serum-derived components are completely resolved by the dissociative gradient centrifugation step, a result which suggests some level of specificity in the interaction between the 65-kDa protein and the mucin. The presence of a component with similar physical properties in canine tracheal mucin supports the possibility of a functional role for this macromolecule.

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REFERENCES

Berman, G., Afford, S. C., Burnett, D., & Stockley, R. A. (1986) *J. Biol. Chem. 261*, 14095–14099.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Creeth, J. M., Bhaskar, K. R., Horton, J. R., Das, I., Lopez-Vidriero, M. T., & Reid, L. (1977) *Biochem. J. 167*, 557–569.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., & Weber, P. (1981) *Anal. Biochem. 118*, 131–137.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.

Haas, J. B., & Kennett, R. H. (1979) in *Monoclonal Antibodies* (Kennett, R. H., McKearn, T. J., & Bechtol, K. B., Eds.) pp 407–411, Plenum, New York and London.

Hager, D. A., & Burgess, R. R. (1980) *Anal. Biochem. 109*, 76–86.

Ringler, N. J., Selvakumar, R., Woodward, H. D., Simet, I. M., Bhavanandan, V. P., & Davidson, E. A. (1987) *Biochemistry 26*, 5322–5328.

Roberts, G. P. (1976) *Arch. Biochem. Biophys. 173*, 528–537.

Rose, M. C., Voter, W. A., Brown, C. F., & Kaufman, B. (1984) *Biochem. J. 222*, 371–377.

Sachdev, G. P., Myers, F. J., Horton, F. O., Fox, O. F., Wen, G., Rogers, R. M., & Carubelli, R. (1980) *Biochem. Med. 24*, 82–94.

Slayter, H. S., Lamblin, G., LeTreut, A., Galabert, C., Houdret, N., Degand, P., & Roussel, P. (1984) *Eur. J. Biochem. 142*, 209–218.

Spencer, R. L., & Wold, F. (1969) *Anal. Biochem. 32*, 185–190.

Tabachnik, N. F., Blackburn, P., & Cerami, A. (1981) *J. Biol. Chem. 256*, 7161–7165.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Williams, C. A., & Chase, M. W. (1971) *Methods Immunol. Immunochem. 3*, 146–160.

Williams, H. R., & Lin, T.-Y. (1971) *Biochim. Biophys. Acta 250*, 603–607.

Woodward, H., Horsey, B., Bhavanandan, V. P., & Davidson, E. A. (1982) *Biochemistry 21*, 694–701.

Woodward, H. D., Ringler, N. J., Selakumar, R., Simet, I. M., Bhavanandan, V. P., & Davidson, E. A. (1987) *Biochemistry 26*, 5315–5322.

Unfolding Free Energy Changes Determined by the Linear Extrapolation Method. 1. Unfolding of Phenylmethanesulfonyl α-Chymotrypsin Using Different Denaturants†

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ABSTRACT: Characteristics and properties of the unfolding free energy change, $\Delta G°_{N\text{-}U}$, as determined by the linear extrapolation method are assessed for the unfolding of phenylmethanesulfonyl chymotrypsin (PMS-Ct). Difference spectral measurements at 293 nm were used to define PMS-Ct unfolding brought about with guanidinium chloride, urea, and 1,3-dimethylurea. All three denaturants were shown to give identical extinction coefficient differences ($\Delta\epsilon_{N\text{-}U}$) between native and unfolded forms of the protein in the limit of zero concentration of denaturant. The independence of $\Delta\epsilon_{N\text{-}U}$ on denaturant supports the linear extension of pre- and postdenaturational base lines into the transition zone, allowing evaluation of unfolding equilibrium constants based on the two-state assumption. An expression, based on the linear extrapolation method, was used to provide estimates of $\Delta G°_{N\text{-}U}$ for the three denaturants using nonlinear least-squares fitting of the primary data, $\Delta\epsilon$ versus [denaturant]. The three $\Delta G°_{N\text{-}U}$ values were identical, within error, suggesting that the free energy change is a property of the protein system and independent of denaturant. It is suggested that the error in $\Delta G°_{N\text{-}U}$ determined from use of the linear extrapolation method is significantly larger than commonly reported in the literature.

Of numerous agents known to denature proteins, guanidinium chloride, urea, and like compounds are believed to cause the most complete unfolding (Tanford, 1968). Many soluble globular proteins can be reversibly unfolded by these agents and exhibit two-state behavior, a condition upon which analyses of unfolding free energy measurements have been based (Saito

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& Wada, 1983; Pace, 1975). Of the methods of thermodynamic analyses developed, the linear extrapolation method is currently the most used. The method was originally based on empirical observation of the linear dependence of observed unfolding free energy changes as a function of denaturant (Green & Pace, 1974) and has received support through thermodynamic derivations (Schellman, 1978, 1987; Schellman & Hawkes, 1980) and theoretical developments (Dill, 1985). The quantity of interest derived from the linear extrapolation is the intercept of the ΔG versus denaturant concentration plot, i.e., the free energy change for protein unfolding at zero denaturant concentration ($\Delta G°_{N-U}$).

