Protein Science (1994), *3*:1236–1244. Cambridge University Press. Printed in the USA.

Ligation alters the pathway of urea-induced denaturation of the catalytic trimer of *Escherichia coli* aspartate transcarbamylase

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(RECEIVED February 7, 1994; ACCEPTED June 1, 1994)

Abstract

We have examined the pathway and energetics of urea-induced dissociation and unfolding of the catalytic trimer (c_3) of aspartate transcarbamylase from *Escherichia coli* at low temperature in the absence and presence of carbamyl phosphate (CP; a substrate), *N*-(phosphonacetyl)-L-Asp (PALA; a bisubstrate analog), and 2 anionic inhibitors, Cl^- and ATP, by analytical gel chromatography supplemented by activity assays and ultraviolet difference spectroscopy. In the absence of active-site ligands and in the presence of ATP, c_3 dissociates below 2 M urea into swollen c chains that then gradually unfold from 2 to 6 M urea with little apparent cooperativity. Linear extrapolation to 0 M urea of free energies determined in 3 independent types of experiments yields estimates for $\Delta G_{dissociation}$ at 7.5 °C of about 7–10 kcal m^{-1} per interface. $\Delta G_{unfolding}$ of dissociated chains when modeled as a 2-state process is estimated to be very small, on the order of $\sim$2 kcal m^{-1}. The data are also consistent with the possibility that the unfolding of the dissociated monomer is a 1-state swelling process. In the presence of the ligands CP and PALA, and in the presence of Cl^-, c_3 dissociates at much higher urea concentrations, and trimer dissociation and unfolding occur simultaneously and apparently cooperatively, at urea concentrations that increase with the affinity of the ligand.

Keywords: analytical gel chromatography; aspartate transcarbamylase; protein folding; subunit dissociation; urea denaturation

Denaturation of multimeric proteins involves more than 1 process because the protein must both dissociate and unfold. If ligands are present, there may be several different dissociation and unfolding reactions. As the concentration of a chemical denaturant increases, the order in which these processes occur and the energetic relationships among them define a pathway of denaturation. We have used both kinetic and equilibrium experiments to explore the pathway of urea denaturation of the c_3 subunit of *Escherichia coli* aspartate transcarbamylase at low temperature in the presence and absence of ligands and anionic inhibitors.

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Abbreviations: AGC, analytical gel chromatography; ATCase, *Escherichia coli* aspartate transcarbamylase; c, catalytic chain; r, regulatory chain; GuHCl, guanidine-HCl; PALA, *N*-phosphonacetyl-L-aspartate; CP, carbamyl phosphate; βME, β-mercaptoethanol; DTT, dithiothreitol; OPA, *o*-phthaldialdehyde; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

ATCase is a large, multisubunit enzyme whose activity is regulated by ligand-induced changes in interchain and intersubunit interactions (for recent reviews, see Allewell [1989], Hervé [1989], and Lipscomb [1992]). The holoenzyme is comprised of 6 c and 6 r polypeptide chains that can be dissociated into 2 trimeric catalytic subunits and 3 dimeric regulatory subunits. Because the holoenzyme (c_6r_6) can be readily reconstituted from isolated r_2 and c_3, and because in genetically engineered *E. coli* the proportions of r_2, c_3, c_6r_4, and c_6r_6 vary according to gene dosage while no other multimeric species are ever observed, the holoenzyme is thought to assemble in vivo from preformed c_3 and r_2 (Schachman, 1983, and references therein).

The catalytic trimer, c_3, is the smallest unit of ATCase that has enzymatic activity. Although active sites are located between c chains, and binding of substrates and substrate analogs triggers a major change in interchain interactions (Howlett & Schachman, 1977), binding of substrates is not cooperative as it is in the holoenzyme. Nucleoside triphosphates, which regulate the holoenzyme heterotropically, inhibit c_3 by binding competitively with carbamyl phosphate at the active site (Porter et al., 1969; Suter & Rosenbusch, 1977; Honzatko & Lipscomb, 1982). The structure of c_3 as it exists in the crystalline holoen-

zyme is shown in Figure 1, viewed down the 3-fold axis relating the monomers.

We have previously studied the thermal denaturation of ATCase and its subunits in the presence and absence of PALA and regulatory nucleotides by differential scanning calorimetry (Edge et al., 1985, 1988). Here we report a parallel analysis of urea-induced dissociation and unfolding of c_3 by analytical gel chromatography, supplemented by ultraviolet difference spectroscopy and activity assays. These approaches allow us to monitor dissociation and unfolding both globally and functionally in the presence and absence of ligands and inhibitors. Although the effect of ligation on thermal denaturation of multimeric proteins has been documented in a number of systems (cf. Edge et al., 1985, 1988; Brandts et al., 1989; Zolkiewski & Ginsburg, 1992), the effect of ligation on chemical denaturation has rarely been explored. We find that in the absence of ligands, c_3 dissociates in urea before it unfolds. In the presence of ligands, the denaturation pathway is altered and c_3 apparently dissociates and unfolds simultaneously. In the presence of anionic inhibitors, the denaturation pathway can be either sequential, as it is in the presence of ATP, or concerted, as it is in the presence of Cl^-.

Results

Urea denaturation of c_3, in contrast to thermal denaturation, is largely reversible (M. Daugherty & N.M. Allewell, unpubl. results). Denaturation of 10 μM c_3 in 7 M urea for 2 h at 0 °C, followed by removal of urea by dialysis, resulted in recovery of 72% of the initial activity and 77% recovery in the presence of 0.25 M NaCl. Because aggregation frequently interferes with the reconstitution of multimeric proteins (Jaenicke & Rudolph, 1986), these levels of recovery are relatively good. Because denaturation is largely reversible at low temperature, all experiments were performed at low temperatures.

