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Cold Denaturation of CheY

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The thermal stability of the bacterial chemotaxis protein CheY from *Salmonella typhimurium* has been examined by thermal denaturation at pH 7.0 in the presence of guanidine–HCl and urea. For both denaturants, thermal denaturation monitored by circular dichroism spectropolarimetry consists of transitions both above and below 25°C, which is strong evidence for a heat capacity change that is $\geq$1500 cal/(mol K) upon unfolding. While many data for chemical and thermal denaturation are consistent with data for CheY from *Escherichia coli*, the observation of cold denaturation for *S. typhimurium* CheY is inconsistent with the small heat capacity change, 600 to 850 cal/(mol K), reported for denaturation of the *E. coli* protein.

Keywords: CheY; denaturation; folding; circular dichroism

The structural basis for the energetics of protein stability is the subject of considerable investigation and discussion (Murphy & Freire, 1992; Privalov & Makhatadze, 1993). A major tool in this area of research is the use of thermodynamics to describe protein stability (Privalov, 1979). One of the strongest arguments for the application of thermodynamics to protein folding is the observation of cold denaturation: this is clear evidence for a positive heat capacity change upon unfolding, $\Delta C_{p,u}$, which has been proposed on the basis of heat denaturation studies (Pace & Tanford, 1968; Nojima *et al.*, 1978; Privalov *et al.*, 1986; Chen & Schellman, 1989; Antonino *et al.*, 1991; Griko & Privalov, 1992; Nishii *et al.*, 1994). Because both heat and cold denaturation are accurately described by a single thermodynamic function, the denatured states for both processes are the same in so far as thermodynamics are concerned. Nevertheless, questions remain concerning the possible structural differences between the heat- and cold-denatured proteins (Tamura *et al.*, 1991; Damaschun *et al.*, 1993). The basis for this is the different molecular mechanisms proposed or implied for heat and cold denaturation: the former is thought to be dominated by configurational entropy effects, while the latter is ascribed to decreases in the hydrophobic contribution to stability (Murphy & Freire, 1992). One limitation to the study of structure in cold-denatured protein is the difficulty of populating this species at experimentally accessible temperatures (Privalov, 1990). The results reported herein for CheY suggest that this protein is a promising subject for these much needed structural studies.

CheY has been the subject of intense investigation because of its role in bacterial signal transduction (Stock *et al.*, 1991) and because it is the prototypical member of a large family of two-component regulatory systems found in both prokaryotes and eukaryotes (Volz, 1993; Chang *et al.*, 1993; Ota & Varshavsky, 1993). High-resolution X-ray analysis of CheY from *Salmonella typhimurium* and *Escherichia coli* reveals a structure consisting of five parallel β-strands surrounded by five amphiphilic helices (Stock *et al.*, 1989; Volz & Matsumura, 1991). Serrano and co-workers have reported a number of studies concerning the stability and folding of *E. coli* CheY (Filimonov *et al.*, 1993; Jiménez *et al.*, 1994; Muñoz *et al.*, 1994), the first of which focused on calorimetric studies of unfolding. A preliminary thermodynamic analysis of CheY from *S. typhimurium* is largely in accord with the results for the *E. coli* protein, most notably in the marked disparity between the extrapolated free energies of unfolding obtained with urea and guanidine hydrochloride (GuHCl; DeKoster *et al.*, 1993). The two studies disagree, however, in the values of $\Delta C_{p,u}$: *E. coli* CheY was assigned values ranging from 600 to 850 cal/(mol K), while a preliminary estimate of about 1700 cal/(mol K) was obtained with the *S. typhimurium* protein. The two proteins differ by conservative substitutions at three out of 128 amino acid residues (Phe51, Ile54, and Ser76 in *S. typhimurium* CheY are replaced by tyrosine, valine, and glycine, respectively, in the *E. coli* protein; Stock *et al.*, 1985) and the three-dimensional structures are very similar, so the discrepancy in $\Delta C_{p,u}$ values probably does not result from differences in sequence or structure.

Abbreviation used: GuHCl, guanidine hydrochloride.