The difficulty with the linear extrapolation method is that under a single set of experimental conditions it is experimentally possible to collect unfolding free energy change data only over a limited concentration range. Thus, the linear extrapolation of these data to zero concentration of denaturant is so long that it raises questions about the validity as well as the accuracy with which the free energy change for unfolding can be measured. Furthermore, Pace and Vanderburg (1979) extended the range of free energy data as a function of denaturant concentration in the horse myoglobin system and found that the data deviate from the extrapolated line at low concentrations of denaturant. While this result does not necessarily invalidate the meaning of $\Delta G°_{N-U}$ obtained by extrapolating the data obtained at high denaturant concentration, it does suggest the need for a more rigorous validation of $\Delta G°_{N-U}$ as a thermodynamic quantity.

We have been interested in comparing the stabilities of closely related proteins in terms of their unfolding free energy changes, $\Delta G°_{N-U}$. In such comparisons it is important to establish the error associated with the extrapolated quantity, $\Delta G°_{N-U}$, and to investigate its characteristics as a thermodynamic quantity. In this paper, we use three denaturants to assess the characteristics of $\Delta G°_{N-U}$ and to evaluate the error associated with measurements of the unfolding of phenylmethanesulfonyl chymotrypsin (PMS-Ct), a covalently modified form of α-chymotrypsin. In a companion paper (Bolen & Santoro, 1988), we will explore how well the $\Delta G°_{N-U}$ quantity conforms to the characteristics required of a thermodynamic function of state.

Materials and Methods

α-Chymotrypsin, salt free and three times recrystallized from Worthington (CDI), was found by active-site titration with the cinnamoylimidazole assay (Schonbaum et al., 1961) to be 90–94% active. Since chromatography of the enzyme on a Sephadex G-75 column with 1 mM hydrochloride acid (Yapel et al., 1966) resulted in no improvement in active-site titration, the enzyme was used without further purification.

Phenylmethanesulfonyl fluoride (PMSF) purchased from Sigma Chemical Co. was used to prepare phenylmethanesulfonyl chymotrypsin (PMS-Ct) by addition of 100 μL of 0.2 M PMSF, dissolved in acetonitrile, to 3 mL of 33 mM pyrophosphate buffer at pH 7.0 containing 50 mg of α-chymotrypsin. Reactions for PMS-Ct preparations were allowed to proceed for at least 30 min, and then the reaction mixture was adjusted to pH 4.0 and applied to a Sephadex G-25 column, preequilibrated with 0.2 M KCl at pH 4.0. The fractions containing the peak of absorbance at 280 nm were pooled and concentrated by a factor of 10–12 times by using an Amicon Model 3 standard cell equipped with a UM 10 ultrafiltration membrane. Experiments indicated no change in molar absorptivity of the protein on forming PMS-Ct, so concentrations were determined at 280 nm by using a molar absorptivity of 50 000 M^{-1} cm^{-1}. Less than 0.5% residual catalytic activity of α-chymotrypsin was detected in the PMS-Ct preparations by use of *N*-acetyl-L-tryptophan ethyl ester as a substrate.

Guanidinium chloride was prepared by addition of concentrated HCl to guanidinium carbonate (supplied by MCB Co.) until no further carbon dioxide was evolved and the pH was approximately 2. The resultant mixture was evaporated to dryness and the guanidinium chloride recrystallized from methanol. The melting point of the guanidinium chloride prepared in this manner was 187–188 °C, which is comparable to that previously reported (Wong et al., 1971). A 6 M solution gave very low absorbance at 230 nm. Concentrations of guanidinium chloride and urea solutions used in this work were determined from refractive index measurements at 25 °C on an Abbe refractometer using equations relating refractive index to concentration as given by Pace (1986).

Ultrapure urea from Schwartz Mann Co. and 1,3-dimethylurea obtained from Sigma Chemical Co. were used after recrystallization from methanol. Melting points of 135 and 108–109 °C for urea and 1,3-dimethylurea, respectively, compare exactly with that given in the *Handbook of Chemistry and Physics* (1985). An equation relating molar solution concentration with refractive index was determined from analytically prepared solutions to have the form [1,3-dimethylurea] = $83.68\Delta n - 115.3\Delta n^2 + 1268\Delta n^3$.

Unfolding of PMS-Ct was evaluated by difference spectroscopy following the change in absorbance at 293 nm as a function of denaturant concentration. A Beckman Acta MVI spectrophotometer was used with two pairs of matched tandem cuvettes supplied by Precision Cells. All measurements were thermostated at 25.00 ± 0.05 °C. For each denaturant concentration in the transition region, the absorbance was recorded for at least 1 h in order to obtain a sufficient base line to extrapolate to zero time of denaturation and to evaluate ΔA. The final protein concentration in the cuvette was around 0.2 mg/mL in the 0.05 M acetate with 0.144 M NaCl.

