Thermodynamic characterization of monomeric and dimeric forms of CcdB (controller of cell division or death B protein)

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The protein CcdB (controller of cell division or death B) is an F-plasmid-encoded toxin that acts as an inhibitor of *Escherichia coli* DNA gyrase. The stability and aggregation state of CcdB have been characterized as a function of pH and temperature. Size-exclusion chromatography revealed that the protein is a dimer at pH 7.0, but a monomer at pH 4.0. CD analysis and fluorescence spectroscopy showed that the monomer is well folded, and has similar tertiary structure to the dimer. Hence intersubunit interactions are not required for folding of individual subunits. The stability of both forms was characterized by isothermal denaturant unfolding and calorimetry. The free energies of unfolding were found to be 9.2 kcal · mol^{-1} (1 cal ≈ 4.184 J) and 21 kcal · mol^{-1} at 298 K for the monomer and dimer respectively. The denaturant concentration at which one-half of the protein molecules are unfolded (C_m) of the dimer is dependent on protein concentration, whereas the C_m of the monomer is independent of protein concentration, as expected. Although thermal unfolding of the protein in aqueous solution is irreversible at neutral pH, it was found that thermal unfolding is reversible in the presence of GdmCl (guanidinium chloride). Differential scanning calorimetry in the presence of low concentrations of GdmCl in combination with isothermal denaturation melts as a function of temperature were used to derive the stability curve for the protein. The value of ΔC_p (representing the change in excess heat capacity upon protein denaturation) is 2.8 ± 0.2 kcal · mol^{-1} · K^{-1} for unfolding of dimeric CcdB, and only has a weak dependence on denaturant concentration.

Key words: CcdB (controller of cell division or death B protein), DSC (differential scanning calorimetry), GdmCl (guanidinium chloride), stability curve.

INTRODUCTION

The protein CcdB (controller of cell division or death B) is encoded by plasmid F [1,2] in *Escherichia coli*, and is involved in its maintenance, along with CcdA. The protein is a poison of DNA gyrase, and is a potent cytotoxin. CcdB is a small, 101-amino-acid homodimeric protein. Since expression of active CcdB protein results in cell death, it is very easy to select for inactive and temperature-sensitive mutants of the protein, and we are currently using this as a model system to understand the molecular basis for temperature-sensitivity. We have so far isolated approximately 20 temperature-sensitive mutants of the protein, and are studying the relationship between protein stability, expression level and the temperature-sensitive phenotype (K. Bajaj, G. Chakshusmathi and R. Varadarajan, unpublished work). In the present study, we report a detailed thermodynamic characterization of the wild-type protein. This thermodynamic data is essential to interpret stability and folding data of temperature-sensitive mutants of CcdB. Intersubunit interactions are typically thought to be important for stabilization of multimeric proteins. Although CcdB is a dimeric protein at neutral pH, in the present work we show that intersubunit interactions are not required for the folding of CcdB. Size-exclusion chromatography was used to characterize the aggregation state of the protein as a function of pH. Whereas CcdB at neutral pH is a dimer, at pH 4.0 it dissociates into folded monomers that have very similar secondary and tertiary structure to the native dimer. Isothermal denaturation studies were used to measure the stabilities of both monomeric and dimeric forms of the protein. This is typically not possible to do for multimeric proteins, because it is difficult to obtain solution conditions where folded monomers are significantly populated. In addition, the stability of the dimeric form was characterized by DSC (differential scanning calorimetry) as a function of denaturant concentration to obtain detailed information on the stability of CcdB as a function of denaturant concentration and temperature.

EXPERIMENTAL

Protein expression and purification

The CcdB gene was amplified from the vector pZero-2 (Invitrogen) and cloned into the expression vector pBAD24 [3]. CcdB was expressed from pBAD24 containing the CcdB gene coding for the 101-amino-acid CcdB polypeptide under control of the *AraC* promoter in *E. coli* strain CSH501. The strain carries the CcdB-resistance mutation $gyrA_{462}$, and was obtained from Dr M. Couturier (Laboratory of Prokaryote Genetics, Institute of Biology and Molecular Medicine, Free University of Brussels, Belgium). This strain also carries the pSC101 plasmid [1], which expresses $lacI^q$ for tight regulation of protein synthesis. The protein was purified by a slight modification of the procedure published previously [2]. Cells were grown to a D_{600} of 0.5, induced with 0.5 % (w/v) arabinose and grown for 4 h at 37 °C. Cells were harvested and sonicated, and the nucleic acids were precipitated with 0.5 % (v/v) polyethylenimine. Ammonium sulphate was added to the solution to a final concentration of 80 %.

Abbreviations used: ANS, 8-anilinonaphthalene-l-sulphonic acid; CcdA/CcdB, controller of cell division or death A (or B) protein; DSC, differential scanning calorimetry; GdmCl, guanidinium chloride.

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The pellet was re-dissolved in 3–6 ml of 20 mM Tris/HCl, pH 8.5, and loaded on to a Sephacryl S-100 gel-filtration column pre-equilibrated with the same buffer. The fractions containing CcdB were pooled, and purified using a Resource Q (6 ml; Amersham Biosciences) ion-exchange column using a gradient of 50–300 mM NaCl. CcdB was typically eluted at approx. 100 mM NaCl. Fractions containing CcdB were pooled and dialysed against 10 mM Hepes, pH 7, concentrated and stored at − 70 °C. Protein purity was checked by SDS/PAGE (15 % gels), and found to be ≈95 % pure. The activity of CcdB was checked by the gyrase inhibition assay [1]. The molar absorption coefficient (ε) at 280 nm was taken to be 1.4 $M^{-1} \cdot cm^{-1}$ for monomeric CcdB [4]. All CcdB concentrations reported here are in monomeric units, unless otherwise stated.

Gel filtration

The oligomeric status of CcdB at pH 7.0 and pH 4.0 was checked by gel-filtration chromatography using a Superdex-75A column (column volume, V_t, of 24 ml) on a Bio-Rad Biologic Duo Flow FPLC system. Buffers used (for gel-filtration studies at pH 7.0 or pH 4.0 respectively) were 10 mM Hepes/NaOH, pH 7.0, containing 200 mM NaCl or 10 mM acetate, pH 4.0, containing 200 mM NaCl. For some of the studies at pH 7.0, urea concentrations ranging from 1–3 M were present. The buffer was degassed, and the column was equilibrated with the buffer before injecting the sample. Samples (50 μg) of protein equilibrated in the appropriate buffer were loaded on to the column and eluted at a flow rate of 0.4 $ml \cdot min^{-1}$. The column was equilibrated with the following set of marker proteins: thioredoxin (11.7 kDa), thioredoxin dimer (23 kDa), ovalbumin (44 kDa) and RNase A (13.7 kDa). Standard calibration curves were obtained by plotting the ratio of elution volume and void volume (V_e/V_o) against log molecular mass [5]. Void volume was measured by running a Blue Dextran marker at both pHs. Gel-filtration profiles of CcdB at pH measurements of 4.2, 4.4, 4.6, 5.0, 6.0, 8.0 and 9.0 were also obtained in a similar fashion using CGH10 buffer (10 mM citrate/10 mM glycine/10 mM Hepes) at the respective values of pH.