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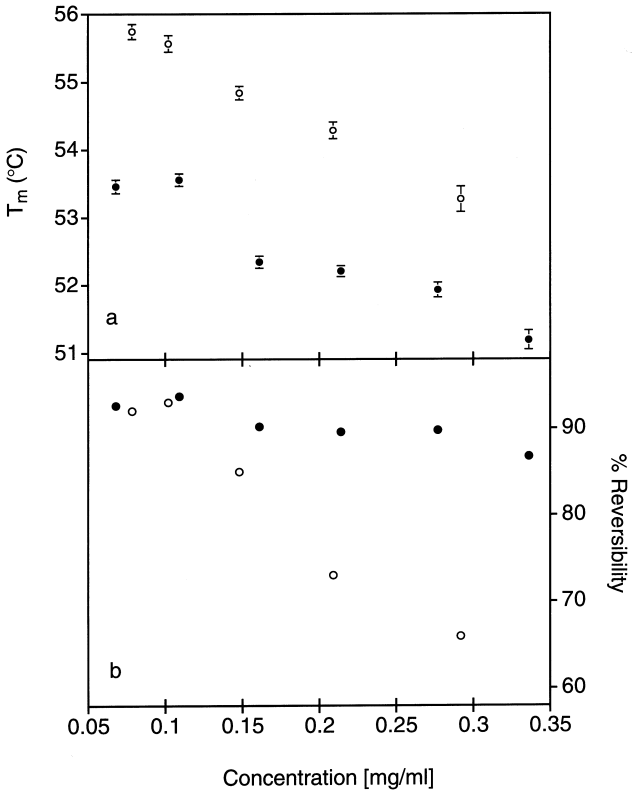

Figure 1. a, The midpoint for thermal denaturation, t_m, (denoted by T_m in the Figure). b, The reversibility of unfolding as a function of CheY concentration in the presence (○) or absence (●) of 0.1 M NaCl. All samples contained 50 mM sodium phosphate, and 0.1 mM EDTA, at pH 7. CheY was purified as described previously (Stock *et al.*, 1985) and protein concentration was determined using an extinction coefficient of 6420 M^{-1} cm^{-1} (DeKoster *et al.*, 1993). Thermal denaturation was monitored by CD at 222 nm using an AVIV 62 DS spectropolarimeter. Samples were stirred continuously in a sealed 10 mm pathlength cuvette, and temperature was monitored with a thermocouple inserted in the solution and positioned just above the beam. Temperature was increased continuously from 0 to 80°C at a rate of 1 deg.C / min. The values of t_m were determined by fitting the data to a modified van't Hoff equation describing a two-state unfolding reaction (equation 2 reported by Swint & Robertson, 1993; DeKoster *et al.*, 1993). Error bars in a represent fitting errors at one standard deviation. The percentage reversibility is $(\Delta\epsilon' / \Delta\epsilon, \times 100)$, where $\Delta\epsilon$ is the difference between the ellipticity of native and denatured CheY, and $\Delta\epsilon'$ is the difference after cooling the sample back to 0°C.

A significant complication in the thermodynamic analysis of CheY stability is the protein's tendency to self-associate at temperatures approaching the thermal denaturation transition. CD and NMR data provide some evidence for non-native structure in the resulting multimer (Filimonov *et al.*, 1993), and this is corroborated by a decrease in the apparent midpoint for thermal denaturation, t_m, with increasing protein concentration (Figure 1). The three-state model used to interpret the calorimetric data for *E. coli* CheY included a dimerization reaction coupled to thermal denaturation. Two results of the calorimetric experiments were the small $\Delta C_{p,u}$ reported for the unfolding of *E. coli* CheY, approximately 600 to 850 cal / (mol K), and the absence of any heat capacity change for the association reaction, $\Delta C_{p,a}$. However, a 14 kDa protein such as CheY might be expected to have a larger $\Delta C_{p,u}$ upon complete unfolding (Privalov, 1979; Murphy & Freire, 1992); and indeed, structural energetic calculations (Murphy & Freire, 1992) based on the X-ray structures of CheY predict a $\Delta C_{p,u}$ of 2100 cal / (mol K), closer to the preliminary estimate of DeKoster *et al.* (1993). Moreover, the reported enthalpies of unfolding for the *E. coli* protein appear to show a temperature dependence consistent with a $\Delta C_{p,u}$ of at least 1600 cal / (mol K) (Filimonov *et al.*, 1993). Alternatively, the smaller $\Delta C_{p,u}$ reported by Filimonov *et al.* (1993) could result from self-association of non-native protein.

