

Urea and Guanidine-HCl Yield Different Unfolding Free Energies for CheY: Which Denaturant Provides the Most Reliable Free Energy Values?

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I. Introduction

Much of the current effort in protein chemistry is aimed at establishing the link between the conformation of proteins and the energetics of protein folding. The free energy of unfolding, ΔG_u , is an often used measure of the thermal stability of proteins and is the basis for quantitative evaluation of perturbations to protein structure, such as mutations or changes in solvent conditions (1). Such perturbations are being used to derive correlations between conformation and energetics, to the extent that we are beginning to describe the atomic-level origins of the macroscopic thermodynamic parameter, ΔG_u (1-3).

A number of methods are available for measuring ΔG_u , the most direct of which is differential scanning calorimetry (DSC)(4). DSC is used to detect the excess heat capacity accompanying thermal denaturation of proteins which, when integrated over temperature, permits determination of the enthalpy of unfolding, ΔH_u . In addition, proteins show a characteristic difference in heat capacity, ΔC_p , between the native and denatured states which can be measured either 1) directly in a single calorimetric scan if the data are of sufficient quality or 2) by monitoring ΔH_u as a function of temperature, where the denaturational temperature is altered by changing the pH of the sample. In conjunction with ΔH_u and the temperature at the midpoint of the thermal transition, T_u , ΔC_p can be used to calculate ΔG_u at any other temperature, T , using a modified Gibbs-Helmholtz equation (4,5):

$$\Delta G_u = \Delta H_u(1 - T/T_u) - \Delta C_p[(T_u - T) + T \ln(T/T_u)] \quad (1)$$

The limited availability of high-sensitivity calorimeters has led to the development of alternative and indirect methods for characterizing the thermodynamics of unfolding (6). One approach is to determine ΔH_u by a van't Hoff analysis of data obtained from thermal denaturation studies in which a

spectroscopic probe is used to follow the progress of unfolding. ΔC_p can, in principle, be determined by varying T_u , as described above for the calorimetric experiments. Unlike the calorimetric approach, however, the spectroscopic methods, and indeed most indirect methods, typically rely on a two-state model for unfolding.

A more recent development is the use of chemical denaturants to determine ΔG_u (6). In these experiments $\Delta G_u(H_2O)$, ΔG_u in the absence of denaturant, is obtained by linear extrapolation of unfolding free energies measured in the unfolding transition, i.e. in the presence of denaturant, back to zero denaturant concentration. This simple and popular approach is summarized in Equation 2:

$$\Delta G_u(H_2O) = \Delta G_u + m[D] \quad (2)$$

where m is the dependence of ΔG_u on the concentration of denaturant, $[D]$.

There are both experimental and theoretical arguments for the validity of the linear extrapolation method (LEM) (7-9). In the few instances where the results of LEM and calorimetric studies have been compared using matched solution conditions, the two methods give essentially identical results (8,9). The LEM, however, is still based largely on empirical observation and relies on assumptions that must be tested with each protein to be studied.

One such assumption is that the chemical denaturants affect only the intrinsic stability of the protein. It follows that if no additional effects are operant, then a variety of denaturants should yield the same extrapolated value for $\Delta G_u(H_2O)$. In fact, it has been demonstrated that GuHCl can act to both stabilize oxidized thioredoxin as a salt and destabilize it as a denaturant (9). Binding of GuHCl appears to stabilize β -lactoglobulin (10). And in the case of rat intestinal fatty acid binding protein, urea-induced denaturation provided a nearly two-fold higher $\Delta G_u(H_2O)$ than that observed with GuHCl (11).

We have obtained very similar results in our studies of CheY, a small (M_r 14,000) globular protein involved in bacterial chemotaxis (12,13). We believe, however, that the ambiguous results with CheY can be resolved in favor of those obtained using GuHCl through examination of 1) the effects of ligand binding and ionic strength and 2) the results from thermal denaturation experiments. This approach may prove to be generally useful in those instances where different chemical denaturants yield different values of $\Delta G_u(H_2O)$ for the same protein.

II. Materials and Methods

CheY from *Salmonella typhimurium* was purified as described previously (14). Protein was stored at -20°C in 50 mM sodium phosphate buffer at pH 7. Protein concentration was determined by measuring absorbance at 280 nm and using an extinction coefficient for the native protein of $6420 \text{ M}^{-1}\cdot\text{cm}^{-1}$, which was determined by the method of Edelhoch (15).