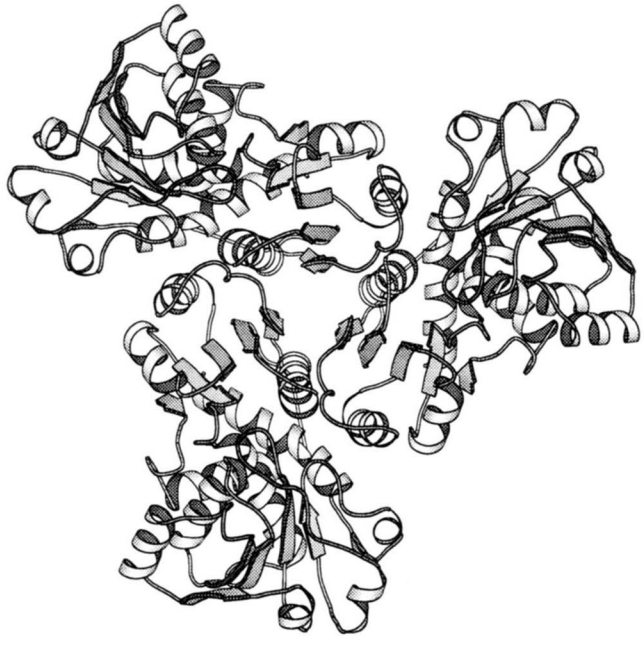

Fig. 1. Structural schematic of a c_3 subunit generated from the coordinates for 1 catalytic trimer in the holoenzyme coordinates in the Brookhaven Protein Data Bank, file 6AT1.

Difference spectroscopy

Incubating c_3 with either GuHCl or urea results in an ultraviolet absorbance difference spectrum relative to native c_3 with a minimum at 288 nm (Burns & Schachman, 1982b). The change in absorbance at 288 nm produced by solvent perturbation primarily monitors the exposure of Tyr residues, especially in Tyr-rich proteins such as c_3 (Donovan, 1973). Difference absorbance at 288 nm was monitored after jumping c_3 from native conditions into various urea concentrations at 14 °C (Fig. 2A). Fitted $t_{1/2}$'s for the ΔA_{288} ranged from 1 min to 39 min. Absorbance changes below ~2 M urea were of the same magnitude as the drift of the spectrophotometer. The equilibrium ΔA_{288} data (Fig. 2B) show a broad transition between about 2 and 5 M urea with a midpoint near 3.5 M. Results at 24 °C were similar (data not shown), suggesting that the process monitored by the change in absorbance does not have a large enthalpy change in this temperature range.

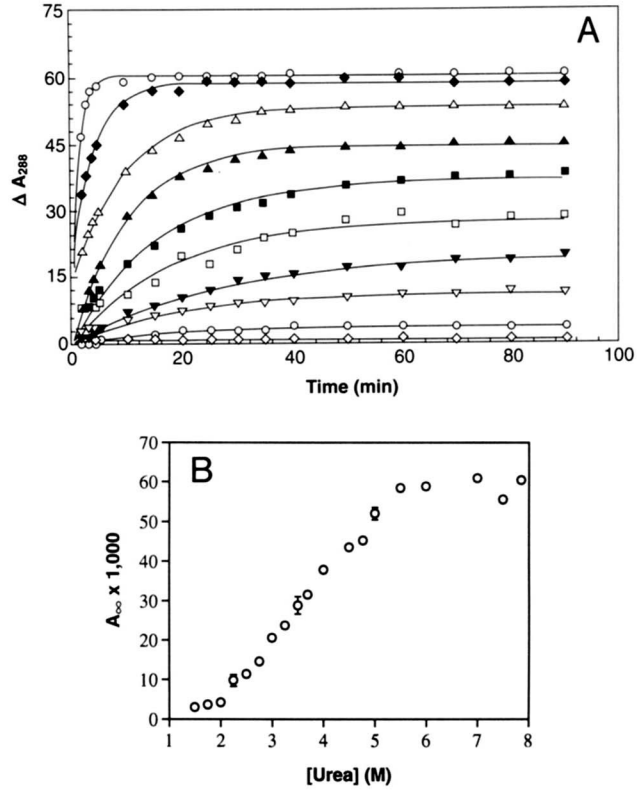

Fig. 2. Kinetics of urea denaturation of c_3 determined by UV difference spectroscopy. **A:** Changes in absorbance at 288 nm × 1,000 are shown after mixing c_3 with urea to final concentrations of 5 μM c_3 in 1.75–7.85 M urea, in a buffer containing 0.1 M K^+ HEPES, 0.2 mM DTT, 0.2 mM EDTA, pH 8.3, at 14 °C. Data shown are (from bottom to top): 1.0 M urea (◇), 2.0 M urea (○), 2.5 M urea (▽), 3.0 M urea (▼), 3.5 M urea (□), 4.0 M urea (■), 4.77 M urea (▲), 5.0 M urea (△), 5.5 M urea (◆), 7.85 M urea (○). **B:** Values of A_∞ × 1,000 determined using Equation 1 are plotted versus [urea]. Error bars are the 67% confidence intervals of the nonlinear fits to the data in panel A.

Analytical gel chromatography

In the absence of ligands

In the absence of ligands, the weight average partition coefficient (σ) increases as [urea] increases up to 1.75 M, then decreases as the protein unfolds at higher urea concentrations (Fig. 3A). The increase in partition coefficient, corresponding to a decrease in molecular Stokes radius at lower [urea], is a common feature of subunit dissociation in multimeric proteins that have been studied by small zone chromatography (cf. Herold & Kirschner, 1990; Toyama et al., 1991; Aceto et al., 1992).

Elution profiles from S-200 Sephacryl confirm that the dominant process at low [urea] is dissociation. These profiles consist of 2 partially resolved peaks whose relative areas shift with protein and urea concentrations in the manner expected for an associating equilibrium (Fig. 4). Control experiments in which the time during which the protein sample was equilibrated in 1.75 M urea before being applied to an S-200 column indicated that elution profiles obtained with preequilibration times of 20–240 min were essentially identical (data not shown). Column calibration indicated that between 1.25 M and 1.75 M urea, the Stokes radii of both species increase by 3–4 Å, indicating that both the monomer and trimer swell with increasing [urea], but that the dissociation of trimer to monomer dominates the shift in the overall weight average σ on the S-300 gel. The broad transition between 2 and 7 M urea, similar to that observed by difference absorbance, indicates that the dissociated protein expands between 2 and 7 M urea.