One possible consequence of a large positive $\Delta C_{p,u}$ is cold denaturation (Privalov, 1990). Experimental observation of cold denaturation often entails using chemical denaturants because many proteins, including CheY, are otherwise too stable; in general, protein stability can be monitored experimentally only when the free energy of unfolding, ΔG_u^0, is within the range ± 1.5 kcal / mol (Pace *et al.*, 1989). Chemical denaturants also decrease the enthalpy of heat denaturation and increase $\Delta C_{p,u}$, which, combined with destabilization, increase the probability of cold denaturation at experimentally accessible temperatures (Privalov, 1990; Makhatadze & Privalov, 1992). In fact, one cause for concern about using denaturants in these experiments might be their effects on $\Delta C_{p,u}$, but data for ribonuclease A and lysozyme suggest that any increase in $\Delta C_{p,u}$ will be no greater than 30% in 2 M GuHCl and 5% in 2 M urea (see Table 4 of Makhatadze & Privalov, 1992).

Simulations using the equations and thermodynamic parameters reported by Filimonov *et al.* (1993) show that CheY in 2 M urea is not expected to undergo cold denaturation above 185 K (Figure 2a). In contrast, the larger estimated $\Delta C_{p,u}$ for *S. typhimurium* CheY leads to the expectation of cold denaturation in the presence of chemical denaturants. This expectation is borne out by experiment: CheY shows evidence for two thermal transitions in the presence of either urea or GuHCl (Figure 3). Cold denaturation is observed with both denaturants, even though chemical denaturation with urea and GuHCl yield very different estimates for ΔG_u^0 at pH 7 and 25°C, 6 to 7 kcal / mol and 3 kcal / mol, respectively (DeKoster *et al.*, 1993; Filimonov *et al.*, 1993). Far-UV CD spectra of CheY in the presence of 2 M urea or 1.5 M GuHCl provide additional evidence for denaturation by both heat and cold (Figure 4).

One interesting feature of the thermal denaturation profiles (Figure 3) and CD spectra (Figures 4c, d) is the difference between the ellipticity of heat and cold denatured CheY: cold-denatured protein shows little residual CD signal, whereas the heat-denatured protein retains nearly 50% of its native ellipticity at