Guanidinium chloride denaturation of PMS-Ct was also monitored by fluorescence emission at 327 nm with excitation at 295 nm. These measurements were performed on a SLM 8000 C spectrofluorometer at 25 °C using protein concentrations of 0.03 mg/mL or less in 0.05 M acetate with 0.144 M NaCl.

Urea gradient gel electrophoresis experiments were performed in much the same way as described by Creighton (1979). The gels (1.5-mm thickness, 20-cm width, 16-cm height) were prepared with a linear horizontal gradient of 0–6 M urea and an inverse gradient of 11–6% acrylamide. The electrophoresis buffer at pH 4 was 0.5 M Tris–acetate, and riboflavin (0.004 mg/mL of gel) was used for photopolymerization of the gel. Native and unfolded samples of PMS-Ct were prepared for electrophoresis by (1) mixing 50 μL of 15 mg/mL of "native" PMS-Ct with 0.5 mL of 20% sucrose in 0.05 M Tris–acetate, pH 4.0 (to be called native or folded PMS-Ct), and (2) mixing 50 μL of 15 mg/mL of native PMS-Ct with 6 M urea solution in 0.05 M Tris–acetate, pH 4.0 (to be called "unfolded" PMS-Ct). From either of these solutions, a 200-μL volume was applied evenly to the top of the gel and electrophoresis begun with 5 min of preparing the sample. In each case, electrophoresis was conducted under constant voltage (135 V) for 1 h in a water-jacketed Bio-Rad Protean II apparatus maintained at 25 ± 1 °C by a refrigerated water bath. The pH of points on the gels were monitored after electrophoresis with a Radiometer GK732936 flat surface pH electrode connected to a Radiometer PHM85 pH meter. It was found that the pH varied with position on the gel (in the direction of the urea gradient) from pH 3.9 to 4.2.

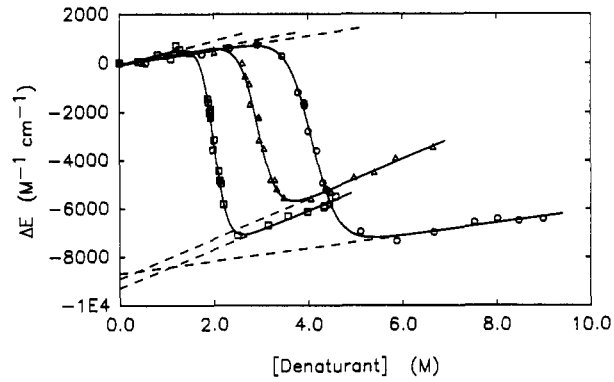

FIGURE 1: Difference spectral measurements of the denaturant-induced unfolding of PMS-Ct at pH 4.0. Data represented are urea (○), 1,3-dimethylurea (△), and guanidinium chloride (□). Solid symbols indicate reversibility points taken as described in the text. The dashed lines for pre- and postdenaturational concentration regions are calculated by using the nonlinear best-fitted estimates of $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, m_N, and m_U as defined in eq 1. The solid lines are the results of nonlinear least-squares best fits for each of the denaturants from eq 4. Reversibility points were not included in the nonlinear least-squares analysis.

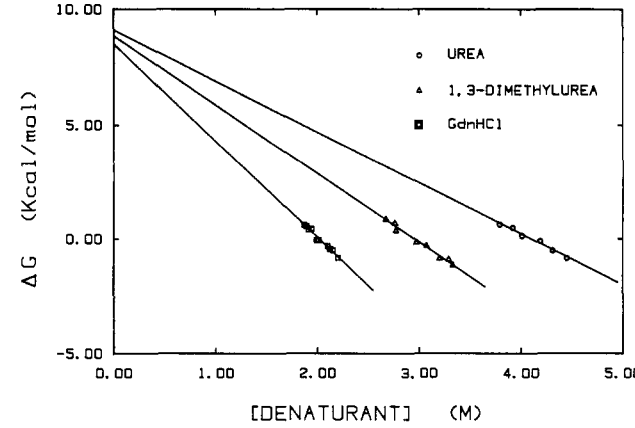

FIGURE 2: Dependence of free energy for unfolding of PMS-Ct as a function of each of the three denaturants given in Figure 1. Solid lines are the consequence of using the $\Delta G^\circ_{N\text{-}D}$ and m_G values obtained from the individual nonlinear least-squares best fitting of the data of Figure 1. Symbols are the same as given in Figure 1.

The gels were stained for visualization as described by Creighton (1979).

Nonlinear least-squares fitting was performed by a program provided by Dr. Michael Johnson at the University of Virginia and described elsewhere (Johnson & Frasier, 1985).