Spectral measurements

Fluorescence emission spectra were collected on a Jasco FP-777 spectrofluorimeter or SPEX Fluoromax-3 using a 1 cm cuvette. Samples were excited at 280 nm and spectra were recorded from 300–400 nm. Typically, 2 μM protein was used in all fluorescence studies, unless otherwise stated. The CD measurements were made on a Jasco-J715A spectropolarimeter using a 0.2 cm path-length for far-UV CD, and a 1 cm path-length for near-UV CD measurements. Spectra were collected with a slit width of 1 nm, a response time of 4 s and a scan speed of 20 $nm \cdot min^{-1}$. The sample concentration was approx. 15 μM for far-UV CD, and approx. 35 μM for near-UV CD studies. Each data point was an average of four accumulations. For ANS (8-anilinonaphthalene-1-sulphonic acid) binding studies, a 10-fold excess of ANS was added to the protein solution (typically 1 μM), and emission spectra were recorded after excitation at 380 nm.

Isothermal denaturation

Equilibrium unfolding as a function of urea/GdmCl (guanidinium chloride) concentration was monitored by fluorescence spectroscopy. Fluorescence measurements were performed using a SPEX Fluoromax-3 spectrofluorimeter with a 1 cm water-jacketed cell. The excitation and emission wavelengths were fixed at 280 nm and 385 nm respectively, with slit widths of 2 nm for both excitation and emission monochromators. Each measurement was an average of four readings.

Isothermal urea-denaturation studies of wild-type CcdB were performed at six temperatures between 10 °C and 43 °C. Isothermal denaturation studies could not be executed at temperatures higher than 43 °C, because of sample aggregation problems. GdmCl-dependent studies were also carried out between 10 °C and 55 °C for CcdB protein. The concentrations of both urea and GdmCl were calculated from measurements of the index of refraction. The values of ΔG° (the free energy change upon protein unfolding at zero denaturant concentration) and m (the linear dependence of free energy upon protein unfolding on denaturant) at a given temperature were estimated according to the linear free energy model [6]. According to the linear free energy model, the changes in free energy and enthalpy upon unfolding depend linearly on denaturant concentration:

$$\Delta X^\circ_D = \Delta X^\circ + m_X[D] \quad (1)$$

where X represents G or H (Gibb's free energy or enthalpy respectively), m is the slope of the transition and ΔX° corresponds to the difference in X between the unfolded and the folded states in the absence of any denaturant (D).

The unfolding of CcdB is described by the following equations:

$$N_2 \overset{K_{eq}}{\rightleftharpoons} 2U \quad (2)$$

$$K_{eq} = [U]^2/[N_2] = 2P_t\left[f_u^{\,2}/(1 - f_u)\right] \quad (3)$$

where N and U represent the native and unfolded states respectively, P_t is the total protein concentration in terms of monomer units and f_u is the fraction of unfolded protein. The equilibrium constant (K_{eq}) and free energy of unfolding (ΔG°) are related by:

$$\Delta G^\circ_D = -RT \ln K_{eq} \quad (4)$$

where R is the gas constant and T is the absolute temperature.

Using eqn (4), eqn (3) can be written as:

$$e^{-\Delta G^\circ D/RT}/2P_t = \left[f_u^{\,2}/(1 - f_u)\right] \quad (5)$$

By rearranging and substituting $e^{-\Delta G^\circ D/RT}/2P_t$ with z in eqn (5), one obtains:

$$f_u = 0.5\left[-z \pm (z^2 + 4z)^{1/2}\right] \quad (6)$$

The spectroscopic signal (Y) of a protein solution (in our case, fluorescence intensity or CD signal) is related to the fraction of unfolded protein (f_u) by:

$$f_u = (Y - Y_f)/(Y_u - Y_f) \quad (7)$$

where Y_f and Y_u are the values of Y for the folded and unfolded protein respectively. These typically change linearly with the denaturant concentration ([D]), as follows:

$$Y_f = y_f + m_f[D] \quad (8)$$

$$Y_u = y_u + m_u[D] \quad (9)$$

where y_f and y_u are the folded and unfolded parameters at zero denaturant concentration. m_f and m_u are the denaturation-dependence of Y for the folded and unfolded state respectively.

Rearranging eqn (7) and substituting from eqns (8) and (9), one obtains:

$$Y = f_u\{y_u + m_u[D] - (y_f + m_f[D])\} + y_f + m_f[D] \quad (10)$$

To obtain the fitting function for the experimental isotherms, f_u in eqn (10) is substituted with eqn (6). Eqn (1) is substituted with $X = G$ into eqn (10), and the data are fitted to obtain the parameters ΔG°, m_G, y_f, m_f, y_u and m_u. At C_m (the denaturant concentration at which f_u is 0.5), from eqn (1) and eqn (5) we obtain:

$$\Delta G^\circ_D = \Delta G^\circ + m_G C_m \quad (11)$$

$$e^{-\Delta G^\circ D/RT}/2P_t = 0.5 \quad (12)$$

Substituting eqn (11) into eqn (12) and rearranging yields:

$$\Delta G^\circ = -RT \ln P_t - m_G C_m \quad (13)$$

Eqn (13) was substituted into eqn (10) and the data were fitted to obtain C_m.

All the data were analysed using Sigmaplot™ for Windows™ scientific graphing software.

Screening procedure for obtaining reversible thermal unfolding

The screen was carried out by incubating CcdB at a final concentration of 150 μg/ml under a variety of different conditions. The variables included pH and various concentrations of additives, such as GdmCl, betaine, sarcosine, glycerol, trehalose and taurine in 50 mM Hepes, pH 7.0. Individual samples were prepared in PCR tubes to a final volume of 100 μl, and placed in the sample block of a PCR machine. Samples were then heated to 75 °C at the rate of 60 °C · h^{-1}. The temperature was maintained for about 10 min at 75 °C, and then reduced to 25 °C at a rate of 180 °C · h^{-1}. Samples were then transferred to an ELISA plate and the absorbance at 405 nm was measured. Irreversible unfolding was typically accompanied by sample precipitation. Precipitation can be detected either visually or by a high value of sample absorbance relative to buffer in the plate reader. Under conditions where there was no visible precipitation (for example, in the presence of 1–2 M GdmCl), the additive was removed by dialysis against 10 mM Hepes, pH 7.0 or by repeated cycles of concentration and buffer dilution using a Microcon ultrafiltration unit. Subsequently samples were analysed for aggregation by 10 % native PAGE [7] and analytical gel filtration on a Superdex-75 (Amersham Biosciences) column.