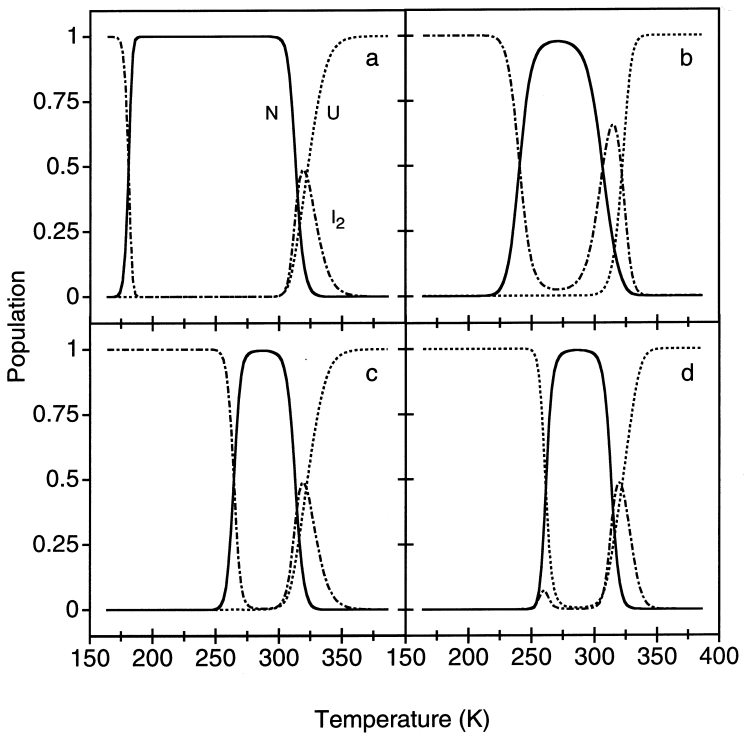

Figure 2. Predicted populations of native, intermediate, and unfolded CheY based on equation (2) and the thermodynamic parameters for the thermal denaturation of *E. coli* CheY at pH 7 in 2 M urea. Populations in all cases were determined using equations 6 through 20 and the thermodynamic parameters reported in Table III of Filimonov *et al.* (1993). These parameters are: P_o, 290 μM (4 mg / ml); t_u, 316 K; t_a, 322 K; ΔH_u, 67 kcal / mol; ΔH_a, −18.5 kcal / mol; $\Delta C_{p,u}$, 600 cal / (mol K); and $\Delta C_{p,a}$, 0 cal / (mol K). To obtain agreement with the calculations presented in Filimonov *et al.* (1993), it is necessary to reduce ΔH_a by one-half and change its sign when using equations 16, 18, and 20. The four panels show predicted populations using: a, thermodynamic parameters of Filimonov *et al.* (1993), and substituting, b, ΔH_a with −40 kcal / mol, c, $\Delta C_{p,u}$ with 1700 cal / (mol K), or, d, $\Delta C_{p,u}$ with 2300 cal / (mol K) and $\Delta C_{p,a}$ with −650 cal / (mol K). The three species of CheY are represented as native (N, continuous line), unfolded (U, broken line), and intermediate (I_2, dot / dashed line).

222 nm. This behavior is similar to that of *Thermus thermophilus* cytochrome *c*-552 (Nojima *et al.*, 1978), and bears some resemblance to the results of small-angle X-ray scattering studies of yeast phosphoglycerate kinase (Damaschun *et al.*, 1993) and calorimetric studies of β-lactoglobulin (Griko & Privalov, 1992), from which it was concluded that the cold-denatured protein more closely resembles a random-coil than does the heat-denatured protein.

How large must the value of $\Delta C_{p,u}$ be in order to observe cold denaturation of CheY? Ideally, thermodynamic parameters would be obtained by fitting the data to a model for the unfolding reaction. However, the contribution of self-association to the overall reaction is not well understood at present, so possible limiting cases will be considered instead. In the simplest case of a two-state reaction, estimates for the heat capacity change can be derived from the temperature dependence of ΔG_u^0 using the modified Gibbs–Helmholtz equation (Pace & Laurents, 1989):

$$\Delta G_u^0(T) = \Delta H_u^0(1 - T / t_m) - \Delta C_{p,u}[(t_m - T) + T \cdot \ln(T / t_m)] \quad (1)$$

where ΔH_u^0 is the enthalpy of heat denaturation and T is the absolute temperature. If ΔG_u^0 is known at two temperatures then, in principle, only ΔH_u^0 or t_m needs to be known in order to estimate $\Delta C_{p,u}$. At pH 7.0 and 25°C, ΔG_u^0 for CheY in 2.5 M urea is between 1.8 and 2.1 kcal / mol (DeKoster *et al.*, 1993; Filimonov *et al.*, 1993). Thermal denaturation of CheY in 2.5 M urea at pH 7 (Figure 3) suggests that ΔG_u^0 at 0°C is $\leqslant$0 kcal / mol. Thus, a *minimum* estimate of 2300 cal / (mol K) for $\Delta C_{p,u}$ is obtained using 1.8 kcal / mol and 0 kcal / mol for ΔG_u^0 at 25°C and 0°C, respectively, and a t_m of 43°C (Figure 3). While uncertainties remain concerning the accuracy of ΔG_u^0 determinations for CheY using chemical denaturants, calculations with the GuHCl data of Figure 3 yield a similar $\Delta C_{p,u}$ of 2200 cal / (mol K).

How might the self-association of CheY affect cold denaturation? The model of Filimonov *et al.* (1993) provides a useful framework for considering this question:

$$2N \leftrightarrows I_2 \leftrightarrows 2U \quad (2)$$

where N and U are native and denatured monomer, respectively, and I_2 is a non-native dimer. Preliminary results of analytical ultracentrifugation studies suggest that the associated species may be larger than a dimer (DeKoster *et al.*, unpublished results), but the basic thermodynamic arguments should hold, regardless of the stoichiometry of the reaction. Mathematical simulations of this model were performed to identify limiting values for three key thermodynamic parameters that would lead to denaturation or formation of the non-native dimer at low temperature: the enthalpy of association (ΔH_a), $\Delta C_{p,a}$ and $\Delta C_{p,u}$. These simulations focused on thermal denaturation at pH 7 in 2 M urea (see the legend to Figure 2), conditions examined in the studies of CheY from both *E. coli* and *S. typhimurium*. Results of the simulations show that both cold

denaturation and cold-induced dimerization are sensitive to variations in the thermodynamic parameters. Indirect evidence suggests that the dimer has a non-native structure (Figure 1) (Filimonov *et al.*, 1993), so the ellipticity changes at low temperature (Figures 3 and 4) may be attributable to cold denaturation, dimerization, or some combination of the two phenomena.

