Unfolding Free Energy Changes Determined by the Linear Extrapolation Method. 2. Incorporation of $\Delta G^\circ_{N\text{-}U}$ Values in a Thermodynamic Cycle†

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ABSTRACT: The linear extrapolation method was used to evaluate the unfolding free energy changes ($\Delta G^\circ_{N\text{-}U}$) for phenylmethanesulfonyl chymotrypsin (PMS-Ct) at pH 6.0. The nonlinear least-squares fits of difference spectral data using urea and guanidinium chloride as denaturants gave identical values for $\Delta G^\circ_{N\text{-}U}$ and $\Delta\epsilon^\circ_U$, the latter being extinction coefficient differences between native and unfolded forms of the protein in the limit of zero concentration of denaturant. The independence of these parameters from the nature of solvent suggests strongly that they are characteristic properties of the protein alone. The $\Delta G^\circ_{N\text{-}U}$ data at pH 6.0 and 4.0, which differ by more than 100-fold in stability of the protein, were incorporated into a thermodynamic cycle involving free energy changes for titration of native and unfolded PMS-Ct from pH 4.0 to 6.0. The purpose of the cycle was to test whether $\Delta G^\circ_{N\text{-}U}$ obtained by use of the linear extrapolation method exhibits the characteristics required of a thermodynamic function of state. Within error, the thermodynamic cycle was found to accommodate the $\Delta G^\circ_{N\text{-}U}$ quantities obtained at pH 4.0 and 6.0 for PMS-Ct.

The free energy change for complete unfolding of a globular protein in aqueous solution ($\Delta G^\circ_{N\text{-}U}$) is a quantity of fundamental interest in nearly all aspects of protein structure and dynamics. The history of evaluation of the quantity known as $\Delta G^\circ_{N\text{-}U}$ spans more than two decades, and at least three procedures involving strong solvent denaturation have been used in evaluation of this quantity. The three procedures are known as the transfer model of Tanford, the denaturant binding model, and the linear extrapolation method (Pace, 1975, 1986). Application of the three procedures to the same set of data results in $\Delta G^\circ_{N\text{-}U}$ values that are not in agreement, and the discrepancies between these free energy changes have been an important subject in reviews of these methods (Pace, 1975, 1986).

Of the three methods employed in evaluation of $\Delta G^\circ_{N\text{-}U}$ the so-called linear extrapolation method appears to enjoy the greatest acceptance. The virtue of the linear extrapolation method over the other two methods resides in the perception that it is more reliable since it gives $\Delta G^\circ_{N\text{-}U}$ values that appear to be independent of whether urea or guanidinium hydrochloride is used as denaturant (Ahmad & Bigelow, 1982; Green & Pace, 1974). By contrast, the other two methods give $\Delta G^\circ_{N\text{-}U}$ values that are more or less dependent on the nature of solvent (Pace, 1975, 1986). Additional support for the linear extrapolation method comes from the work of Schellman (1978, 1987), Schellman and Hawkes, (1980) and Dill (1985), who have provided thermodynamic arguments and theoretical results in support of a linear dependence of unfolding free energy changes with denaturant concentration.

Despite the advantage of $\Delta G^\circ_{N\text{-}U}$ being independent of denaturant, as one would expect of a free energy quantity, the linear extrapolation method has serious experimental limitations that can be traced to the limited denaturant concentration range over which data can be obtained. Since cooperative equilibrium unfolding is experimentally accessible only over a small range of denaturant concentration in the limit of high urea or guanidinium chloride concentration, the linear extrapolation of the apparent free energy changes to zero denaturant is exceedingly long. Given the potential for nonideal solution behavior in high concentrations of denaturants, there is little reason to expect that the apparent unfolding free energy change would be rigorously linear with denaturant concentration. In point of fact, in an analysis of solvent-induced unfolding of myoglobin, Pace and Vanderburg (1979) have suggested curvature does occur in the ΔG vs denaturant plot for this protein, a result more consistent with the denaturant binding model than the linear extrapolation method. Schellman (1987) has shown the relationship between the linear extrapolation and denaturant binding models and demonstrated that the linear extrapolation and the (logarithmically dependent) weak, selective denaturant binding models cannot be distinguished within the accessible range of experimental data normally available. Since the two methods give different values for $\Delta G^\circ_{N\text{-}U}$, the ambiguity in which quantity to use tends to erode confidence in this term as an accurate expression of the stability of the protein. In short, the experimental foundation for determining $\Delta G^\circ_{N\text{-}U}$ is weak, and the characteristics and usefulness of these terms as thermodynamic quantities are largely untested.