DSC

Calorimetric measurements were performed using a model VP-DSC microcalorimeter (MicroCal. Inc., Northampton, MA, U.S.A.) with 0.6 ml cells [8]. DSC measurements were carried out as a function of scan rate, protein concentration and GdmCl concentration. All samples were dialysed against the desired concentrations of GdmCl in CGH10 buffer (see above), pH 7 [9]. All samples were centrifuged and degassed for approx. 15 min before loading, and measurements were performed under 27–29 lbf/in^2 (1 lbf/in^2 ≈ 6.9 kPa) pressure. Data were analysed using the Origin software supplied by the DSC manufacturer. The raw data of the calorimetric scans were baseline-corrected and concentration-normalized. Progress baseline connection of the pre- and post-transition baselines was used for estimation of the heat capacity change. Analysis of the DSC scans was carried out using a model for a single two-state transition with subunit dissociation, as described in the manual supplied by the manufacturer.

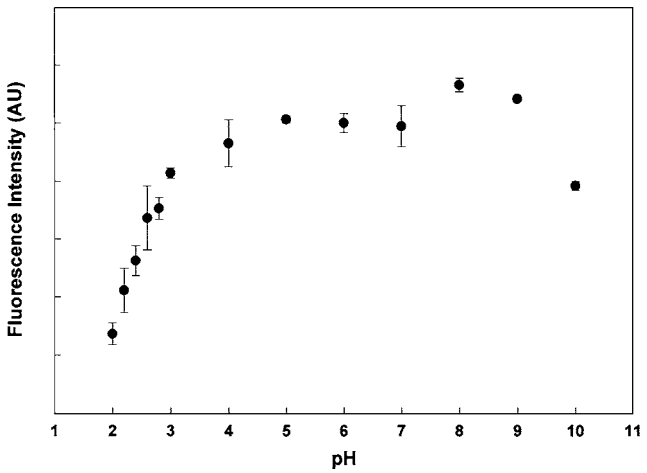

Figure 1 Fluorescence spectra of CcdB at different pH values

Fluorescence intensity at 340 nm in arbitrary units (AU) as a function of pH in the range 2–10. The protein concentration and temperature were 2 μM and 298 K respectively in all cases. The buffer used was CGH10 buffer, adjusted to indicated pH values with either NaOH or HCl. The data point show the mean values; error bars represent ± S.D.

RESULTS AND DISCUSSION

pH-mediated unfolding of CcdB

The structure of CcdB as a function of pH was examined by fluorescence spectroscopy and gel-filtration studies. The intrinsic fluorescence spectrum was relatively unchanged between pH 3 and pH 9. However, at lower and higher pH values there were changes in the fluorescence intensity, suggesting that the protein may be partially denatured (Figure 1). Gel-filtration studies using a Superdex-75 gel-filtration column were performed in the pH range 4–9 (results not shown). There was an appreciable change in the position of the CcdB peak at pH 4 relative to higher pH measurements, and the elution volume declined progressively from 13.9 ± 0.06 ml to 12.4 ± 0.03 ml in the range of pH 4–5. In the pH range 6–9, the elution volume was independent of pH. At pH 4.0, the protein was eluted at a position corresponding to a monomeric folded protein of molecular mass 11 kDa, identical with that of one of the marker proteins, *E. coli* thioredoxin (Figure 2A). At pH 7.0, CcdB elutes as a dimer of molecular mass 23 kDa, identical with that of the thioredoxin dimer. The latter protein consists of two thioredoxin monomers linked by two disulphide bonds (S. Sridhar and R. Varadarajan, unpublished work) with a molecular mass confirmed by ESI–MS (electron spray ionization–mass spectrometry). Molecular-mass analysis was carried out from standard calibration curves at pH 7.0 and pH 4.0, as shown in Figures 2(B) and 2(C) respectively.

Secondary and tertiary structure are similar for CcdB at pH 4 and 7

Two optical probes (fluorescence and CD spectroscopy) were used to characterize the structure of CcdB. The fluorescence, far- and near-UV CD spectra were identical for both pH 4.0 and 7.0 (results not shown). The crystal structure [10] of wild-type CcdB has been solved at a very high resolution of 1.4 Å (1 Å ≡ 0.1 nm). The structure revealed that CcdB exists as a dimer, with the dimer interface being formed by residues from strand S6 (amino acid residues 68–72), several loop segments and the three

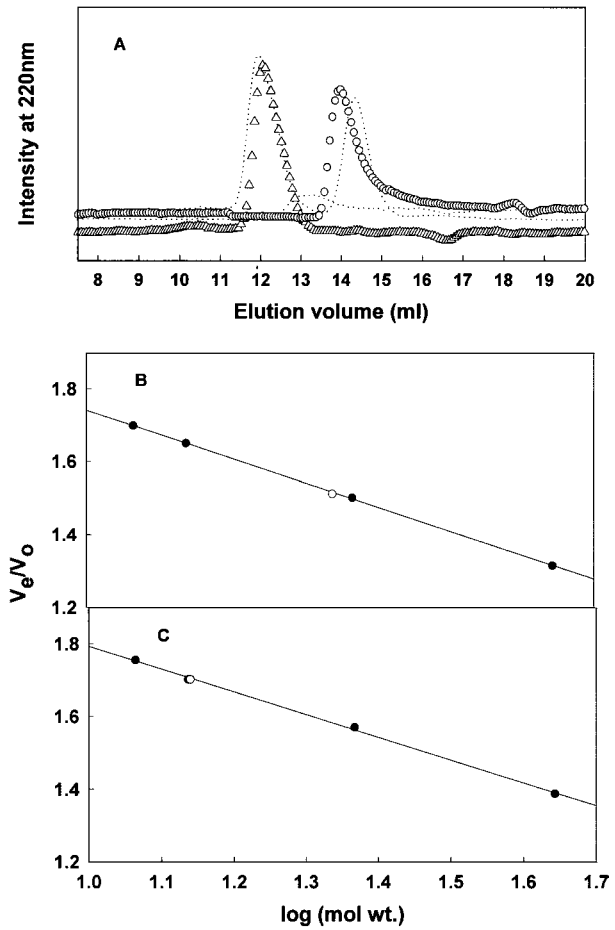

Figure 2 Gel-filtration profiles at pH 4.0 and 7.0

(**A**) The profiles were obtained by injecting protein on to a Superdex-75 analytical gel-filtration column using FPLC. Gel filtration chromatograms of CcdB at pH 7.0 (△) and pH 4.0 (○). Elution profiles of thioredoxin dimer (23 kDa) at pH 7.0 and thioredoxin monomer (11.7 kDa) at pH 4.0 are also shown in dotted lines as marker proteins. (**B**, **C**) Gel-filtration standard calibration curves at pH 7.0 (**B**) and pH 4.0 (**C**) were used to determine the molecular masses of CcdB at pH 7.0 and pH 4.0 respectively. Elution volumes for CcdB at both pHs are indicated by open circles (○), and for marker proteins with filled circles (●). Marker proteins used were ovalbumin (44 kDa), RNase A (13.7 kDa), thioredoxin dimer (23 kDa) and thioredoxin monomer (11.7 kDa). V_e and V_0 are peak elution volume and column void volumes respectively.