Spectral quality guanidine hydrochloride (GuHCl) was purchased from Heico Chemicals, Inc. Ultra-pure urea was purchased from Boehringer Mannheim. The concentration of GuHCl and urea was determined by refractive index measurements (16).

Denaturant-induced unfolding was followed using an AVIV 62DS circular dichroism (CD) spectropolarimeter equipped with a thermoelectric temperature controller equilibrated at $25 \pm 0.1^\circ\text{C}$. Unfolding was achieved by addition of concentrated denaturant to the protein solution in a semi-micro cuvette (4 mm width x 10 mm path-length) equipped with a stir bar. During the experiment, the CheY concentration was diluted from 0.2 mg/ml to 0.03 mg/ml in 50 mM sodium phosphate, pH 7 (plus any added salts). The CD signals at 222 nm and 400 nm were recorded for 2.5 minutes; the mean value at 400 nm was then subtracted

from that at 222 nm. An additional correction was made by subtracting the signal of a buffer solution lacking protein. All CD data are reported as mean residue ellipticity, $[\Theta]$ ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$).

Linear extrapolations were determined by a least squares fit of the CD data to the following equation, which describes the transition as well as pre- and post-translational baselines (17):

$$y_{\text{obs}} = \frac{[(y_n + m_n[D]) + (y_u + m_u[D])(\exp\{-\Delta G_u(\text{H}_2\text{O})/RT + m[D]/RT\})]}{[1 + \exp\{-\Delta G_u(\text{H}_2\text{O})/RT + m[D]/RT\}]} \quad (3)$$

where y_{obs} is the observed ellipticity, $[D]$ is the concentration of denaturant, T is the absolute temperature, R is the gas constant [$1.987 \text{ cal}/(\text{mol}\cdot\text{deg})$], m_n and m_u are the slopes of the native and denatured baselines, respectively, y_n and y_u are the intercepts at zero denaturant concentration for the native and denatured baselines, respectively, m is the slope of the line describing the dependence of ΔG_u on $[D]$, and $\Delta G_u(\text{H}_2\text{O})$ is the free energy of unfolding extrapolated to zero denaturant concentration. Nonlinear least squares fitting of the data was performed as described previously (17). The reported errors are a single standard deviation.

The enthalpy of unfolding (ΔH_u) and the midpoint of the thermal transition (T_u) were determined by van't Hoff analysis of the thermally-induced unfolding of CheY as followed by UV-CD. Protein ($32 \mu\text{g}/\text{ml}$) in 50 mM sodium phosphate, pH 7, was stirred continuously in a 10 mm path-length cuvette. Temperature was monitored with a probe inserted in the solution and positioned just above the beam; the cuvette was sealed to prevent evaporation. In an effort to minimize the time during which protein was exposed to high temperatures, the temperature was increased continuously from 0 to 80°C at rates varying from $5.0^\circ\text{C}/\text{min}$ to $0.25^\circ\text{C}/\text{min}$; ΔH_u and T_u reached constant values at scan rates below $0.5^\circ\text{C}/\text{min}$. Thermal denaturation under these conditions was approximately 90% reversible. Thermodynamic parameters were determined by a least squares fit of the data to the following equation:

$$y_{\text{obs}} = \frac{[(y_n + m_n T) + (y_u + m_u T)(\exp\{-\Delta H_u/RT + \Delta S_u/R\})]}{[1 + \exp\{-\Delta H_u/RT + \Delta S_u/R\}]} \quad (4)$$

where the baseline parameters are as described for Eqn. 3. ΔH_u and ΔS_u are the enthalpy and entropy of unfolding, respectively.

III. Results

CD spectra for native and chemically denatured CheY are shown in Figure 1. The spectrum of native protein shows minima at 222 and 208-210 nm, characteristic of the α -helical peptide chromophore. CheY is an α/β protein consisting of approximately equal numbers of residues in α -helical and β -strand conformations (18,19), but the mean residue ellipticity observed for the helical conformation is much stronger than that seen for β sheets (20). The spectra of CheY in concentrated GuHCl and urea are similar, although urea has a slightly more negative signal below 235 nm. We chose to follow the progress of chemical denaturation at 222 nm, as this is the wavelength at which the maximum difference between native and chemically denatured CheY is observed.