In the preceding paper (Santoro & Bolen, 1988), the linear extrapolation method was used in analysis of unfolding data on phenylmethanesulfonyl chymotrypsin (PMS-Ct) to show that the $\Delta G^\circ_{N\text{-}U}$ quantity is independent of the efficacy and nature of the denaturant that brings about unfolding. This is consonant with results of Ahmad and Bigelow (1982), who found by the linear extrapolation method similar independence of the nature of solvent in the unfolding of ribonuclease A, lysozyme, α-lactoalbumin, and myoglobin. Such independence of $\Delta G^\circ_{N\text{-}U}$ from experimental conditions is expected and required of well-behaved thermodynamic parameters and is a necessary but insufficient characteristic in establishing $\Delta G^\circ_{N\text{-}U}$ as a valid thermodynamic quantity. In this paper, we address the separate question of whether the $\Delta G^\circ_{N\text{-}U}$ quantities obtained by the linear extrapolation method have the properties of predictability and independence of path required of all thermodynamic functions of state.

Materials and Methods

The sources, purification, and methods of determining purity and concentration of α-chymotrypsin, urea, 1,3-dimethylurea, guanidinium chloride, and phenylmethanesulfonyl fluoride

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were described in the preceding paper (Santoro & Bolen, 1988).

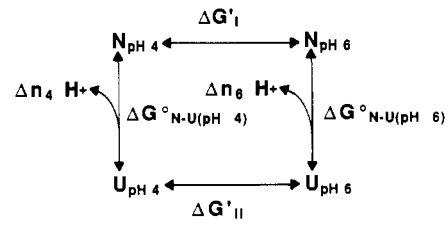
Phenylmethanesulfonyl chymotrypsin (PMS-Ct) used in difference spectral measurements was prepared as previously described (Santoro & Bolen, 1988), but that used in potentiometric titration experiments was obtained by using the following protocol: α-Chymotrypsin (50 mg) was dissolved in unbuffered 0.2 M KCl solution held at neutral pH by adding small volumes of 1.0 M NaOH. A 100-μL aliquot of 0.2 M phenylmethanesulfonyl fluoride dissolved in acetonitrile was added to the α-chymotrypsin solution over a 10-min period and the reaction allowed to proceed for at least 30 min. The resulting reaction mixture was then adjusted to pH 4.0 with small volumes of 1.0 M HCl 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 with the aid of 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 1/(mol·cm). Less than 0.5% residual catalytic activity of chymotrypsin was detected in the PMS-Ct preparations by use of *N*-acetyl-L-tryptophan ethyl ester as a substrate.

Denaturation 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 change in absorbance at zero denaturation time was obtained by recording the absorbance for at least 1 h and extrapolating the postdenaturation base line to zero time of denaturation. The final protein concentration in the cuvette was around 0.2 mg/mL in 0.05 M PIPES buffer (pH 6.0) containing 0.173 M NaCl.

Potentiometric titrations were made by using either a Metrohm E 415 pH-Stat or a Corning Model 125 pH meter equipped with a calomel combination electrode from Arthur Thomas Co. The titration vessel was thermostated at 25.0 ± 0.1 °C and flushed with purified nitrogen gas previously passed through successive scrubbers of alkaline $BaCl_2$ and 2 M phoshoric acid, and a third scrubber containing a solution identical in composition with that used in the titration vessel. The electrode and instrument were calibrated immediately prior to experiments by using a set of certified standard buffer solutions at pH 3.00, 7.00, and 10.00 (and at 1.09, 1.68, and 3.00 in the case of batch apparent activity coefficient measurements). The pH response was linear over the calibrated pH range, and the readings were stable and reproducible. A plot of pH vs electrode output in millivolts gave a slope very close to the expected theoretical value predicted by the Nernst equation. Apparent hydrogen ion activity coefficients were determined for each titrant by using the batch method described by Tanford (1950, 1955) and Nozaki and Tanford (1967a). In the case of titrations of the "native" state of PMS-Ct, the acid used for titration contained 0.2 M KCl as did the protein solution. The concentration of acid titrant (ca. 0.14 M HCl) was determined by using freshly prepared (CO_2 free) Trizma base as a primary standard in 0.2 M KCl. Equivalence points were determined by appropriate Gran plots (Rossotti & Rossotti, 1965).

