX2 exchange behavior can be detected in the SUPREX experiment without prior knowledge of the protein's biophysical properties. The results of this work also reveal that such problems with non-EX2 exchange behavior can generally be minimized if appropriate H/D exchange times are employed in the SUPREX experiment to yield SUPREX curve transition midpoints at chemical denaturant concentrations less than 2 M. (J Am Soc Mass Spectrom 2006, 17, 1535–1542) © 2006 American Society for Mass Spectrometry

Amide H/D exchange (HX) techniques provide an attractive means by which to study protein folding and stability, and they are often used to evaluate the free-energy values associated with protein unfolding reactions (i.e., ΔG_u values). When such techniques are used for the evaluation of ΔG_u values, an assumption of so-called EX2 exchange behavior is required (i.e., the protein's refolding rate must be significantly faster than the intrinsic exchange rate of an unprotected amide proton, k_{int}). The accuracy of ΔG_u values determined by HX methods can be compromised if data is acquired under conditions where amide hydrogen exchange is not exclusively EX2.

Recently, we developed an H/D exchange- and mass spectrometry-based technique termed stability of unpurified proteins from rates of H/D exchange (SUPREX) for evaluating the ΔG_u and *m* values (-$\delta\Delta G_u/\delta$[denaturant] values) of protein folding reactions. The potential for inaccurate ΔG_u and *m* value determinations in the SUPREX experiment due to non-EX2 exchange behavior is high as the technique relies on measurements of amide H/D exchange rates at a range of different chemical denaturant concentrations. At low denaturant concentrations, EX2 exchange mechanisms are usually dominant under the conditions that proteins typically fold into their native-like structures (i.e., pH 7 and 298 K). Under these conditions, k_{int} values are about $\sim 5\ s^{-1}$, and the refolding rates of many proteins are much faster [1, 2]. However, with increasing denaturant concentrations, k_{cl} values decrease, k_{op} values increase, and k_{int} values are relatively constant. Thus, at increasing denaturant concentrations, k_{cl} values can approach k_{int} values and the HX mechanism can switch away from EX2. When k_{cl} values decrease to values that approach k_{int}, a regime is reached that is neither EX2 nor EX1 (i.e.,

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k_{cl} is much smaller than k_{int}). This regime has been and will be referred to here as the EXX regime [3].

The goal of the work described here was to determine the magnitude of the error introduced into ΔG_u and *m* values when SUPREX data collected in the EXX regime is used for their calculation. Ubiquitin was one model protein system used in this work. It was chosen because the biophysical properties of its folding/unfolding reaction are well known by a variety of experimental techniques including those that exploit the HX exchange (e.g., SUPREX and NMR) and those that exploit other biophysical characteristics (e.g., CD or fluorescence spectroscopy) [1, 4–6]. As part of this work, experimentally derived k_{cl} and k_{op} values, originally reported by Sivaraman and coworkers [1] using a magnetization transfer technique, were used to calculate ΔG_u values at a wide range of denaturant concentrations. These ΔG_u values were then compared with theoretical ΔG_{HX} values. The theoretical values were obtained using the classic hydrogen exchange model [7] based on eq 1 with no assumption of EX1 or EX2 in the evaluation of the observed hydrogen exchange rate, k_{ex}.

$$\text{Closed}(\text{NH}) \underset{k_{cl}}{\overset{k_{op}}{\Leftrightarrow}} \text{Open}(\text{NH}) \overset{k_{int}}{\rightarrow} \text{Open}(\text{ND}) \tag{1}$$

Ultimately, the theoretical ΔG_{HX} values obtained were compared with experimentally determined SUPREX values (i.e., ΔG_{SUPREX}), to experimentally determined CD values (i.e., ΔG_{CD}), and to ΔG_u values determined using magnetization transfer data reported by Sivaraman and coworkers [1]. Our results with ubiquitin reveal only small difference between the ΔG_u, ΔG_{HX}, ΔG_{SUPREX}, and ΔG_{CD} values.

As part of this work, we also explored the relationship between ΔG_u values and theoretical ΔG_{HX} values for a series of hypothetical protein systems with a range of different k_{op}, k_{cl}, and k_{int} values. A comparison of the ΔG_u values defined by various "hypothetical" combinations of biophysical parameters to theoretical ΔG_{HX} values for the hypothetical protein systems enabled us to determine if there were specific combinations of k_{op}, k_{cl}, and k_{int} values that would lead to especially large discrepancies between a protein's "true" ΔG_u and the ΔG_{HX} value expected from SUPREX. Our results reveal that large discrepancies between the "true" ΔG_u and the ΔG_{HX} expected from SUPREX are likely to exist for unstable proteins (e.g., ΔG_u ~4.1 kcal mol^{-1}) and/or proteins analyzed under high pH conditions (e.g., pH 9.0). This was not surprising as such conditions are known to promote non-EX2 exchange behavior in proteins. What is more significant about the results reported here is that they reveal a means by which potential problems with non-EX2 exchange behavior can be detected in the SUPREX experiment without prior knowledge of the protein's biophysical properties. Our results also indicate that such problems can generally be avoided if appropriate H/D exchange times are employed in the SUPREX experiment to yield SUPREX curve transition midpoints at chemical denaturant concentrations less than 2 M.