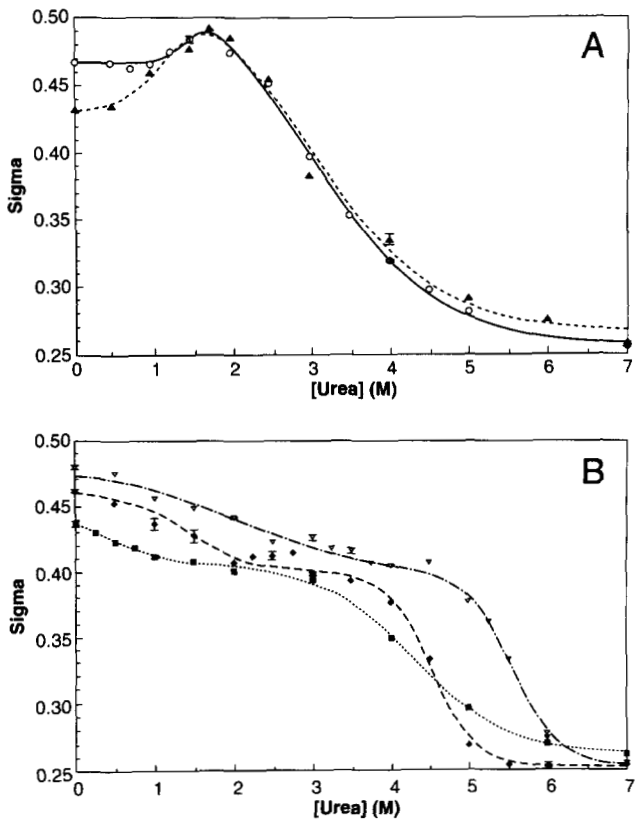

Fig. 3. A, B: AGC experiments showing the dependence of the partition coefficient, σ, on urea concentration in the absence of ligands (○) and in the presence of 50 mM ATP (▲), 5 mM CP (◆), 56 μM PALA (▽), or 0.25 M NaCl (■). Five-micromolar c_3 was incubated for >2 h at 0 °C in 0.1 M K^+ HEPES, 0.2 M βME, 0.2 mM EDTA, pH 8.3, and the appropriate concentration of urea and ligand, as indicated, before loading onto an S-300 Sephacryl column (1.5 × 50 cm) equilibrated in the same buffer and maintained at 7.5 °C.

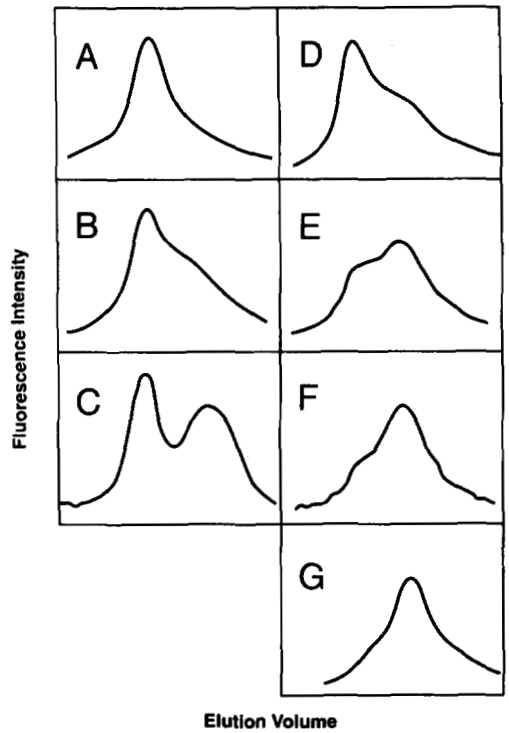

Fig. 4. A–C: Dependence of elution profile on concentration of c_3 in 1.25 M urea on S-200 Sephacryl. Initial concentrations of c_3: (A) 12.7 μM, (B) 5 μM, and (C) 1.3 μM. **D–G:** Effect of varying urea concentration on the elution profile of 5 μM c_3 on S-200 columns. Urea concentrations: (D) 1.25 M, (E) 1.5 M, (F) 1.625 M, and (G) 1.75 M. All experiments were carried out at 7.5 °C in 0.1 M K^+ Hepes, 0.2 M βME, 0.2 mM EDTA, pH 8.3. Samples were equilibrated in urea for >4 h before loading.

In the presence of active-site ligands

In the presence of CP (a substrate) and PALA (a bisubstrate analog; Collins & Stark, 1971), the increase in partition coefficient at low urea concentrations does not occur and the concentration of urea required to produce unfolding increases (Fig. 3B). Because the active sites are located at the interfaces between monomers, stabilization of c_3 by CP and PALA is not unexpected. What is striking is that the ligand-induced stabilization of the trimer changes the apparent pathway of denaturation. The increase in Stokes radius that accompanies unfolding is no longer preceded by a well-defined dissociation process. The denaturation process remains biphasic in the presence of ligands, but with a gradual decrease in σ at low [urea] followed by a relatively cooperative phase above 4 M urea. Note that in the absence of ligands, the protein is almost completely unfolded by 4 M urea. In the presence of PALA, a shoulder appears in the elution profile between 5 and 6 M urea. The σ values in Figure 3B in this region are the weight average σ's including the shoulder. This shoulder may represent a swollen trimeric species with PALA

bound that is in slow equilibrium with the dissociated, unfolded monomer at high [urea].