RESULTS

Solvent-induced protein unfolding can be detected as a function of denaturant concentration by use of any observable giving a detectable difference between the native and unfolded states of the protein. Figure 1 provides such data on three solvent systems that induce unfolding of PMS-Ct as monitored by the maximum difference in extinction coefficient ($\Delta\epsilon_{293}$) between native and unfolded protein. Evaluation of equilibrium constants in the transition region requires extensions of the pre- and postunfolding base lines into the transition region. Mathematically, these extensions are represented by eq 1,

$$\Delta\epsilon_N = \Delta\epsilon^\circ_N + m_N[D] \qquad \Delta\epsilon_U = \Delta\epsilon^\circ_U + m_U[D] \qquad (1)$$

where $\Delta\epsilon_N$ and $\Delta\epsilon_U$ represent the difference extinction coefficients for native and unfolded protein, respectively, evaluated at specified denaturant concentrations in the transition region and are functions of denaturant concentration [D]. $\Delta\epsilon^\circ_N$ and $\Delta\epsilon^\circ_U$ are intercepts, and m_N and m_U are slopes of the pre- and postunfolding regimes, respectively. Equation 2 defines how

$$K_{obsd} = (\Delta\epsilon_N - \Delta\epsilon)/(\Delta\epsilon - \Delta\epsilon_U) \qquad (2)$$

these base lines serve as points of reference in the transition zone in the determinations of equilibrium constants for unfolding at given concentrations of denaturant and corresponding values of $\Delta\epsilon$. K_{obsd} represents the equilibrium ratio of unfolded/native protein species at any specified denaturant concentration. Two requirements for this analysis are that the system must be reversible and that only two states of the protein, native and unfolded, exist in significant population in the transition region.

The linear extrapolation method to be discussed here is commonly used for evaluating the free energy change for conversion of native to unfolded protein in the absence of denaturant ($\Delta G^\circ_{N\text{-}U}$). The method consists of converting the observed equilibrium constants evaluated in the transition region to free energy data (ΔG_{obsd}), plotting these data as a function of denaturant as in Figure 2, and extrapolating the data to zero denaturant concentration. This is expressed mathematically by eq 3, where $\Delta G^\circ_{N\text{-}U}$, the intercept, is the

$$\Delta G_{obsd} = \Delta G^\circ_{N\text{-}U} + m_G[D] \qquad (3)$$

free energy change for unfolding in the absence of denaturant, m_G is the slope of the plot in Figure 2, and $\Delta G_{obsd} = -RT \ln K_{obsd}$.

We have combined eq 1–3 and the relationship $\Delta G_{obsd} = -RT \ln K_{obsd}$ into a single expression (eq 4) relating $\Delta\epsilon$ and

$$\Delta\epsilon = [(\Delta\epsilon_N + m_N[D]) + (\Delta\epsilon_U + m_U[D]) * \exp -(\Delta G^\circ_{N\text{-}U}/RT + m_G[D]/RT)]/[1 + \exp -(\Delta G^\circ_{N\text{-}U}/RT + m_G[D]/RT)] \qquad (4)$$

denaturant concentration, [D], with $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, m_N, m_U, m_G, and $\Delta G^\circ_{N\text{-}U}$ as fitting parameters. The nonlinear least-squares best fits to eq 4 for each of the three solvent systems are given by the solid lines in Figure 1. The extrapolated dashed lines are extensions of the pre- and postunfolding base lines based upon the nonlinear least-squares best-fitted values of $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, m_N, and m_U. Empirically, it is observed that the preunfolding intercepts, $\Delta\epsilon^\circ_N$, center around zero for each of the denaturants, while the postunfolding intercepts, $\Delta\epsilon^\circ_U$, cluster around -9000 M^{-1} cm^{-1} with a deviation in $\Delta\epsilon$ for the three sets of data of only about ±3%. Additionally, the observed $\Delta G^\circ_{N\text{-}U}$ values for the three denaturants also appear to cluster around a common value. With these observations in mind, a simultaneous fitting of the data for all three denaturants was performed with $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, and $\Delta G^\circ_{N\text{-}U}$ as common fitting parameters for all denaturants, while m_N, m_U, and m_G for each denaturant were fitting parameters unique to the particular denaturant used. A compilation of $\Delta G^\circ_{N\text{-}U}$ and m_G values obtained from nonlinear least-squares fitting of each individual denaturant as well as simultaneous fitting of data from all denaturants is reported in Table I.

Along with the best-fitted estimates of $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, m_N, and m_U, and $\Delta G^\circ_{N\text{-}U}$ and m_G data from the individual nonlinear least-squares fits of the three denaturants were used to generate the solid lines in Figure 2. This figure illustrates how the function given in eq 4, along with the parameters evaluated from the nonlinear least-squares method, maps into the usual manner of presentation of the linear extrapolation method.

In terms of $\Delta\epsilon$, reversibility of denaturant-induced unfolding was observed by unfolding PMS-Ct with denaturant concentration in the posttransition region, followed by dilution of the fully unfolded protein to a final guanidinium chloride concentration within the transition region as noted in Figure 1.