Materials and Methods

The ΔG_u values for both ubiquitin and the hypothetical proteins in this work were obtained from the linear extrapolation of apparent ΔG_u values theoretically calculated at different [denaturant] concentrations according to eq 2

$$\Delta G_u(\text{apparent}) = \Delta G_u - m_{eq}[\text{Denaturant}] \tag{2}$$

In eq 2, m_{eq} is defined as $\delta\Delta G_u/\delta$[denaturant]. The apparent ΔG_u values at different [denaturant] were calculated from appropriate k_{op} and k_{cl} values using eq 3

$$\Delta G_u = -RT\ln(k_{op}/k_{cl}) \tag{3}$$

In the case of ubiquitin, the k_{op} and k_{cl} values used in our calculations were taken directly from the log(k_{op} or k_{cl}) versus [denaturant] plots previously reported for ubiquitin by Sivaraman and coworkers [1]. For the hypothetical proteins, k_{op} values were arbitrarily assigned values between 10^{-8} to 10^{-3} s^{-1}, and k_{cl} were arbitrarily assigned values between 1 to 10^4 s^{-1}, respectively. The denaturant dependences of k_{op} and k_{cl} (i.e., m_{cl} and m_{op} values in eqs 4 and 5 [8–10], were also arbitrarily assigned values in the range 0.3 to 3 kcal $mol^{-1}M^{-1}$ for the hypothetical proteins).

$$k_{op} = k_{op}^{0} e^{m_{op}[D]/RT} \tag{4}$$

$$k_{cl} = k_{cl}^{0} e^{-m_{cl}[D]/RT} \tag{5}$$

In eqs 4 and 5, k^0_{op} and k^0_{cl} represent the rate constants for the opening and closing reactions (respectively) in the absence of denaturant. Note that the sum of m_{op} and m_{cl} is equal to the m_{eq} value in eq 2.

The ΔG_{HX} values in this work were obtained from the linear extrapolation of apparent ΔG_{HX} values that were theoretically calculated at different denaturant concentrations using a method analogous to that described in eq 2. The slope of this linear extrapolation (i.e., the m_{eq} value in eq 2) was defined as m_{eqHX}. The apparent ΔG_{HX} values at different [denaturant] were calculated using eq 6 [8]

$$\Delta G_{HX} = -RT\ln K_{open} = -RT\ln(k_{ex}/(k_{int} - k_{ex})) \tag{6}$$

where k_{ex} is the theoretical H/D exchange rate and k_{int} is the intrinsic exchange rate of an unprotected amide proton. All k_{int} values in this work were assigned values based on the following relationship (10^{pH-5} min^{-1}) [11]. The k_{ex} values used in eq 7 for the apparent ΔG_{HX} calculations were determined using eq 7