Effects of anionic inhibitors

AGC as a function of [urea] was also performed in the presence of ATP (Suter & Rosenbusch, 1977) and Cl^- (added as NaCl), an anionic inhibitor believed to be competitive with CP (Kleppe, 1966; Jacobson & Stark, 1975). Both 50 mM ATP and 0.25 M NaCl reduce σ in the absence of urea, probably as a result of their high ionic strength, which either shields ionic interactions between the column and the protein below 2 M urea or produces solvation-related changes in the protein's apparent size. However, the major effects of the 2 inhibitors are different: ATP does not suppress the dissociation step below 2 M urea, while NaCl does. In contrast to the results obtained in the presence of CP or PALA, the second "phase" in the denaturation with NaCl does not appear cooperative.

Stabilization of the trimer by NaCl was confirmed by further AGC experiments carried out on S-200 gels, ultraviolet difference spectroscopy, and differential scanning calorimetry (data not shown). In experiments analogous to those shown in Figure 4, the elution profiles in the presence of 0.25 M NaCl were observed to consist only of a single high molecular weight peak. Addition of increasing NaCl to a solution of c_3 and 5 M urea titrated and ultimately eliminated the ΔA_{288} observed by ultraviolet difference spectroscopy. Differential scanning calorimetry of c_3 in HEPES, pH 7, indicates a broad transition between 60 and 70 °C; addition of 0.25 M NaCl, however, sharpens the transition and increases T_m by several degrees (data not shown).

Activity assays

The experiments described above indicate that, in the absence of ligands, dissociation of c_3 into swollen but folded monomers can be studied in 1.25–1.75 M urea. In order to characterize these processes further, we made use of the fact that folded monomers are inactive (Burns & Schachman, 1982a) and used activity to monitor the formation or disappearance of c_3 in experiments in which the association equilibrium was perturbed by altering [urea]. When c_3 is assayed for activity in the presence of 1.25–1.75 M urea, activity is initially elevated to 120–140% of that observed in the absence of urea, as reported by Dreyfus et al. (1984). This initial activation occurs in less than a minute and is followed by a slow loss of activity, which plateaus at activities below that observed in 0 M urea.

Experiments in which c_3 is "jumped" into urea concentrations between 1.25 and 1.75 M (Fig. 5A) result in a decay to an equilibrium level of activity that decreases with increasing [urea] and decreasing total protein concentration. Fitted values of $t_{1/2}$ ranged from 1 to 1.8 h for all but 1 condition ($t_{1/2} = 2.8$ h for 1.25 M urea, 1 μM c_3).

Estimating the energetics of dissociation and unfolding

From AGC on S-300

Data were modeled with 3 different dissociation-unfolding models that differ in the proposed pathway of unfolding (see Materials and methods for a full description). Returned free energies and parameter values for fits to these models are shown in Table 1. All 3 models assume that all free energies are linearly

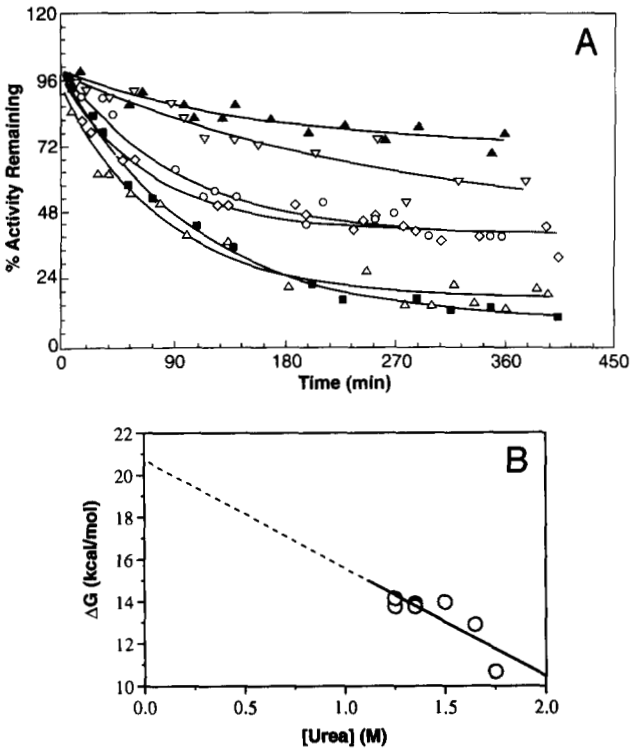

Fig. 5. A: Kinetics of activity loss of c_3 after exposure to low concentrations of urea. Conditions shown are: 1.75 M urea, 0.5 μM c_3 (■); 1.65 M, 1 μM c_3 (△); 1.5 M, 1 μM c_3 (◇); 1.35 M urea, 1 μM c_3 (○); 1.25 M urea, 1 μM c_3 (▽); 1.25 M urea, 5 μM c_3 (▲). **B:** Trimer dissociation free energies as a function of [urea] estimated assuming the activity losses in panel A are due solely to dissociation of the trimer. Linear extrapolation to 0 M urea is also shown.

dependent on urea concentration, and they all predict free energies for 2-state transitions among 3 molecular species

$$N \leftrightarrow I \leftrightarrow U,$$

where N is the native state, I is an intermediate state, and U is the unfolded state. The definitions of N, I, and U differ with the model (see Materials and methods). The curves shown in Figure 3 correspond to the model-dependent fits. In the absence of ligands, the free energy of c:c interactions in the trimer is estimated at about −9.7 kcal/mol.

Besides being model dependent, the values in Table 1 must be regarded as approximate because of changes in concentration due to axial dispersion and problems inherent in studying associating systems using small zone chromatography (Zimmerman & Ackers, 1971). Because native c_3 was diluted 8-fold on the column, the error was estimated by calculating ΔG_d with the initial concentrations of protein and 1/8 that concentration. These values differed by ~2 kcal m^{-1}.