Table I: Free Energy Changes for Unfolding of PMS-Ct[a]

denaturant	$\Delta G^{\circ}_{N\text{-}U}$ (kcal/mol)	$-m_G$ [kcal/(mol·M)]
guanidinium chloride	8.45 (7.41, 9.54)	4.19 (3.66, 4.74)
	8.69 (±0.44)[b]	
urea	9.10 (8.03, 10.21)	2.22 (1.96, 2.49)
	9.19 (±0.45)[b]	
1,3-dimethylurea	8.85 (7.64, 10.18)	3.00 (2.59, 3.44)
	8.83 (±0.56)[b]	
simultaneous fit	8.78 (8.07, 9.54)	

[a] Results of nonlinear least-squares fitting of data to eq 4 with 67% confidence intervals in parentheses. [b] Denotes $\Delta G^{\circ}_{N\text{-}U}$ and m_G values with 67% confidence limits calculated in the conventional manner by (1) performing linear least-squares fits on the pre- and postunfolding base lines and extending them into the transition zone, (2) evaluating apparent equilibrium constants for unfolding in the transition zone by assuming the base lines are errorless quantities and then calculating apparent free energy changes for unfolding, and (3) performing a linear least-squares fit of the ΔG (apparent) vs denaturant plot and extrapolating to zero denaturant concentration to get $\Delta G^{\circ}_{N\text{-}U}$.

The ability to achieve the same $\Delta\epsilon_{293}$ in the transition region regardless of whether the protein was initially in the native or unfolded state is taken as evidence of reversibility. If one unfolds PMS-Ct at high denaturant concentration and immediately dilutes the solution to the transition range or below, the system is found to be totally reversible.

It is noted, however, that the extent of PMS-Ct reversibility in all denaturants decreases with the length of time the protein remains in the unfolded state. This time dependence involving irreversibility can be correlated with the absorbance changes following unfolding in the transition region. In the transition zone, $\Delta\epsilon_{293}$ is observed to decrease slowly with time following unfolding, and this decrease parallels the slow hydrolysis of the phenylmethanesulfonyl moiety from the unfolded state, a process known to occur with the unfolded state in acidic solution (Gold & Fahrney, 1964). The extent of desulfonylation can be monitored with time by diluting aliquots of the denaturing media to a nondenaturing reference concentration and assaying for the α-chymotrypsin (product of desulfonylation) catalytic activity toward *N*-acetyl-L-tryptophan ethyl ester. We find that 15% of PMS-Ct reverts to α-chymotrypsin within 1 h following complete unfolding of PMS-Ct in guanidinium chloride at pH 4.0 but reverts only 2% on unfolding for 1 h at pH 6.0.

The resulting α-chymotrypsin generated by loss of the PMS moiety unfolds at lower denaturant concentration than does PMS-Ct. The conversion of PMS-Ct to α-chymotrypsin results in a continuing change of $\Delta\epsilon_{293}$ with time despite the fact that an equilibrium mixture of native and unfolded PMS-Ct is reached quickly. The continuing change in $\Delta\epsilon_{293}$ is believed to be a consequence of the redistribution of the PMS-Ct unfolding equilibrium toward the unfolded state as unfolded PMS-Ct is hydrolyzed to form unfolded α-chymotrypsin. It is noted that the linear continuous time dependence of $\Delta\epsilon_{293}$ is evident only in the transition zone and does not occur at higher denaturant concentrations, where both PMS-Ct and α-chymotrypsin are fully unfolded, or at lower denaturant concentrations, where only native PMS-Ct is present. Such time dependence is not due to autolysis by α-chymotrypsin created as a result of PMS-Ct desulfonylation since we, as well as others, find no autolysis accompanying urea denaturation of α-chymotrypsin at pH values below 4.5 (Martin & Frazier, 1963; Martin, 1964).

The slow linear continuous time dependence of $\Delta\epsilon_{293}$ in the transition zone is handled experimentally by extrapolation to zero time of unfolding, and the $\Delta\epsilon_{293}$ values reported have all been evaluated in that manner.

Table II: Tryptophan and Tyrosine Residues of PMS-Ct Exposed to 6 M Guanidinium Chloride[a]

protein	tryptophan		tyrosine	
	calcd	obsd	calcd	obsd
PMS-Ct	8	8.05	4	3.74
chymotrypsinogen	8	8.0[b]	4	3.85[b]

[a] Absorbance measurements at 280 and 288 nm were made on 12.5 nM PMS-Ct in 0.02 M phosphate buffer at pH 6.5 containing 6 M guanidinium chloride. The absorbances were corrected for contributions due to the five cystines in PMS-Ct and the observed numbers of tryptophan and tyrosine evaluated by using the equations and molar absorptivities given by Edelhoch (1967). [b] Data from Edelhoch (1967).

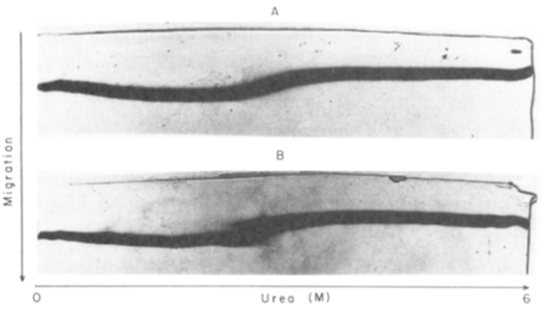

FIGURE 3: Urea gradient gel electrophoresis of PMS-Ct originating in either the native folded form (A) or the unfolded form (B) at pH 4 and 25 °C. Experimental conditions for the electrophoresis are given under Materials and Methods.