C-terminal residues. Three methionine residues (Met^{32}, Met^{68} and Met^{97}) from each monomer form a cluster that extends over the central hydrophobic core of the dimer. Both of the tryptophan residues and all four tyrosines in each monomer are buried. CcdB is predominantly a β-sheet protein. The peak of negative ellipticity at 208 nm in a CD spectrum is characteristic of the β-sheet content. The native fluorescence spectra at both pH 7.0 and pH 4.0 show an emission λ_{max} at 340 nm. When the protein is unfolded in urea or GdmCl, the emission λ_{max} is red-shifted to 357 nm, similar to that of free tryptophan, and there is an increase in the quantum yield. In the native state at pH 7.0, CcdB does not bind to ANS. However, ANS binding is observable at pH 4 (results not shown). Taken together with the gel-filtration studies, these results suggest that, at pH 4, CcdB exists as a folded monomer with very similar secondary and tertiary structure to that of dimeric CcdB at pH 7.0. In order to characterize the stability of the protein at both pH values, isothermal urea-denaturation melts were performed. Urea-induced unfolding was found to be reversible at both pH 7.0 and pH 4.0.

Table 1 Thermodynamic parameters obtained from equilibrium GdmCl denaturation melts for CcdB at pH 7.0

The m value derived from the global fit was -5.6 kcal · mol^{-1} · M^{-1}. For all experiments, the protein concentration used was 2 μM.

Temperature (°C)	ΔG° (kcal · mol^{-1})	C_m (M)
10	18.9 ± 0.4	2.09
15	19.7 ± 0.4	2.22
20	20.8 ± 0.4	2.38
25	21.2 ± 0.4	2.44
30	20.3 ± 0.4	2.25
35	20.1 ± 0.4	2.20
40	19.6 ± 0.4	2.08
45	19.0 ± 0.3	1.94
50	19.0 ± 0.3	1.93
55	17.9 ± 0.4	1.70

Two-state denaturant mediated unfolding at pH 7.0

The overall unfolding reaction of CcdB starts with a folded dimer (N_2), which dissociates into two unfolded monomers (2U). The unfolding is thus described by the scheme $N_2 \leftrightarrow 2U$.

GdmCl-induced equilibrium unfolding studies were performed in the temperature range 10°–55 °C, monitoring the unfolding by intrinsic fluorescence at 385 nm (Table 1). Urea-induced denaturation was performed between 10 °C and 43 °C using a protein concentration of 2 μM. In all cases, for both urea and GdmCl, denaturation reversibility was greater than 90 %. Representative data for urea denaturation are shown in Figure 3(A). Both urea and GdmCl denaturation data fitted well to a model describing a two-state unfolding coupled with subunit dissociation, as described in the Experimental section. The denaturant-mediated unfolding reaction follows a single and coincident transition when followed by changes in either fluorescence or CD (Figure 3B). This also suggests that only two states are significantly populated in the denaturation-transition zone. As shown previously [11], and undertaken by ourselves in the present study (although results not shown), the C_m of dimeric CcdB increases at 298 K from 5.08 ± 0.04 at 1 μM to 5.58 ± 0.04 at 20 μM protein concentration, consistent with an $N_2 \leftrightarrow 2U$ denaturation transition. Accordingly, the equilibrium denaturation curves were fitted for a dimeric system, as described in the Experimental section, to obtain ΔG°, m and C_m. The unfolding free energy ΔG° in the absence of denaturant was obtained using the linear extrapolation model [6]. The data at each temperature was first fitted independently with the 'm' value as an adjustable parameter. Since the m value did not appear to be temperature-dependent, subsequently the data were globally fitted using a single m value. Both methods gave virtually identical values of ΔG° and C_m. The values at 298 K from the global fit are 22.3 ± 0.7 kcal · mol^{-1} (1 cal ≈ 4.184 J), -2.8 ± 0.2 kcal · mol^{-1} · M^{-1} and 5.2 ± 0.04 M for ΔG°, m and C_m respectively. At a protein concentration of 2 μM, the C_m was found to vary from 4.6 ± 0.04 at 43 °C to 5.27 ± 0.04 at 20 °C, with an average free energy of unfolding, ΔG°, of 21 ± 0.7 kcal · mol^{-1}. We could not perform the equilibrium unfolding measurements with urea at higher temperatures because of sample aggregation and baseline errors. In order to determine ΔG° as a function of temperature (the stability curve) [12], it is essential to know the heat capacity change (ΔC_p) and the change in molar enthalpy (ΔH°) upon unfolding. Hence DSC studies were performed to supplement the thermodynamic data available from isothermal denaturation studies, and denaturation data from both studies were jointly fitted to obtain the stability curve.

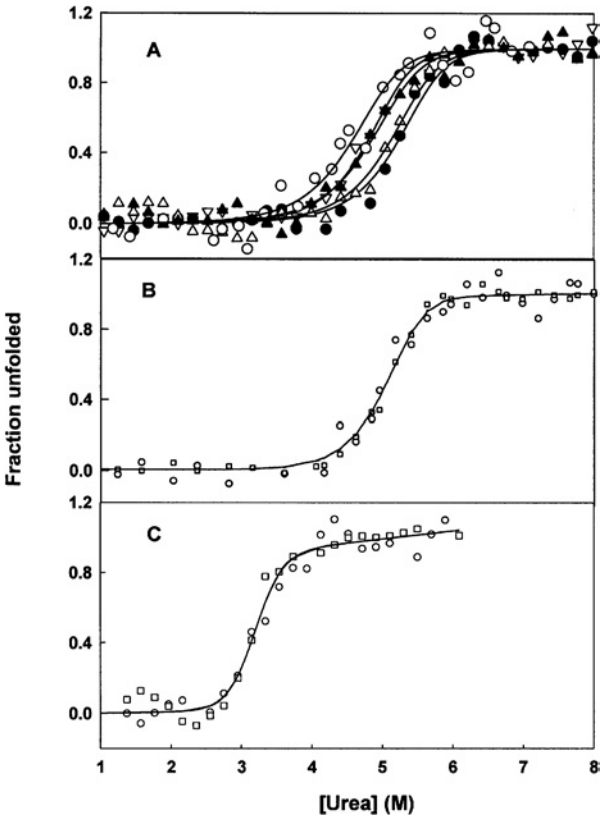

Figure 3 Equilibrium urea denaturation of CcdB at pH 7.0 and pH 4.0

(**A**) Equilibrium urea denaturation profiles of CcdB at pH 7.0 at 10 °C (▽), 20 °C (●), 25 °C (△), 40 °C (▲) and 43 °C (○). The isothermal melts were carried out in 10 mM Hepes, pH 7.0 using 2 μM protein. The theoretical curves were obtained by fitting all the melts together to a global fit function with a single *m* value of two-state unfolding in conjunction with subunit dissociation for a dimeric proteins. (**B**, **C**). Two-state unfolding of CcdB at pH 7.0 (**B**) and pH 4.0 (**C**). Urea denaturation was monitored by fluorescence (○) and CD spectroscopy (□). The protein concentration used was 6 μM.