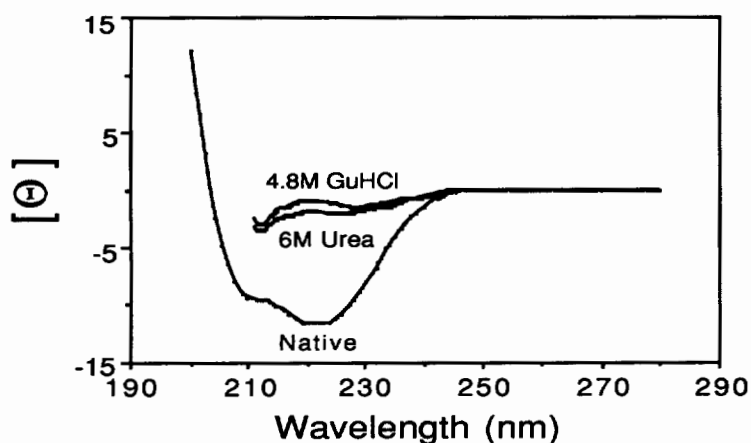


Figure 1. CD spectra of native and chemically denatured CheY. The protein concentration is 15 μM in 50 mM sodium phosphate, pH 7, at 25°C. The samples were in a 2 mm pathlength cuvette. The units for $[\Theta]$ are $[\text{deg}/(\text{cm}^2 \cdot \text{dmol}^{-1}) \times 10^{-3}]$.

A plot of $[\Theta]_{222}$ versus denaturant concentration is shown in Figure 2, along with curves representing least squares fits to Eqn. 3. The parameters derived from the fitting procedure are presented in Table I. The observed agreement between the data and fitted curves is representative of all of the experiments discussed below. The most striking result from these experiments is that the values of $\Delta G_{\text{D}}(\text{H}_2\text{O})$ obtained from GuHCl- and urea-induced denaturation of CheY are approximately 3 kcal/mol and 7 kcal/mol, respectively (Table I).

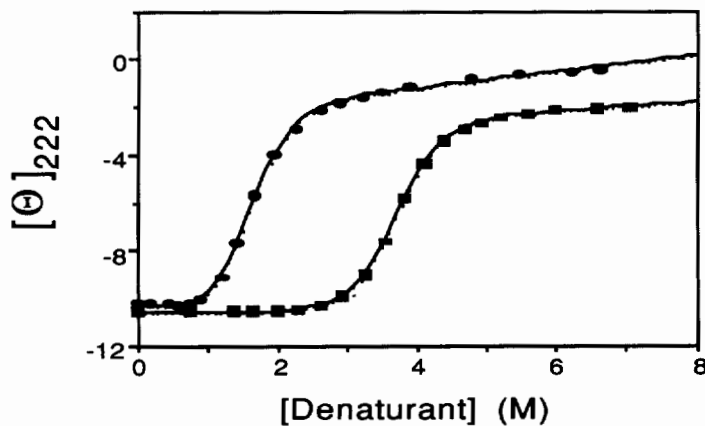


Figure 2. Chemical denaturation of CheY by GuHCl (circles) and urea (squares) in 1 mM EDTA, 50 mM sodium phosphate, pH 7, at 25°C. The thin line represents the least squares fit of the data to Equation 3. The units for $[\Theta]_{222}$ are $[\text{deg}/(\text{cm}^2 \cdot \text{dmol}^{-1}) \times 10^{-3}]$.

Table I. Chemical Denaturation of CheY at pH 7.0, 25°C^a

Additions	m (cal/mol/M)	[D] _{1/2} (M) ^b	ΔG(H ₂ O) (kcal/mol)
GuHCl			
	1990 ±470	1.56 ±0.37	3.1 ±0.9
1mM EDTA	1950 ±190	1.50 ±0.17	2.9 ±0.4
1mM EDTA	1940 ±200	1.55 ±0.17	3.0 ±0.4
1M NaCl	1560 ±350	2.10 ±0.23	3.3 ±0.2
1M NaCl	1550 ±190	2.19 ±0.17	3.4 ±0.4
10mM MgCl ₂	2590 ±320 ^c	1.70 ±0.19 ^c	4.4 ±0.6 ^c
Urea			
	1790 ±480	3.73 ±0.38	6.7 ±1.8
	1930 ±680	3.72 ±0.49	7.2 ±2.5
1mM EDTA	1840 ±100	3.66 ±0.08	6.7 ±0.4
1mM EDTA	1810 ±60	3.71 ±0.04	6.7 ±0.2
1M NaCl	1430 ±150	6.05 ±0.15	8.7 ±0.9
1M NaCl	1440 ±180	5.80 ±0.17	8.3 ±1.0
10mM MgCl ₂	1630 ±140	4.34 ±0.12	7.1 ±0.6
10mM MgCl ₂	1720 ±180	4.22 ±0.14	7.3 ±0.7

^a All samples contain 50 mM sodium phosphate.