Titrations of the unfolded form of the PMS-Ct were performed in 6.0 M guanidinium chloride containing 0.2 M KCl, using a titrant of about 0.014 M HCl in 6.0 M guanidinium chloride containing 0.2 M KCl. The exact concentration of hydrogen ion in the titrant was determined against Trizma base as a primary standard dissolved in 6.0 M guanidinium chloride containing 0.2 M KCl. The apparent hydrogen ion activity coefficient of the titrant was determined by the batch method [see Tanford (1950, 1955) and Nozaki and Tanford (1967a)]. The number of H^+ ions bound to the protein on titration in the acid pH range was determined by the method of Nozaki and Tanford (1967a), using the apparent activity coefficients determined as described above.

Scheme I



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

$\Delta G^\circ_{N\text{-}U}$ as a thermodynamic function must obey the rules of any thermodynamic function of state. Scheme I defines a thermodynamic cycle that requires $\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)} = \Delta G'_I - \Delta G'_{II}$, where $\Delta G^\circ_{N\text{-}U(pH4)}$ and $\Delta G^\circ_{N\text{-}U(pH6)}$ represent unfolding free energy changes for PMS-Ct obtained at pH 4 and 6 by use of the linear extrapolation method and $\Delta G'_I$ and $\Delta G'_{II}$ represent the free energy changes for titration of native and unfolded forms of PMS-Ct between pH 4 and 6. The quantities Δn_4H^+ and Δn_6H^+ are the numbers of moles of hydrogen ion taken up per mole of protein on unfolding PMS-Ct at pH 4 and 6, respectively. Independent measurements of each of the four sides of the thermodynamic cycle will test whether $\Delta G^\circ_{N\text{-}U}$ values obtained by linear extrapolation satisfy the condition required of the thermodynamic cycle.

The molar absorptivity difference between native and unfolded forms of the protein at 293 nm was used to monitor PMS-Ct unfolding in the presence of either urea or guanidinium chloride. Figure 1 gives the denaturation profile of PMS-Ct at pH 6.00 illustrating the dependence of the unfolding transition in these denaturants. The unfolding transitions were completely reversible at either pH 4 or 6 [pH 4 data are given in the preceding paper (Santoro & Bolen, 1988)]. The data for guanidinium chloride and urea were fitted by nonlinear least-squares analysis using the linear extrapolation model as presented previously (Santoro & Bolen, 1988). This model results in an expression (eq 1) relating the

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

molar extinction difference ($\Delta\epsilon$) to 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. $\Delta\epsilon^\circ_N$ and $\Delta\epsilon^\circ_U$ represent the intercepts and m_N and m_U the slopes of the pre- and postunfolding base lines, respectively. These base lines provide the basis for determining equilibrium constants for unfolding at specific denaturant concentrations in the transition zone assuming two-state behavior for unfolding. The remaining fitting parameters, m_G and $\Delta G^\circ_{N\text{-}U}$, are the slope and intercept of the linear ex-

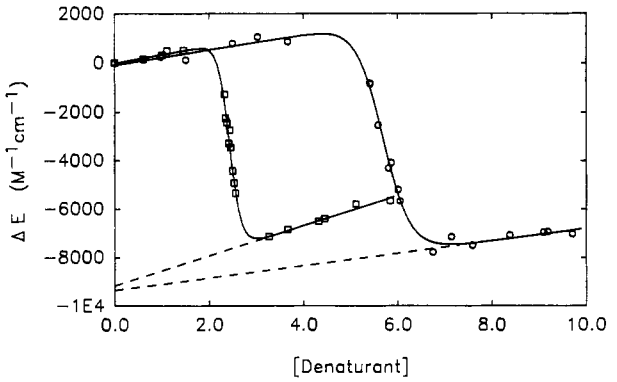


FIGURE 1: Difference spectral measurements of the denaturant-induced unfolding of PMS-Ct. Data represented are urea (○) and guanidinium chloride (□). The dashed lines for pre- and postdenaturational concentration regions are calculated by using the nonlinear best-ffitted estimates of $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, m_N, and m_U as described in eq 1. The solid lines are the result of nonlinear least-squares best fits for each of the denaturants.