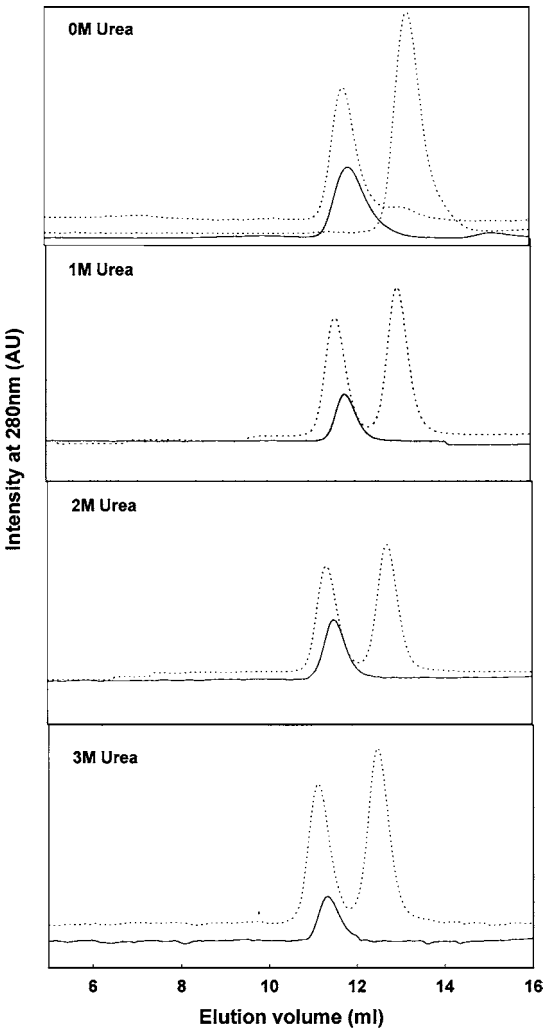

Figure 4 Gel-filtration profiles as a function of added urea concentration at pH 7.0

Elution profiles of CcdB are shown as continuous lines, and those of the marker proteins, thioredoxin dimer and monomer, as broken lines. In all cases, CcdB elutes close to the position of the thioredoxin dimer at urea concentrations within the folded baseline, derived from equilibrium melts monitored by fluorescence spectroscopy. AU, arbitrary units.

Two-state denaturant-mediated unfolding at pH 4.0

The gel-filtration studies described above indicated that CcdB was a monomer at pH 4.0. Hence the C_m for denaturant-mediated unfolding should be independent of protein concentration, in contrast with the situation at pH 7.0. This is indeed the case, and C_m was found to be independent in the range of protein concentrations studied (1–12 μM), with an average value of 3.2 ± 0.1 M (results not shown). Denaturation data at pH 4.0 were fitted to a scheme $N \leftrightarrow U$. The two-state nature of the transition was confirmed by the observation that denaturation profiles monitored by two different probes (CD spectroscopy at 222 nm and fluorescence intensity at 385 nm) were superimposable (Figure 3C). The average values are 9.2 ± 0.6 kcal $\cdot$ mol^{-1}, -2.9 ± 0.2 kcal $\cdot$ mol^{-} M^{-1} and 3.2 M for ΔG°, *m* and C_m respectively. Both the gel-filtration data in Figure 2 and the concentration-dependence of C_m are consistent with CcdB being a dimer at pH 7.0 and a monomer at pH 4.0. In order to confirm further that denaturation at pH 7.0 does not go through the pathway $N_2 \leftrightarrow 2N \leftrightarrow 2U$, gel-filtration studies were carried out as a function of urea concentration using urea concentrations in the native baseline region between 0 M and 3 M (Figure 4). In all cases, the native protein was found to be dimeric in the native baseline region, confirming further that denaturation by chemical denaturants at pH 7.0 involves a co-operative transition between the native dimer and unfolded monomers.

Thermal stability as a function of pH

The thermal stability in the pH range 2.0–9.5 was monitored by DSC (results not shown). Unfortunately, the thermal unfolding was irreversible at all pH values examined. At pH 2, the protein appears to be in an acid unfolded state, since it does not show any thermal transition. At pH 4, the apparent T_m (the temperature at which $f_u = 0.5$) is somewhat lower than at neutral pH, and the transition is considerably broadened relative to the unfolding transitions observed at higher pH. However, the irreversible nature of the thermal transitions prevented further analysis of the data.

Conditions for reversible thermal denaturation

The vast majority of stability studies have focused on the thermodynamic characterization of small, monomeric globular

proteins [13,14]. There are far fewer studies on oligomeric systems, because of the high degree of irreversibility in unfolding and the complexity of the overall process [15]. DSC is one of the most powerful methods for characterizing protein stability [16]. In principle, this technique permits measurement of all relevant thermodynamic parameters (ΔH°, ΔG° and ΔC_p) from a small set of experiments. Although it may often be possible to obtain reversible thermal unfolding in the presence of suitable additives, such as low concentrations of GdmCl [17,18], sample limitations often restrict the number of solution conditions that can be examined by DSC. We have developed a procedure that uses very small amounts of sample to find conditions for reversible thermal unfolding of CcdB, and have used DSC to obtain the stability curve for the protein at neutral pH.

At physiological pH in 10 mM Hepes buffer, CcdB unfolds irreversibly with visible precipitation upon heating. This prevented the determination of thermodynamic parameters by DSC under these conditions. We therefore designed a screening procedure, which consumes very little protein, to determine reversible conditions for thermal unfolding. The effect of various additives was examined. It was found that, at pH 7, aggregation was prevented by addition of GdmCl at concentrations of 0.8 M and above. Aggregation was also prevented in 5 M sarcosine, 4 M trimethylamine *N*-oxide and very high concentrations of glycerol. Native PAGE under conditions where there was no visible aggregation showed very clearly that the final folded form under all these conditions was identical with that of the native folded protein (results not shown). Detailed calorimetric studies were performed at neutral pH as a function of GdmCl concentration.

Thermal denaturation

We have characterized the stability of CcdB as a function of GdmCl by high-sensitivity DSC. The transitions were highly reversible, as demonstrated by repeated scans of the same sample in the presence of GdmCl. Figure 5(A) shows the buffer-corrected partial molar excess heat capacity data for 25 μM CcdB in CGH10 buffer/1.5 M GdmCl, pH 7.0, scanned at 90 °C h^{-1}. The dotted line indicates the calculated baseline of the excess heat capacity of the system. Examination of areas under the scan and rescan reveal a high reversibility of 85 %. To check the effect of scan rate, a DSC scan in 1.5 M GdmCl was performed at a scan rate of 60 °C · h^{-1} (Table 2). The data indicate that both T_m and the molar unfolding enthalpy at T_m [ΔH_D° (T_m)] are independent of scan rate. Figure 5(B) shows a set of DSC scans for wild-type CcdB obtained in a GdmCl concentration range of 1–2 M in CGH10, pH 7.0. It is clear from Figure 5(B) that the transition temperature decreases with increasing GdmCl concentration. Each of the DSC scans was analysed as described in the Experimental section. The number of subunits (n) was found to be two, in accordance with the dimeric status of CcdB [4] and the present study. Co-operative unfolding of the dimer occurs without any detectable intermediates. Thermodynamic parameters obtained from DSC data are listed in Table 2. Denaturation at pH 4.0 was irreversible, even in the presence of GdmCl, so it was not possible to characterize this state calorimetrically.

