Thermodynamic Analysis of the Chemotactic Protein from *Escherichia coli*, CheY†

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Received June 28, 1993; Revised Manuscript Received August 26, 1993[⊕]

ABSTRACT: CheY, the 129 amino acid chemotactic protein from *Escherichia coli*, is a good model for studies of folding of parallel α/β proteins. We report here the thermodynamic characterization of the wild-type CheY at different pH values and in different buffers and denaturation conditions. The denaturation of CheY by urea monitored by circular dichroism and fluorescence fits the two-state unfolding model. The stability of the protein is ionic strength dependent, probably due to the presence of three Asp residues in very close proximity in its active site. The presence of a Mg^{2+} ion, which seems to interact with Asp 13 in the active site, stabilizes the native structure by up to 6.9 kJ mol^{-1}. The CheY maximum stability (31.7 ± 2.1 kJ mol^{-1}), without magnesium, is reached at pH 5.1. Analysis of scanning calorimetry data has shown that temperature-induced unfolding of CheY is not a two-state process and proceeds through a highly populated intermediate state, corresponding to protein dimers, as was subsequently confirmed by direct cross-linking experiments. According to circular dichroism, fluorescence, nuclear magnetic resonance, and ANS binding experiments, this "intermediate dimer" at pH 2.5 exhibits all known characteristics of the "molten globule" state. The reversible dimerization of "molten globules" might explain such peculiarities as the increased stability or the cooperative unfolding found for the molten globule state of some proteins.

All the information necessary for the formation of three-dimensional structures in proteins is contained in the amino acid sequence (Anfisen & Scheraga, 1975). However, the mechanism by which a three-dimensional structure is formed from the primary structure is not well understood. It is clear that the number of possible conformations available to a polypeptide is so enormous that a random-search mechanism for protein folding is excluded (Levinthal, 1968; Wetlaufer, 1973). Consequently, the most interesting aspect of folding studies is to characterize the possible intermediate conformations that lie between the unfolded and folded states. Several different analyses have been done on various proteins for this purpose [for a review, see Lecomte and Matthews (1993)]. Our group has decided to characterize the folding pathway or pathways present in the open α/β parallel family of proteins, using one of the smallest members of the family, the chemotactic protein from *Escherichia coli*, CheY,[1] as a model case (Stock *et al.*, 1990).

CheY is a 129 amino acid α/β parallel protein, belonging to the signal transduction protein family in bacteria (Stock *et al.*, 1990). It functions as a phosphorylation-activated response regulator that controls bacterial chemotaxis [for a review, see Stock *et al.* (1990)]. The X-ray three-dimensional structures of CheY from *E. coli* (Volz & Matsumura, 1991) and *Salmonella typhimurium* (Stock *et al.*, 1989), as well as the nuclear magnetic resonance (NMR) solution structure of CheY from *E. coli* (Bruix *et al.*, 1993), have been recently determined at high resolution, and they are quite similar to flavodoxin (Bowie *et al.*, 1990) and to the EF-Tu, P21-ras family of proteins (Chen *et al.*, 1990). CheY is composed of a central five-stranded parallel β-sheet surrounded by five α-helices. There are two hydrophobic cores on each side of the β-sheet that result from the packing of the helices against the β-sheet. It has two trans Pro residues and one cis Pro (Pro110), and no cysteine residues (Matsumura *et al.*, 1984). There is a single Trp residue (Trp58) which is half-exposed to the solvent (48 Å^2). The active center of CheY is composed of three Asp residues (Asp12, Asp13, and Asp57) and one Lys residue (Lys109) clustered in an acidic pocket at the edge of the β-sheet. Two of the Asp residues are buried (Asp12 and Asp57; 4 and 0 Å^2 of surface-accessible area, respectively). Asp57 is the residue that is phosphorylated (Sanders *et al.*, 1989). CheY binds tightly 1 mol of Mg^{2+} per 1 mol of protein. It has been proposed that the Mg^{2+} interacts with Asp12 and Asn59 (Volz & Matsumura, 1991).

In the present work we have characterized the thermodynamic properties of CheY, in different solvent conditions, using a wide variety of techniques: fluorescence, circular dichroism, temperature-gradient gels, calorimetry, nuclear magnetic resonance, and cross-linking studies.

EXPERIMENTAL PROCEDURES

Chemicals. 1,4-Piperazinediethanesulfonic acid (Pipes), tris(hydroxymethyl)aminomethane, (Tris), ethylenediaminetetraacetic acid (EDTA), 3-cyclohexylamino-1-propanesulfonic acid (CAPS), and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. Sodium phosphate, glycine, formic acid, and sodium acetate were purchased from

† P.L.M acknowledges financial support from the DGYCYT (Spain) (Grant PB90-0876). J.M.H. is a fellow of the DGYCYT (Spain).

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⊕ Abstract published in *Advance ACS Abstracts,* October 15, 1993.

[1] Abbreviations: PCR, polymerase chain reaction; NMR, nuclear magnetic resonance; CD, circular dichroism; 2D NMR, two-dimensional NMR; 3D NMR, three-dimensional NMR; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tris-HCl, tris equilibrated with hydrochloric acid; CAPS, (3-cyclohexylamino-1-propanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; ANS, 8-Anilino-1-naphthalenesulfonic acid, ammonium salt; HOD, deuterated water; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

0006-2960/93/0432-12906$04.00/0

Merck. 8-Anilinonaphthalene-1-sulfonic acid, ammonium salt (ANS), was purchased from Fluka. Double-distilled water was used in all the experiments. Q Sepharose and the HiLoad 26/60 Superdex 75 column were purchased from Pharmacia. Centripep 10 and 30 concentrators were from Amicon. Ultrapure urea and guanidine hydrochloride (GdnHCl) were from BRL.

Recombinant CheY. The wild-type *E. coli* gene for CheY was obtained by using the PCR (polymerase chain reaction) technique (Clackson *et al.*, 1991). *Bam*HI and *Hin*dIII sites were introduced at the 5′ and 3′ ends, respectively, using the following primers: primer 1, TGAAAGGATCCGATAAA-GAACTTAAATTTTTGGTG; primer 2, ATAGAAGCT-TCCTCACATGCCACTTTC. The vector used for cloning the CheY gene was obtained by PCR from the barnase (ribonuclease from *B. amiloliquefaciens*) plasmid (Serrano *et al.*, 1990). This vector is derived from the ptz18U plasmid (Pharmacia). It contains the lacZ promoter and a ribosome binding site before the alkaline phosphatase signal peptide and the barnase gene (Serrano *et al.*, 1990). *Bgl*II and *Hin*dIII sites were introduced, at the 5′ and 3′ ends, respectively, using the following PCR primers: primer 3, ATAGTAGATCT-CACTTTATTTTCTCCACGGATC; primer 4, AATGG-GAGATGAACAATATGG. The *Bgl*II site was introduced between the first and second amino acids of the barnase construct. The construction was verified by DNA sequencing of the entire coding sequence (Sanger *et al.*, 1977).

Protein Expression and Purification. (a) Expression. The plasmid was cloned into the JM109 strain of *E. coli*. Bacteria was grown in L-broth, at 310 K, from a single colony until an OD, at 600 nm, of ~0.4 was reached. Then IPTG was added to a final concentration of 0.5 mM, and the bacteria were grown overnight at 37 °C. Cells were harvested and concentrated by centrifugation (10000*g*), and the paste was washed and resuspended in 50 mM Tris-HCl, pH 8.0 (4 mL per liter of culture). The cells were broken up by sonication, and the cell debris was removed by centrifugation at 15000*g* for 20 min.

(b) Purification of the Soluble Protein. The supernatant of the last centrifugation was spun down at 100000*g* for 2 h, filtered on a 22-μm filter, and loaded into a Q Sepharose column equilibrated with 5 mM sodium phosphate buffer, pH 7.0. The column was washed with several volumes of the same buffer, and the protein was eluted with 100 mM NaCl in the same buffer. Fractions containing the protein were pooled and concentrated on Centripep 30 concentrators. The concentrated protein was then loaded into a HiLoad 26/60 Superdex 75 column equilibrated in 5 mM sodium phosphate buffer, pH 7.0, and the fractions containing the pure CheY protein were concentrated, flash frozen in liquid nitrogen, and stored at 203 K. The purified protein was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

(c) Purification of the Insoluble Protein. The bacterial debris was washed with 10 mM Tris-HCl, pH 8.0, and resuspended in 6 M urea (4 mL per liter of starting culture), and the insoluble fraction was removed by centrifugation at 100000*g* for 40 min. The supernatant was diluted 10 times with ice-cold 10 mM Tris-HCl, pH 8.0, and then centrifuged at 100000*g* for 30 min. The supernatant was filtered through a 22-μm filter, concentrated on Centripep 30 concentrators, and loaded into the HiLoad 26/60 Superdex 75 column. The purified protein was treated in the same manner as the soluble protein.

Non-Denaturing Gel Electrophoresis. Proteins were electrophoresed at 4 °C on a 15% (w/v) polyacrylamide gel in 0.37 M Tris-HCl, pH 8.8, with a constant current of 30 mA until the dye had run out of the gel. The gel was prerun for 20 min before the samples were loaded. A stacking gel was made of 3.7% (w/v) polyacrylamide gel in 0.125 M Tris-HCl, pH 8.8. For temperature-gradient gels 200 μg of CheY in 100 μL of 50 mM sodium glycinate, pH 2.5, was loaded onto a 15% polyacrylamide gel in 50 mM sodium glycinate, pH 2.5, buffer, in which a temperature gradient had been created perpendicular to the migration of the protein with two baths at 293 and 333 K. The protein was run for 1 h at a constant current of 50 V and then for 6 h at a constant current of 200 V. After the protein was run, the temperature gradient was measured by sticking a temperature probe, every centimeter, into the gel.

Protein Concentration Determination. The molar extinction coefficient of CheY was determined by following the method of Gill and Hippel (1989). Briefly, CheY protein was diluted into 6 M guanidine hydrochloride and 20 mM sodium phosphate buffer, pH 6.5, and the absorbance at 280 nm was determined. The same experiment was done in the absence of guanidine hydrochloride. Since CheY has one Trp and two Tyr residues, a molar extinction coefficient of 8250 was used for the denatured protein. The following formula was used:

$$e_{M,nat} = A_{nat} e_{M,GdnHCl} / A_{GdnHCl}$$

where $e_{M,nat}$ is the molar extinction coefficient and A_{nat} is the absorbance of the native protein. $e_{M,GdnHCl}$ and A_{GdnHCl} are the molar extinction coefficient and the absorbance of the denatured protein, respectively. It was found that 1 unit of absorbance, in a 1-cm path length cuvette, at 280 nm is equivalent to 1.5 mg/mL CheY protein.

Sequence and Mass Spectroscopy Analysis. The purified CheYs corresponding to both the insoluble and soluble fractions were analyzed by mass spectroscopy, and the first five amino acids were determined by protein sequencing, at the EMBL Peptide & Protein Service.

Spectroscopy. The intrinsic fluorescence of CheY decreases upon denaturation, as there is a shift in the maximum toward the red. The fluorescence yield of the native protein, with an excitation wavelength of 290 nm and emission recorded at 315 nm, is flat with respect to concentration of urea or guanidine hydrochloride. On the other hand, the fluorescence yield of the denatured protein increases constantly with increasing concentrations of urea (Pace *et al.*, 1992) but not guanidine hydrochloride. The maximal relative change in fluorescence upon chemical denaturation is obtained with an excitation wavelength of 290 nm and an emission wavelength of 315 nm. An Aminco Bowman Series 2 luminescence spectrometer was used for the equilibrium studies, with an excitation slit width of 2 nm for chemical denaturation or 1 nm for temperature denaturation and an emission slit width of 8 nm. The molar ellipticity at 222 nm of the folded and the unfolded protein is flat with respect to urea, and the fraction of folded vs unfolded protein at a given urea concentration is simply the ratio of the observed ellipticity change to the maximal ellipticity change. A Jobin-Yvon CD-VI machine was used for the CD equilibrium studies.

Titration of magnesium binding to CheY was done by monitoring the quenching of tryptophan fluorescence as a result of increasing concentrations of magnesium (Lukat *et al.*, 1990). The experiments were done at 293 K, using 5–10 μM protein solution in 50 mM Tris-HCl at pH 7.0 (Kar *et*

al., 1992). Stock magnesium chloride solutions were prepared at concentrations that allowed complete titration with no more than 4% dilution of the sample. The data were treated according to the Scatchard equation for equilibrium binding (Segel, 1975).

Chemical Denaturation Experiments. Urea and guanidine hydrochloride solutions were prepared gravimetrically in volumetric flasks. The solutions were divided into 750-mL aliquots with an Eppendorf Multipette loaded with 12.5-mL Combitips and stored at 253 K. For each data point in the experiment, 100 μL of the stock CheY solution in 425 mM of the appropriate buffer was diluted with a 750-mL aliquot, using a 100-μL Hamilton syringe. The final CheY concentration ranged from 3 to 8 μM. The samples were allowed to equilibrate at 298 K for at least 1 h. Spectroscopic measurements were carried out in a thermostated cuvette holder at 298 K. The temperature inside the cuvette was monitored with a thermocouple probe immersed inside the cuvette. Chemical unfolding was monitored by fluorescence or circular dichroism (CD). Unfolding with urea was found to be totally reversible after dilution 10 times in 10 mM Tris-HCl, pH 8.0.