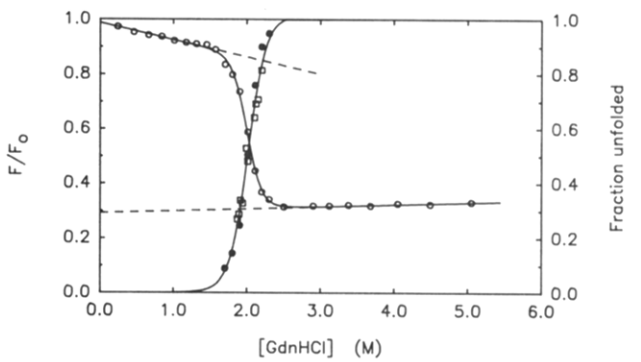

FIGURE 4: Guanidinium chloride induced unfolding of PMS-Ct as monitored by fluorescence emission and, also, expressed as fraction unfolded. Fluorescence emission at 327 nm after excitation at 295 nm (○) is given as the ratio of fluorescence of 0.02 mg/mL PMS-Ct under the guanidinium chloride conditions indicated to that in the absence of guanidinium chloride. Measurements were made at 25 ± 0.05 °C in 0.05 M acetate buffer, pH 4.00, containing 0.144 M NaCl. The fraction of unfolded PMS-Ct in guanidinium chloride was calculated from these fluorescence data (●) and from $\Delta\epsilon$ data (□) of Figure 1 by using linear extensions of the pre- and postunfolding base lines (dashed lines).

An indication of the extent of protein unfolding in guanidinium chloride is given in Table II, in which the number of exposed tyrosine and tryptophan residues in the protein is estimated from molar absorptivities of model compound data following the method of Edelhoch (1967).

Two-state behavior is an important assumption in analysis by the linear extrapolation method. Figures 3 and 4 provide limited evidence in support of this assumption for PMS-Ct unfolding in urea and guanidinium chloride. Parts A and B of Figure 3 represent urea gradient gels (Creighton, 1979, 1980) performed on the native and (6 M urea) unfolded forms of PMS-Ct, respectively, at pH 4. The transition observed in panel A is continuous, indicating rapid equilibration between states, and the midpoint of the transition occurs at the same urea concentration as the transition in panel B. Electrophoresis of the unfolded form also gives a continuous transition but with

a spur indicating existence of a fraction of the population of unfolded species that cannot refold rapidly at the urea concentrations in the transition region. This pattern is characteristic of two states, native and unfolded, with fast- and slow-refolding populations comprising and unfolded state (Creighton, 1980).

Guanidinium chloride induced unfolding of PMS-Ct at pH 4 was also monitored by fluorescence emission, and the results are presented in Figure 4. Linear extensions of the pre- and postunfolding base lines of this figure permit evaluation of the fraction unfolded in the transition zone, and these data along with fraction-unfolded data derived from difference spectral measurements in Figure 1 are presented together in Figure 4.

Discussion

The linear extrapolation method is a construct for evaluating the stability of the folded form of a protein relative to unfolded form in the limit of zero concentration of denaturant. The quantity of interest, $\Delta G°_{N-U}$, is believed to represent the free energy change for complete unfolding of the native protein in a purely aqueous buffered medium. In applying this method to the study of protein denaturation, several conditions are either assumed or approximated. How well these conditions hold will affect how appropriate it is to describe the $\Delta G°_{N-U}$ quantity as a free energy term endowed with the properties expected of such a term. This study is an attempt to assess the adequacy of several of the approximations and assumptions associated with the linear extrapolation method and to evaluate the error in the extrapolated quantity known as $\Delta G°_{N-U}$.

Unfolding/refolding reversibility and two-state behavior are conditions required for application of the linear extrapolation method. The unfolding of PMS-Ct with any of the three denaturants used in the present work is found, within error, to be completely reversible within the time it takes to fully unfold the protein and return to renaturing conditions. There are, however, time-dependent phenomena occurring with the unfolded form of PMS-Ct that result in formation of irreversibly denatured protein as a function of the length of time the protein is in the unfolded form. This slow, time-dependent irreversibility is believed to be associated with cis–trans isomerization of one or more of the nine proline residues in PMS-Ct. The accumulation of slow-refolding denatured species has been observed in the two-state urea unfolding of α-chymotrypsin at pH 4 (Creighton, 1979, 1980).