Determination of ΔC_p (the change in excess heat capacity upon protein denaturation) for CcdB

As an accurate estimate of the ΔC_p from a single DSC scan is not possible [19] because of the errors associated with baseline calculation, ΔC_p was estimated as shown in Figures 6 and 7. Stability curves shown in Figure 6 were obtained by jointly fitting extrapolated free energies from GdmCl isothermal denaturation

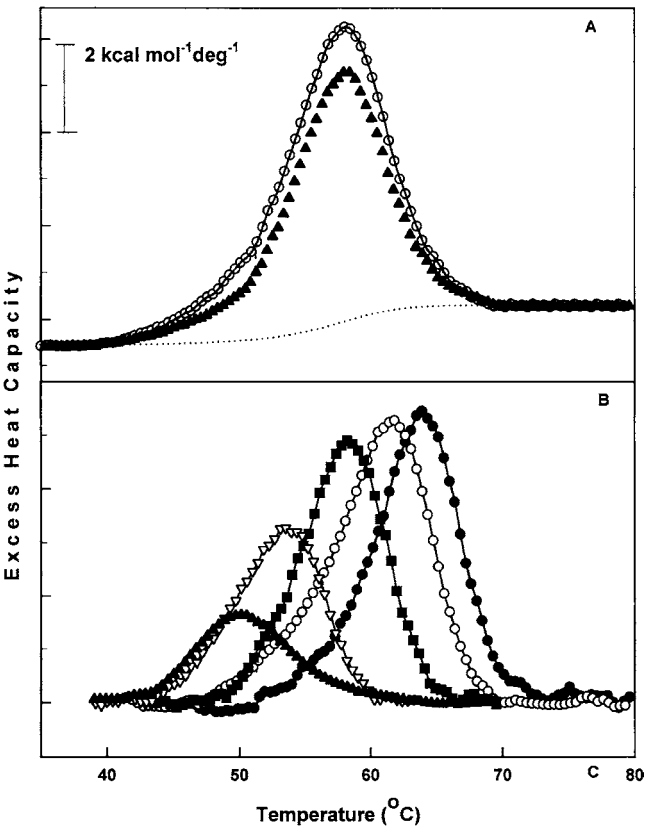

Figure 5 Representative DSC profiles of CcdB in the presence of GdmCl

(**A**) Scan (○) and rescan (▲) of 25 μM CcdB at pH 7 in the presence of 1.5 M GdmCl. The two-state with subunit dissociation model fitted to the data is shown by the continuous line, and the baseline by the dotted line. (**B**) Baseline-subtracted DSC scans of CcdB as a function of [GdmCl]. GdmCl concentrations used are 2.0 M (▲), 1.8 M (▽), 1.5 M (■), 1.2 M (○) and 1.0 M (●). The continuous lines represent the theoretical curves generated from the fit of two-state unfolding in conjunction with subunit dissociation.

Table 2 Thermodynamic parameters obtained from DSC for the unfolding of CcdB as a function of [GdmCl] at pH 7.0

[GdnCl] (M)	C_t (μM)	ΔH_D° (T_m) (kcal · mol^{-1})	T_m (K)	ΔG_D° (T_m) (kcal · mol^{-1})	ΔC_{pD} (kcal · mol^{-1} · K^{-1})
2.0	16	100	326.4	7.2	5.74
1.8	16	123	331.9	7.3	5.0
1.5	16	132	336.5	7.4	4.12
1.5*	16	126	336.8	7.4	2.32
1.5	16	135	336.7	7.4	4.28
1.2	16	137	340.3	7.5	4.71
1.0	16	141	342.5	7.5	4.64

* DSC carried out at a scan rate of 60 °C · h^{-1}. All other DSC experiments were carried out at a scan rate of 90 °C · h^{-1}.

data in temperature range 10–55 °C and DSC data points. Values of ΔG_D° (T) were estimated in the transition zone for thermal unfolding for each calorimetric scan using the following equations:

$$\Delta G_D^\circ(T) = \Delta H_D^\circ(T_m) - T\{[\Delta H_D^\circ(T_m) - \Delta G_D^\circ(T_m)]/T_m\} + \Delta C_{pD}(T - T_m - T \ln T/T_m) \quad (14)$$

$$K = 2P_t\left[f_u^2/(1 - f_u)\right] \quad (15)$$

$$\Delta G_D^\circ(T_m) = -RT_m \ln K \quad (16)$$

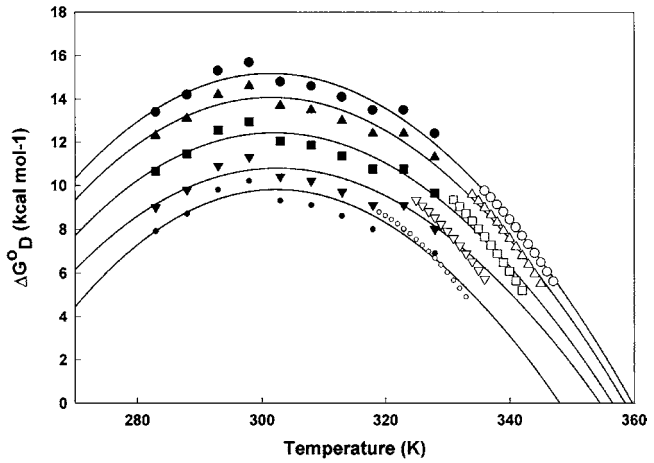

Figure 6 ΔC_p estimation for unfolding of CcdB

Protein stability curves for CcdB at pH 7.0 as a function of GdmCl concentrations. From the top to the bottom trace, the GdmCl concentrations are 1 M (●, ○), 1.2 M (▲, △), 1.5 M (■, □), 1.8 M (□, ▽) and 2 M (●, ○). The solid symbols are obtained from extrapolation of the data from isothermal GdmCl denaturation studies data at the indicated temperature. The open symbols represent data from the transition regions of the calorimetric scans, as described in the text. The solid curves through the data points represent the fit to eqn (17) for data at each GdmCl concentration.

Here, T_m is the reference temperature where the fraction unfolded (f_u) = 0.5, K is the equilibrium constant for the unfolding reaction and P_t is the total protein concentration.