Under no circumstances does the variation of ΔH_a alone, while fixing all other thermodynamic parameters at values reported in Table III by Filimonov *et al.* (1993), lead to cold denaturation of CheY, and cold-induced dimerization is expected only when ΔH_a is $\leqslant -40$ kcal / mol (Figure 2b), which is about twice the value reported for the *E. coli* protein. Such a scenario shifts the temperature of maximal stability to about 5°C, well below the experimentally observed maximum of about 25°C (Figures 3 and 4). Varying the value of $\Delta C_{p,u}$ alone leads to cold-induced dimerization at temperatures above 273 K only when its value exceeds 1700 cal / (mol K) (Figure 2c); no cold denaturation is observed. Similarly, no cold denaturation is observed when only $\Delta C_{p,a}$ is varied. Furthermore, only positive values of $\Delta C_{p,a}$ ($\geqslant$900 cal / mol K) lead to cold-induced dimerization, but positive ΔC_p values are highly unlikely for a protein *association* reaction (Ross, 1986; Murphy *et al.*, 1993; Spolar & Record, 1994; Burrows *et al.*, 1994). Thus, within the framework of the model described by equation (2), the $\Delta C_{p,u}$ value reported for *E. coli* CheY cannot be reconciled with the experimental observation of cold denaturation or dimerization.

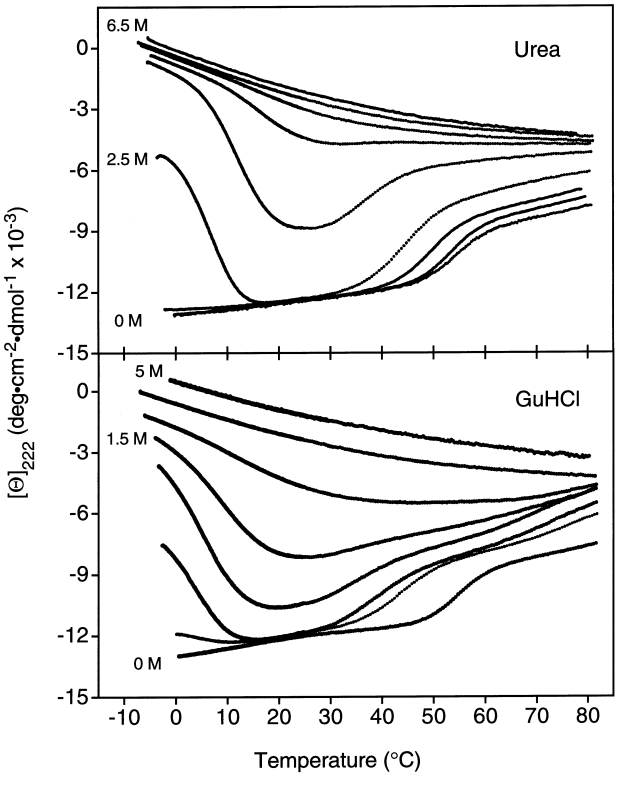

Figure 3. Thermal denaturation of 4.1 to 6.2 μM (57 to 87 μg / ml) CheY in 50 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, and various concentrations of urea (top panel) and GuHCl. Urea samples contained 0, 0.75, 1.5, 2.5, 3.5, 4.25, 4.9, 5.5 and 6.5 M denaturant and GuHCl samples contained 0, 0.75, 1.0, 1.25, 1.5, 2.0, 3.0 and 5.0 M denaturant. The thermal scan rate was 0.1 deg.C / min below 25°C, and 1 deg.C / min above 25°C; one low-temperature scan at 0.05 deg.C / min in 1.5 M GuHCl was superimposable on the 0.1 deg.C / min scan, while faster scan rates led to significant deviations below 25°C. Data were collected at 0.1 and 0.4 deg.C intervals below and above 25°C, respectively. For all experiments, cold denaturation was completely reversible, and heat denaturation was at least 85% reversible.