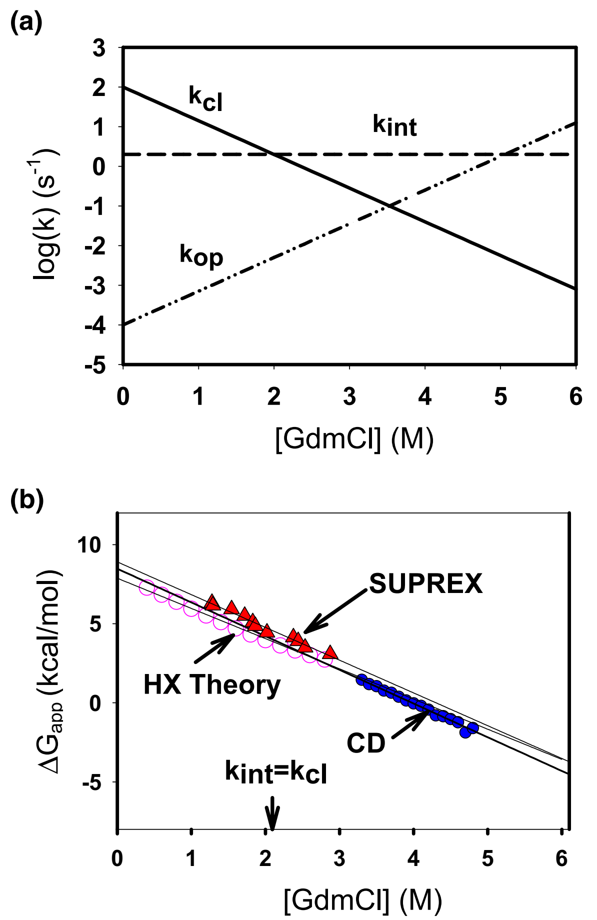

Figure 1. Analysis of ubiquitin. (**a**) Logarithm of k_{cl}, k_{op}, and k_{int} versus [GdmCl] plots. The solid line represents the logarithm of k_{cl}, the dashed-dotted line represents the logarithm of k_{op}, and the dashed line represents the logarithm of k_{int}. The kcl and kop data were reconstructed from reference [1]. (**b**) Apparent unfolding free-energy versus [GdmCl] plots. The open circles represent the theoretical HX data calculated using the k_{cl}, k_{op}, and k_{int} values at each [denaturant], the triangles represent experimental data acquired by SUPREX, and the filled circles represent experimental data acquired by CD. The three solid lines are the results of linear least-squares analyses of the HX, SUPREX, and CD datasets.

$$k_{ex} = \frac{k_{op}k_{int}}{k_{op} + k_{cl} + k_{int}} \tag{7}$$

where k_{op}, k_{cl}, and k_{int} are as defined above. Note that the derivation of eq 6 requires the assumption that k_{cl} is much greater than k_{int} and that the protein is stable (i.e., $K_{op} = k_{op}/k_{cl}$ is much smaller than 1). However, in our calculations of k_{ex} using eq 7, no assumptions were made regarding the relative magnitudes of k_{cl} and k_{int}. We only assumed that the protein was stable (i.e., $K_{op} < 0.01$) [12]. Thus, apparent ΔG_{HX} values were only calculated in the denaturant concentration ranges where k_{op} was <100-fold smaller than k_{cl}.

The ΔG_{SUPREX} and m_{SUPREX} values reported in this work for ubiquitin were taken directly from data that has been previously reported [4].

The ΔG_{CD} and m_{CD} values for ubiquitin were experimentally determined in conventional chemical denaturant-induced equilibrium unfolding studies using CD spectroscopy as a structural probe. These were carried out on an Applied Photophysics π*-180 spectrometer (Applied Photophysics Ltd., Leatherhead, Surrey, UK). The CD signal was monitored at 220 nm. Titrations were set up by mixing 0 M and 6 M GdmCl solutions containing the protein in 20 mM tris (pH 7.4) buffer. The mixing time was 1 min, there was a delay of 5 s, ~5000 CD signals were collected over the course of 30 s, and the signals were averaged. The averaged CD signals were used to generate the ΔG_{CD} and m_{CD} values according to the linear extrapolation method (LEM) [13] that exploits the well-documented linear relationship between a protein's apparent ΔG_u and the denaturant concentration as described above in eq 2.

Results and Discussion

Ubiquitin

Shown in Figure 1a are logk_{int}, logk_{op}, and logk_{cl} versus [denaturant] plots we generated for ubiquitin. These plots were reconstructed from ubiquitin data previously reported [1]. An average k_{int} value for ubiquitin was determined from the pH (i.e., where $k_{int} = 10^{\text{pH-5}}$ min^{-1} or 1.7 s^{-1} at pH 7.0) [11]. Values for k_{int}, have been shown to very slightly change (e.g., <10-fold) with denaturant concentration [14]. However, for the purposes of this work, no denaturant dependence was assigned to k_{int} values.

From the data in Figure 1a, clearly k_{cl} approaches k_{int} at around 2 M GdmCl. In this region, HX is neither in the EX2 regime nor in the EX1 regime. It is also apparent that the transition midpoint of a GdmCl-induced equilibrium unfolding curve for ubiquitin should be close to 3.5 M as k_{cl} is equal to k_{op} at this denaturant concentration. This is close to that observed in the GdmCl-induced equilibrium unfolding data collected here; 4.0 M (see the denaturant concentration at which the apparent ΔG_u value is 0 in Figure 1b). The 0.5 M discrepancy is relatively small and likely due to the inaccuracies associated with reconstructing the data in Sivaraman et al. [1].

The apparent unfolding free energies determined by SUPREX, HX theory, and CD (i.e., apparent ΔG_{SUPREX}, ΔG_{HX}, and ΔG_{CD} values, respectively) were plotted as a function of denaturant concentration (see Figure 1b).

Table 1. Thermodynamic data obtained for ubiquitin

	ΔG_u (kcal mol^{-1})	m_{eq} (kcal mol^{-1}M^{-1})
Literature data[a]	8.1[b]	2.3[b]
Theoretical HX data	7.9	1.9
SUPREX data	8.7 ± 0.2	2.1 ± 0.2
CD data	8.5 ± 0.3	2.1 ± 0.2

[a]From reference [1].
[b]Values were determined based on data taken from reference [1].