For a dimeric system, although at T_m $f_u = 0.5$, the free energy of unfolding, ΔG°, is not equal to zero at T_m. Using eqns (15) and (16), one can calculate the free energy of unfolding in the presence of GdmCl at T_m [ΔG°_D (T_m)]. Using ΔH°_D (T_m) (from DSC), ΔG°_D (T_m) (calculated) and ΔC_{pD} (from DSC) listed in Table 2, values of ΔG°_D at each GdmCl concentration (1.0–2.0 M) in the transition region ($f_u = 0.1$–0.9) were calculated. For values of T close to T_m, the contribution of the ΔC_{pD}-containing term is small, and hence the ΔG°_D values are insensitive to errors in calorimetric ΔC_{pD} resulting from errors in baseline determination. For each isothermal denaturation melt, values of ΔG°_D at GdmCl concentrations between 1 and 2 M were calculated from the known m and ΔG° values at that temperature. At a given denaturant concentration, values of ΔG°_D derived from DSC and isothermal melts were fitted to following equation:

$$\Delta G^\circ_D(T) = \Delta H^\circ_{gD}(1 - T/T_g) + \Delta C_{pD}[T - T_g - T \ln(T/T_g)] \quad (17)$$

where the subscript indicates the parameter at a reference temperature, T_g, where $\Delta G^\circ = 0$. Values of ΔC_{pD} obtained at each concentration of GdmCl from these stability curves were linearly extrapolated to estimate ΔC_p in the absence of denaturant. ΔC_p was estimated to be 2.8 ± 0.4 kcal · mol^{-1} · K^{-1}, and was found to be virtually independent of GdmCl (results not shown). It is clear from Figure 6 that values of ΔG°_D obtained from DSC are in good agreement with the isothermal GdmCl data for the few temperatures where ΔG°_D could be estimated by both methods.

Using an alternate approach, values of ΔG°_D were calculated from DSC data as described above at five different temperatures in the range 326–342 K. The data points at each of the five temperatures were globally fitted to the linear extrapolation model using a single m value. This m value was found to be -5.6 ± 0.3 kcal · mol^{-1} · M^{-1}, which is similar to the m value obtained from the global fitting of the GdmCl isothermal denaturation data

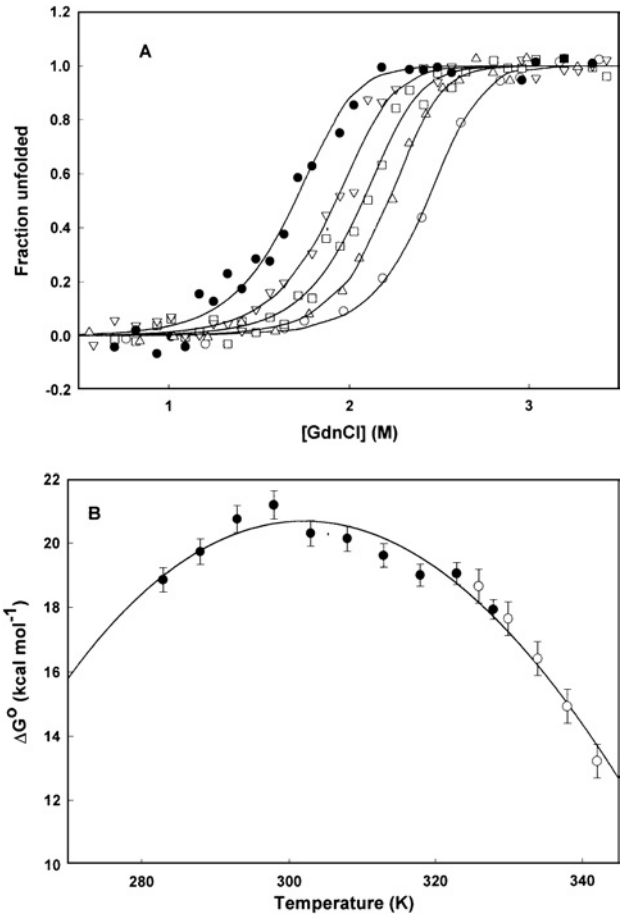

Figure 7 GdmCl denaturation of CcdB at pH 7.0

(**A**) Equilibrium GdmCl denaturation profiles of CcdB at pH 7.0 at 15 °C (△), 25 °C (○), 40 °C (□), 50 °C (▽) and 55 °C (●). The isothermal melts were carried out in 10 mM Hepes, pH 7.0, using 2 μM protein. The theoretical curves were obtained by fitting all the melts together to a global fit function with a single m value of two-state unfolding in conjunction with subunit dissociation for a dimeric proteins. (**B**) Stability curve of CcdB at pH 7.0. Stability curve of CcdB at pH 7.0 (solid line) as derived by fitting the GdmCl-mediated isothermal denaturation data points (●) and DSC data points (○) obtained by extrapolation to zero denaturant, as described in the text for eqn (1). The error bars in (**B**) show ± S.E.M.

(Table 1). ΔG° obtained at each of these temperatures and ΔG° values derived from GdmCl denaturation melts in the range 283–328 K (Table 1 and Figure 7A) were jointly fitted to eqn (17), as shown in Figure 7(B). This provides estimates of $\Delta H_g = 213.4 \pm 9.2$ kcal · mol^{-1}, $T_g = 371.7 \pm 2.2$ K and $\Delta C_p = 2.8 \pm 0.2$ kcal · mol^{-1} · K^{-1}. Previous studies that have examined the denaturant-dependence of ΔC_{pD} (summarized in [20]) showed no definite trends in the denaturant dependence of ΔC_{pD} in different proteins. However, with GdmCl as a denaturant, ΔC_{pD} was either independent of denaturant or increased with increasing GdmCl concentrations, whereas m values showed both increases and decreases with temperature. In the present case, ΔC_{pD} appears to be almost independent of GdmCl, and m values also appear to be independent of temperature, within experimental errors. The high value of ΔC_p (per mol residue) indicates that there is no residual structure in the denatured state, unlike the case for several other oligomeric proteins [21]. The high value of ΔG° is also relevant to the biological function of CcdB, as discussed previously [4]. Since accurate thermodynamic data are available for only a small number of multimeric systems [15,22–24], the present work is a useful addition to this dataset. An interesting feature of the present

system is the presence of a stable, folded, monomeric form of the protein at low pH.

Comparison of stabilities at pH 4.0 and 7.0

The values of ΔG° for CcdB unfolding at pH 7.0 and 4.0 as measured from urea denaturation studies are approx. 21 and 9.2 kcal respectively at 298 K.

The comparison of stabilities of monomeric and dimeric forms can best be illustrated by breaking up the overall free energy of unfolding of the dimer into two hypothetical steps, as shown below:

$$N_2 \xrightarrow{1} 2N \xrightarrow{2} 2U$$

where N_2, N and U represent folded dimer, folded monomer and denatured monomer respectively. The total free energy of unfolding $\Delta G^\circ(\text{total}) = \Delta G^\circ(1) + 2\Delta G^\circ(2)$, where $\Delta G^\circ(1)$ and $\Delta G^\circ(2)$ represent the standard molar free energy changes for steps 1 and 2 respectively [22]. Of the two steps, it is the first step that depends on choice of standard state. $\Delta G^\circ(1)$ is related to the dissociation constant between native dimer and native monomer. It is reasonable to assume that $\Delta G^\circ(1) > 0$ (for 1 M choice of standard state), since native dimer is certainly more populated than native monomer at molar concentrations of total protein. Hence $0.5\Delta G^\circ(\text{total}) \geqslant \Delta G^\circ(2)$ for this choice of standard state.