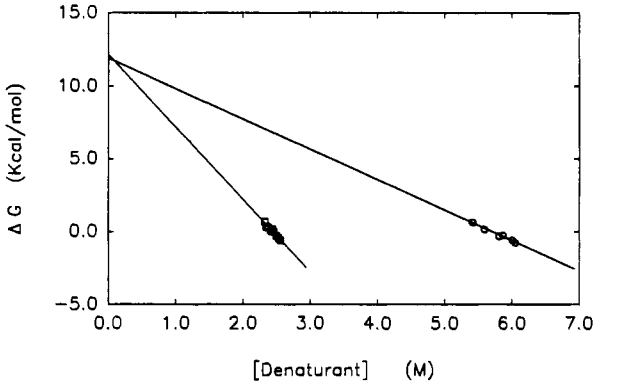


FIGURE 2: Dependence of free energy for unfolding of PMS-Ct as a function of the two denaturants given in Figure 1. Solid lines are the consequence of using $\Delta G^\circ_{N\text{-}U}$ and m_G values obtained from the individual nonlinear least-squares best fitting of the data in Figure 1. Symbols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

trapolation of unfolding free energy changes versus denaturant concentration. The solid lines in Figure 1 represent the nonlinear least-squares best fits of eq 1 to urea and guanidinium chloride data, and 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. It is noted that the pre- and postunfolding base lines extrapolated to zero denaturant give the same extinction coefficient difference of about $-9100\ M^{-1}\ cm^{-1}$ between native and unfolded protein in aqueous solution regardless of which denaturant is used. This extinction coefficient difference is identical within error with that obtained at pH 4.0 by using three denaturants (Santoro & Bolen, 1988).

Figure 2 demonstrates how the fitted parameters m_G and $\Delta G^\circ_{N\text{-}U}$ map onto the usual way of presenting the linear extrapolation data. A simultaneous fitting of the data for urea and guanidinium chloride unfolding data at pH 6.0 with $\Delta\epsilon^\circ_N$, $\Delta\epsilon^\circ_U$, and $\Delta G^\circ_{N\text{-}U}$ as parameters common to both denaturants was performed, and the results of the individual and simultaneous fittings are listed in Table I along with $\Delta G^\circ_{N\text{-}U}$ data obtained at pH 4.0.

The difference in free energy for unfolding at pH 4 and 6 ($\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$) and accompanying errors were determined in two ways: by directly subtracting the $\Delta G^\circ_{N\text{-}U}$ determined from the simultaneous fits of data obtained at pH 4 and 6 and from a global fit of the difference $\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$, using all five sets of the pH 4 and 6 unfolding data. The results of these analyses are given in Table I. In

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

denaturant	$\Delta G^\circ_{N\text{-}U}$ (kcal/mol)	confidence interval (67%)	$-m_G$ [kcal/(mol·M)]
pH 4.0			
simultaneous fit (three denaturants)	8.78[a]	(8.07, 9.54)	4.36 (4.0, 4.7)[d] 2.14 (1.97, 2.3)[e] 2.97 (2.75, 3.2)[f]
pH 6.0			
simultaneous fit (two denaturants)	11.9	(10.7, 13.1)	4.89 (4.4, 5.4)[g] 2.1 (1.88, 2.3)[h]
$\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$ (individual simultaneous fits)	−3.1[b]	±1.4	
$\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$ (global fit for difference)	−3.3[c]	(−2.1, −4.6)	
$\Delta G'_I - \Delta G'_{II}$	−3.1	(−2.9, −3.4)	

[a] From Santoro and Bolen (1988). [b] Evaluation of $\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$ ($\Delta\Delta G^\circ_{N\text{-}U}$) by taking the difference in simultaneous fits of pH 4.0 and 6.0 data and propagating error assuming symmetrical confidence intervals of ±0.7 and ±1.2 kcal/mol for the pH 4.0 and 6.0 data, respectively. [c] Evaluation of $\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$ by a global fit of the combined pH 4.0 and 6.0 data with the constraint of common (best fit) values for $\Delta G^\circ_{N\text{-}U(pH4)}$ for the pH 4.0 data and ($\Delta G^\circ_{N\text{-}U(pH4)} + \Delta\Delta G^\circ_{N\text{-}U}$) for the pH 6.0 data. [d-f] m_G values and corresponding confidence intervals from simultaneous fit of pH 4.0 data to eq 1 for guanidinium chloride, urea, and 1,3-dimethylurea, respectively. [g,h] m_G values and corresponding confidence intervals from simultaneous fit of pH 6.0 data to eq 1 for guanidinium chloride and urea, respectively.

the global fitting procedure, PMS-Ct unfolding data in urea, 1,3-dimethylurea, and guanidinium chloride at pH 4.0 [see previous paper, Santoro and Bolen (1988)] were used together with urea and guanidinium chloride data at pH 6 given in Figure 1. All of these data were constrained to the same (best fitted) $\Delta\epsilon^\circ_N$ and $\Delta\epsilon^\circ_U$ intercepts for the pre- and postunfolding base lines. $\Delta G^\circ_{N\text{-}U(pH4)}$ and $\Delta G^\circ_{N\text{-}U(pH6)}$ were constrained as parameters common to the pH 4 and 6 data, respectively, while all other parameters were allowed to float. The difference, $\Delta G^\circ_{N\text{-}U(pH4)} - \Delta G^\circ_{N\text{-}U(pH6)}$, accounts for two of the four steps in the thermodynamic cycle of Scheme I, and the confidence interval gives an estimate of the accuracy with which free energy differences can be distinguished for protein unfolding transition by using multiple sets of data.