The results of these simulations are highly dependent on the choice of thermodynamic parameters for the unfolding and self-association of CheY. Many combinations of enthalpies, heat capacities, and thermal midpoints generate results that are consistent with experimental results. However, two general trends for the model are clear. First, for cold *denaturation* to be observed near 0°C, $\Delta C_{p,u}$ must be large and positive and $\Delta C_{p,a}$ must be large and negative (Figure 2d). Second, significant cold-induced *dimerization* above 273 K results from large negative values of ΔH_a or combinations of large positive $\Delta C_{p,u}$ values with small values of $\Delta C_{p,a}$ (Figure 2b, c). The first scenario appears most plausible, given the current knowledge of the energetics of protein–protein interactions (Ross, 1986; Murphy *et al.*, 1993; Spolar & Record, 1994; Burrows *et al.*, 1994). Thus, the reported ΔC_p values for the unfolding and self-association of *E. coli* CheY, 600 to 850 cal / (mol K) and 0 cal / (mol K), respectively, are probably incorrect. And while the estimated $\Delta C_{p,u}$ for *S. typhimurium* CheY, 1700 to 2300 cal / (mol K), is consistent with the observation of cold denaturation, this value may be subject to modification as more is learned about self-association.

Do these results provide any insight into the discrepancy between values of ΔG_u^0 obtained with urea and GuHCl? Accurate determination of ΔG_u^0 by linear extrapolation requires that interactions between protein and denaturant be linear in denaturant activity (Schellman, 1987). Furthermore, a valid ΔG_u^0 will be obtained for CheY only if this applies to the self-association reaction as well. Thus, a simple qualitative explanation for the disagreement in ΔG_u^0 is that at least one of the denaturants has effects on the denaturation or self-association of CheY that are non-linear in denaturant activity. At the molecular level, this could be manifested as strong stoichiometric binding of denaturant by one of the three states of CheY (i.e. native, denatured or multimeric; Schellman, 1987). For example, self-association appears to be linked to the destabilization of native CheY, so denaturants that bind to CheY multimers will appear to be more potent than those that do not. In contrast, denaturants that bind to native monomer will appear to be weak denaturants and provide high estimates for ΔG_u^0. Finally, any denaturant that binds to denatured protein will yield

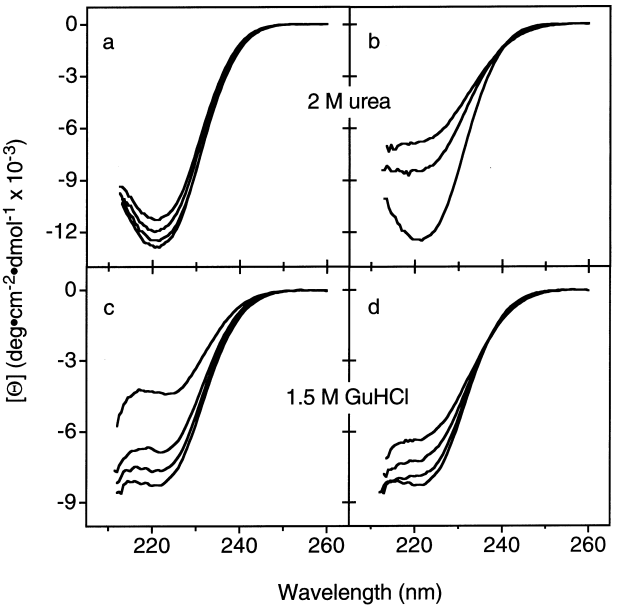

Figure 4. Temperature dependence of the far UV–CD spectrum of 50 μM (0.7 mg / ml) CheY in 50 mM sodium phosphate (pH 7.0), 0.1 mM EDTA and either 2 M urea (a,b) or 1.5 M GuHCl (c,d). At 25°C, CheY is completely native in 2 M urea and about 50% denatured in 1.5 M GuHCl (DeKoster *et al.*, 1993). Spectra were collected at the following temperatures, from bottom to top: a, 10°C, 25°C, 5°C and –3°C. b, 25°C, 50°C and 75°C. c, 25°C, 15°C, 10°C and 0°C. d, 25°C, 35°C, 50°C and 75°C. Spectra were collected in a 2 mm pathlength cuvette. Data were acquired at 0.5 nm intervals with a 2 to 4 s averaging time. Buffer blanks were subtracted from all spectra. Spectra in c and d were smoothed over an interval of three data points.

a low estimate for ΔG_u^0. Urea yields larger values of ΔG_u^0 than GuHCl does, so possibilities for the discrepancy include: (1) Strong binding of GuHCl by multimers; (2) strong binding of GuHCl by denatured CheY; and (3) urea binding to native monomers. In truth, self-association is ill-characterized at present, and an understanding of denaturant action will follow only from a more complete characterization of the self-association reaction and its relationship to CheY stability.

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