Linear extrapolation of the SUPREX data, the CD data, and the theoretical HX data yielded the ΔG and m values summarized in Table 1. There is good agreement between the values determined by SUPREX and by CD, as would be expected for a two-state folding protein like ubiquitin. Significantly, the agreement is good despite the fact that the SUPREX data were collected in the 1.2 to 2.8 M denaturant concentration range where ubiquitin is in the EXX exchange regime. It is also noteworthy that the SUPREX and HX theory data points in Figure 1b are very similar in the denaturant concentration range in which they overlap (i.e., between 1.2 to 2.8 M).

Our results on the ubiquitin system suggest that the EXX regime did not compromise the accuracy of SUPREX. The assumption of EX2 exchange behavior in our SUPREX analysis of ubiquitin did not significantly affect our ability to accurately measure the protein's folding/unfolding free-energy.

Hypothetical Proteins

To test the generality of our findings with ubiquitin, we set out to compare ΔG_{HX} values calculated for a series of hypothetical proteins to their ΔG_u values that were defined by a range of k_{cl}, k_{op}, m_{cl}, m_{op}, and m_{eq} values. The hypothetical proteins included three classes of proteins in which each class was defined by a series of twelve proteins with the same m_{eq} value, either 1.0 (Class 1), 2.0 (Class 2), or 4.0 (Class 3) kcal $mol^{-1}M^{-1}$. The twelve proteins in each class were arbitrarily assigned k_{op} values that varied from 10^{-8} to 10^{-3} s^{-1} and assigned k_{cl} values that varied from 10^4 to 10^0 s^{-1}. A total of four different combinations of k_{op} and k_{cl} values were used to define four different ΔG_u value conditions (i.e., 4.1 (Condition 1), 6.8 (Condition 2), 9.5 (Condition 3), and 16.4 (Condition 4) kcal mol^{-1}), and at each ΔG_u value condition one of three different m_{cl} values were assigned (either 0.3, 0.5, or 0.7 kcal $mol^{-1}M^{-1}$ for m_{eq} = 1 kcal $mol^{-1}M^{-1}$; either 0.5, 1.0, or 1.5 for m_{eq} = 2.0 kcal $mol^{-1}M^{-1}$; and either 1.0, 2.0, or 3.0 kcal $mol^{-1}M^{-1}$ for m_{eq} = 4.0 kcal $mol^{-1}M^{-1}$).

Plots of $\ln k_{cl}$ versus [denaturant] and of apparent ΔG_{HX} value versus [denaturant] were generated for the 36 hypothetical proteins in this study assuming one of three different pH conditions including pH 5.0, 7.0, and 9.0. These plots generated for the 3 hypothetical proteins with a ΔG_u value of 6.8 kcal mol^{-1} and an m_{eq} value of 2.0 kcal $mol^{-1}M^{-1}$ (Class 2, Condition 2) at pH 7 are shown in Figure 2, and the plots that were generated for the three hypothetical proteins with a ΔG_u value of 9.5 kcal mol^{-1} and an m_{eq} value of 2.0 kcal $mol^{-1}M^{-1}$ (Class 2, Condition 3) at pH 7 are shown in Figure 3.

Ultimately, the data points in the ΔG_{app} versus [denaturant] plots (like the ones shown in Figures 2b and 3b) of all the proteins in this study were subject to a linear least-squares analysis to determine a y-intercept and slope that were taken as the ΔG_{HX} and m_{eqHX} values, respectively. The ΔG_{HX}, m_{eqHX} and correlation coefficients, R^2 values, obtained from these linear least-squares analysis were tabulated according to their class (i.e., m_{eq} value) and assumed solution pH (i.e., pH 5.0, 7.0, and 9.0). The values obtained for the proteins in classes 1, 2, and 3 (i.e., for the proteins with m_{eq} values of 1.0, 2.0, and 4.0 kcal $mol^{-1}M^{-1}$, respectively) at pH 7.0 are summarized in Tables 2, 3, and 4, and the values obtained for the proteins in each class at the two additional pHs in this study (i.e., pH 5.0 and 9.0) are summarized in Tables 5, 6, 7, 8, 9, and 10.