Thermal Denaturation Experiments. Thermally induced unfolding was also monitored by fluorescence spectroscopy using 5 mM CheY in either 50 mM sodium glycinate buffer at pH 2.5 or 50 mM Na-Pipes, pH 7.0, with different urea concentrations (0.4–2.5 M urea), or by thermal gradient electrophoresis experiments using the same buffers. The pK_a of Pipes is temperature dependent, and the pH varies from 7.0 to 6.8 over the temperature range used (298–353 K). It was found that the stability of CheY does not change significantly over this pH range. The pK of glycine is temperature dependent, and the pH varies from 2.5 to 1.9 over the temperature range used (298–344). The CD and fluorescence spectra of the protein do not change noticeably over this pH range. Reversibility after cooling was shown by reappearance of the initial fluorescence and CD spectra in the near-UV.

Chemical Denaturation. This analysis is for a two-state model of denaturation where only the native and unfolded states are significantly populated. The equilibrium constant for unfolding, K_u, in the presence of a denaturant is calculated from eq 1,

$$K_u = (F_f - F)/(F - F_u) \quad (1)$$

where F is the observed fluorescence and F_f and F_u are the values of the fluorescence of the folded and unfolded forms of the protein. Since the fluorescence yield of the denatured protein increases with urea, the fluorescence yield of the unfolded protein in water needs to be extrapolated from higher urea concentrations. The slope of the urea effect on the fluorescence yield was calculated from several experiments and used to calculate F_u in water. The transition corresponds to a two-state process. It has been found experimentally that the free energy of unfolded proteins in the presence of urea is linearly related to the concentration of the denaturant (Pace, 1986).

$$\Delta G_u = \Delta G_{H_2O} - m[\text{denaturant}] \quad (2)$$

The values of m and ΔG_{H_2O}, the apparent free energy of unfolding in the absence of denaturant, may be calculated from eq 1, because $\Delta G_u = -RT \ln K_u$. We directly fit the entire data set from the fluorescence-monitored urea denaturation with the nonlinear regression analysis program Kaleidograph (by Abelbeck Software), using eq 3 (which is derived from eqs 1 and 2).

$$F = F_f - (F_f - F_u) \exp\{(m[\text{urea}] - \Delta G_{H_2O})/RT\}/\{1 + \exp([m[\text{urea}] - \Delta G_{H_2O})/RT]\} \quad (3)$$

There is a long extrapolation to 0 M urea from measurements made over the range 2.5–4.5 M urea. Consequently, small errors in m result in large errors in the estimation of ΔG_{H_2O}. At least three repetitions were done for each experimental condition in order to have an accurate value of m.

Thermal Denaturation. Fluorescence of the native and denatured states varies linearly with the temperature. We found that the transition between these two states did not have a great amplitude or a big slope compared to the linear change with temperature. Consequently, fitting of the curve with six variables resulted in a great uncertainty in the calculation of the temperature at which 50% of the protein was denatured. In order to have a better fit, we used the ratio of the fluorescence emission values at two wavelengths. In this case we obtained flat baselines, and we only used four variables for the fitting. Similar results were obtained by both methods. Calculation of K_u as a function of temperature was similar to that for the chemical denaturation, assuming a two-state transition.

Calorimetry Experiments. Scanning calorimetry experiments (DSC) were performed with the computerized version of the DASM-4M microcalorimeter (Privalov & Potehkin, 1986), with a cell volume of 0.47 ml, at heating rates of 0.5, 1, and 2 K/min. The protein concentration was between 2 and 6 mg/mL. The samples were extensively dialyzed before measurement against the corresponding buffer. The following buffers were used: at acidic pH, either glycine or acetate; at neutral pH, either Pipes or phosphate; and at alkaline pH, either glycine or CAPS. The ionic strength used was either 5 or 10 mM. To avoid contamination of the protein with heavy metals, 1 mM EDTA was added to the buffers. In some cases the buffers contained 2 M urea or 0.5 M GdnHCl.

The partial heat capacity of the protein was calculated from calorimetric records, as previously described (Privalov & Potehkin, 1986). The protein specific volume used was calculated from the amino acid composition (0.73 mL/g). We used 14 000 Da for the molecular mass. The baseline of the instrument was recorded routinely before and after the experiment with both calorimeter cells filled with the corresponding buffer.

Analysis of Scanning Calorimetry Data. The temperature dependence of the molar heat capacity of CheY was further analyzed using SCAL software developed at the Institute of Protein Research (Russian Academy of Sciences, Pushchino) (Filimonov et al., 1982) or using the curve-fitting option of SIGMAPLOT software (Jandel Co.).

To analyze the calorimetric data, the excess heat capacity ($\Delta C_{p,0}$) over the heat capacity of the initial state ($C_{p,0}$) was calculated after the latter was approximated by a linear function of the temperature, as will be described in the Results section. The $\Delta C_{p,0}$ function consists of two terms, as follows:

$$\Delta C_{p,0}(T) = C_p(T) - C_{p,0}(T) = C_p^{exc}(T) + \Delta C_{p,0}^{int}(T) \quad (4)$$

where C_p^{exc} is the excess of the heat absorbance and $\Delta C_{p,0}^{int}$ is the excess of the chemical or intrinsic C_p over the initial state. Since the attempts to fit CheY data to a monomolecular sequential process failed (see Results), the $\Delta C_{p,0}$ was fitted to the following "operative" three-state model of unfolding:

$$2N \overset{K_u}{\rightleftharpoons} 2U \overset{K_a}{\rightleftharpoons} I_2 \quad (5)$$

where N corresponds to the native state, U to the unfolded state, and I_2 to a protein dimer, the conformation of which might be different from that of N and U. Then K_u, the monomolecular unfolding constant, and K_a, the bimolecular association constant, are expressed as

$$K_u = [U]/[N] = f_u/f_n \quad (6)$$

$$K_a = 1/K_d = [I_2]/[U]^2 = f_i/2P_0f_u^2 \quad (7)$$

where f_n, f_u, and f_i are the fractions of protein molecules in the corresponding states, P_0 is the total concentration of protein in solution, and K_d is the dissociation constant. For the fractions we have

$$f_n = [N]/P_0;\ f_u = [U]/P_0;\ f_i = 2[I_2]/P_0;\ f_n + f_u + f_i = 1 \quad (8)$$

If we define $q = 1 + K_u$ (the partition function of the unfolding step) and solve a simple quadratic equation, we get for the fractions

$$f_n = 2/[q + (q^2 + 8P_0K_aK_u^2)^{1/2}] \quad (9)$$

$$f_u = K_uf_n \quad (10)$$

$$f_i = 1 - qf_n \quad (11)$$

Following the definitions used by Freire and Biltonen (1978), we can write for the excess enthalpy function

$$\langle \Delta H_0(T) \rangle = \Delta H_u f_u + (\Delta H_u + \Delta H_a)f_i = \Delta H_u(1 - f_n) + \Delta H_a f_i \quad (12)$$

Here ΔH is the positive enthalpy change of unfolding, and $\Delta H_a = -\Delta H_d$ is the negative enthalpy change of association. The derivative of the enthalpy excess is $\Delta C_{p,0}$, and its two terms (see eq 4) are expressed as

$$\Delta C_{p,0}^{\ \text{int}} = \Delta C_{p,u}(1 - f_n) + \Delta C_{p,a}f_i \quad (13)$$

$$C_p^{\text{exc}} = \Delta H_a\, df_i/dT - \Delta H_u\, df_n/dT \quad (14)$$

The enthalpy changes per mol of monomer and other thermodynamic functions were calculated under the assumption of a constant ΔC_p using the following equations:

$$\Delta H_u(T) = \Delta H_u(T_u) + \Delta C_{p,u}(T - T_u) \quad (15)$$

$$\Delta H_a(T) = \Delta H_a(T) + \Delta C_{p,a}(T - T_a) \quad (16)$$

$$\Delta S_u(T) = \Delta H_u(T_u)/T_u + \Delta C_{p,u} \ln (T/T_u) \quad (17)$$

$$\Delta S_a(T) = \Delta H_a(T_a)/T_a + \Delta C_{p,a} \ln (T/T_a) \quad (18)$$

$$K_u(T) = \exp[(-\Delta H_u/T + \Delta S_u)/R] \quad (19)$$

$$K_a(T) = 1/K_d = (1/P_0) \exp[2(-\Delta H_a/T + \Delta S_a)/R] \quad (20)$$

As the apparent association constant depends on the total protein concentration, T_a corresponds to the temperature where $K_a = 1/P_0$, whereas T_u is the temperature at which $K = 1$. The temperature (usually different from T_u) where $\Delta C_{p,0}$ has its maximal value is denoted as T^*.

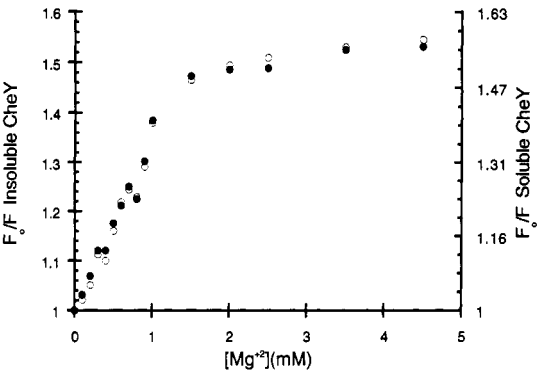

FIGURE 1: Stern–Volmer plots of the soluble (○) and insoluble (●) fractions of CheY. The quenching experiments were performed in 20 mM Tris-HCl, pH 7.0, at 293 K, as indicated by Kar *et al.* (1992), by addition of a stock 0.1 M $MgCl_2$ solution to a 7.5 μM solution of the soluble or the insoluble fraction of CheY. The excitation wavelength was 290 nm, and the emission was recorded at 348 nm. F_0 and F correspond to the fluorescence intensity at 346 nm in the absence and in the presence of increasing concentrations of $MgCl_2$, respectively.

ANS Binding. An ANS stock concentration of 28 mM was made in water. The final concentration of ANS in all the experiments was 250 μM, and the molar ANS/protein ratio was 250/1 (unless otherwise indicated). The excitation wavelength was 396 nm, and the emission signal was recorded at 495 nm.

Cross-Linking Experiments. CheY protein, at a final concentration of 0.75 mg/mL, was equilibrated for 5 min at different temperatures (298–338 K). Then formaldehyde (35%. w/v) was added to a final concentration of 1%, w/v, and the samples were incubated at the selected temperature for 10 min. The reaction was stopped by the addition of 5× Laemmli buffer (Serrano & Avila, 1985).

Nuclear Magnetic Analysis. All spectra were recorded on a Bruker AMX-600 spectrometer in the phase-sensitive mode using the time-proportional phase-incrementation mode (Marion & Wutrich, 1983). Water suppression was achieved by selective presaturation, placing the carrier on the H_2O or HOD resonance.

RESULTS

Genetic Construction and Expression of Proteins. Ligation of the CheY PCR product resulted in the substitution of Ala2 by the sequence Arg-Ser. The new sequence of the protein is Met-Arg-Ser-Asp-etc. instead of the wild-type sequence, Met-Ala-Asp-etc. Inspection of the three-dimensional structure of *E. coli* CheY (Volz & Matsumura, 1991) indicates that the first two residues are not interacting with the rest of the protein and are far away from the active center. Nuclear magnetic resonance determination of the structure in solution of the engineered form of CheY indicates that the modification of the amino-terminal end of the protein does not affect its structure (Bruix *et al.*, 1993).

Protein Purification and Characterization. About two-thirds of the protein expressed in *E. coli* at 310 K is found in inclusion bodies (insoluble protein), while the rest is found in the soluble fraction of *E. coli* lysate (soluble protein). The insoluble protein can be renatured after denaturation with urea and dilution in 10 mM Tris-HCl, pH 8.0. Both the soluble and insoluble proteins can be easily purified to homogeneity, and they exhibit identical properties with respect to molecular weight chromatography, fluorescence and CD spectrometry, non-denaturing gel electrophoresis at pH 8.8 (data not shown), and magnesium binding (Figure 1). We

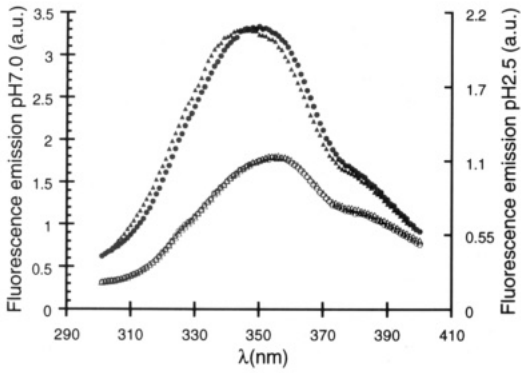

FIGURE 2: Fluorescence emission spectra of different conformations of CheY. Protein concentrations were 10 μM in either 50 mM Na-Pipes, pH 7.0, or sodium glycinate, pH 2.5. (●) CheY at 298 K, pH 7.0. (▲) CheY at 298 K, pH 2.5. (○) CheY in 6 M urea at 298 K, pH 7.0. (△) CheY in 6 M urea at 298 K, pH 2.5. a.u. = arbitrary units.

also sequenced the first five residues of both the soluble and insoluble forms of CheY. In both cases the sequence was the one expected, and a Met was found as the first residue. Mass spectrometry analysis indicated that the mass was the one expected from the amino acid sequence and, consequently, from the absence of postranslational modifications in both the soluble and insoluble forms of CheY (data not shown). Moreover, X-ray crystallography (Bellsolell and Coll, manuscript in preparation) and 2D and 3D NMR analysis (Bruix *et al.*, 1993) of the protein purified from inclusion bodies revealed the same structure as the previously published X-ray structure (Volz & Matsumura, 1991). Scatchard analysis of the binding of magnesium to the soluble and insoluble CheY proteins indicates that there is 1 mol of Mg^{2+} bound per 1 mol of protein with a K_d of 0.56 ± 0.05 mM (data not shown). These values are identical to the ones described for the metal-free form of CheY from *E. coli* (Lukat *et al.*, 1990; *Kar et al.*, 1992; Needham *et al.*, 1993).