The existence of two-state behavior markedly simplifies thermodynamic analysis of protein unfolding. We have tried to assess the validity of the assumption in PMS-Ct unfolding induced by urea and guanidinium chloride by using urea gradient gel electrophoresis and by monitoring more than one spectral property sensitive to unfolding induced in guanidinium chloride. The urea gradient gels in Figure 3 suggest the existence of native and unfolded states but with fast- and slow-refolding species within the unfolded population (Creighton, 1980). This conclusion is consistent with Creighton's (1979, 1980) description of urea-induced unfolding of chymotrypsinogen and α-chymotrypsin and with our observations that the extent of solvent-induced reversibility is dependent upon the length of time the protein remains in the unfolded state. It should be emphasized that during the 1-h course of the electrophoresis about 15% of the unfolded form of the protein becomes desulfonylated, and in Figure 3B, the PMS-Ct has existed in the unfolded form for several minutes prior to and during electrophoresis. These two conditions do not hold for the difference spectral and fluorescence data (reported in Figures 1 and 4) that were extrapolated to zero time in order to reduce or eliminate the effects of desulfonylation and buildup of slow-refolding species.

Two-state behavior can be ruled out if different extents of unfolding are observed when observables sensitive to different properties of the protein are used. A popular means of illustrating two-state behavior consists of a congruent plot of fraction unfolded versus denaturant obtained by using two or more spectral techniques (Pace, 1975). Figure 4 shows that both UV absorption difference and fluorescence measurements result in very close correspondence of fractional denaturation profiles in guanidinium chloride. However, this type of test is not positive evidence for two-state behavior; it simply does not rule out that possibility. The visual inspection of fraction-unfolded data in Figure 4 can be deceptive in that the individual nonlinear least-squares fits of the difference spectral and fluorescence data provide different values of $\Delta G°_{N-U}$ that, though they overlap in their confidence intervals, may possibly be distinguishable. Using statistical methods, we are currently trying to establish criteria that provide a more stringent test of two-state behavior.

For the purpose of the analyses provided here, two-state behavior remains an assumption; i.e., the data we have provided are supported but do not prove two-state behavior.

A measure of the extent of PMS-Ct unfolding in guanidinium chloride was estimated in terms of the method of Edelhoch (1967), in which the number of tyrosine and tryptophan residues in PMS-Ct exposed to 6 M guanidinium chloride is calculated from absorbances at 280 and 288 nm. Table II gives a quantitative accounting of the exposure of tryptophan and tyrosine residues. These data are in agreement with values reported by Edelhoch (1967) on unfolding of chymotrypsinogen and lend support to the notion that PMS-Ct unfolded in guanidinium chloride is complete and that there is little indication of residual structure in the unfolded species.

The linear extrapolation method has characteristics to be assessed in addition to the complete, reversible, two-state unfolding conditions discussed. These characteristics involve the adequacy of linear extensions of the pre- and postunfolding base lines into the transition zone and the proper treatment of the data in assessing the accuracy of the $\Delta G°_{N-U}$ and m_G parameters obtained from the method.

The usual two-state method of analysis of such data involves extensions of pre- and postdenaturation base lines into the transition zone to serve as indices of native and unfolded protein extinction coefficient differences as a function of denaturant concentration. From these indices, equilibrium constants for protein unfolding are constructed as discussed under Results. The rationale for linear extension of the base lines follows from difference spectral data obtained on model aromatic amino acids (Bigelow & Geschwind, 1960; Hamaguchi et al., 1963). It is important to note that within ±3% the extrapolations of the postdenaturational base lines for all three denaturants intersect at a common $\Delta\epsilon$. Such behavior is expected if all of the denaturants produce the same unfolded state of the protein, whose molar extinction coefficient is linearly dependent on denaturant concentration. The $\Delta\epsilon°_U$ of around -9000 M^{-1} cm^{-1} observed in the absence of denaturant and common to all denaturants is, then, an intrinsic property of the unfolded protein. Furthermore, the common values of $\Delta\epsilon°_N$ and $\Delta\epsilon°_U$ observed for PMS-Ct unfolding, regardless of denaturant, give further support to the use of the extension of base lines in calculating unfolding equilibrium constants.

The usual practice in evaluating $\Delta G°_{N-U}$ is to convert the equilibrium constants for unfolding obtained in the transition

zone to free energy changes and to plot these data as a function of denaturant concentration. The linear extrapolation is then performed to evaluate the unfolding free energy change at zero denaturant concentration. The data of Figure 2 illustrate that the three denaturants result in much the same value for $\Delta G°_{N-U}$ with only about 0.6 kcal/mol separating the values. The fact that such agreement exists in solutions of guanidinium chloride and urea compounds that differ greatly in ionic character, solution properties, and efficacy in denaturation gives compelling evidence that $\Delta G°_{N-U}$ is a property of the protein system, essentially independent of denaturant. In a study similar to this one, Ahmad and Bigelow (1982) found very good agreement between $\Delta G°_{N-U}$ values obtained from the linear extrapolation using three denaturants with ribonuclease A, lysozyme, α-lactoalbumin, and myoglobin. Such behavior is required of any method of analysis resulting in evaluation of a function of state, and the linear extrapolation method meets that requirement with a significant number of proteins.

