A stable intermediate in the equilibrium unfolding of *Escherichia coli* citrate synthase

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Abstract

Urea-induced unfolding of *Escherichia coli* citrate synthase occurs in two phases, as monitored by circular dichroism at 222 nm (measuring secondary structure) or by tryptophan fluorescence. In this paper we characterize the intermediate state, which retains about 40% of the ellipticity of the native state, and is stable between 2.5 M and 5.5 M urea, approximately. This intermediate binds significant amounts of the probe for hydrophobic surfaces, anilinonaphthalene sulfonate, but forms aggregates at least as high as an octamer, as shown by transverse urea gradient polyacrylamide electrophoresis. Thermal denaturation of *E. coli* citrate synthase also produces an intermediate at temperatures near 60 °C, which also retains about 40% of the native ellipticity and forms aggregates, as measured by electrospray-ionization/time-of-flight mass spectrometry. We have used a collection of “cavity-forming” mutant proteins, in which bulky buried hydrophobic residues are replaced by alanines, to explore the nature of the intermediate state further. A certain amount of these mutant proteins shows a destabilized intermediate, as measured by the urea concentration range in which the intermediate is observed. These mutants are found in parts of the citrate synthase sequence that, in a native state, form helices G, M, N, Q, R, and S. From this and other evidence, it is argued that the intermediate state is an aggregated state in which these six helices, or parts of them, remain folded, and that formation of this intermediate is also likely to be a key step in the folding of *E. coli* citrate synthase.

Keywords: citrate synthase; folding intermediate; protein folding

The most thorough investigations of protein folding and stability have been done with unusually small proteins, which are folded into single domains and display simple two-state unfolding processes (Jackson, 1998, and references therein). It is very desirable, however, to extend studies to larger, more complex, and therefore more typical proteins as far as possible. CS has attracted attention as a suitably more complex subject. The vertebrate examples are dimers of identical subunits, each of which consists of two distinct folding domains (Remington et al., 1982; Liao et al., 1991). The fold is all- α , and the active site is in the cleft between the domains. Residues from both subunits contribute to each active site, so that the dimer is the catalytically functional unit. The pig CS protein displays a simple two-state unfolding profile, as urea or guanidine HCl is added, which may be monitored easily by fluorescence or CD. This denaturation is not reversible upon simple dilution (West et al., 1990), although some renaturation can be

achieved using slow dialysis (Kelly & Price, 1992). Perhaps more interesting, renaturation of CS is greatly assisted by the addition of chaperonins, and pig CS is, in fact, a widely used substrate for chaperonin studies (Buchner et al., 1991; Zhi et al., 1992). It has been noted that pig CS undergoes thermal aggregation, making it an ideal chaperonin target (Ehrnsperger et al., 1997). Very recently, pig CS has been shown to be a suitable substrate for “artificial chaperones” (Daugherty et al., 1998).

CS has also been studied as a model for understanding how protein structure is adjusted to the needs of organisms that live at extremes of temperature. Thus, Danson, Hough, and colleagues have compared in detail the structure of the mesophilic pig CS with the thermophilic and extremely thermophilic CS proteins from two archaea, *Thermoplasma acidophilum* and *Pyrococcus furiosus*, respectively (Russell et al., 1994, 1997). These two archaeal CS proteins, only 28% identical in amino acid sequence to pig CS, are also homodimers and have basically the same secondary and tertiary structure, apart from the fact that the pig CS has an N-terminal extension and looser, more extensive looping between the helices (Russell et al., 1997). Recently, these studies have been extended to include a dimeric, cold-active CS from an Antarctic bacterium (Russell et al., 1998).

An even more complex folding problem is presented by another CS, that from Gram-negative bacteria such as *Escherichia coli*. *E. coli* CS is a mixture of dimers and hexamers of identical sub-

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Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; CD, circular dichroism; CS, citrate synthase; ESI, electrospray ionization; MS, mass spectrometry; TFBA, 3,3,3-trifluoro-1-bromoacetone; TOF, time of flight.