Denaturation brings about pK changes of ionizable groups on the protein and, in the acid region, uptake of hydrogen ion accompanies the denaturation event. The stoichiometry of hydrogen ion uptake can be determined at a fixed pH by mixing unbuffered protein solution with unbuffered denaturant solution, with both solutions having been adjusted to precisely the same fixed pH prior to mixing. The resultant mixed solution can then be back-titrated to the original pH, and back-titration of a control experiment containing the same components but without protein is also performed. The difference (in hydrogen ion concentration) of back-titrations for the control and protein-containing solution is expressed in moles of hydrogen ion absorbed per mole of protein. Figure 3 gives the results of such mixing experiments at pH 4.00 as a function of the final concentration of guanidinium chloride. The uptake of hydrogen ion (Δn_4) accompanying denaturation at pH 4.00 was found to be 2.26 ± 0.08 mol of H^+/mol of protein, giving the following stoichiometric equation for PMS-Ct denaturation under these conditions:

$$N + 2.26H^+ \leftrightarrow D \qquad (2)$$

The standard-state free energy changes for titration of native ($\Delta G'_I$) and unfolded forms of the protein ($\Delta G'_{II}$) are obtained

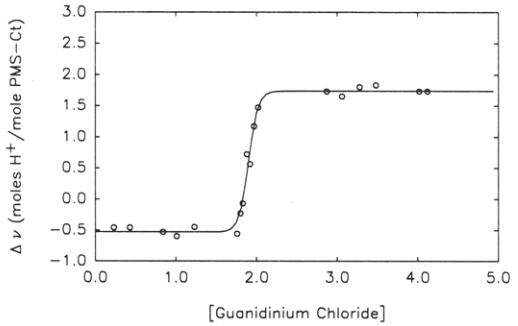


FIGURE 3: Determination of hydrogen ion uptake by PMS-Ct on unfolding induced by guanidinium chloride. Data were obtained following the protocol described under Results. An uptake of 2.26 ± 0.08 mol of H^+/mol of PMS-Ct was calculated by the difference between the pre- and postunfolding base lines.

from integration of plots of pH versus the relative number of hydrogen ions (ν) bound to the native and unfolded species, respectively. The exact relationship is given by eq 3, which

$$\Delta G'_{\mathrm{I}} \text{ or } \Delta G'_{\mathrm{II}} = -2.303RT \int \nu \, \mathrm{dpH}(\nu) \qquad (3)$$

has been derived by a number of researchers (Pfeil & Privalov, 1976; Schellman & Hawkes, 1980; Hermans & Acampora, 1967; Chu et al., 1984). Potentiometric titration curves were determined for native and unfolded forms of PMS-Ct with ν arbitrarily referenced to a value of zero for the native form of the protein at pH 6.0. Since hydrogen ions are taken up on denaturation at pH 4, the unfolded form of the protein is more highly protonated than native protein by 2.26 hydrogen ions at this pH. Thus, the titration plot for unfolded protein is frame shifted by 2.26 hydrogen ions at pH 4.00 relative to the titration curve for native protein. The titration free energy changes for native and unfolded protein species are represented by the areas under the titration curves in Figure 4. The standard free energy difference between native and unfolded protein over the pH range 4.0–6.0 ($\Delta G'_{\mathrm{I}} - \Delta G'_{\mathrm{II}}$) represented by the area between the titration curves was determined to be −3.1 kcal/mol with a 67% confidence interval of (−2.9, −3.4) kcal/mol.