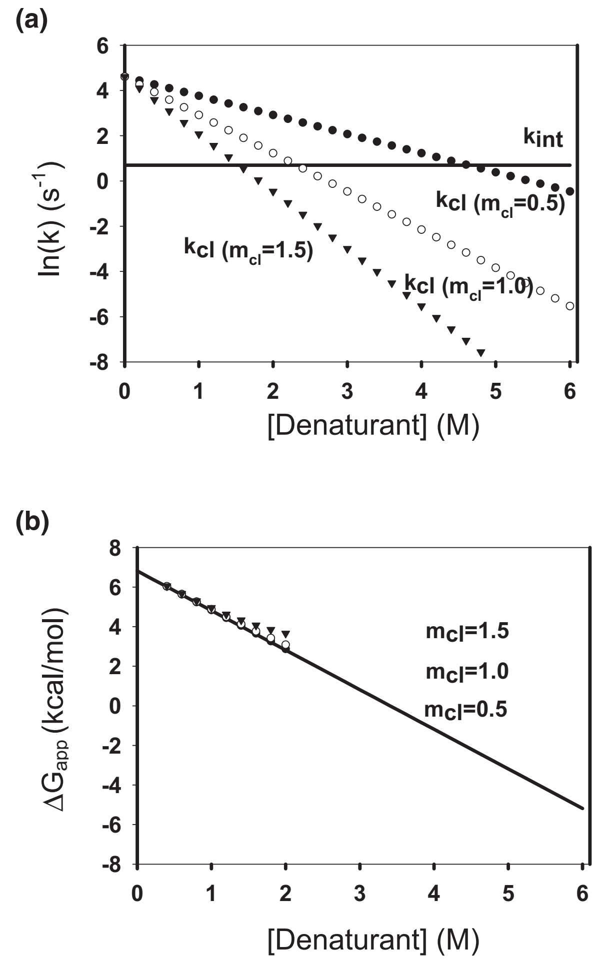

Figure 2. Theoretical analysis of hypothetical proteins in Class 2 (pH 7.0) under Condition 2 (ΔG_u = 6.8 kcal mol^{-1}) (**a**) $\ln k_{cl}$ versus [denaturant] plots generated using three different m_{cl} values indicated. The horizontal line represents k_{int}. (**b**) Apparent ΔG_{HX} values versus [denaturant] plots for three different m_{cl} values indicated. The ΔG_{HX} values were calculated starting from 0.4 M denaturant, at each denaturant concentration with 0.2 M interval and only in the denaturant concentration range where k_{op} is smaller than $k_{cl}/100$. The line represents the apparent ΔG_u values. In each figure, inverted filled triangle, open circle, and filled triangle represent m_{cl} = 1.5, 1, and 0.5 kcal mol^{-1} M^{-1}, respectively.

The main goal of the work on the hypothetical protein systems described here was to determine if there was a significant discrepancy between the theoretical ΔG_{HX} and m_{eqHX} values in Tables 2 to 10 and the

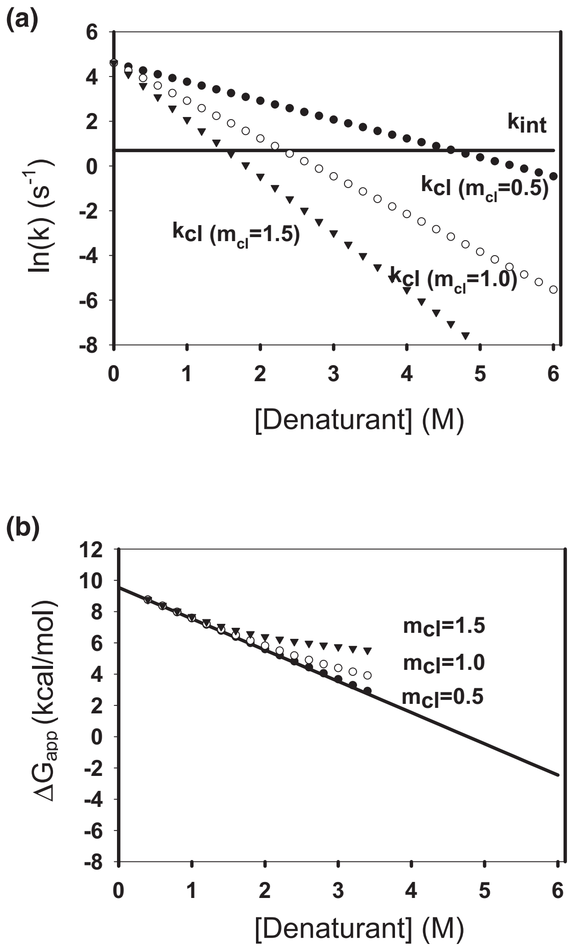

Figure 3. Theoretical analysis of hypothetical proteins in Class 2 (pH 7.0) under Condition 3 (ΔG_u = 9.5 kcal mol^{-1}) (**a**) $\ln k_{cl}$ versus [denaturant] plots for the three different m_{cl} values indicated. The horizontal line represents k_{int}. (**b**) Apparent ΔG_{HX} values versus [denaturant] plots for the three different m_{cl} values indicated. ΔG_{HX} values were calculated starting from 0.4 M denaturant, at each denaturant concentration with 0.2 M interval and only in the denaturant concentration range where k_{op} is smaller than $k_{cl}/100$. The line represents the apparent ΔG_u values. In each figure, inverted filled triangle, open circle, and filled circle represent m_{cl} = 1.5, 1, and 0.5 kcal mol^{-1} M^{-1}, respectively.