Chemical Denaturation Monitored by Fluorescence Spectroscopy and Circular Dichroism Analysis. (a) Fluorescence Spectroscopy Analysis. Fluorescence emission spectra of tryptophan residues in CheY were used to monitor qualitatively any changes in the environment of Trp58 upon unfolding of the protein. Figure 2 shows the fluorescence emission spectrum of CheY in 50 mM Pipes at pH 7.0 and 298 K. The maximum of the spectrum is found at 349 nm. This is what would be expected from a tryptophan residue in a somewhat polar environment (Burstein *et al.*, 1973), as is the case for Trp94 in CheY (Stock *et al.*, 1989; Volz & Matsumura, 1991). In the presence of 6 M urea there is a decrease in the intensity of the fluorescence emission and a red shift of the maximum to 356 nm, indicating that the Trp is completely exposed to the solvent (Burstein *et al.*, 1973). The maximum ratio between the fluorescence in the native and the unfolded form of CheY was found to be around an emission wavelength of 315 nm. At pH 2.5 there is an unspecific quenching of Trp58 and a 2-nm blue shift of the spectrum (Figure 2). This shift is not produced by a nonspecific effect of the low pH on the Trp fluorescence since the spectrum at pH 2.5 in 6 M urea is similar to that at pH 7.0 in 6 M urea (Figure 2).

(b) CD Analysis. Circular dichroism spectra, at 298 K, in the near- and far-UV were measured as indicators of relative differences in secondary and tertiary structure between the native and unfolded states of the protein. Figure 3A shows the far-UV spectra of CheY, at pH 7.0 and 2.5, in the absence or in the presence of 8 M urea. The protein spectrum at pH 7.0 has three well-defined ellipticity minima at 182, 211, and

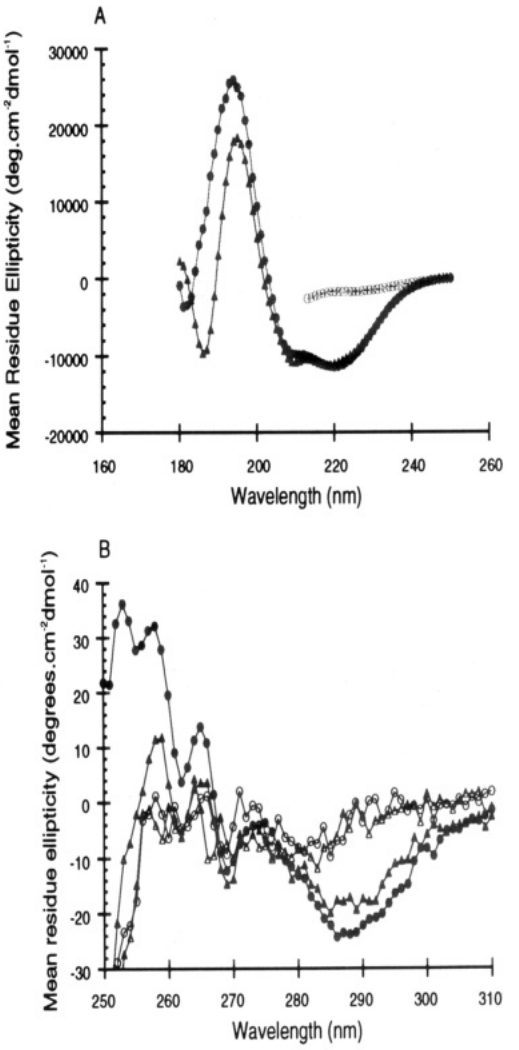

FIGURE 3: CD spectra of different conformations of CheY. (A) Far-UV CD spectra. (B) Near-UV CD spectra. (●) Native CheY in 5 mM sodium phosphate, pH 7.0. (▲) CheY in 50 mM sodium glycinate, pH 2.5. (○) Unfolded CheY in 5 mM sodium phosphate, pH 7.0. (△) Unfolded CheY in 50 mM sodium glycinate, pH 2.5. The buffer for the unfolded protein contained 6 M urea (identical spectra were obtained in the presence of 4 M guanidine hydrochloride). Since the spectra in the far-UV of the denatured protein at pH 2.5 and 7.0 are identical, we show only the one at pH 7.0. The spectra were recorded at 298 K. The concentration of the protein for the far-UV spectra was 10 μM, while for the near-UV it was 70 μM. A 0.2-cm light path cell was used for recording the spectra.

220 nm and a maximum at 193 nm. The ratio between the minima at 220 and 211 nm is 1.16. In the presence of 8 M urea or 4 M GdnHCl (data not shown) there is a 10-fold decrease of the negative minimum at 220 nm and a displacement of the minimum to 223 nm. The far-UV spectrum at pH 2.5 is slightly different from that found at pH 7.0. The three minima have moved to 188, 209, and 219 nm, and the maximum is at 194 nm. The ellipticity at 220 nm is less negative, while at 209 nm it is more. The ratio between the minima at 219 and 209 nm is 1.01. This indicates either that a conformational rearrangement of the protein has occurred at pH 2.5 or that a small proportion of the protein is denatured. Since the spectrum of CheY at pH 2.5 is almost independent of the temperature in the range 273–298 K (data not shown), that strongly argues in favor of the existence of a small conformational rearrangement. Figure 3B shows the near-UV spectra of CheY at pH 7.0 and 2.5, in the absence or in the presence of 6 M urea. The near-UV spectrum of CheY at pH 7.0 has a well characterized minimum at 287 nm

(probably corresponding to the Trp residue) and four maxima around 253, 258, 265, and 275 nm that could correspond to the Phe and Tyr residues. In the presence of 6 M urea the minimum and the maxima disappear, indicating that the tertiary interactions responsible for the asymmetric environment of the aromatic residues are broken. At pH 2.5 the spectrum is also slightly different from the one found at pH 7.0. The first maximum disappears, while the other three maxima and the minimum remain well defined although less pronounced. In the presence of 6 M urea the signal disappears, as happened at pH 7.0 (Figure 3B). Disappearance of the first maximum is clearly due to some conformational rearrangement of the protein. However, the less pronounced maxima and minimum could be due to the presence of a small proportion of non-native protein at 298 K and pH 2.5.

(c) Stability Analysis. Figure 4A shows the change in fluorescence of CheY upon titration with urea in 50 mM Pipes, pH 7.0. Fitting of the data to eq 3 gave values of ΔG_{H_2O} = 23.4 kJ mol^{-1} and m = 6.69 kJ mol^{-1} M^{-1}. The midpoint of unfolding (K_u = 1) is at 3.48 ± 0.03 M urea (Table I). The same transition was observed by using the changes in the ellipticity at 222 nm, indicating that it is a global process of denaturation (Table I).

Analysis of the free energy of unfolding of CheY, determined by urea denaturation, at different pH values indicates that the maximum stability is around pH 5.1 (Table I). However, at pH 5.1 the protein exhibits a high tendency to aggregate, especially at high ionic strength. Consequently, this pH was not chosen as the normal working pH. The stability of the protein decreases quite dramatically below pH 3.7. Unfortunately, at pH values lower than 3.5 there is a strong pH dependence on the urea concentration. Consequently, the estimation of m and ΔG is not valid. The only reliable value is the concentration of urea at which 50% of the protein is denatured (Pace *et al.*, 1992). Fitting of the denaturation curve at pH 2.5 indicates that there is a cooperative transition and that around 5% of the protein (5 ± 2.5%; the error is high since the extrapolation is not reliable), at 298 K, is in a non-native state (Figure 4A).

Increasing the ionic strength of the buffer with sodium chloride results in an enhancement of the stability, that could be up to 4.2 kJ mol^{-1} (Table II). Unfortunately, at salt concentrations higher than 0.6 M the protein easily aggregates, and this precludes the use of higher ionic strengths. The presence of millimolar concentrations of Mg^{+2} greatly enhances the stability of the protein, and at 4 mM Mg^{2+} there is an enhancement of 2.72 kJ mol^{-1} (Table II). This is in agreement with the fact that there is a high-affinity binding site for Mg in the protein (Lukat *et al.*, 1990; Kar *et al.*, 1992). Also the use of phosphate buffer, instead of Na-Pipes, at pH 7.0 results in a slight enhancement of the stability of CheY (Table I).

Figure 4B shows the change in fluorescence of CheY upon titration with GdnHCl in 50 mM Pipes, pH 7.0. In the case of the guanidine hydrochloride denaturation, ΔG_{H_2O} = 14.2 kJ mol^{-1} and m = 9.4 kJ mol^{-1} M^{-1}. The midpoint of unfolding (K_u = 1) is at 1.48 ± 0.05 M GdnHCl (Table I). This is a big discrepancy since it has been indicated that a deviation of around 10–15 % at zero concentration of denaturant can be found, due to the different ways in which both denaturant agents interact with the protein (Makhatadze & Privalov, 1992), while in CheY it is 40%. Also the m value is half of what has been described for proteins of similar size (barnase has an m value of 19 kJ mol^{-1} M^{-1} under similar conditions; Kellis *et al.*, 1989). One reason for this discrepancy could be

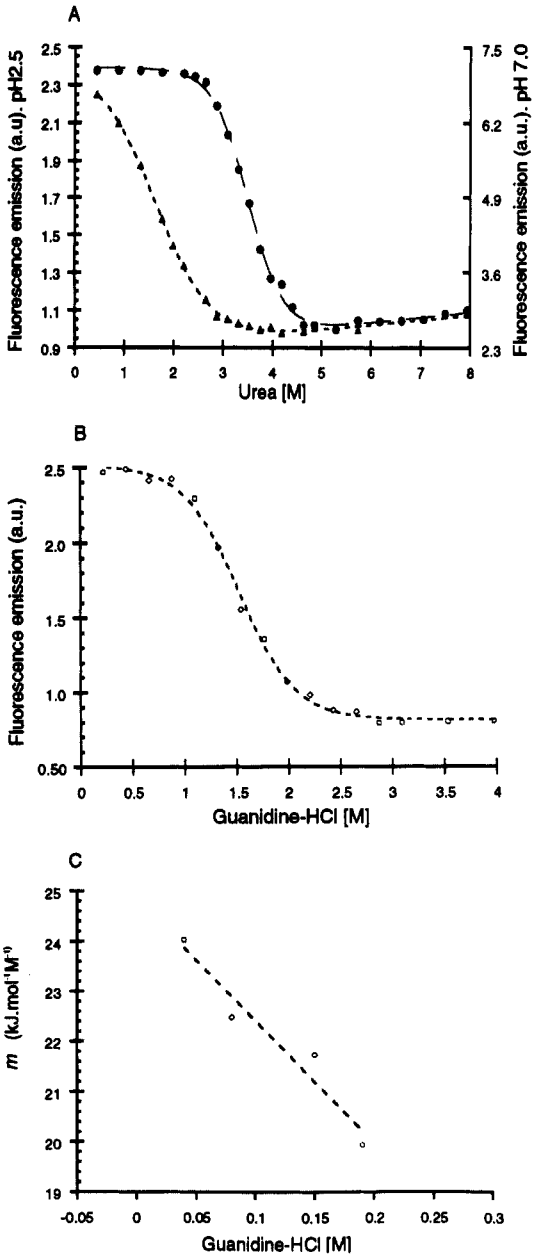

FIGURE 4: Equilibrium unfolding of CheY protein by urea or guanidine hydrochloride. The excitation wavelength was 290 nm, and the fluorescence emission was recorded at 315 nm. CheY concentration was 10 μM, and the measurements were taken at 298 K. (A) (●) CheY in 50 mM Na-Pipes, pH 6.3. (▲) CheY in 50 mM sodium glycinate, pH 2.5. Denaturation was done with urea. (B) CheY in 50 mM Na-Pipes, pH 6.3. Denaturation was done with guanidine hydrochloride. (C) Changes in the m value produced by increasing the concentration of guanidine hydrochloride. CheY in 5 mM Na-Pipes, pH 6.3, was denatured by urea in the presence of increasing concentrations of guanidine hydrochloride (0–0.2 M). The differences in free energy of unfolding between the denaturation experiment in the absence and in the presence of increasing amounts of guanidine hydrochloride, were determined. By applying eq 2, we calculate the changes in the m value upon increasing the concentration of guanidine hydrochloride.

that CheY is more unfolded in urea than in GdnHCl. However, the CD spectra in the near- and far-UV of urea- or GdnHCl-denatured CheY are identical. Another explanation can be found, given the fact that CheY is stabilized at high ionic strength and GdnHcl is a salt. Consequently, one would expect that upon an increase of the GdnHCl concentration there would be two opposite effects, a stabilization due to an increase in the ionic strength and a destabilization due to the GdnHCl denaturing effect. The same type of mixed

Table I: pH Dependence of CheY Free Energy of Unfolding as Determined by Chemical Denaturation[a]

buffer	m[b] (kJ mol^{-1} M^{-1})	[urea]$_{50\%}$[c] (M)	ΔG_{H_2O}[b] (kJ mol^{-1})
sodium glycine, pH 2.5[d]	4.47 ± 0.25	1.66	7.40 ± 0.9
sodium formate, pH 3.7[d]	6.94 ± 0.42	2.98	20.7 ± 1.5
sodium formate, pH 4.4	7.27 ± 0.50	3.66	26.6 ± 1.9
sodium acetae, pH 5.1	7.98 ± 0.42	3.98	31.8 ± 2.3
sodium acetate, pH 5.1[e]	8.03 ± 0.51	4.05	32.5 ± 2.0
Na-MES, pH 6.3	6.69 ± 0.33	3.51	23.5 ± 1.4
Na-MES, pH 6.3[f]	9.44 ± 0.42	1.48	14.2 ± 0.67
sodium phosphate, pH 7.0	6.56 ± 0.33	3.64	23.9 ± 1.4
Na-Pipes, pH 7.0	6.69 ± 0.51	3.48	23.2 ± 1.9
Na-Hepes, pH 8.0	6.52 ± 0.37	3.36	21.9 ± 1.4

[a] Denaturation was performed under Experimental Procedures. All the buffers were prepared at 50 mM. CheY concentration was 10 μM. [b] ΔG_{H_2O} and m are calculated by assuming that the fluorescence yield of the folded state is independent of [urea], while that of the unfolded state is dependent on [urea]. [c] [urea]$_{50\%}$ is unaffected by this model. [d] The determination of m and ΔG_{H_2O} at pH values lower than 4.0 is not very precise due to the fact that at high urea concentrations there is a significant decrease of the pH in the transition region. On the other hand, the [urea]$_{50\%}$ value is less sensitive to the changes in the slope, m, and is a better reporter for the changes in protein stability (Pace *et al.*, 1992). [e] Chemical denaturation monitored by CD. The ellipticity at 222 nm was measured at each urea concentration, and the denaturation curve was fitted as described under Experimental Procedures. The ellipticity of the folded and unfolded states did not change with different urea concentrations. [f] Guanidine hydrochloride denaturation.