The possibility of ionic-strength and ligand-specific effects on the σ values also complicates the quantitative analysis of these data. Partition coefficients for c_3 in the absence of urea change in the presence of NaCl, saturating at a [NaCl] of about 0.4 M and a $\Delta\sigma$ of −0.015. In the presence PALA, σ changes in the opposite direction, saturating at 50 μM PALA and a $\Delta\sigma$ of 0.017. The change with PALA reflects known quaternary

Table 1. *Parameter values derived by fitting AGC results to various denaturation models*[a]

Model 1 (Equations 3 and 4)

Parameter	No ligands	+56 μM PALA	+5 mM CP	+50 mM ATP	+0.25 M NaCl
A_{NI}	−3.6 (−7.1, −1.7)	−0.67 (−0.82, −0.53)	−0.67 (−1.9, −1.1)	−1.9 (−3.2, −0.98)	−1.7 (−2.2, −1.1)
A_{IU}	−0.70 (−0.61, −0.77)	−1.9 (−2.2, −1.6)	−2.0 (−2.5, −1.4)	−0.7 (−0.79, −0.57)	−0.95 (−1.0, −0.90)
ΔG_{NI}	5.5 (2.8, 10.9)	1.4 (1.2, 1.6)	2.0 (1.6, 2.7)	2.5 (1.3, 4.1)	0.73 (0.26, 1.2)
ΔG_{IU}	2.1 (1.7, 2.4)	10.5 (8.9, 12.0)	9.0 (6.5, 11.3)	2.0 (1.7, 2.3)	4.1 (3.8, 4.3)
σ_N	0.467 (0.464, 0.470)	0.4797[b]	0.461[b]	0.430 (0.418, 0.443)	0.444 (0.435, 0.452)
σ_I	0.550 (0.518, 0.578)	0.399 (0.393, 0.405)	0.401 (0.397, 0.404)	0.550[c]	0.406 (0.404, 0.409)
σ_U	0.256 (0.251, 0.260)	0.254 (0.247, 0.262)	0.253 (0.249, 0.257)	0.266 (0.256, 0.276)	0.263 (0.261, 0.266)

Model 2 (Equations 5 and 6)

Parameter	No ligands $[c_3]$ = (0.5 mg/mL)	No ligands $[c_3]$ = (0.0625 mg/mL)	+50 mM ATP $[c_3]$ = (0.5 mg/mL)	+50 mM ATP $[c_3]$ = (0.0625 mg/mL)
A_d	−10.1 (−11.2, −4.7)	−10.0 (−11.7, −4.67)	−3.6 (−6.0, −1.8)	−3.6 (−6.0, −1.8)
A_{IU}	−0.70 (−0.78, −0.62)	−0.70 (−0.78, −0.62)	−0.7 (−0.79, −0.60)	−0.69 (−0.79, −0.60)
ΔG_d	28.0 (20.0, 29.7)	30.2 (22.3, 32.7)	17.4 (14.7, 20.9)	19.7 (17.1, 22.8)
ΔG_{IU}	2.1 (1.75, 2.45)	2.1 (1.7, 2.5)	2.0 (1.8, 2.3)	2.0 (1.8, 2.3)
σ_N	0.467 (0.464, 0.470)	0.467 (0.464, 0.470)	0.426 (0.413, 0.443)	0.426 (0.414, 0.443)
σ_I	0.542 (0.516, 0.570)	0.542 (0.516, 0.570)	0.550[c]	0.550[c]
σ_U	0.256 (0.251, 0.261)	0.256 (0.252, 0.260)	0.266 (0.256, 0.275)	0.265 (0.256, 0.275)

Fits to Model 3 (Equations 7 and 8)

Parameter	+56 μM PALA $[c_3]$ = (0.5 mg/mL)	+56 μM PALA $[c_3]$ = (0.0625 mg/mL)
A_{NI}	−0.68 (−0.81, −0.51)	−0.69 (−0.85, −0.53)
A_d	−4.5 (−7.0, −2.7)	−4.5 (−7.2, −2.8)
ΔG_{NI}	1.4 (1.2, 1.6)	1.4 (1.2, 1.7)
ΔG_d	38 (28, 52)	40 (31, 55)
σ_N	0.4797[b]	0.4797[b]
σ_I	0.340 (0.389, 0.407)	0.400 (0.392, 0.407)
σ_U	0.256 (0.246, 0.266)	0.256 (0.246, 0.266)

[a] Values in parentheses are the upper and lower 67% confidence intervals of the nonlinear regression.
[b] Constrained to the observed value.
[c] Constrained to value determined in independent experiments (Bromberg, 1990).

changes (Howlett & Schachman, 1977); the change with NaCl is unexplained. Compared with starting σ's of about 0.45 and overall $\Delta\sigma$'s of about 0.22 for full denaturation, these are relatively minor shifts. They do, however, suggest the possibility either of interactions between c_3 and the Sephacryl, which are altered with increasing ionic strength, or the possibility that NaCl as well as PALA induces alterations in the protein's apparent size.

From AGC on S-200

If the area under each peak in Figure 4 is proportional to the concentration of c chains, then $[c_3]/[c] = \text{area}_1/3 \cdot \text{area}_2$, where the subscripts indicate the order of elution. In addition, $3[c_3] + [c] = [c_{tot}]$, the total concentration of protein expressed as c chains. K is then given by

$$K = [c]^3/[c_3] = (c_{tot})^2(\text{area}_2)^3/[\text{area}_1 \cdot (\text{area}_1 + \text{area}_2)^2].$$

Using the concentration of the protein in the sample applied and 1/8 that concentration as limiting values and assuming linear dependence of ΔG_d on [urea] results in estimates of the free energy of dissociation of 23.5–24 kcal mol^{-1}. The c:c interchain interaction energy is therefore ≈ -8 kcal (mol interface)$^{-1}$.

From activity assays

Figure 5B is a plot of the free energies calculated from the dissociation equilibrium constants determined from the plateaus of the activity loss plots in Figure 5A (see Materials and methods). Loss of activity was monitored after jumping c_3 into 1.25–1.75 M urea. The extrapolation to 0 M urea yields a free energy of 20.8 kcal m^{-1} for the dissociation reaction, giving a free energy of stabilization of the c:c interface of −7 kcal mol^{-1}.