^b $[D]_{1/2} = \Delta G(H_2O)/m$

^c These values are the mean and standard deviation for three independent measurements.

CheY contains a binding site for divalent cations (21), hence contaminating metals in the urea might explain an elevated value of $\Delta G_u(H_2O)$ in this denaturant (see below). This appears to be unlikely, as the addition of 1 mM EDTA leads to little or no change in $\Delta G_u(H_2O)$. Moreover, repeated recrystallization of urea resulted in no change in $\Delta G_u(H_2O)$ (data not shown). The results of additional experiments, described below, are consistent with the conclusion that contaminating metals cannot explain the differences in extrapolated free energies.

Another possible explanation resides in a fundamental physical difference between GuHCl and urea: the former is a salt and the latter is not. To be consistent with our results, high ionic strength (i.e. high GuHCl) would have to destabilize CheY. It was found that, in fact, adding 1 M NaCl to the experiments in which urea is the denaturant resulted in an apparent *increase* in the stability of CheY (Table I). Ionic strength effects thus do not explain the discrepancies in $\Delta G_u(H_2O)$.

In principle, one predictable property of CheY is an increase in stability in the presence of divalent cation (22). CheY has a single binding site for divalent cation, so the predicted increase in $\Delta G_u(H_2O)$ in the presence of ligand (L), $\Delta\Delta G_u(H_2O + L)$, can be calculated using Eqn. 5.

$$\Delta\Delta G_u(H_2O + L) = \Delta G_u(H_2O + L) - \Delta G_u(H_2O) = RT \ln(1 + [L]/K_D) \quad (5)$$

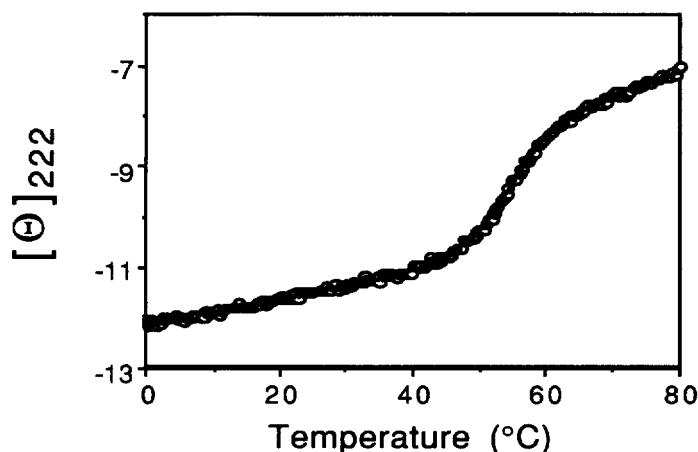


Figure 3. Thermal denaturation of CheY in 50 mM sodium phosphate, pH 7. The thin continuous line represents the least squares fit of the data to Equation 4. The units for $[\Theta]_{222}$ are $[\text{deg}/(\text{cm}^2 \cdot \text{dmol}^1)] \times 10^{-3}$.

Dissociation constants for a variety of metals have been determined under conditions similar to those employed in the present studies (21). For example, the K_D for magnesium at pH 7 and 20°C is 0.45 mM, so addition of 10 mM MgCl_2 to dilute CheY should result in an increase in $\Delta G_u(\text{H}_2\text{O})$ of about 1.8 kcal/mol under these conditions. At pH 7 and 25°C, an increase in $\Delta G_u(\text{H}_2\text{O})$ of about 1.4 kcal/mol is observed in GuHCl while no significant increase is seen when urea is the denaturant (Table I). There is clearly an increase in the $[D]_{1/2}$ for urea in the presence of magnesium, but m has undergone a compensatory decrease. These results suggest that denaturation by urea is not providing reliable values of $\Delta G_u(\text{H}_2\text{O})$.