Table II: Metal Concentration Dependence of CheY Free Energy of Unfolding[a]

NaCl		$MgCl_2$	
concn (mM)	$\Delta\Delta G$ (kJ mol^{-1})	concn (mM)	$\Delta\Delta G$ (kJ mol^{-1})
0	0	0	0
30	0.4	24	2.7
80	1.0	10	5.4
193	1.6	20	5.5
327	2.9	30	5.7
570	3.7	80	6.9

[a] Denaturation was performed in 5 mM Na-Pipes, pH 7.0, at 298 K, as described under Experimental Procedures. CheY concentration was 10 μM. A 5 M NaCl or a 1 M $MgCl_2$ stock solution was used in order to obtain the final metal concentration. The excitation wavelength was 290 nm, and the fluorescence emission was recorded at 315 nm. The curve was fitted as indicated under Experimental Procedures. The standard error for all the determinations is between 0.1 and 0.2 kcal mol^{-1}.

effect has been recently described for ribonuclease T1 (Mayr & Schmid, 1993). This mixed effect should result in a decrease of the m value with increasing GdnHCl concentrations and consequently in a wrong estimation of ΔG_{H_2O}. The free energy of unfolding in water, calculated by the linear extrapolation procedure, will then be higher or lower than the value in urea depending of the result of both effects on the region from which the extrapolation is made. In order to determine whether this is the case, we have done urea-denaturation experiments in the presence of increasing concentrations of GdnHCl. By determining the effect of GdnHCl on the stability of the protein and applying eq 2, we can calculate the changes in the m value with increasing concentrations of GdnHCl (Figure 13). As expected if our hypothesis is correct, the m value decreases with increasing GdnHCl concentrations. In any case, due to this double effect of GdnHCl, we have performed all the following experiments with urea.

Thermal Denaturation Monitored by Fluorescence Spectroscopy and Circular Dichroism Analysis. Thermal unfolding of CheY at pH 7.0 with an ionic strength of 50 mM resulted in irreversible denaturation as monitored by optical scattering and recovery of fluorescence (data not shown). Reducing the ionic strength, under the same conditions, to 5 mM resulted in a partial reversibility of the denaturation proccess as long as the protein was not heated above 338 K. Addition of urea increased the temperature (up to 353 K) and ionic strength range (up to 50 mM) in which thermal denaturation of the protein was found to be more than 90% reversible (data not shown). The same phenomenon is present in the pH range 3.5–8.0. Only at pH 2.5 in sodium glycinate buffer or at pH 10 in CAPS buffer were we able to heat up and cool down the protein in a fully reversible manner.

(a) Fluorescence Analysis. Fluorescence analysis of CheY in 2 M urea and 50 mM Na-Pipes, pH 7.0, at different temperatures (273–363 K) shows a continuous decrease of the fluorescence emission at 348 nm with a very small transition (data not shown). This transition can be better shown by plotting the ratio between the fluorescence emissions at 348 and 360 nm (Figure 5A). The apparent T_m of the transition is 330 ± 5 K (Table IV). Fluorescence analysis at different temperatures (273–363 K) of CheY in 50 mM glycine buffer, pH 2.5, shows a continuous decrease of the fluorescence emission at 344 nm with a very small transition centered around 334 ± 3 K (Table IV). This transition can be better shown by plotting the ratio between the fluorescence emissions at 344 and 356 nm (Figure 5B).

(b) CD Analysis. CD analysis of the temperature-induced denaturation of CheY, in phosphate buffer, pH 7.0, and 2 M urea, exhibits a behavior compatible with a two-state transition with a midpoint at 311 ± 4 K (Figure 6A). There is a simultaneous change in the ellipticities at 222 and 290 nm. The CD minimum at 287 nm in the near-UV disappears completely at 323 K, while there is still considerable negative ellipticity at 222 nm in the far-UV.

In glycine buffer, pH 2.5, there is a small transition around 307 K in the ellipticity at 222 nm (data not shown). This transition can be better shown by plotting the ratio between the ellipticities at 221 and 209 nm (Figure 6B). The CD signal at 290 nm disappears completely at 323 K, while there is still considerable negative ellipticity at 222 nm. Within experimental error there is a simultaneous change in the ellipticities at 222 and 290 nm. The far-UV CD spectrum in glycine buffer at 315 K shows that the helical content of the protein has decreased but is still very high (Figure 7A). The

Table III: Scanning Calorimetry Thermodynamic Parameters of Temperature-Induced Unfolding of CheY[a]

pH	T_u (K)	ΔH_u (kJ mol^{-1})	$\Delta C_{p,u}$ (kJ K^{-1} mol^{-1})	ΔG_u^{298} (kJ mol^{-1})	T_a (K)	ΔH_a (kJ mol^{-1})	T^* (K)
2.5	318 ± 1	245 ± 20	3.7 ± 1	12.1 ± 2	335 ± 2	155 ± 20	312
10.3	328 ± 1	280 ± 20	3.5 ± 1	20.5 ± 2	360 ± 3	135 ± 20	320
7.0	331 ± 3	350 ± 30	2.9 ± 1.5		389 ± 5	165 ± 30	318
7.0 (2 M urea)	316 ± 3	280 ± 20	2.5 ± 1.5		322 ± 5	155 ± 30	313

[a] T_u is the unfolding temperature, determined from the calorimetric model. ΔH_u is the enthalpy of unfolding, determined from the calorimetric model. T_a is the temperature at which the dimers dissociate, determined from the calorimetric model. ΔH_a is the enthalpy of dissociation and unfolding of the dimers, determined from the calorimetric model. T^* corresponds to the observed maximum of C_p. ΔG_u^{298} was calculated by extrapolating ΔH_u and ΔS_u to 298 K, using the $\Delta C_{p,u}$ values.

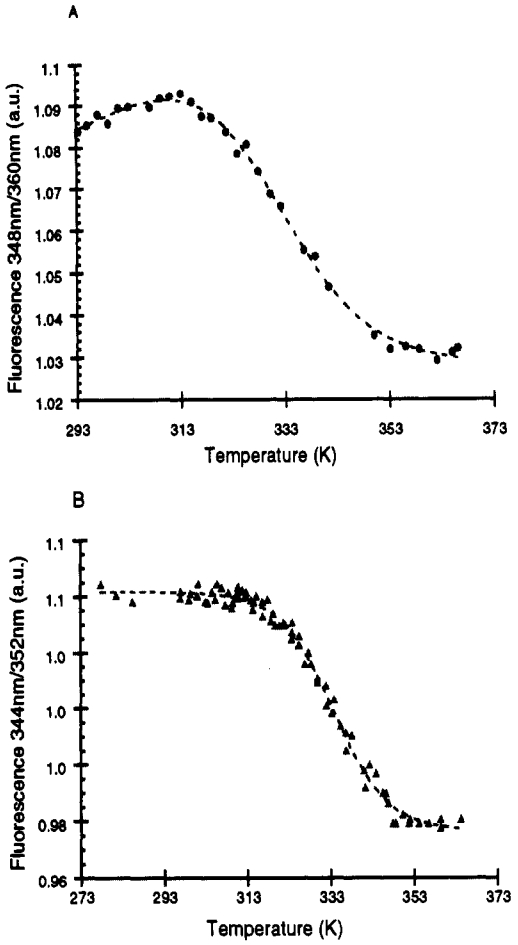

FIGURE 5: Temperature denaturation of CheY monitored by fluorescence. (A) Temperature-induced conformational changes of CheY, at a protein concentration of 10 μM in 50 mM sodium phosphate, pH 7.0, and 2 M urea, monitored by the changes in the relationship between the fluorescence emissions at 348 and 360 nm. (B) Temperature-induced conformational changes of CheY, at a protein concentration of 10 μM in 50 mM sodium glycinate, pH 2.5, monitored by the changes in the relationship between the fluorescence emissions at 344 and 352 nm.

Table IV: Comparison of Different Thermodynamic Parameters for the Temperature-Induced Unfolding of CheY Obtained from DSC, Fluorescence, Circular Dichroism, Temperature-Gradient Gels, and Cross-Linking Studies[a]

	pH 2.5		pH 7.0 (2 M urea)	
method	T^* (K)	T_a (K)	T^* (K)	T_a (K)
calorimetry	312	335 ± 2	313	323 ± 5
gradient gels	317 ± 4			
fluorescence		334 ± 3		330 ± 5
CD	306 ± 4		311 ± 4	
cross-linking	318 ± 5		318 ± 5	

[a] The thermodynamic parameters shown in this table have been obtained from the previous corresponding figures (Figures 5, 6, 8, and 12). The temperature-gradient gels were run as indicated in Experimental Procedures. The temperature at which 50% of the protein was in the native state was determined by gel densitometry.

near-UV spectrum of the protein (Figure 7B) is very similar to that found in the presence of 6 M urea (Figure 3B), indicating the absence of the tertiary interactions responsible for the assymetric environment of the aromatic residues.

(c) Thermal Denaturation Monitored by Scanning Calorimetry. The DSC experiments with CheY were carried out at pH 2.5–3.0, 7.0, and 10.0–10.3. The typical temperature functions of the partial molar heat capacity are shown in Figure

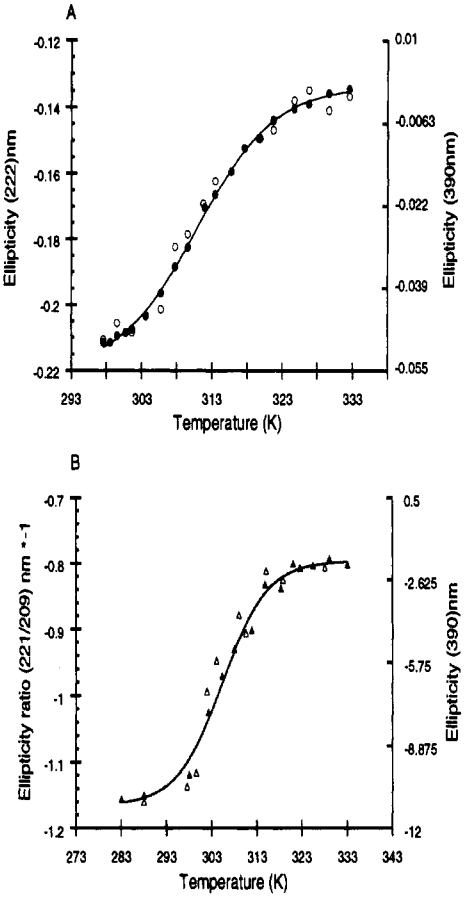

FIGURE 6: Effect of temperature on CheY ellipticity at 222 and 290 nm. The temperature-induced conformational changes of CheY at a concentration of 8 μM in (A) 50 mM sodium phosphate buffer, pH 7.0 and 2 M urea or (B) 50 mM sodium glycinate, pH 2.5, were monitored at 222 nm (●, ▲) or 290 nm (○, △).

8. Calorimetric records were highly reversible on reheating and, within experimental uncertainty, independent of scan rate at both acidic and alkaline pH. At these acidic and basic pHs we could register two well-defined endothermic transitions. At neutral pH, however, we were able to register only one asymmetric peak, which was reversible when the heating was stopped just after the peak maximum was passed and became irreversible when the heating was kept up to higher temperatures. By optical measurements it was shown that such irreversibility at neutral pH was caused by aggregation. At pH 7.0 the process is highly irreproducible, and for that reason we only show in Figure 8 the "reversible" part of the C_p function. Although addition of 2 M urea to the protein improved the reversibility at pH 7.0, it was not possible to obtain a complete analysis due to the presence of a strong endothermic effect of the urea. It should be noticed that due to rather small heat effects accompanying the very broad unfolding processes of CheY, the calorimetric data were recorded with relatively large uncertainty even at complete equilibrium conditions of acidic and alkaline pH. To get reliable results, we had to work at protein concentrations of about 4–6 mg/mL; at lower concentrations the experimental errors became unacceptable, while at higher concentrations the reversibility decreased after the samples were heated to high temperatures (383 K).