Discussion

Figure 3 clearly demonstrates that ligation of c_3 alters the order and energetics of the processes of dissociation and unfolding in urea. In the absence of ligands, the trimer dissociates into swollen monomers in 1.25–1.75 M urea. Dissociation is detected as an increase in the weight average partition coefficient on S-300 Sephacryl (Fig. 3A), as 2 peaks on S-200 Sephacryl (Fig. 4), and as the loss of activity that is concurrent with dissociation (Fig. 5). The observed increase in σ is a signature of subunit dissociation

(cf. Herold & Kirschner, 1990; Toyama et al., 1991; Aceto et al., 1992). Slight shifts in the partition coefficients of monomers and trimers in the region of [urea] where dissociation occurs indicate that both simultaneously swell.

That the dissociated species is primarily the swollen monomer is inferred from its Stokes radius, determined by calibrating the column with monomeric proteins of known Stokes radii, which is greater than that expected for the folded monomer, but less than that of a folded dimer. The swollen monomer may be analogous to the "molten globules" reported in other systems. Previous studies of monomeric c chains (Burns & Schachman, 1982a, 1982b), performed before compact denatured states of proteins were widely known, characterized monomers in chaotropic salts as having many of the same attributes as compact denatured states of monomers described in recent studies of other monomers derived from multimeric proteins (e.g., Leistler et al., 1992; Silva et al., 1992; Flynn et al., 1993).

In the presence of CP and PALA, which bind at the active sites between c chains, instead of first dissociating, the trimer swells, producing a gradual decrease in the partition coefficient, then undergoes concurrent and cooperative dissociation and unfolding (Fig. 3B). Although other models cannot be ruled out, the combined concerted model fits the data and is one of the simplest. Because c_3 is not active above 2 M urea, we can identify the swollen trimer as a compact denatured state, stabilized by ligation. Because the free energy of the combined process includes a contribution from the free energy of binding of the ligand, it occurs at higher urea concentrations than in the absence of ligands, and we observe that the shift in relative midpoint is correlated with the free energy of binding of the specific ligand. Values of ΔG_{IU} in the fits to model 1 (Equations 3 and 4 in Materials and methods) in the absence and presence of 0.25 M NaCl, 5 mM CP, and 56 μM PALA show an increase in stability of 2.1, 6.9, and 8.4 kcal m^{-1}, respectively. The denaturation profile in the presence of Cl^- is less cooperative and occurs at lower [urea], possibly reflecting the relatively weak binding of Cl^-. Fine structure in the change in partition coefficient as the ligated trimer swells suggests that the trimeric denatured state undergoes further structural changes.

The fits of these data to the denaturation models outlined in Equations 3–8 provide estimates of the corresponding free energy changes. These values must be regarded as preliminary and approximate because of their model dependence and because of the multiple effects of axial dispersion in small zone experiments (Zimmerman & Ackers, 1971). In the absence of ligands, the free energy of c:c interactions in the trimer is estimated at −9.7 kcal m^{-1} (67% confidence limits: −6.7 to −10.9 kcal m^{-1}). Values estimated from peak areas on S-200 Sephacryl (−8 kcal m^{-1}) and from activity assays (−7 kcal m^{-1}) fall within the confidence limits of the small zone chromatography value.

ATP, which also binds at the active site, behaves quite differently from the other ligands. The model-dependent fits suggest that ATP decreases the stability of the trimer to urea denaturation. Destabilization of the c:c interface by ATP has also been inferred from measurement of rates of subunit exchange (Yang & Schachman, 1987), rates of tritium exchange (Lennick & Allewell, 1981), and negative cooperativity of binding (Suter & Rosenbusch, 1977). In thermal denaturation experiments, where the temperature dependence of assembly and/or the presence of phosphate prevents dissociation, ATP also binds preferentially to the denatured trimer (Edge et al., 1985, 1988).

Characteristics of the unfolding of the swollen monomers in the absence of ligands and in the presence of ATP suggest that the unfolding may not be 2-state. Although it is convenient to assume 2-state cooperativity and obtain free energies for N ↔ D transitions from sigmoidal denaturation curves, the decrease in σ above 2 M urea in the absence of ligands (Fig. 3A) is not sigmoidal. The parameter A_{IU} for the unliganded fit from Table 1 is small compared to theoretical and experimental urea denaturation slopes for monomeric proteins at 20–25 °C (Alonso & Dill, 1991); and ΔG_{IU} (the estimated monomer unfolding free energy) at ~2 kcal/mol is small compared to the range of free energies for 2-state denaturation of monomeric proteins (Pace, 1975; Privalov & Gill, 1988). Unfolding curves obtained at 7.5 °C (Fig. 3A), 14 °C (Fig. 2B), and at 24 °C (difference absorbance data not shown) show little temperature dependence, suggesting that the processes they monitor involve little net enthalpy change.

Consideration of compact denatured states in the light of heteropolymer theory (Dill & Shortle, 1991) suggests an alternative model for unfolding of the monomer

$$c_3 \leftrightarrow 3c_U$$

in which the monomers swell after dissociation, increasing in solvent exposure as conditions become more favorable for the solvation of hydrophobic residues. In this model, there is no energy barrier separating any different denatured thermodynamic states of the monomer. Much of the present data are consistent with such a process occurring above 2 M urea in the absence of ligands and in the presence of ATP. In favorable cases, rapid size-exclusion chromatography can discriminate between 2-state (N ↔ D) and 1-state (swelling) unfolding transitions (Uversky, 1993) and may be useful in further defining the process that occurs above 2 M urea in Figure 3A.