Thermal denaturation experiments were performed to provide an additional test of the validity of the $\Delta G_u(\text{H}_2\text{O})$ values obtained with the two denaturants (Figure 3). A van't Hoff analysis of these data (Equation 4) yielded a ΔH_u of 59 kcal/mol and a T_u of 54°C. These parameters and $\Delta G_u(\text{H}_2\text{O})$ can be used in Eqn. 1 to estimate ΔC_p for CheY: ΔC_p estimates from the GuHCl and urea experiments are 1.7 kcal/(mol·deg) and -1.3 kcal/(mol·deg), respectively. The latter value is clearly unrealistic and provides an additional argument against the use of urea in characterizing the thermodynamics of CheY unfolding.

Preliminary results of fluorescence studies, in which additional denaturants are being investigated, are consistent with the results presented above. Urea-induced unfolding yields a $\Delta G(\text{H}_2\text{O})$ value (7.4 kcal/mol) similar to that observed in the analogous CD experiments, while the value for $\Delta G(\text{H}_2\text{O})$ obtained with 1,3-dimethylurea as the denaturant, about 4 kcal/mol, is closer to that seen with GuHCl -induced unfolding as monitored by CD. Additional experiments are underway to determine if the difference in $\Delta G(\text{H}_2\text{O})$ obtained with 1,3-dimethylurea and GuHCl is significant.

IV. Discussion

There is a significant difference in the value of $\Delta G_u(\text{H}_2\text{O})$ for CheY when GuHCl and urea are used as denaturants: 3.0 kcal/mol and 6.7 kcal/mol, respectively

(Table I). Plausible explanations for this discrepancy include 1) destabilization of CheY by high ionic strength, 2) contamination of urea by divalent cation which, as a result of binding, is stabilizing CheY, and 3) preferential binding of urea by native CheY.

The first hypothesis was tested by adding 1 M NaCl to the experiments in which urea was used as the denaturant. The resulting $\Delta G_u(\text{H}_2\text{O})$ value was, in fact, higher than that observed in the absence of salt, so high ionic strength appears to stabilize CheY. The presence of EDTA does not significantly alter the values of $\Delta G_u(\text{H}_2\text{O})$, which suggests that the second hypothesis is likewise untrue. These results do not allow us to rule out completely the possibility of some contaminating ligand in the urea, but this ligand would have to either bind with great avidity or be present at very high concentrations to explain the approximately 4 kcal/mol difference in the extrapolated free energies.

Two additional observations lead us to conclude that denaturation by urea is not providing reliable estimates of $\Delta G_u(\text{H}_2\text{O})$. First, $\Delta G_u(\text{H}_2\text{O})$ does not show the expected increase in the presence of MgCl_2 when urea is the denaturant, whereas such an increase is observed with GuHCl as the denaturant. And second, when $\Delta G_u(\text{H}_2\text{O})$, thermal denaturation data, and Equation 1 are used to estimate ΔC_p , the result is an unrealistically negative value when using values of $\Delta G_u(\text{H}_2\text{O})$ obtained in the urea experiments. We feel that the simplest, and as yet unproven, explanation for the results with urea is the preferential binding of urea by native CheY.

The question remains as to whether or not the data obtained with GuHCl are providing better estimates of ΔG_u . The results with added MgCl_2 and preliminary fluorescence studies suggest that GuHCl is providing values of $\Delta G_u(\text{H}_2\text{O})$ that are reasonable estimates of ΔG_u . The addition of 1 M NaCl to the urea denaturation experiments leads to an increase in $\Delta G_u(\text{H}_2\text{O})$, so the extrapolated free energies obtained using GuHCl may be too high. We currently are attempting to control for possible ionic strength effects (9).

A van't Hoff analysis of data obtained from the thermal denaturation of CheY provides estimates for ΔH_u and T_u which, along with $\Delta G_u(\text{H}_2\text{O})$, have been used to calculate a ΔC_p of about 1.7 kcal/(mol·deg). This value is very close to those obtained by a variety of purely calculational approaches (23,24). There are, moreover, a number of proteins similar in size to CheY for which ΔC_p values, ranging from 1 to 2 kcal/(mol·deg), have been determined (25). If we use these ΔC_p values and the estimates for ΔH_u and T_u for CheY determined in this study, then estimates for ΔG_u at 25°C obtained using Eqn. 1 range from 2.6 kcal/mol to 3.9 kcal/mol. Thermally- and GuHCl-induced unfolding of CheY are thus providing results that are consistent with the expected properties of a globular protein the size of CheY.

More generally, it appears that thermal denaturation experiments can provide valuable information in cases where the LEM provides ambiguous results. The utility of this approach will of course depend on the magnitude of the observed difference in extrapolated free energies and the errors in ΔH_u and T_u .

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