From the calorimetric data presented in Figure 8 it is clear that CheY thermal denaturation does not follow a two-state model. The next simplest model would correspond to a monomolecular three-state equilibrium mechanism. Nevertheless, it is impossible to fit the calorimetric data to that

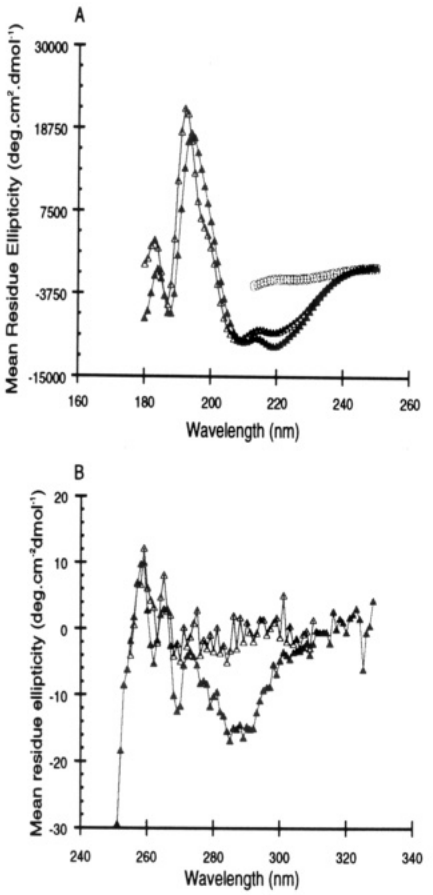

FIGURE 7: CD spectra of CheY in glycine, pH 2.5, at 298 and 314 K. (A) Far-UV spectra of 10 μM CheY in 50 mM glycine buffer, pH 2.5, at 298 K (▲) and 314 K (△) and in the presence of 6 M urea (○). (B) Near-UV spectra of 70 μM CheY in 50 mM glycine buffer, pH 2.5, at 298 K (▲) and 314 K (△).

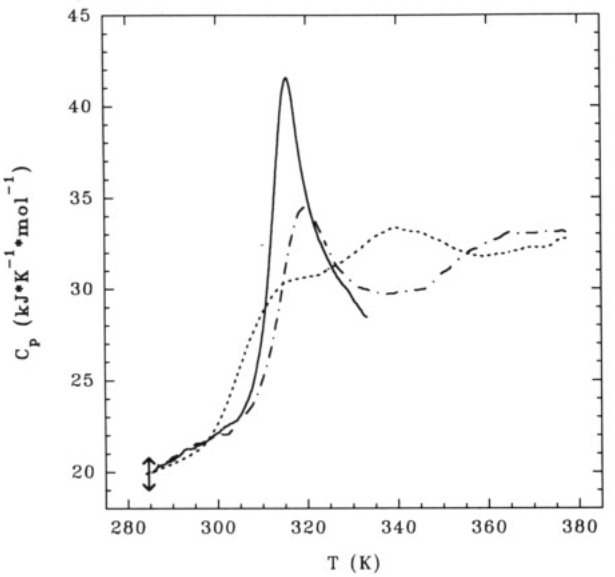

FIGURE 8: Temperature dependencies of the molar partial heat capacity of CheY at pH 7.0 (—), pH 2.5 (---), and pH 10.3 (· – ·). The double-headed arrow shows the error in the absolute value of C_p at 283 K.

scheme, or to any monomolecular one, because the van't Hoff monomolecular enthalpy calculated for the first peak at pH 10 or 7 (at pH 2.5 such an estimation is somewhat complicated) is approximately 3-fold higher than the calorimetric one. In addition, the heat effect estimated from the area under the peak was extremely low (100–140 kJ mol^{-1}), whereas the van't Hoff enthalpy estimations gave very reasonable values (300–380 kJ mol^{-1}) for a protein of this size. Hence, since a monomolecular mechanism contradicts our calorimetric data, we have assumed the next simplest model with a three-state denaturation scheme including a dimeric state of the protein (the formulations are described in Experimental Procedures). Thus, it has been found that all the calorimetric curves obtained under experimental conditions which exclude irreversibility of unfolding fit adequately into the model for equilibrium unfolding displayed in eq 5. As seen from Figures 9 and 10, the quality of the fit is rather high at all pH values.

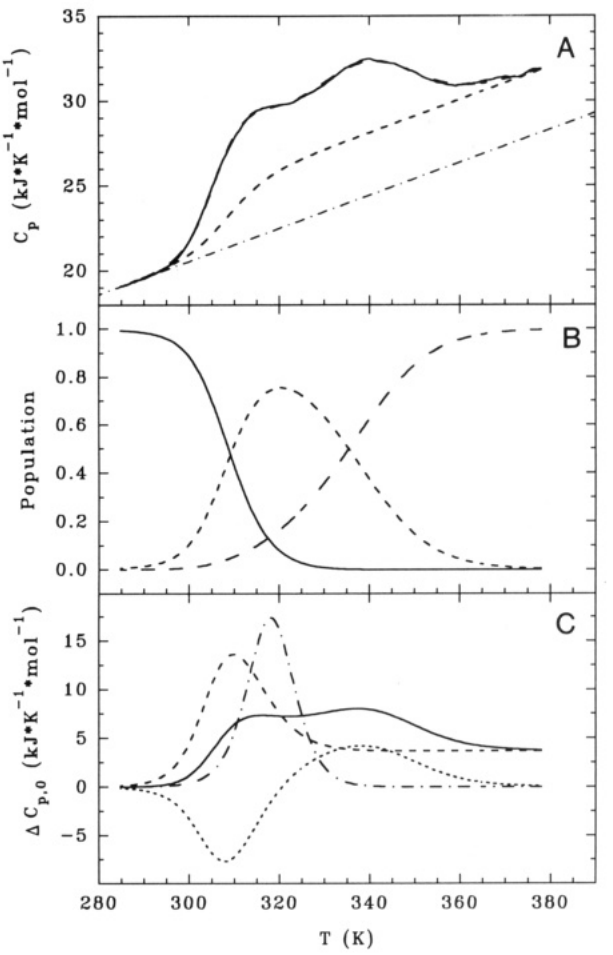

FIGURE 9: Analysis of DSC at pH 2.5 (for the best-fit parameters, see Table III). (A) Temperature functions of the experimental C_p (—) and of its best fit to eq 5 (– –). The $C_{p,0}$ function is drawn as explained in the text (· – ·). $\Delta C_{p,0}^{int}$ was calculated by the fitting procedure under the assumption of $\Delta C_{p,a} = 0$ (---). (B) Populations of the N state (—), the U state (– –) and the I_2 state (---) as found by fitting the C_p function to eq 5. (C) Best fit of the $\Delta C_{p,0}$ function (—) plotted together with its unfolding term $C^{unf}(T) = -\Delta H_u\, df_n/dT + \Delta C_{p,u}(1 - f_n)$ (– –) and the association/dissociation term, $C^{ass}(T) = \Delta H_a\, df_i/dT$ (---). For comparison, the C_p^{exc} for a single two-state transition with the same parameters of unfolding is plotted (· – ·).

The model displayed in eq 5, used by us for calorimetric data analysis, describes structural aspects of unfolding rather than the melting mechanism, since both transitions in such a presentation of the three-state equilibrium process have a clear structural interpretation. Namely, the first transition and its parameters describe protein unfolding, whereas the second one describes association–dissociation equilibrium between the unfolded polypeptide and the "intermediate dimer". The real sequence of events could be understood from consideration of plot B in Figure 9, where the populations of the three states are shown, calculated by fitting the experimental curve at pH 2.5 into eq 5. Initially, only the native state is significantly

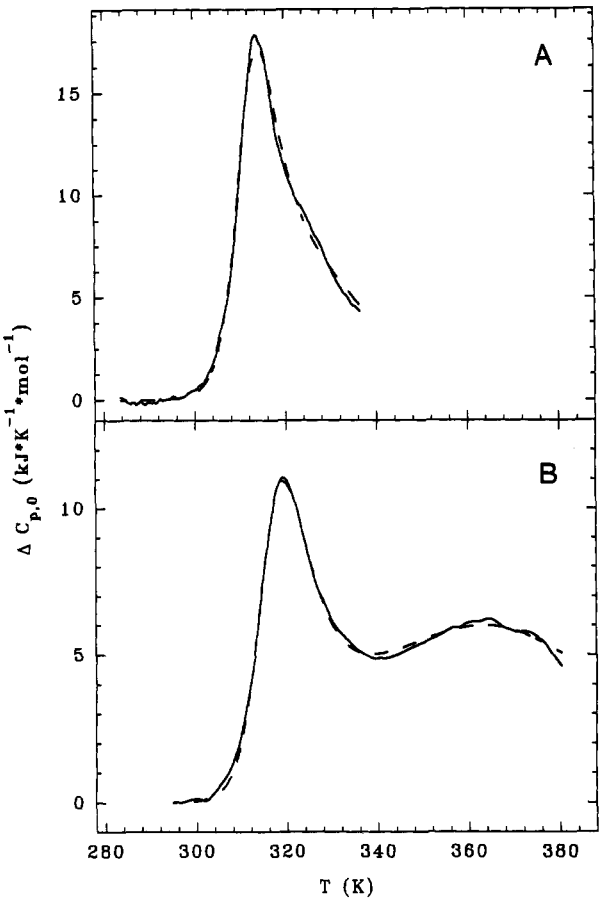

FIGURE 10: Temperature dependencies of $\Delta C_{p,0}$ (—) and their best fits (– –), based on the calorimetric model (see Results), for CheY at pH 7.0 with 2 M urea (A) and at pH 10.3 (B).

populated. When the temperature increases, it is replaced by the dimeric form (10–20% of the protein at 298 K and pH 2.5), the population of which reaches up to 75% of the protein at acidic pH (at neutral and alkaline pH this state becomes almost completely populated since dimers appear to be more stable). Finally, the intermediate state is replaced by the unfolded conformation at very high temperatures. This mechanism of unfolding, as well as the existence of intermediate dimers, was confirmed by thermal gel analysis and cross-linking experiments (see below). For that reason the structural scheme of eq 5 should be replaced by the scheme

$$2N \overset{K_{na}}{\rightleftharpoons} I_2 \overset{K_d}{\rightleftharpoons} 2U \qquad (21)$$

in which the same three states are placed in the order of increasing enthalpy and consequently in the order of their appearance upon heating of the solution. However, whereas the second transition in the latter scheme reflects the dimer dissociation and the denaturation of the intermediate, the first one (K_{na}) corresponds to a complex process of conformational transition accompanied by dimerization. Therefore its thermodynamic parameters do not have a clear structural interpretation.

To explain such a big discrepancy between the small peak area and the high peak sharpness of the first transition in plot C of Figure 9, the terms of eq 14 are shown together with the resulting best-fit curve. It is seen that the endothermic "unfolding" is highly compensated by the exothermic dimerization, since the unfolded molecules immediately start to form intermediate dimers. At higher temperatures these dimers dissociate into the unfolded form with a positive heat effect, giving rise to the second peak of C_p. The unfolding and association processes are strongly coupled, and thus the high tendency to dimerization existing at low temperature facilitates unfolding. This destabilizing effect is clearly seen from a comparison of the actual peak of unfolding with a two-state one having the same thermodynamic parameters. This two-state peak is much higher than the actual one, and its maximum (T_u) is shifted by about 6 K to a higher temperature with respect to T^*. The differences are even more drastic at neutral and alkaline pH, where the unfolding enthalpy is greater and the stabilities of the native and dimeric states are higher.

To reduce the number of variables, the fitting was done under the assumption that the ΔC_p of dimerization/dissociation is negligible. This assumption was supported by the observation that, within the analysis errors, ΔH_a did not depend on T_a even when $\Delta C_{p,a}$ was not assumed to be zero. To calculate $\Delta C_{p,0}$, one has to extrapolate $C_{p,0}$ over the whole transition range as shown in plot A of Figure 9. The uncertainty of such a linear extrapolation might lead to large errors, particularly in those cases when melting starts at a low temperature and the unfolding process is broad, which is exactly the case of CheY. So, we have calculated an average slope of the initial C_p from all our calorimetric records, which was found to be 0.12 ± 0.02 kJ K^{-1} mol^{-1}; this slope was used in the subsequent calculations of $\Delta C_{p,0}$. The average $C_{p,0}$ at 298 K was 20 ± 1 kJ K^{-1} mol^{-1}, as shown in Figure 8. The thermodynamic parameters calculated by applying the three-state schemes in eqs 5 and 21 to the analysis of scanning calorimetry data are given in Table III. These values will be discussed in more detail in the Discussion section.

If the scheme in eq 21 is correct, then we should expect that under those conditions in which there is a significant proportion of the dimer (i.e., pH 2.5, 298 K) an increase in protein concentration will shift the equilibrium toward dimer formation. In order to determine whether that was the case, we followed the changes in fluorescence when protein concentration was increased from 1 μM to 1 mM, at pH 2.5 and 298 K. Since there is a concentration-dependent decrease of the fluorescence above 2 units of absorbance, it is not possible to obtain data susceptible to a quantitative analysis. However, it is possible to see qualitative changes that reflect dimer formation (Figure 11). There is a shift to the blue of the fluorescence maximum with increasing protein concentration that is reflected in the ratio between the emission at 346 and 360 nm. At low protein concentrations the greatest part of the protein is in a monomeric state, in agreement with the chemical denaturation data that estimates that around 95% of the protein is in a native conformation. At the protein concentration used in calorimetry the amount of protein that is in a native conformation is smaller and agrees well with the percentage of native protein (around 80–90%) (Figure 11).