It should be noted that the titration curve for the native state of PMS-Ct was found to be a small but discernible function of protein concentration over the range from 0.7 to 12 mg/mL, while titration of the unfolded form of PMS-Ct proved to be independent of protein concentration over the same range. The titration dependence on protein concentration for the native state is believed to be due to the dimerization of PMS-Ct known to occur in the pH range 4–6 (Horbett & Teller, 1973; Neet & Brydon, 1970; Gorbunoff et al., 1978). Neet and Brydon (1970) observed that PMS-Ct associated to the same degree as α-chymotrypsin at pH 5.4 and 6.2, while Gorbunoff et al. (1978) found the PMS-Ct dimerization constant to be about 10-fold lower than that of α-chymotrypsin at pH 4.12. By use of these data, it is found that at a PMS-Ct concentration of 0.7 mg/mL the protein is better than 90% in the monomer form over the pH range of interest. Since the difference between the titration curves for 0.7 and 12 mg/mL PMS-Ct was too small to provide titration data in the limit of zero protein concentration, the titration curve for 0.7 mg/mL was used for the analysis presented in Figure 4. The titration of unfolded PMS-Ct was carried out in 6 M guanidinium chloride with slight modification following the methods reported by Roxby (1970) and Roxby and Tanford (1971). Similar to data reported by these workers, an apparent hydrogen ion activity coefficient of −0.69 was determined in 6 M guanidinium chloride (in the presence of 0.2 M KCl). This

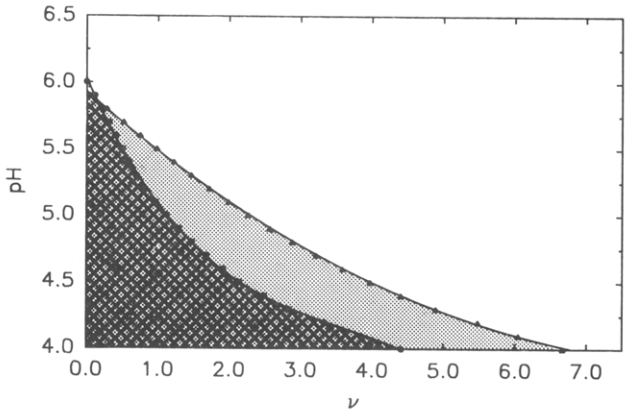


FIGURE 4: Evaluation of the free energy difference for titration of native (○) and unfolded (△) forms of PMS-Ct between pH 4.0 and 6.0. The areas under the curves represent the free energy change for titration from pH 4.0 to 6.0 of the native (cross-hatched) and unfolded (stippled and cross-hatched) protein according to eq 3. The pH 4.0 and 6.0 titration free energy changes for native ($\Delta G'_{\mathrm{I}}$) and unfolded ($\Delta G'_{\mathrm{II}}$) forms of the protein were obtained by fitting the respective titration data to third-degree polynomials and then integrating the polynomial equations between the pH 4.0 and 6.0 limits. The hydrogen ion uptake at pH 4.00 of 2.26 ± 0.08 mol of H^+/mol of PMS-Ct (see Figure 3) is the largest source of error in evaluation of $\Delta G'_{\mathrm{I}}$ and $\Delta G'_{\mathrm{II}}$, and the nonlinear confidence interval is based on the area under the unfolded titration curve obtained by ±0.08 displacement of the curve.

apparent activity coefficient was used in the calculation of ν, the number of moles of hydrogen ion bound per mole of protein as presented in Figure 4.

Discussion

Below pH 4.5, α-chymotrypsin undergoes reversible thermal- or solvent-induced unfolding, but above this pH, autolysis of the enzyme results in irreversible denaturation (Martin & Frazier, 1963; Martin, 1964; Tischenko et al., 1974). By use of urea gradient gel electrophoresis, Creighton (1979) has shown that the two-state approximation adequately describes urea-induced α-chymotrypsin unfolding. Results reported in the companion paper (Santoro & Bolen, 1988) indicate that PMS-Ct unfolding in guanidinium chloride at pH 4.0 is also two-state. The phenylmethanesulfonyl moiety attached to Ser-195 of the active site renders this derivative catalytically inactive, and we have found that PMS-Ct can undergo reversible unfolding at pH values above 4.5 without autolysis. These characteristics permit the use of PMS-Ct in the pH-dependent study described here.

Unfolding of PMS-Ct induced by urea and guanidinium chloride at pH 6.0 exhibits the same features reported at pH 4.0 (Santoro & Bolen, 1988) except for the magnitudes of $\Delta G^\circ_{\mathrm{N\text{-}U}}$ and m_{G} obtained at the higher pH. The plots of $\Delta\epsilon$ vs [denaturant] are highly cooperative, and the base lines of the unfolded forms are observed to extrapolate to a common difference extinction coefficient of about −9100 M^{-1} cm^{-1}, identical with that obtained at pH 4.0. The average of −9070 ± 130 M^{-1} cm^{-1} in $\Delta\epsilon^\circ_{\mathrm{U}}$ from the five independent measurements (three at pH 4 and two at pH 6) gives ample demonstration that $\Delta\epsilon^\circ_{\mathrm{U}}$ is a property of the protein, independent of the degree of unfolding cooperativity (m_{G} values) and the magnitude of $\Delta G^\circ_{\mathrm{N\text{-}U}}$ derived from the unfolding data (see Table I). Moreover, $\Delta\epsilon^\circ_{\mathrm{U}}$ is independent of solution conditions such as the nature of the denaturing solvent and pH (within the pH range 4.0–6.0). These characteristics reinforce the interpretation given in the previous paper that $\Delta\epsilon^\circ_{\mathrm{U}}$ represents the hypothetical difference extinction expected for complete unfolding of PMS-Ct in the absence of denaturant.