assigned ΔG_u and m_{eq} values. The ΔG_{HX} and m_{eqHX} values corresponded to those that would be expected in SUPREX analyses of the hypothetical proteins. The assigned ΔG_u and m_{eq} values would correspond to the value expected in a more conventional non-HX-based technique (i.e., the "true" value). In our comparative analyses, we considered discrepancies of greater than 10% to be significant, as the relative standard deviations of experimentally determined ΔG_{SUPREX} and m_{SUPREX} values are typically about 10%.

Our results at pH 7 (see Tables 2-4) reveal that 14 of the 36 hypothetical proteins yielded ΔG_{HX} values and m_{eqHX} values that were consistent with both the assigned ΔG_u and m_{eq} values, and 26 of the 36 hypothetical proteins had ΔG_{HX} values that were consistent with the assigned ΔG_u value. The data in Figure 2b illustrated the relative small discrepancies that were observed. At pH 7.0 we also observed that all the proteins stabilized by 4.1 kcal mol^{-1} (Condition 1 in Tables 2, 3, and 4) had large discrepancies between the ΔG_{HX}, m_{eqHX} values and the assigned ΔG_u, m_{eq} values, as did the proteins with relatively large m_{cl} values (i.e., >~1.5 kcal mol^{-1} M^{-1}). An example of these larger discrepancies can be seen in the m_{cl} = 1.5 data points in Figure 3b which are not co-linear, and which do not coincide

Table 2. Theoretical thermodynamic parameters for hypothetical proteins in Class 1 (i.e., m_{eq} = 1.0 kcal mol^{-1}M^{-1}) at pH 7.0

		m_{cl} (kcal mol^{-1}M^{-1})		
Conditions		0.3	0.5	0.7
Condition 1	m_{eqHX}	0.8[a]	0.6[a]	0.4[a]
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	4.7[a]	4.7[a]	4.7[a]
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	1	0.9997	0.9986
Condition 2	m_{eqHX}	1.0	0.9	0.8[a]
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	6.8	6.8	6.6
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	0.9994	0.9934
Condition 3	m_{eqHX}	1.0	0.8[a]	0.6[a]
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	9.5	9.3	8.9
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	0.9998	0.9937	0.9621
Condition 4	m_{eqHX}	1.0	0.9	0.7[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.4	16.1	15.5
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	1	0.9971	0.9622

[a]Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.

Table 3. Theoretical thermodynamic parameters for hypothetical proteins in Class 2 (i.e., m_{eq} = 2.0 kcal mol^{-1}M^{-1}) at pH 7.0

		m_{cl} (kcal mol^{-1}M^{-1})		
Conditions		0.5	1	1.5
Condition 1	m_{eqHX}	1.6[a]	1.2[a]	0.7[a]
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	4.7[a]	4.7[a]	4.7[a]
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	1	1	1
Condition 2	m_{eqHX}	2.0	1.8	1.5[a]
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	6.8	6.7	6.5
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	0.9994	0.9881
Condition 3	m_{eqHX}	1.9	1.6[a]	1.0[a]
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	9.5	9.2	8.7
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	0.9999	0.9935	0.9459
Condition 4	m_{eqHX}	2.0	1.8	1.2[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.4	15.9	14.9
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	1	0.9927	0.9247

[a]Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.

Table 4. Theoretical thermodynamic parameters for hypothetical proteins in Class 3 (i.e., m_{eq} = 4.0 kcal $mol^{-1}M^{-1}$) at pH 7.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		1	2	3
Condition 1	m_{eqHX}	ND[b]	ND	ND
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	ND	ND	ND
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	ND	ND	ND
Condition 2	m_{eqHX}	3.9	3.6	2.7[a]
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	6.8	6.7	6.3
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	0.9994	0.988
Condition 3	m_{eqHX}	3.9	3.2[a]	1.9[a]
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	9.5	9.1	8.5
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	1	0.9944	0.9536
Condition 4	m_{eqHX}	4.0	3.5[a]	2.2[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.3	15.8	14.6[a]
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	1	0.9917	0.9202

[a]Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.
[b]ND = not determined. Proteins with an m_{eq} = 4.0 kcal $mol^{-1}M^{-1}$ are not likely to have such low stability.

with the "true values" (represented by the solid line), especially at high denaturant concentrations (i.e., >2 M).