(d) Cross-Linking Experiments. If our calorimetric model is correct, then one would expect to see the formation of dimers or higher complexes at or close to the melting temperature (Tables III and IV). In order to check this hypothesis, we looked at the cross-linking of the protein, in different buffers and temperatures, with formaldehyde. This cross-linking agent has been previously used with tubulin and was found to be quite specific (Serrano & Avila, 1985). As a negative control, we performed the cross-linking reaction in the presence of 6 M urea (Figure 12D), and as would be expected if the cross-linking reaction were specific for protein complexes being formed, no cross-linking of CheY was observed. As a positive

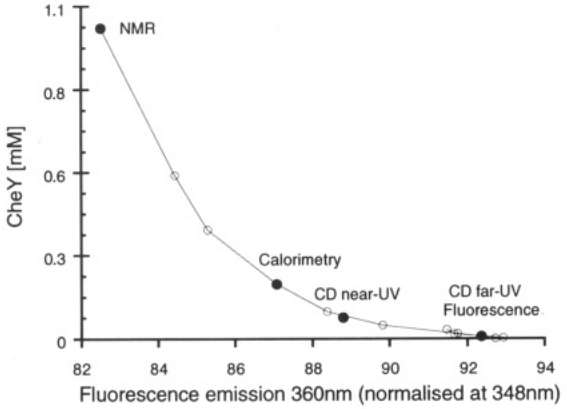

FIGURE 11: Fluorescence changes dependent on protein concentration. One milliliter of a CheY solution in 50 mM glycine buffer, pH 2.5, at 298 K, in the concentration range 1 μM to 1 mM, was excited at 290 nm, and the fluorescence emission was recorded between 300 and 450 nm. The same solution without protein was analyzed in the same manner in order to eliminate the Raman effect. The spectra were normalized at 346 nm, and the emission fluorescence at 360 nm was plotted against protein concentration (○). We also show the protein concentrations used in the following experiments (●): chemical denaturation monitored by fluorescence (Fluorescence), circular dichroism in the near-UV (CD near-UV) or far-UV (CD far-UV), scanning calorimetry (Calorimetry), and nuclear magnetic resonance (NMR).

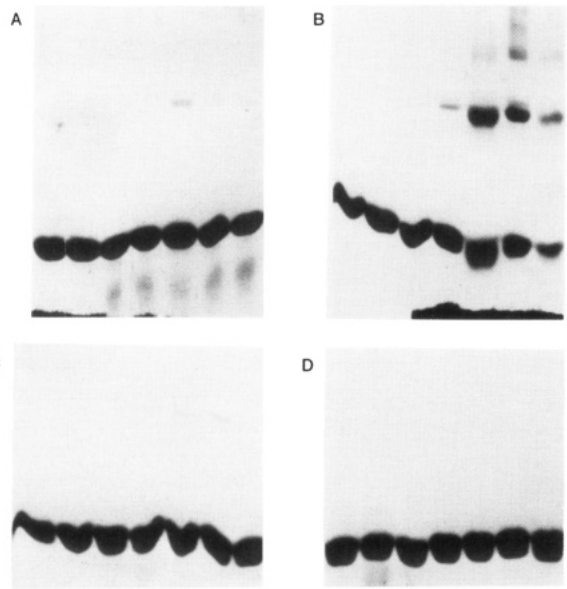

FIGURE 12: Chemical cross-linking of CheY in different buffers at different temperatures. Fifty-microliter aliquots of CheY protein (0.75 mg/mL) in (A) 50 mM sodium glycinate buffer, pH 2.5, (B) 50 mM phosphate buffer, pH 7.0, (C) 50 mM phosphate buffer, pH 7.0, with 2 M urea, and (D) 50 mM phosphate buffer, pH 7.0, with 6 M urea were preincubated for 5 min at different temperatures, and then formaldehyde was added to a final concentration of 1%, w/v. After 10 min, 5× Laemmli buffer was added, and the samples were boiled for 2 min. The lanes from left to right in all panels correspond respectively to 298, 307, 312, 317, 323, 328. and 338K.

control, we cross-linked the protein in 50 mM phosphate buffer, pH 7.0 (Figure 12B). We knew that under these conditions the protein, once heated above 320 K, aggregates in an irreversible manner. As expected, we found that once the protein reached 318 K, there was formation of dimers and higher aggregates. If 2 M urea was present in the experiment, then we found that only dimers were formed at temperatures higher than 318 K, which disappeared at higher temperatures (Figure 12C). In 5 mM glycine buffer, pH 2.5, there is dimer formation at 318 K that slowly disappears at higher temperatures (Figure 12A), as was predicted by the calorimetric model (eq 21).

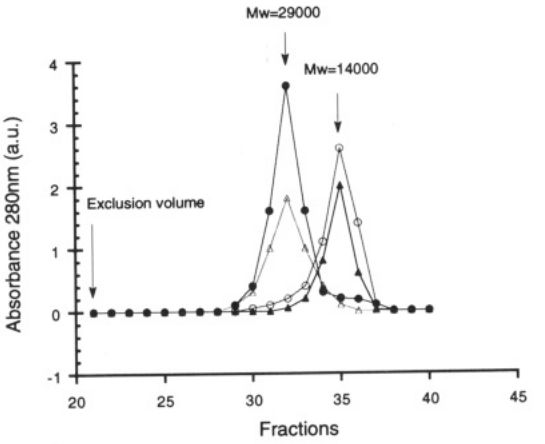

FIGURE 13: Molecular weight chromatographic analysis of CheY at different temperatures. A HiLoad 16/60 Superdex 75 column (Pharmacia) was equilibrated in 5 mM phosphate buffer with 2 M urea. The temperature of the column was controlled by a temperature bath. One hundred microliters of 1 mg/mL carbonic anhydrase (△) (29 000 Da), 100 μL of 1 mg/mL α-lactalbumin (▲) (14 000 Da), or 100 μL of CheY (3.2 mg/mL) in the same buffer was loaded indepently and run into the column equilibrated at 298 and 320 K. The absorbance at 280 nm was monitored. The elution position of CheY changed significantly with the temperature. At 298 K it eluted at its normal position (○) (14 000 Da). However, at 320 K the majority of the protein eluted at the position of carbonic anhydrase, with a small amount still eluting at its normal position (●). The exclusion volume and the elution positions of carbonic anhydrase and α-lactalbumin are indicated by arrows.

(e) Molecular Weight Characterization of the Intermediate. Cross-linking experiments confirm in a very graphic way the calorimetric model, but they cannot prove that the postulated intermediate is a dimer and not something bigger, or a mixture of dimers, trimers etc. In order to demonstrate that this is the case, we have used the same molecular weight column used to purify CheY. The matrix of this column (see Experimental Procedures) can be used in the pH range 3–11 and can withstand temperatures up to 328 K. In the cross-linking experiments we found that there was dimer formation in 2 M urea at pH 7.0 around 320 K. Consequently, we equilibrated the column in 5 mM phosphate buffer, pH 7.0 and 2 M urea and calibrated it with carbonic anhydrase (29 000 Da) and lactalbumin (14 000 Da). We loaded CheY in this buffer and ran it at 298 K; then we raised the temperature in the column to around 320 K and reloaded CheY. In Figure 13we show the elution profile for CheY at the two temperatures indicated above, as well as those for the two molecular weight markers. It seems clear that CheY at 320 K behaves as a molecule with double its molecular weight, thus confirming the calorimetric model.

(f) Thermal Denaturation Monitored by Nuclear Magnetic Resonance. ^{1}H nuclear magnetic resonance spectra of CheY at different pHs and temperatures were measured to obtain information on differences in secondary and tertiary structures. Panels A and B of Figure 14show the spectrum of CheY at pH 7.0 and 308 K in H_2O between 10.5 and 6.8 and between 0.6 and 0 ppm, respectively. Although there must be a significant bleaching of the resonances due to saturation transfer, the strong dispersion of the resonances of both the amide protons (between 10 and 7 ppm) and the methyl protons (between 1.5 and –0.2 ppm) indicates well-defined tertiary structure, as expected for a native conformation. In contrast, in the spectrum in 6 M urea at pH 7.0, the dispersion of the amide (Figure 14C) and the methyl protons (Figure 14D) is

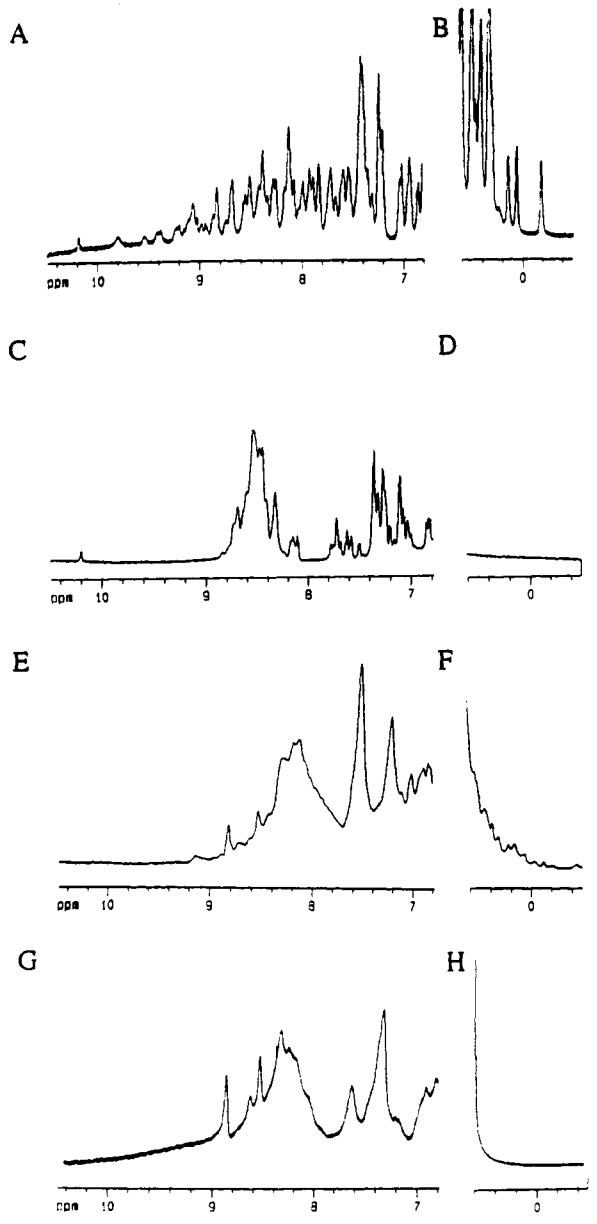

FIGURE 14: Nuclear magnetic resonance analysis of CheY. ^{1}H NMR spectra of CheY, at around 1 mM, were obtained at different temperatures as indicated in methods. The regions corresponding to the amide (A, C, E, G) and methyl protons (B, D, F, H) of CheY at pH 7.0 (A, B, C, D) and pH 2.5 (E, F, G, H) are shown. In panels C and D, we show the spectra corresponding to the protein in 6 M urea. Panels A and B, 311 K; C and D, 281 K; E and F, 294 K; G and H, 316 K. The pH of the CheY solution at a protein concentration of 20 mg/mL was adjusted to pH 2.5 with dilute HCl and to pH 7.0 with phosphate buffer.

dramatically reduced (there is only a small signal left around 10 ppm, and there are no resonances upfield of 8.8 ppm or downfield of 0.6 ppm), as expected for a denatured protein. At pH 2.5 and 298 K, the spectrum is a mixture of the native and a non-native protein conformation (Figure 14E,F). This is what we were expecting on the basis of the calorimetric model and the fluorescence experiment suggesting that, at the protein concentration used in NMR (Figure 11), the greatest part of the protein should be in a non-native dimer conformation. The spectrum of CheY at 313 K and pH 2.5 has no resonances upfield of 9.0 ppm or downfield of 0.6 ppm, indicating that the protein has very little tertiary structure left (Figure 14G,H), as was found by the CD analysis. However, the fact that the line width of the resonances is broader than in 6 M urea and that the dispersion of the chemical shift is larger indicates that the protein is not in a fully random coil conformation. This result, together with the CD analysis of the protein in similar conditions that reveals the presence of considerable secondary but no tertiary structure, strongly suggests that CheY at pH 2.5 and 313 K could be in a molten globule conformation (Eder *et al.*, 1993).

(g) ANS Binding. It has been indicated that the fluorescent compound ANS binds with high affinity to nonpolar ligand pockets of proteins in the folded state and to the so-called molten globule conformation, while it interacts very poorly with fully unfolded proteins (Semisotnov *et al.*, 1991). Since some of the properties of CheY in glycine buffer at pH 2.5 resemble those of other proteins in the molten globule state, we decided to analyze the interaction of ANS with the protein.

The fluorescence emission of ANS is pH dependent, with the maximum below pH 2.5 (Figure 15A). Above pH 4.7 the fluorescence emission is almost the same in the presence or in the absence of protein. When the protein is heated in the presence of 2 M urea at pH 7.0, there is no increase in the ANS fluorescence compared to ANS without the protein (data not shown). On the other hand, when we looked at the ANS binding to CheY at pH 7.0 in the presence of increasing concentrations of urea, we found that in the transition region there is a small increase in the fluorescence emission of ANS that does not disappear completely at higher urea concentrations (Figure 15B). Similar phenomena have been found in other proteins, and it has been indicated that this is due to the fact that any intermediate will be maximal in the transition region (Semisotnov *et al.*, 1991). Addition of increasing concentrations of urea to the protein at pH 2.5 results, as expected, in an almost complete disappearance of the ANS binding, as determined by the fluorescence emission in the absence of protein (data not shown).