Application of the linear extrapolation method to the urea and guanidinium chloride data at pH 6.0 results in a common value of 11.9 kcal/mol for $\Delta G°_{N-U}$ and, like the data at pH 4.0, again demonstrates the extrapolated quantity $\Delta G°_{N-U}$ is independent of denaturant. The solvent independence of $\Delta\epsilon°_U$ and $\Delta G°_{N-U}$ for the pH 4.0 and 6.0 unfolding data provides a useful characterization of the linear extrapolation method that is consistent with previous work showing solvent independence of the term $\Delta G°_{N-U}$ (Ahmad & Bigelow, 1982; Pace, 1986). Such independence from solution conditions is required of any parameter that is claimed to be a thermodynamic quantity.

Despite the above-mentioned consistencies in support of the linear extrapolation quantity $\Delta G°_{N-U}$ being called a thermodynamic quantity, the property of independence of solvent is necessary but insufficient for classifying the term as a free energy quantity. A more stringent criterion of authenticity and character can be tested by incorporating it in a thermodynamic cycle. If it is indeed a thermodynamic function of state, $\Delta G°_{N-U}$ must obey the fundamental rules obligated by the type of cycle as given in Scheme I.

This thermodynamic cycle requires the free energy difference $\Delta G°_{N-U(pH4)} - \Delta G°_{N-U(pH6)}$ to be equivalent to $\Delta G'_I - \Delta G'_{II}$, the difference in titration free energies between pH 4 and 6 for the native and unfolded forms of the protein. The difference of −3.3 (−2.1, −4.6) kcal/mol for $\Delta G°_{N-U(pH4)} - \Delta G°_{N-U(pH6)}$ represents a change of more than 2 orders of magnitude in stability of PMS-Ct between these two pH values. By comparison, −3.1 (−2.9, −3.4) kcal/mol evaluated from the integration of data given in Figure 4 was obtained fo $\Delta G'_I - \Delta G'_{II}$. Thus, $\Delta G°_{N-U(pH4)} - \Delta G°_{N-U(pH6)}$ is found to be in agreement with $\Delta G'_I - \Delta G'_{II}$ as required by the cycle, but the agreement is compromised by the magnitude of the error determined in the evaluation of $\Delta G°_{N-U(pH4)} - \Delta G°_{N-U(pH6)}$. The plausibility of $\Delta G°_{N-U}$ being a function of state is genuine, but it is clear from the data that a definitive statement on the matter will not be compelling until the magnitude of the error is reduced. The error associated with the linear extrapolation method is a most serious deficiency, delaying an experimental exploration of the $\Delta G°_{N-U}$ quantity as well as masking insight into effects responsible for the method.

While we have focused on $\Delta G°_{N-U(pH4)}$ and $\Delta G°_{N-U(pH6)}$ as the quantities of interest in Scheme I, it is equally important to provide an analysis of the experimental evaluation of $\Delta G'_I$ and $\Delta G'_{II}$. Potentiometric titration of proteins and evaluation of the free energy change for protein titration have a long and distinguished history, and several reviews have appeared on the subject [see, e.g., Steinhardt and Zaiser (1955), Tanford (1955), and Linderstrom-Lang and Nielsen (1959)]. Free energy changes for titration expressed in eq 3 have been determined for several proteins (Privalov, 1976; Hermans & Acampora, 1967), and the difference in titration free energy due to the Bohr effect has been determined for oxy- and deoxyhemoglobin over a limited pH range by using an integration similar to that given in Figure 4 (Chu et al., 1984). Potentiometric titration of the unfolded forms of at least two proteins have been studied in detail by Tanford and associates (Nozaki & Tanford, 1967c; Roxby & Tanford, 1971), and it was found from studies using model amino acids that the intrinsic pK's of titratable side chains in 6 M guanidinium chloride are quite close to the corresponding pK values in dilute salt solutions (Nozaki & Tanford, 1967b).