At low pH (i.e., pH 5.0) where k_{int} is small and EX2 mechanisms are dominant, our data reveals that HX derived ΔG_{HX} and m_{eqHX} values are generally consistent with "true" values (see Tables 5–7). Only one of the 36 hypothetical proteins examined at pH 5.0 had significant discrepancies between the HX derived ΔG_{HX} and m_{eqHX} values and the "true" value (see the one m_{eqHX} value in Table 7 marked with an asterisk). It is also noteworthy that the one observed discrepancy was in the *m* value and it was only 15%.

At pH 9.0, where k_{int} is relative large and EX1 mechanisms are dominant, the ΔG_{HX} values and m_{eqHX} values obtained for a large fraction of the hypothetical

Table 5. Theoretical thermodynamic parameters for hypothetical proteins in Class 1 (i.e., m_{eq} = 1.0 kcal $mol^{-1}M^{-1}$) at pH 5.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		0.3	0.5	0.7
Condition 1	m_{eqHX}	1.0	1.0	1.0
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	4.1	4.1	4.1
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	1	1	1
Condition 2	m_{eqHX}	1.0	1.0	1.0
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	6.8	6.8	6.8
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	1	0.9999
Condition 3	m_{eqHX}	1.0	1.0	1.0
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	9.5	9.5	9.5
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	1	1	0.9994
Condition 4	m_{eqHX}	1.0	1.0	1.0
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.4	16.4	16.4
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	1	1	1

Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.

Table 6. Theoretical thermodynamic parameters for hypothetical proteins in Class 2 (i.e., m_{eq} = 2.0 kcal $mol^{-1}M^{-1}$) at pH 5.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		0.5	1.0	1.5
Condition 1	m_{eqHX}	2.0	2.0	1.9
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	4.1	4.1	4.1
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	1	1	1
Condition 2	m_{eqHX}	2.0	2.0	2.0
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	6.8	6.8	6.8
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	1	1
Condition 3	m_{eqHX}	2.0	2.0	1.9
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	9.5	9.5	9.4
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	1	1	0.9981
Condition 4	m_{eqHX}	2.0	2.0	1.9
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.4	16.4	16.1
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	1	1	0.9943

Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.

Table 7. Theoretical thermodynamic parameters for hypothetical proteins in Class 3 (i.e., m_{eq} 4.0 kcal $mol^{-1}M^{-1}$) at pH 5.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		1	2	3
Condition 1	m_{eqHX}	ND[a]	ND	ND
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	ND	ND	ND
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	ND	ND	ND
Condition 2	m_{eqHX}	4.0	4.0	4.0
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	6.8	6.8	6.8
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	1	1
Condition 3	m_{eqHX}	4.0	4.0	3.8
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	9.5	9.5	9.4
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	1	1	0.9986
Condition 4	m_{eqHX}	4.0	4.0	3.4[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.4	16.3	15.6
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	1	1	0.9791

Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.
[a]ND = not determined.

Table 8. Theoretical thermodynamic parameters for hypothetical proteins in Class 1 (i.e., m_{eq} = 1.0 kcal $mol^{-1}M^{-1}$) at pH 9.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		0.3	0.5	0.7
Condition 1	m_{eqHX}	0.7[a]	0.5[a]	0.3[a]
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	7.1[a]	7.1[a]	7.1[a]
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	1	1	1
Condition 2	m_{eqHX}	0.8[a]	0.6[a]	0.3[a]
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	7.3	7.3	7.3
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	0.9998	0.9989	0.996
Condition 3	m_{eqHX}	0.7[a]	0.5[a]	0.3[a]
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	10.0	10.0	10.0
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	0.9998	0.9991	0.9972
Condition 4	m_{eqHX}	1.0	0.9	0.7[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.3	16.2	16.0
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	0.9999	0.9967	0.9733

[a]Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.

proteins in this work were in poor agreement with the "true" values (see values marked with an asterisk in Tables 8–10). In general, the m_{eqHX} values were more in error than the ΔG_{HX} values. At pH 9.0 only four out of the 36 hypothetical proteins had ΔG_{HX} values and m_{eqHX} values that were in reasonable agreement with the assigned ΔG_u and m_{eq} values. These four proteins were all the most stable proteins in our study (i.e., their ΔG_u values were 16.4 kcal mol^{-1}).

Table 9. Theoretical thermodynamic parameters for hypothetical proteins in Class 2 (i.e., m_{eq} = 2.0 kcal $mol^{-1}M^{-1}$) at pH 9.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		0.5	1	1.5
Condition 1	m_{eqHX}	1.5[a]	1.0[a]	0.5[a]
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	7.1[a]	7.1[a]	7.1[a]
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	1	1	1
Condition 2	m_{eqHX}	1.6[a]	1.1[a]	0.6[a]
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	7.4	7.3	7.2
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	0.9999	0.9993	0.9968
Condition 3	m_{eqHX}	1.6[a]	1.0[a]	0.5[a]
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	10.0	10.0	9.9
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	0.9999	0.9994	0.9982
Condition 4	m_{eqHX}	1.9	1.3[a]	0.7[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.2	15.5	15.0
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	0.9993	0.9829	0.9322

[a]Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.