In the previous experiments we found that CheY at pH 2.5 and 298 K is in an equilibrium between native monomer and non-native dimers. The NMR results indicate that this non-native dimer could be in a molten globule state. If that is the case, then we should expect it to bind ANS very strongly. If we assume that ANS is mainly binding to the non-native dimer, then the presence of a high concentration of ANS (250/1 molar ratio) will shift the equlibrium toward dimer formation:

$$CheY_{mon} \rightleftharpoons CheY_2 + ANS \rightleftharpoons CheY_2\text{–}ANS \quad (22)$$

If this hypothesis is correct, then we should find that the changes in ANS fluorescence upon dilution of a sample containing ANS and CheY at pH 2.5 and 298 K are not linear (Semisotnov *et al.*, 1991). Figure 15C represents this dependence on a log scale and shows that the dependence is not linear. Extrapolation of the final linear part of this dependence to a zero dilution of the mixture indicates that the intensity of ANS is 15 times less than the initial intensity. This intensity is close to the one found for CheY at pH 7.0 and 298 K, indicating that ANS is binding preferentially to the dimer.

DISCUSSION

We are interested in the analysis of the folding of parallel α/β proteins due to the fact that this class of folding motif has been very extensively studied from a theoretical point of view. CheY has been chosen as a model protein for this folding motif, due to its regularity and small size, and our purpose in this work is to present a detailed thermodynamic characterization, under equilibrium conditions, prior to its kinetic characterization (V. Munoz, E. Lopez, and L. Serrano, manuscript in preparation).

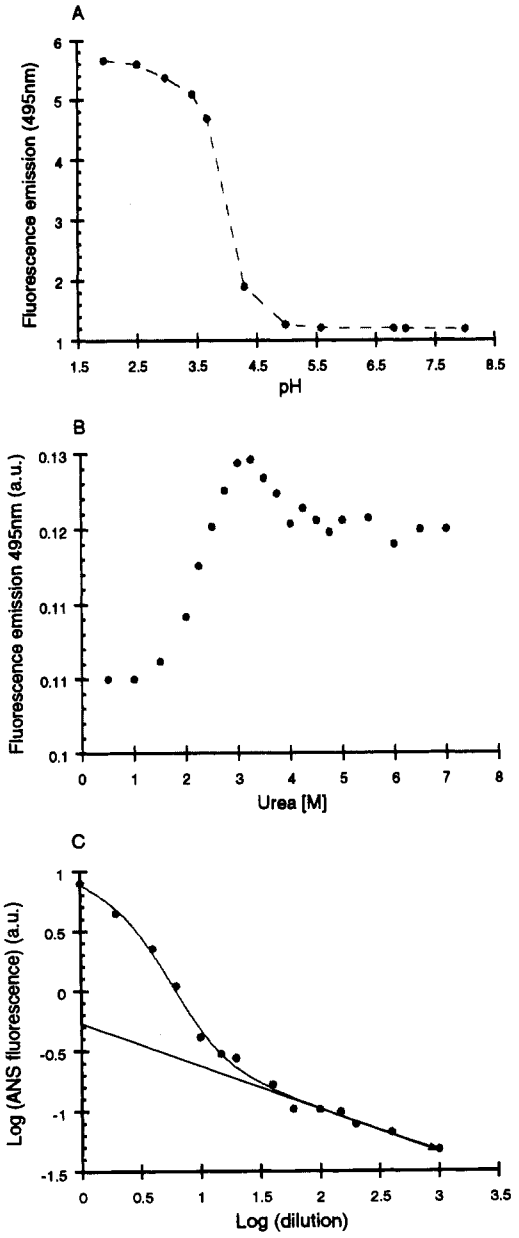

FIGURE 15: ANS interaction with CheY. The concentration of CheY was 1 μM, and that of ANS, 250 μM. In order to monitor any changes in the emission fluorescence of ANS induced by changes in the experimental conditions, the same experiments were carried out without CheY. The emission values of ANS without CheY were subtracted from those of ANS with CheY, and the difference was plotted. (A) pH dependence of the interaction of ANS with CheY. (B) CheY in 50 mM Na-Pipes, pH 7.0, and different urea concentrations. (C) Dilution-induced changes in the fluorescence emission of ANS. A 1 μM CheY solution with 250 μM ANS, in 50 mM glycine buffer at 298 K, was diluted in the range 0–1000 times with 50 mM glycine buffer, and the intensity of the fluorescence emission was recorded. On the y-axis we present the log of the intensity of the fluorescence emission; on the x-axis, the log of the dilution factor.

Integrity of the Purified Protein. CheY has been previously purified from *E. coli* by other groups (Matsumura *et al.*, 1984; Stock *et al.*, 1989). It has been indicated that *E. coli* CheY can be purified in the metal-bound (CheY1) or the metal-free form (CheY2) (Kar *et al.*, 1992). These two forms differ in their metal binding affinities as well as in the chemical shifts of some aromatic residues. In order to prepare metal-free CheY, the protein needs to be washed several times with excess EDTA, and once the metal is removed, the protein does not return to its original conformation by re-adding the metal (Kar *et al.*, 1992). Expression of CheY protein in our hands resulted in the formation of inclusion bodies which make up around 80% of the protein. This protein can be easily renatured by denaturing it with 6 M urea and diluting the urea 10 times in Tris-HCl, 10 mM, at pH 8.0. On the other hand, the soluble protein is purified to homogeneity without being exposed to any chelating agent, and consequently, according to Kar *et al.* (1992), it should have a Mg^{2+} ion bound to it. We could not find any significant difference in the properties of the two CheY preparations with any of the methods we used. Moreover, the binding constant for magnesium, using the same conditions described by Kar *et al.* (1992), is identical for both preparations and to that of the so-called free-metal CheY2, and it is also very similar to the results found by other groups for *E. coli* CheY (Lukat *et al.*, 1990; Needham *et al.*, 1993). Mass spectroscopy analysis of both fractions did not reveal any Mg atom bound to the protein. The fact that we could not find any differences between the two CheY preparations could be due to the different purification methods used. In our case we used a method similar to that used to purify CheY from *S. typhimurium* (Stock *et al.*, 1989; Lukat *et al.*, 1990). This method involves using an anion-exchange resin before a molecular weight column. Kar *et al.* (1992), on the other hand, used a cibacron blue column prior to the molecular weight column. We attempted the purification of CheY on a cibacron blue column, but in our hands we could not detect any specific binding. We cannot rule out the possibility that only the magnesium-free protein will bind to the MonoQ column and, consequently, that we are losing the magnesium-bound form of CheY. NMR (Bruix *et al.*, 1993) and crystallography studies (Bellsolell *et al.*, manuscript in preparation) on the insoluble purified fraction of CheY indicate that the secondary and tertiary structures are almost identical to those previously found with X-ray crystallography. Consequently, all our studies have been performed on what Kar *et al.* (1992) have denominated the CheY2 conformation.

CheY Stability. The ΔG_{H_2O} value is a measure of the conformational stability of the protein. CheY is maximally stable around pH 5.1. In CheY there are no His residues, and the only groups that could titrate around pH 5.0 should be the three Asp residues in the active site. This is probably so because they are very close to each other (the distances between the side-chain $O_{\delta 1}$a and $O_{\delta 2}$ groups range from 3 to 6 Å), two of them are buried (Asp12 and Asp57), and only Asp57 makes a good salt bridge to Lys109. Asp12 makes two hydrogen bonds to two water molecules, while Asp13 makes one hydrogen bond to a water molecule and its own main-chain NH group (Volz & Matsumura, 1991). Consequently, there is an excess negative charge in a very small and confined space that should raise the pK_a of at least one of the three Asp residues. The fact that CheY stability is increased by almost 4.2 kJ mol^{-1} at high ionic strength (0.6 M NaCl) or by up to 6.9 kJ mol^{-1} in the presence of 80 mM $MgCl_2$ confirms that the repulsion of the three Asp residues is one of the reasons for the low stability of CheY at pHs greater than 5.1. The stability of the protein does not change noticeably between pH 6.3 and 8.0, and it decreases dramatically below pH 4.4. At low pH, where CheY will have a large positive charge, the pKs of the carboxyl groups in the folded state are expected to be lower than in the unfolded protein, and consequently the unfolded protein will bind protons more tightly than the native state (Nozaki & Tanford, 1967).

How Unfolded Is the Chemically Denatured State? There is increasing evidence that proteins do not unfold completely in 8 M urea or 6 M GdnHCl [for a review, see Shortle (1993)].

It has been found by NMR studies that the structures present in the unfolded states are due mainly to hydrophobic clustering (Evans *et al.*, 1991). The presence of residual structure in the denatured state results in a smaller value for the free energy of unfolding and also a smaller value of m since it is related to the average fractional change in degree of exposure of residues on denaturation (Foss & Schellman, 1959). The m value at pH 6.3 for CheY is somehow small when compared with proteins of equivalent size (110 versus 129 residues) such as barnase ($m = 7.9 \pm 0.3$ kJ mol^{-1} M^{-1} at pH 6.3) (Pace *et al.*, 1992; Horovitz *et al.*, 1992). There is also the fact that the unfolded protein binds ANS at pH 7.0. These data could indicate that there is some residual structure left in 8 M urea at pH 7.0. ^{1}H NMR analysis of the denatured protein does not reveal the presence of any residual structure, although this does not demonstrate its total absence.

CheY Conformation at pH 2.5 and 7.0. How different are the conformations of the protein at pH 2.5 and 7.0? Fluorescence analysis of CheY indicates that Trp58 is in a slightly more hydrophobic enviroment at acidic pH. This means either that a negatively charged group close to Trp58 (Asp57 or Asp64) is not charged anymore or that a small conformational rearrangement has taken place. CD analysis of CheY at pH 2.5 and 7.0 indicates that the protein is essentially folded at pH 2.5 and 298 K, although there are minor conformational rearrangements detected in the near-UV spectra.

Urea denaturation shows that CheY unfolding at pH 2.5 and 298 K exhibits a cooperative behavior indicative of a folded structure and that around 5% of the protein is already in a non-native conformation.

Temperature denaturation of CheY at pH 2.5 or 7.0, monitored by CD, indicates the existence of an early transition corresponding to the loss of the tertiary interactions responsible for the asymmetric enviroment of the aromatic residues. This transition can be detected by a native temperature gradient gel at pH 2.5 (data not shown), in which the temperature at which 50% of the protein is native corresponds to that at which 50% of the protein retains the aromatic tertiary interactions (Table IV). At the same time, the fluorescence emission of the protein decreases linearly and there is a small blue shift of the maximum indicating less exposure of the Trp residue. Then there is a second transition, which is detected only by fluorescence, in which the Trp becomes as exposed to the solvent as in 6 M urea. The analysis of calorimetric data at pH 2.5 clearly reveals two subsequent cooperative transitions that coincide with the ones detected by CD and fluorescence (Table IV), the first one being similar to the one at pH 7.0. In addition, calorimetric analysis shows that at pH 2.5 and 298 K around 10–20% of the protein is already in a non-native conformation.

The main difference between pH 2.5 and 7.0, at 298 K, is the fact that ANS binds strongly to CheY at pH 2.5. However, as we have seen previously, this binding is concentration dependent. ANS binds very strongly to a protein complex at pH 2.5, while it binds to the monomeric form of the protein at pH 2.5 to a similar extent as to CheY at pH 7.0.

All these data suggest that the conformation of CheY at pH 2.5 and 298 K is quite similar to the one at pH 7.0.

Temperature Denaturation. The thermodynamic analysis of the reversible thermal denaturation of CheY is complicated due to the pH-dependent formation of dimers during protein unfolding, as shown by the cross-linking and molecular weight chromatography experiments. At acidic and alkaline pH values the formation of dimers is a reversible process, which permits a thermodynamic analysis. At neutral pH and low ionic strength the thermal process is reversible as long as the protein is not exposed to temperatures higher than 318 K, that is to say, before the formation of irreversible aggregates. Formation of dimers explains why, upon heating the protein at low pH or at neutral pH in the presence of 2 M urea, there is no significant change in the fluorescence spectrum apart from a slight shift of 2 nm to the blue. The only fluorescence changes that we can detect correspond to the complete unfolding of the protein that should take place after or simultaneously with the dissociation of the dimer. The formation of dimers is an exothermic process which adds negatively to the endothermic denaturation process.

From a thermodynamic point of view nothing is unusual in such an unfolding pathway, particularly when the scheme in eq 21 is considered instead of that in eq 5: upon heating, the CheY molecule passes through equilibrium states with increasing enthalpy. Our analysis has shown that only one intermediate state is highly populated, and this intermediate should be a dimer, since for a trimer the fitting error, as given by the fitting program, is three times higher than for a dimer (for higher aggregates the error is higher than for a trimer; data not shown). This hypothesis has been confirmed by the molecular weight chromatographic experiment. At 308–318 K this dimer is separated from both the native and the unfolded state by an enthalpy change of about 150 kJ mol^{-1}, and it is much more stable than the native conformation. For these reasons two well-separated peaks are visible in the thermograms, and it is possible to cross-link the dimers at selected temperatures.