The application of nonlinear least-squares methods to the linear extrapolation method results in a significantly larger error associated with $\Delta G°_{N-U}$ than is commonly cited by most workers in the field. The usual practice in the field is to use linear least-squares analysis to extend the pre- and postdenaturation base lines into the transition zone and to consider these lines error free in evaluating equilibrium constants for unfolding. In mapping the resulting equilibrium constants into the free energy versus denaturant concentration plot and performing a least-squares linear extrapolation of these data to zero denaturant concentration, an underestimate of the error in $\Delta G°_{N-U}$ is obtained. For the quality of $\Delta\epsilon_{293}$ data given in Figure 1 the error analysis commonly used in the field results in 67% confidence limtis of $\pm$0.4–0.5 kcal/mol (see Table I).

In contrast to the usual practice of evaluating equilibrium constants from the primary data and replotting the results in a ΔG (apparent) versus denaturant concentration plot, the nonlinear least-squares method performs the analysis on the primary data alone and considers error in all parameters, including the extrapolated base lines. Table I shows that application of the nonlinear least-squares method results in a 67% nonlinear confidence interval from 2 to 2.5 times that obtained by the statistical proocedures commonly cited. We believe the nonlinear least-squares method provides a more realistic assessment of the error in $\Delta G°_{N-U}$.

It is possible to improve the precision of $\Delta G°_{N-U}$ by a simultaneous fit of the data from all three denaturant profiles. The fitting was performed with $\Delta G°_{N-U}$, $\Delta\epsilon°_N$, and $\Delta\epsilon°_U$ as parameters common to all three sets of data and with all points weighted equally. $\Delta G°_{N-U}$ was found to be 8.8 kcal/mol with a significantly smaller confidence interval in comparison with the individual fits and a smaller variance of fit than the individual fits. Accordingly, a simultaneous fit assuming $\Delta G°_{N-U}$, $\Delta\epsilon°_N$, and $\Delta\epsilon°_U$ as common parameters is justified since these parameters obtained from the individual fits of the three denaturants cannot be distinguished statistically from one another.

In the case of solvent-induced PMS-Ct unfolding, $\Delta G°_{N-U}$ obtained by use of the linear extrapolation method has properties expected of a thermodynamic quantity. It appears to be independent of the type and efficacy of strong denaturant used to bring about unfolding, and the difference in extinction coefficient of native and unfolded protein obtained from denaturant data is observed to be an intrinsic property of the protein, independent of denaturant. However, while these are necessary characteristics for a thermydynamic function of state in the context of the experimental observable used, they are not sufficient. Further work is necessary to establish how well values for $\Delta G°_{N-U}$ obtained through the linear extrapolation method hold up in tests for thermodynamic functions of state.

References

Ahmad, A., & Bigelow, C. C. (1982) *J. Biol. Chem. 257*, 12935–12938.

Bigelow, C. C., & Geschwind, I. I. (1960) *C. R. Trav. Lab. Carlsberg 31*, 283–304.

Bolen, D. W., & Santoro, M. M. (1988) *Biochemistry* (following paper in this issue).

Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235–264.

Creighton, T. E. (1980) *J. Mol. Biol. 137*, 61–80.

Dill, K. A. (1985) *Biochemistry 24*, 1501–1509.

Dixon, G. H., & Neurath, H. (1957) *J. Biol. Chem. 225*, 1049–1059.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Gold, A. M., & Fahrney, D. (1964) *Biochemistry 3*, 783–791.

Green, R. F., & Pace, C. N. (1974) *J. Biol. Chem. 249*. 5388–5393.

Hamaguchi, K., Kurono, A., & Goto, S. (1963) *J. Biochem. (Tokyo) 54*, 259.

Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301–342.

Martin, C. J. (1964) *Biochemistry 3*, 1635–1643.

Martin, C. J., & Frazier, A. R. (1963) *J. Biol. Chem. 238*, 3268–3273.

Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.

Pace, C. N. (1986) *Methods Enzymol. 131*, 266–280.

Pace, C. N., & Vanderburg, K. E. (1979) *Biochemistry 18*, 288–292.

Saito, Y., & Wada, A. (1983) *Biopolymers 22*, 2123–2132.

Schellman, J. A. (1978) *Biopolymers 17*, 1305–1322.

Schellman, J. A. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 115–137.

Schellman, J. A., & Hawkes, R. B. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 331–343, Elsevier, New York.

Schonbaum, G. R., Zerner, B., & Bender, M. L. (1961) *J. Biol. Chem. 236*, 2930–2935.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–275.

Weast, R. C., Ed. (1985) *Handbook of Chemistry & Physics*, 66th Ed., p C-540, Chemical Rubber Co., Cleveland, OH.

Wong, K. P., Roxby, R., & Tanford, C. (1971) *Anal. Biochem. 40*, 459–464.

Yapel, A., Han, M., Lumry, R., Rosenberg, A., & Shiao, D. (1966) *J. Am. Chem. Soc. 88*, 2573–2584.