Table 10. Theoretical thermodynamic parameters for hypothetical proteins in Class 3 (i.e., m_{eq} = 4.0 kcal $mol^{-1}M^{-1}$) at pH 9.0

		m_{cl} (kcal $mol^{-1}M^{-1}$)		
Conditions		1	2	3
Condition 1	m_{eqHX}	ND[b]	ND	ND
ΔG_u = 4.1 kcal mol^{-1}	ΔG_{HX}	ND	ND	ND
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 1 s^{-1}	R^2	ND	ND	ND
Condition 2	m_{eqHX}	3.2[a]	2.1[a]	1.1[a]
ΔG_u = 6.8 kcal mol^{-1}	ΔG_{HX}	7.3	7.2	7.2
k_{op}/k_{cl} = 10^{-3} s^{-1}/ 10^2 s^{-1}	R^2	1	0.9998	0.9993
Condition 3	m_{eqHX}	3.1[a]	2.1[a]	1.0[a]
ΔG_u = 9.5 kcal mol^{-1}	ΔG_{HX}	10.0	9.9	9.9
k_{op}/k_{cl} = 10^{-5} s^{-1}/ 10^2 s^{-1}	R^2	0.9999	0.9998	0.9995
Condition 4	m_{eqHX}	3.7	2.5[a]	1.3[a]
ΔG_u = 16.4 kcal mol^{-1}	ΔG_{HX}	16.2	15.2	14.6[a]
k_{op}/k_{cl} = 10^{-8} s^{-1}/ 10^4 s^{-1}	R^2	0.9988	0.9851	0.955

[a]Significantly (i.e., >10%) different than the corresponding ΔG_u or m_{eq} value.
[b]ND = not determined.

Practical Implications

The analyses performed here were all done on proteins with known or assigned biophysical parameters (i.e., k_{op}, k_{cl}, and m_{cl} values). In many applications of SUPREX, such values have not been assigned for the protein under study. Thus, it is difficult to predict a priori whether or not large errors attributable to problems associated with EXX exchange behavior would be introduced into the SUPREX-derived ΔG_{HX} values and m_{eqHX} values. The results presented here, however, suggest that it may be possible to detect such EXX exchange behavior problems by close examination of the data used in the linear extrapolation of apparent ΔG_{SUPREX} values to obtain ΔG_{SUPREX} and m_{SUPREX} values.

Plots of apparent ΔG_{SUPREX} versus [denaturant] that have nonlinearities such as the nonlinearities observed for data points in Figures 2b and 3b may signal such EXX problems. The slight curvature in the data points in Figure 2b is likely to be obscured in real SUPREX experiments by the inherent error associated with a SUPREX curve midpoint determination (~0.1 to 0.3 M units); but fortunately the error introduced by the EXX behavior in such cases is small. On the other hand, the m_{cl} = 1.5 kcal mol^{-1} M^{-1} data in Figure 3b has pronounced nonlinearity when the problems associated with EXX behavior arise (i.e., at denaturant concentrations >2 M). Such a pronounced nonlinearity is likely to be detected in real SUPREX data. Also, note the relatively poor correlation coefficients obtained in the nonlinear least-squares analysis of the m_{cl} = 1.5 datasets in Table 3. In theory, the calculation of such poor correlation coefficients in the fitting of SUPREX data to the

SUPREX equation [4] could signal the EXX exchange problems discussed here.

It is also important to note that all the pronounced nonlinearities that we observed for the 36 hypothetical proteins in this study occurred at denaturant concentrations greater than 2 M (e.g., see Figures 2b and 3b). Thus, the accuracy-related problems presented by EXX exchange behavior could be avoided or at least minimized if apparent ΔG_{SUPREX} values at lower denaturant concentrations (less than 2 M denaturant) were exclusively used to derive ΔG_{SUPREX} and m_{SUPREX} values from the linear extrapolations.

Conclusions

The results of these studies suggest that SUPREX analyses at lower pH conditions are more likely to yield accurate ΔG_u and m values. This is not surprising as such conditions generally favor EX2 exchange behavior in proteins. More importantly, our results reveal how potential problems with non-EX2 exchange behavior can be detected in the SUPREX experiment without any prior knowledge of the proteins' biophysical parameters. Our results also reveal that potential accuracy-related problems arising from non-EX2 exchange behavior can be minimized by using longer H/D exchange times in the SUPREX experiment to ensure that the transition midpoints of the SUPREX curves used to obtain ΔG_{SUPREX} and m_{SUPREX} values are at lower denaturant concentrations (i.e., less than 2 M). These findings promise to be of great practical importance to practitioners of the SUPREX technique.

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