In the present case, isothermal denaturation studies show that ΔG° (total) at 298 K, pH 7.0, is approx. 21 kcal $\cdot$ mol^{-1} (assuming 1 M standard choice of standard state), and that $\Delta G^\circ(2)$ at 298 K, pH 4.0, is approx. 9.2 kcal $\cdot$ mol^{-1}. Assuming that $\Delta G^\circ(2)$ at pH 4.0 is similar to that at pH 7.0, $\Delta G^\circ(1) = \Delta G^\circ(\text{total}) - 2\Delta G^\circ(2) \approx 3$ kcal/mol. This is a surprisingly small number, because it would imply that the dissociation constant between folded dimer and folded monomer is only $e^{-(3/0.6)} = 6.7 \times 10^{-3}$ M. Hence at concentrations below millimolar levels, CcdB should be a folded monomer rather than a folded dimer. Since this is not the case, it suggests that $\Delta G^\circ(2)$ must depend on pH, and should be smaller at pH 7 than at pH 4. A different choice of standard state would not alter this conclusion, although the numerical value of $\Delta G^\circ(1)$, and units of the corresponding dissociation constant, would depend on the choice of standard state.

For most multimeric proteins, it is not possible to find conditions in which the folded monomer is stable with respect to the denatured state. This has given rise to the belief that intersubunit contacts make a very large contribution to the stability of multimeric proteins, and that these interactions are important for subunit folding (see, for example, [22] and references therein). In the case of CcdB, we are able to find a stable folded monomer at pH 4. This demonstrates that, in this particular case, intersubunit interactions are not required for subunit folding. Somewhat surprisingly, denaturation at pH 7.0 proceeds directly from the folded dimer to the unfolded monomer. No folded monomer was detectable at any of the urea concentrations in the folded baseline (Figure 4). In multimeric systems, folding is typically coupled with subunit association [22]. Partially folded, monomeric forms of a multimeric protein have been observed previously in a few isolated cases. The dimer of porcine mitochondrial malate dehydrogenase can be dissociated by relatively mild changes in pH or protein concentration. However, unlike in the present case, the monomeric form has different fluorescence properties. The monomeric form of the malate dehydrogenase enzyme present at pH 5.0 was recently shown by hydrogen exchange to be folded into a conformation similar to that adopted by the native dimer at pH 7.0 [25]. The dimeric enzyme 4-aminobutyrate aminotransferase also dissociates into monomers at pH 5.0 [26]. The monomers have similar secondary structure to the dimer. The *E. coli* enzyme glycinamide ribonucleotide transformylase (GART) also undergoes pH-dependent dimerization. It is monomeric in solution at pH 7.5 over a wide range of protein concentrations, and is a closely packed dimer at pH 6.75 or less, as demonstrated by analytical ultracentrifugation studies and dynamic light scattering [27–31].

However, for none of the above cases was a detailed thermodynamic comparison of monomer and dimer stabilities carried out. Monomeric, equilibrium intermediates have also been detected during the equilibrium unfolding of multimeric proteins, such as the dimeric procaspase-3 [32], trp apo-repressor [33], *E. coli* CRP (cyclic 3′,5′-phosphate receptor) protein [34], cod alkaline phosphatase [35] and *Bacillus stearothermophilus* SHMT (serine hydroxymethyltransferase) [36]. However, in these studies, it is typically difficult to find conditions where only the monomeric species is populated and to directly characterize its properties. In most cases, the properties of such intermediates are inferred or derived from complex, multi-parametric fitting procedures. In addition, such species are more often observed in large, complex, multidomain proteins that can fold independently of intersubunit interactions [22]. In contrast, in the present study we have been able to characterize the equilibrium stabilities of a small, single domain protein, CcdB, in both monomeric and dimeric forms. The monomeric form is formed at pH 4. Dimer dissociation probably occurs at this pH because of increased electrostatic repulsion between the two subunits. The total charge on CcdB as a function of pH was calculated using the isoelectric program in the GCG package. The charge increases from -0.53 at pH 7 to 8.69 at pH 4.0. There is a steep charge increase in the pH range 5.0–4.0 from 2.48 to 8.69. Thus it is likely that the dimer-to-monomer transition occurs in this pH range. It was not possible to follow the transition spectroscopically, since the monomer and dimer have very similar spectral properties. However, denaturation as well as gel-filtration studies at pH values of 7.0, 6.0 and 5.0 (results not shown) also are consistent with this suggestion. The elution volume (12.4 ± 0.03 ml), as well as the C_m (4.06 ± 0.04 M) at pH 5.0, are significantly different from the corresponding values at pH 6 and 7 (C_m being 4.94 ± 0.04 M and 5.09 ± 0.04 M, and elution volumes being 12.3 ± 0.03 ml and 12.2 ± 0.06 ml, at pH 6 and pH 7 respectively), suggesting that monomers begin to be populated at this pH. Analysis of denaturation data in the pH range 5.0–4.0 is complicated, because the unfolding transition involves mixtures of native monomers and dimers with very similar spectroscopic properties, and hence was not attempted. Analysis of the CcdB dimer structure reveals that the only charged residue close to the dimer interface is Asp^{67}. However, this residue is quite exposed (accessibility of 33 %). In addition, since the interface is primarily hydrophobic, it appears unlikely that titration of this single aspartate residue alone is responsible for the dimer-to-monomer transition between pH 5 and 4; rather, it is probably due to titration of several residues distributed all over the molecule. Further mutational studies will attempt to address this issue.

The dissociated monomer is surprisingly stable, and has a value of ΔG° of approx. 9 kcal $\cdot$ mol^{-1}, comparable with that of many stable, naturally occurring monomeric proteins. The amount of surface area buried upon subunit association of folded monomers is 1235 Å^2 per monomer. This is quite extensive as compared with other dimeric proteins of similar size, e.g. dimeric subtilisin inhibitor, with a subunit molecular mass of 10.9 kDa, has an interface area of 750 Å^2, and cytochrome c' with 13.9 kDa has an interface area of 810 Å^2 [37]. Despite this, intersubunit interactions are not essential for subunit folding. The present work shows that the monomeric form of the protein is stable and folded at pH 4.0. It is therefore reasonable to suppose that folded, rather than unfolded, monomers must be the primary species in equilibrium

with the folded dimers at neutral pH in aqueous solution. Since very low levels of CcdB are required for biological activity, this gives rise to the intriguing possibility that folded monomers may be the biologically relevant form of the protein *in vivo*. The possibility that a monomer might be involved in an interaction complex with GyrA had been previously considered [38], because dimeric CcdB is too large to fit into the GyrA structure without conformational rearrangement. However, such a model was felt by the authors to be inconsistent with the known dimeric structure of CcdB. Future studies will attempt to address this issue, and to determine whether monomeric, folded CcdB is formed as a kinetic intermediate during the folding process at neutral pH.

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