Figure 6 shows proposed pathways for denaturation of c_3 in the presence and absence of ligands as a thermodynamic cycle. Large zone AGC experiments currently in progress will improve the quantitation substantially. It may be that the primary ener-

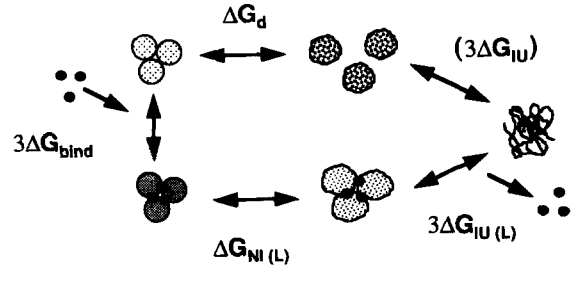

Fig. 6. Schematic depiction of the proposed pathways for denaturation of c_3 in the absence of ligands and in the presence of PALA, CP, and Cl^-. The black dots represent ligands, while the larger shaded circles represent monomers. The first process in the sequential (upper) pathway involves dissociation of the trimer and swelling of the monomers, followed by unfolding of the monomer. The "concerted" (lower) pathway involves only a swelling of the trimer in the first step, followed by a simultaneous dissociation, unfolding, and dissociation of ligand. The macroscopic free energies depicted on this schematic linkage correspond to those modeled in Table 1. If unfolding of the monomer proceeds via a 1-state swelling process the free energy ΔG_{IU} would not exist (see text). Both pathways produce a denatured particle with the same Stokes radius.

getic stabilization of the "native" folded form of the protein is from interchain rather than intrachain interactions, as appears to be the case in the *trp* repressor system (Fernando & Royer, 1992). One of the principal reasons why monomers of the catalytic subunit are not observed in vivo may be that trimer formation is necessary for complete folding, and that ligation stabilizes the trimer even further.

Conservation of energy dictates that unless it is specifically linked to other processes, binding of a ligand will stabilize a protein to denaturation. Examples of systems in which ligation is not linked to any other process are, however, rare. Often ligand binding is energetically linked, either positively or negatively, to subunit dissociation through long-range effects. For example, in the ATCase holoenzyme, binding of ATP to the regulatory subunits weakens the c:c interface (Edge et al., 1985, 1989), although the nucleotide binding site is far from the interface. In c_3, the linkage is probably *steric*, rather than *allosteric*. Because ligands bind between subunits, some of the energy of ligation can be used *directly* to stabilize subunit association. The contrasting effects of Cl^- and ATP may be due to the fact that Cl^- can fit easily into the active site of c_3 and promote subunit association by reducing the positive charge at the interface, whereas ATP does not fit fully into the active site (Honzatko & Lipscomb, 1982) and, while reducing the positive charge, may disrupt other interactions.

Although the experiments illustrate the potential of AGC in analyzing the energetics and pathways of folding and assembly and provide clear evidence for a change in pathway upon ligation, they suffer from the shortcomings of small zone experiments. Large zone experiments utilizing HPLC now in progress will improve the quantitation and allow the denaturation pathways in the presence and absence of ligands to be characterized in greater detail and with greater certainty.

Materials and methods

Materials

PALA was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. Purity was determined as described previously (Bromberg et al., 1990). Ultrapure urea was obtained from Schwarz/Mann Biotech. ATP (grade II, dilithium salt) was obtained from Sigma Chemical Company. All other chemicals and proteins used were obtained from Pharmacia Fine Chemicals or Sigma. The pH of all buffers was adjusted at room temperature.

Purification of c_3, verification of its purity, storage, desalting and exchange into experimental buffer (0.1 M K^+ HEPES, 0.2 mM DTT, 0.2 mM EDTA, pH 8.3, unless otherwise noted), and determination of protein concentration were performed as described previously (Bromberg et al., 1990, and references therein). Plasmids EK1104 and pEK17 were kindly provided by Dr. Evan Kantrowitz (Boston College, Chestnut Hill, Massachusetts).

Spectroscopy

Ultraviolet difference spectra were obtained with either a Hitachi model 100-60 or an EU700 series GCA/McPherson spectrophotometer. Two-compartment quartz mixing cells were employed and spectra were recorded from 230 to 320 nm before and after jumping c_3 from native conditions into various concentrations of urea at 14 °C in 0.1 M K^+ HEPES, 0.2 mM DTT, 0.2 mM EDTA, pH 8.3, and a final concentration of 5 μM c_3. The data in Figure 2A were fit to a single phase exponential decay

$$A(t) = A_\infty(1 - \exp^{-k_{app}t}), \quad (1)$$

where A_∞ is the fitted limiting value of $A(t)$ at 288 nm.

AGC

Methods were those of Ackers (cf. Ackers, 1970; Valdes & Ackers, 1979), modified as described by Bromberg et al. (1990). Resins were S-200 and S-300 Sephacryl from Pharmacia. Columns were thermostatted at 7.5 °C with a circulating waterbath. Excluded and included volume markers were blue dextran and glycine, respectively. Protein and glycine were postcolumn derivatized with OPA and detected with a Gilson Spectraglo fluorometer fitted with OPA filters. Blue dextran was detected at 620 nm. Experiments with CP were performed in a chromatographic chamber thermostatted at 12 °C. Colorimetric phosphate assays (Fiske & Subbarow, 1929; Spector et al., 1957) indicated that the concentration of CP eluting from the column was >2 mM.

The partition coefficient, σ, is defined as

$$\sigma = (V_e - V_0)/(V_i - V_0),$$

where V_e is the elution volume of the sample, V_i is the elution volume of glycine (the included volume of the gel), and V_0 is the elution volume of blue dextran (void volume). In small zone experiments, 0.5 mL of protein, blue dextran, and glycine were always loaded as a mixture, allowing the elution volume differences $(V_i - V_0)$ and $(V_e - V_0)$ to be determined directly in each column run from the positions of the 3 eluted peaks.

Previous experiments carried out with several single-site mutants of c_3 required high concentrations of βME to prevent aggregation and precipitation of the protein (Bromberg et al., 1990). The present experiments were also performed in the presence of 0.2 M βME. Titrations of c_3 with βME at 1 M urea show that 0.2 M βME destabilizes the association of the trimer by 0.5–1 kcal/mol (Bromberg, 1990).