With respect to the meaning of the parameters in Table IV and their correlation with other thermodynamic data we should point out, as was mentioned above, that the second broad peak on the thermograms corresponds exclusively to the dimer dissociation. Its presence on the analyzed curve is necessary for justification of the association/dissociation parameters, which is why the parameters found by curve fitting at acidic and alkaline pH are reliable. On the contrary, at pH 7 the parameters were calculated with some uncertainty due to the lack of the dissociation peak and the relatively high errors in measurement of C_p. This uncertainty concerns mostly the T_a and T_u values, which have a large error. It was also useless to calculate ΔG from pH 7 data, whereas the values of ΔH_u and ΔH_a turned out to be much less sensitive to experimental errors and to the absence of the second peak from the thermograms. As pointed out before, the actual position of the first peak maximum (T^*) does not, in general, coincide with T_u and, depending on solvent conditions and the magnitude of the association constant, might be shifted from T_u to lower temperatures by several degrees. However, T^* is the value that should be compared with the transition temperatures registered by other techniques (Table IV). As we have stated above, there is very good agreement between the T^* and T_a values, at pH 2.5 and 7.0 with 2 M urea, and the transition temperature values determined from CD, fluorescence, cross-linking, and calorimetry.

What Is The Nature of the Dimer Formed upon Thermal Denaturation of the Protein? It is generally assumed in equilibrium denaturation studies of proteins that the system behaves as a two-state one in which only the folded and unfolded forms of the protein are significantly populated. However, an equilibrium intermediate has been detected in a number of different proteins under very acidic or basic conditions, in different salt concentrations, or in the presence of organic solvents; in proteins modified by removing a ligand

or a prosthetic group, or by breaking disulfide bridges; and in chemically denatured proteins upon heating or in some temperature-denatured proteins at neutral pH (Denton *et al.*, 1981; Ohgusi & Wada, 1983; Dolgikh *et al.*, 1984, 1985; Brahzhnikov *et al.*, 1985; States *et al.*, 1987; Kuwajima, 1989; Baker et al., 1992; Bismuto *et al.*, 1992; Gottfried & Haas, 1992; Eder *et al.*, 1993). In all cases these intermediates share some common properties (Kuwajima, 1989), which are present in the dimer formed upon thermal unfolding of CheY at pH 2.5: (a) The fluorescence spectrum of the complex measured at 318 K is very similar to that of the native protein, the main difference being a blue shift of around 2 nm, indicating that the Trp is slightly less exposed in that state. (b) CD analysis of CheY, at temperatures at which the dimer is supposed to be the major component, shows that it has a high secondary structure content with little or no native tertiary structure. (c) Nuclear magnetic resonance studies of the protein at pH 2.5 and at a temperature at which almost 100% of the protein should be dimerized indicate that the structure of the complex is intermediate between the folded and unfolded states. There is considerable loss in the dispersion of the amide and methyl regions of its ^1H nuclear magnetic resonace spectrum, but less than in 6 M urea and with broader line widths. (d) As we have seen from the dilution experiments, ANS binds mainly to the dimeric state, as expected if it is in a molten globule state conformation. (e) The calorimetric data alone are not sufficient to elucidate the nature of these dimers, namely, to answer the question, is their conformation very different from that of the unfolded state? One cannot exclude the possibility that there is no conformational change when the dimers are formed from a hypothetical kinetic intermediate state between the folded state and the dimer. Then all the heat effect would only arise from the formation of the contact area from the already existing elements of structure. It must be noted, however, that structures participating in the contact melt cooperatively at relatively high temperature with a heat effect corresponding to about half of the overall unfolding enthalpy of ribonuclease A at 318 K (Privalov & Khechinashvili, 1974). This is a rather high heat effect, taking into account the fact that ribonuclease has a similar molecular weight to CheY and also one of the highest specific enthalpies of unfolding at low temperature. Therefore, judging only from the thermodynamic parameters, it seems that both transitions in eq 21 should be accompanied by rather large conformational changes.

The nuclear magnetic resonance, CD, and fluorescence data indicate that the dimer at pH 2.5 could then be considered the result of the specific interaction of two molten globule monomers. That would fit well with the way we postulate ANS interacts with CheY at low pH. On the other hand, it is difficult to reconcile the idea of a molten globule state with a specific interaction between two molecules and with the great heat effect found upon dissociating the complex. One of the plausible explanations for these results is that the region of the molecule involved in the interaction is a localized region of supersecondary structure that consequently possesses a certain degree of cooperativity. These regions have been found in the intermediate state of α-lactalbumin (Kuwajima, 1989), and their existence has been suggested also in the molten globule of subtilisin (Eder *et al.*, 1993).

At pH 7.0 the interpretation is not easy since the fluorescence of ANS in the presence of CheY is almost insignificant (although it is higher at those urea concentrations where we would expect the presence of an intermediate), and there is no increase of ANS binding with temperature. NMR is not an appropriate technique at this pH because of the irreversible aggregation of the protein at high concentrations. The fact that in many other proteins ANS fluorescence is much higher in the presence of acidic molten globules than with neutral ones (Semisotnov, 1991) could be due to the anionic character of ANS and its weaker binding to negatively charged molecules. At neutral pH the thermal denaturation of the protein also seems to be monitored by the reversible formation of the complex, which at this pH gives rise on heating to the irreversible formation of aggregates. Nevertheless, once the main cooperative transition has occurred, the protein loses its native structure but still possesses significant secondary structure. At alkaline pH the situation appears to be qualitatively similar to that at acidic pH, although the dissociation temperature appears to be somewhat higher, i.e., the dimers are more stable. Therefore, to all ends and purposes, it is reasonable to assume that the nature of the intermediate complex formed by partially unfolded monomers should be very similar at all pH values investigated and corresponds to a dimer in the molten globule state. This is in accord with the well-known tendency of the molten globule to undergo association processes (Kuwajima, 1986).

The formation of the molten globule dimer was not detected at low temperatures in the case of chemical denaturation, where a simpler mechanism, with a very unstable intermediate only detectable by ANS binding in the transition region, would operate.

An important question that is left open is whether the intermediate dimer detected upon thermal denaturation is obligatory to the folding of CheY. Kinetic analysis of the folding and unfolding reactions of CheY (V. Muñoz, E. Lopez, and L. Serrano, manuscript in preparation) indicates that there is no concentration dependence in the folding and unfolding rates and consequently that if formation of the dimer is obligatory in the folding reaction, it does not affect, within experimental error, the rate constants of both reactions.

CONCLUSIONS

The thermodynamic analysis of CheY has revealed that the protein is not very stable compared to other proteins of similar size. This lack of stability is probably due to the strong electrostatic repulsion potential around the three Asp residues of the active site. Chemical denaturation of the protein at neutral pH suggests that some residual structure is present in the unfolded state. The analysis of the temperature-induced denaturation of CheY has been shown to be particularly complicated due to the existence of a very stable dimeric intermediate. At neutral pH the protein melting is accompanied by irreversible aggregation of the intermediate dimers, but dimer formation can be reversed as long as the protein is not heated above 318 K or if 2 M urea is present. The intermediate dimers formed at pH 2.5, and probably at alkaline and neutral pH, have the majority of the properties of the molten globule state. This mechanism of temperature denaturation, which includes a dimeric molten globule, can play a role in the unfolding of many other proteins. For example, it could explain why molten globule states of some proteins have increased molecular weight as well as unusually high stability and cooperativity of unfolding.

ACKNOWLEDGMENT

We would like to thank Dr. T. Creighton for his useful remarks and suggestions concerning this manuscript. We are grateful to Victor Muñoz and Eva Lopez for supplying some of the CheY protein and to Dr. P. Matsumura and Dr. Stock

for providing the X-ray crystal coordinates of CheY from *E. coli and S. typhimurium*, respectively. We would like to thank Deborah Sanders for technical assistance. We are also very grateful to the EMBL Peptide Service for the amino acid sequence and mass spectroscopy analyses.

REFERENCES

Anfisen, C. B. & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Baker, D., Sohl, J. L. & Agard, D. A. (1992) *Nature 356*, 263.
Baum, J., Dobson, C. M., Evans, P. A., & Hanley, C. (1989) *Biochemistry 28*, 7.
Bismuto, E., Sirangelo, I., & Irace, G. (1992) *Arch. Biochem. Biophys. 298*, 624.
Bowie, J. U., Clarke, N. D., Pabo, C. O., & Sauer, R. T. (1990) *Proteins: Struct., Functi., Genet. 7*, 257.
Brazhnikov, E. V., Chigadze, D. A., Dolgikh, D. A., & Ptitsyn, O. B. (1985) *Biopolymers 24*, 1899.
Bruix, M., Pascual, J., Santoro, J., Prieto, J., Serrano, L., & Rico, M. (1993) *Eur. J. Biophys.* (in press).
Burstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.
Chen, J. M., Murphy, R. B., Brandt-rauf, P. W., & Pincus, M. R. (1990) *Int. J. Pept. Protein Res. 36*, 11.
Clackson, T., Gussow, D., & Jones, P. T. (1991) in *PCR: A practical approach* (McPherson, M. J., Quirke, P., & Taylor, G. R., Eds.) pp 187–214, IRL Press, Oxford.
Denton, J. B., Konishi, Y., & Scheraga, H. (1982) *Biochemistry 21*, 5155.
Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Bushuev, V. N., Gilmanshin, R. I., Lebedev, Yu. O., Semisotnov, G. V., Tiktopulo, E. I., & Ptitsyn, O. B. (1984) *Eur. J. Biophys. 13*, 109.
Dolgikh, D. A., Kolomiets, A. P., Bolotina, I. A., & Ptitsyn, O. B. (1984) *FEBS Lett. 165*, 88.
Eder, J., Rheineker, M., & Fersht, A. R. (1993) *Biochemistry 32*, 18.
Evans, P. A., Topping, K. D., Woolfson, D. N., & Dobson, C. M. (1991) *Proteins: Struct., Funct., Genet. 9*, 248.
Ewbank, J. J., & Creighton, T. E. (1992) *Nature 350*, 518.
Filimonov, V. V., Potekhin, S. A., Matveyev, S. V., & Privalov, P. L. (1982) *Mol. Biol. (Moscow) 16*, 551.
Foss, J. G., & Schellman, J. A. (1959) *J. Phys. Chem. 63*, 2007.
Freire, E., & Biltonen, R. L. (1978) *Biopolymers 17*, 446.
Gill, S. C., & Hippel, P. H. (1989) *Anal. Biochem. 182*, 319.
Goto, Y., & Fink, A. L. (1989) *Biochemistry 28*, 945.
Gottfried, D. S., & Haas, E. (1992) *Biochemistry 31*, 12353.
Horovitz, A., Matthews, J., & Fersht, A. R. (1992) *J. Mol. Biol. 227*, 560.
Kar, L., De Croos, P. Z., Roman, S. J., Matsumura, P., & Johnson, M. E. (1992a) *Biochem. J. 287*, 533.
Kar, L., Matsumura, P., & Johnson, M. E. (1992b) *Biochem. J. 287*, 521.
Kellis, J. T., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 4914–4922.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631.
Kuwajima, K. (1986) *Proteins 6*, 87.
Laemmli, U. K. (1970) *Nature 227*, 680.
Lecomte, J. T. J., & Matthews, C. R. (1993) *Protein Eng. 6*, 1.
Levinthal, C. (1968) *J. Chim. Phys. Phys.-Chim. Biol. 65*, 44.
Lukat, G. S., Stock, A. M., & Stock, J. B. (1990) *Biochemistry 29*, 5436.
Makhatadze, G. I., & Privalov, P. L. (1992) *J. Mol. Biol. 226*, 491.
Matsumura, P., Rydel, J. J., Linzmeier, R., & Vacante, D. (1984) *J. Bacteriol. 160*, 36.
Mayr, L. M., & Schmid, F. X. (1993) *Biochemistry 32*, 7994.
Mulqueen, P. M., & Kronman, M. J. (1982) *Arch. Biochem. Biophys. 215*, 28.
Needham, J. V., Chen, T. Y., & Falke, J. J. (1993) *Biochemistry 32*, 3363.
Nozaki, Y., & Tanford, C. (1967) *Methods Enzymol. 11*, 715.
Ohgusi, M., & Wada, A. (1983) *FEBS Lett. 164*, 21.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266.
Pace, C. N., Laurents, D. V., & Erickson, R. E. (1992) *Biochemistry 31*, 2728.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665.
Privalov, P. L., & Potekhin, S. A. (1986) *Methods Enzymol. 113*, 4.
Privalov, P. L, Tiktopulo, E. I., Venyaminov, S. Yu., Griko, Yu. V., Makhtadze, G. I., & Khechinashvili, N. N. (1989) *J. Mol. Biol. 205*, 737.
Sanders, D. A., Gillece-Castro., B. L., Stock, A. M., Burlingame, A. L., & Koshland, D. E., Jr. (1989) *J. Biol. Chem. 264*, 21770.
Sanger, F., Nicklen, S., & Coulson, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Segel, I. H. (1975) in *Enzyme Kinetics: Behaviour Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 218–220, Wiley, New York.
Semisotnov, G. V, Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A.F., & Gilmanshin, R. I. (1991) *Biopolymers 31*, 119.
Serrano, L., & Avila, J. (1985) *Biochem. J. 230*, 551.
Serrano, L., Horovitz, A., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *Biochemistry 29*, 9343.
Shortle, D. (1993) *Curr. Opin. Struct. Biol. 3*, 66.
States, D. J., Dobson, C. M., Karplus, M., & Creighton, E. (1987) *J. Mol. Biol. 195*, 731.
Stock, A. M., Mottonen, J. M., Stock, J. B., & Schutt, C. E. (1989) *Nature 337*, 745.
Stock, J. B., Stock, A. M., & Mottonen, J. M. (1990) *Nature 344*, 395.
Volz, K., & Matsumura, P. (1991) *J. Biol. Chem. 266*, 15511.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697.