Guanidinium chloride is a suitable solvent for acid–base titrations, and titration curves of unfolded forms of such proteins as ribonuclease (Nozaki & Tanford, 1967c) and lysozyme (Roxby & Tanford, 1971) demonstrate that they are completely predictable on the basis of model compound pK values in this solvent. The observation that the pK values of the ionizable residues on a protein in 6 M guanidinium chloride are essentially unperturbed from the pK's of model amino acids has been taken to suggest that the polypeptide chain in this solvent is without important noncovalent interactions (Nozaki & Tanford, 1967b). Many of the pK's of amino acid residues of a protein are known to be perturbed in the native state, and when these pK's are normalized on unfolding in guanidinium chloride at a fixed pH, hydrogen ion uptake or release can be expected to occur. Nozaki and Tanford (1967a) have shown that the magnitude of the hydrogen ion change upon unfolding at any pH can be evaluated either by overlaying the complete titration curves of both the native and (guanidinium chloride) unfolded forms of the protein or by direct determination of the hydrogen ion uptake or release using the method described and illustrated in Figure 3. Both of the methods were found by these authors to give identical results.

Since we anticipate problems with hydrolysis of the PMS moiety at low pH (Santoro & Bolen, 1988), we have not attempted to determine complete titration curves for native and unfolded PMS-Ct, but rather have determined the titration curves over a limited pH range and established their relative positions using the technique whose results are illustrated in Figure 3. The free energy change for titration of the native and unfolded PMS-Ct over the pH range of interest was carried out by integration as described.

We have attempted to identify features of the linear extrapolation method that would either support or challenge the notion that $\Delta G°_{N-U}$ is a legitimate free energy quantity that exhibits behavior expected of a thermodynamic term. The results of the studies presented here support that position in terms of the following characteristics: (1) Difference spectral measurement of PMS-Ct unfolding in different denaturants show a common $\Delta\epsilon$ in the limit of zero denaturant concentration that is independent of the nature and efficacy of the denaturant. (2) The evaluated $\Delta G°_{N-U}$ quantities obtained from the linear extrapolation method are, within error, independent of denaturant. (3) Incorporation of $\Delta G°_{N-U}$ values in a thermodynamic cycle show that, within error, the $\Delta G°_{N-U}$ quantities exhibit the additive properties expected of a thermodynamic function of state. The evaluations of $\Delta G°_{N-U}$ are, however, plagued by the magnitude of the error associated with this term, and it is clear there is a continuing need for experimental exploration of the limits of the linear extrapolation method and an evaluation of the meaning (at the molecular level) of the $\Delta G°_{N-U}$ term given by this method.

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Crystal Structure of Nitric Oxide Inhibited Cytochrome *c* Peroxidase†

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ABSTRACT: We have collected X-ray diffraction data from a crystal of cytochrome *c* peroxidase (CCP) complexed with the inhibitor nitric oxide to a resolution of 2.55 Å. A difference Fourier map shows density indicating the NO ligand is bound to the heme iron at the sixth coordination site in a bent configuration. Structural adjustments were determined by least-squares refinement that yielded an agreement residual of $R = 0.18$. The orientation of the ligand, tilting toward Arg-48, causes adjustment in the position of this nearby polar side chain. As a model for the substrate hydrogen peroxide, this geometry is consistent with the suggestion that Arg-48 serves to polarize the O–O peroxide bond to promote heterolytic cleavage of the bond [Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199–8205]. Strong difference density is also observed near residues 190–194, especially around the indole ring of Trp-191. The density indicates movement of the indole ring away from the proximal His-175 imidazole ring by about 0.25 Å, which appears to cause perturbation of the neighboring residues. The response of Trp-191 on the proximal side of the heme to binding nitric oxide on the distal side probably results from delocalization of the electron density of the ligand. Relevant to this is the recent finding that a mutant in which Trp-191 is replaced by phenylalanine has dramatically reduced activity, less than 0.05% of the parent activity [Mauro, J. M., Fishel, L. A., Hazzard, J. T., Meyer, T. E., Tollin, G., Cusanovich, M. A., & Kraut, J. (1988) *Biochemistry 27*, 6243–6256]. Characterization of this mutant showed in particular that electron transfer from cytochrome *c* was severely hindered. This mutagenesis result combined with the sensitivity of the position of Trp-191 to the electronic character of the sixth coordination site ligand leads us to speculate on the role of Trp-191 in electron transfer.

Cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5; CCP)[1] is easily isolated from the mitochondria of yeast (Yonetani & Ray, 1965). Early experiments demonstrated its ability to rapidly catalyze the cleavage of organic hydroperoxide molecules into alcohol and

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure.

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