Model-based analysis of AGC data

Data were fit with the nonlinear least-squares program NONLIN (Johnson & Frasier, 1985) on a Vax 8550. Following Pace (1986), free energies were assumed to be linear functions of [urea] such that ΔG_{xy}, the free energy of the equilibrium $x \leftrightarrow y$ at a given urea concentration, is given by

$$\Delta G_{xy} = \Delta G_{xy}{}^0 + A_{xy}[\text{urea}], \quad (2)$$

where $\Delta G_{xy}{}^0$ is the free energy in the absence of urea and A is the slope of the [urea] dependence.

The first model fit makes no assumptions about the processes involved and uses dimensionless equilibrium constants:

$$\mathrm{N} \overset{K_{\mathrm{NI}}}{\leftrightarrow} \mathrm{I} \overset{K_{\mathrm{IU}}}{\leftrightarrow} \mathrm{U}, \quad (3)$$

where N is the native protein, I is an intermediate, and U is the unfolded protein.

Because σ_{obs} is a weight average and $[c_{tot}] = [c_N] + [c_I] + [c_U]$, where $[c_{tot}]$, $[c_N]$, $[c_I]$, and $[c_U]$ are concentrations of total protein, N, I, and U, respectively, σ_{obs} is given by

$$\sigma_{obs} = \frac{\sigma_N + K_{NI}\sigma_I + K_{NI}K_{IU}\sigma_U}{1 + K_{NI} + K_{NI}K_{IU}}, \quad (4)$$

where σ_N, σ_I, and σ_U are partition coefficients of native c_3, intermediate, and unfolded c chains, respectively.

Data obtained in the absence of ligand and in the presence of ATP were fit to a model with a dissociation equilibrium constant for the first transition:

$$c_3 \overset{K_D}{\leftrightarrow} 3c_I \overset{K_U}{\leftrightarrow} 3c_U, \quad (5)$$

where c_3 is the native trimer, c_I is the swollen monomer, and c_U is the unfolded monomer. Here σ_{obs} is given by

$$\sigma_{obs} = \frac{3[c_I]^2\sigma_T/K_D + \sigma_I + K_U\sigma_U}{3[c_I]^2/K_D + 1 + K_U}. \quad (6)$$

$[c_I]$, the molar concentration of folded monomer, was approximated for each iteration by an interval division routine.

Data obtained in the presence of PALA, where the protein appears to dissociate and unfold simultaneously near 5.5 M urea, were analyzed in terms of a model with a dissociation equilibrium constant for the second transition:

$$c_3 \overset{K_{NI}}{\leftrightarrow} T_I \overset{K_D}{\leftrightarrow} 3c_U, \quad (7)$$

where c_3 is the native trimer, T_I is the swollen trimer, and c_U is the unfolded protein, and where σ_{obs} is given by

$$\sigma_{obs} = \frac{3[c_U]^2\sigma_{T_I}/K_{NI}K_D + 3[c_U]^2\sigma_{T_I}/K_D + \sigma_U}{3[c_U]^2/K_{NI}K_D + 3[c_U]^2/K_D + 1.0}. \quad (8)$$

Partition coefficients for the modeled molecular species did not vary with varying models, although they did vary with different ligands/inhibitors. For several fits, one of the σ values had to be constrained in order to obtain a physically meaningful fit. Parameter values for free energies and urea dependence of steps that were identical between models also did not vary. Including more than 1 intermediate in the models did not improve the quality of the fit to data obtained in the absence of ligands or in the presence of ATP or NaCl. Results for CP and PALA suggest an additional intermediate between 2 and 3 M urea; however, the number of points and the amplitude of the variations in σ were not sufficient to obtain convergence for models with more than 1 intermediate.

Activity measurements

Because activity was monitored over several hours, the colorimetric assay of Bencini et al. (1983) was modified to minimize the contribution of CP hydrolysis. Fifty-microliter aliquots of 80 mM CP, 0.32 M L-Asp, 0.1 M K^+ HEPES, 0.2 mM EDTA, pH 8.3, were frozen in microcentrifuge tubes and stored at −70 °C until needed. Before each assay, 0.75 mL 0.1 M K^+ HEPES, 0.2 mM EDTA, pH 8.3, containing various concentrations of urea, was added to the frozen substrate to give final concentrations of CP and L-Asp of 5 mM and 20 mM, respectively. Five micrograms of enzyme was added and the tube was incubated at 7 °C. After varying amounts of time, 0.75 mL of the assay solution was added to 3.0 mL of the colorimetric reagent (100 mM zinc acetate, 15 mM ammonium molybdate, pH 5) and incubated on ice for 3 min. OD_{350} was determined with a Spectronic 20 spectrophotometer kept in a cold chamber at 10 °C in the presence of desiccant. Phosphate standard curves obtained in the presence and absence of urea were identical, ruling out a reaction between urea and the colorimetric reagents.

Kinetic data (Fig. 5A) were fit to a single exponential decay

$$P_{obs(t)} = \Delta P(\infty)\exp^{-k_{app}t} + a, \quad (9)$$

where P is the experimental parameter (% activity), ΔP is the activity lost at infinite time, and a is the activity remaining at infinite time. Putative dissociation equilibria (K) can be calculated from the plateau value of the activity loss after jumping c_3 into low [urea] if one assumes that the loss of activity is a direct measure of the amount of dissociation:

$$K = (3P[c_{tot}])^3/(a[c_{tot}]), \quad (10)$$

where P is the percent activity lost at infinite time and a is the percent activity remaining at infinite time.

Acknowledgments

Part of this work is from a Ph.D. thesis submitted to Wesleyan University by S.B. This work was supported by NIH grant DK-17335 (to N.M.A.), a Connecticut High Technology Scholarship (to S.B.), and NIH NRSA F32-GM14400 (to V.J.L.). We thank Margaret Daugherty for performing the renaturation experiments; Clarinda MacLow, Kelly Morgan, and Anjulika Chawla for technical assistance; Hiroki Morizono and Dr. Ken Dill for critical reading of the manuscript; and Dr. Clare Woodward for helpful suggestions.

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