units, 426 amino acids in length. The dimer-hexamer equilibrium is affected by such factors as pH, ionic strength, and the presence of the allosteric inhibitor, NADH (Tong & Duckworth, 1975; Ayed et al., 1998). No X-ray crystallographic structure of *E. coli* CS has yet appeared, but the amino acid sequence is 28% identical with that of pig CS, and all the active site residues, and many other strongly conserved residues, can be recognized. A model for the folded subunit of *E. coli* CS, based on the pig CS structure and the sequence similarity, has been described (Duckworth et al., 1987). The roles of most active site residues of *E. coli* CS in catalysis have been verified by structure-function studies using mutant proteins (Pereira et al., 1994). Although no work on the interaction of *E. coli* CS with chaperonins has yet been published, the protein, like pig CS, is almost certainly a substrate for the GroE chaperone system, because *E. coli* cells not expressing GroE are also devoid of folded or soluble CS protein (Horwich et al., 1993).

In this paper we report a detailed study of the unfolding of *E. coli* CS by urea or heat and draw conclusions about the nature of a stable, partly unfolded state that appears at intermediate urea concentrations or temperatures.

Results

Unfolding of wild-type *E. coli* CS

The CD spectrum of *E. coli* CS is typical of all- α -helical proteins (Adler et al., 1973), with a double minimum at 208 and 222 nm (Fig. 1). The unfolding of structure, in response to the addition of urea, was measured by decrease in negative ellipticity at 222 nm. Unfolding occurred in two stages. The first stage, in which about 60% of the CD ellipticity was lost, had a midpoint at 1.75 M urea. This gave an intermediate state, which predominated in the range 2.5 to 5.5 M urea. Most of the remaining ellipticity was lost in a second process, with a midpoint at 6.5 M urea (Fig. 1). The same kind of two-stage unfolding occurred with GdnHCl, with midpoints of about 1 and 3 M for the two transitions (data not shown). Because the unfolding studies were conducted with a subunit concentration of about 2 μ M, where *E. coli* CS is partly hexameric, the contribution of hexamer subunit interactions to stability was tested by preparing the TFBA-modified derivative, which is completely dimeric under these conditions (Ayed et al., 1998). Only a slight effect on the midpoint of the first transition was seen (data not shown).

It was also possible to monitor *E. coli* CS denaturation by urea using tryptophan fluorescence. Again, a two-stage unfolding was seen, with about two-thirds of the change occurring in the first stage and the rest in the second. The fluorescence emission maximum of a protein gives information about the environment of the tryptophans. *E. coli* CS has three tryptophan residues, at positions 260, 391, and 395 (Ner et al., 1983). For native CS, $\lambda_{max} = 320$ nm, for the intermediate state $\lambda_{max} = 330$ nm, and for denatured protein $\lambda_{max} = 360$ nm (Fig. 2); thus, it appears that all three tryptophans are in regions that remain largely folded in the intermediate state.

Reversibility of unfolding

Full reversibility of urea denaturation was tested by dialyzing the denatured form (equilibrated with 9 M urea) directly into buffer. Protein precipitated, and no activity was recovered. To test the

Fig. 1. Urea denaturation of wild-type *E. coli* CS as monitored by CD. (A) MRE₂₂₂, normalized to the value in the absence of urea, as a function of urea concentration; (B) CD spectra at zero (●), 4 M (○), and 9 M (▲) urea.

reversibility of the second transition only, the dialysis was repeated, but now against 4 M urea (in the middle of the range where the intermediate is stable). An amount of ellipticity was recovered that matched the amount remaining when native CS was equilib-

Fig. 2. Tryptophan fluorescence emission maxima for CS equilibrated with different urea concentrations. Excitation was at 280 nm.

brated with 4 M urea; that is, by the criterion of CD spectrum, the second transition is reversible (data not shown). Dialyzing "intermediate" (prepared by equilibrating with 4 M urea) against buffer again failed to give any CS activity, or any increase in CD ellipticity, so the first transition is not reversible by this method.

Thermal denaturation

Thermal unfolding of wild-type CS was monitored by heating a single sample to a series of temperatures in a jacketed cell in the CD spectrometer, starting at 22 °C and ending at 90 °C, and measuring the ellipticity at each temperature. Although the main features of the temperature–ellipticity profile were reproduced in different experiments, profiles were not identical from one trial to the next, probably because heating rate was difficult to reproduce with the manually controlled temperature bath we used. Also, the protein always precipitated upon cooling again, showing that a real equilibrium was not being achieved. Nonetheless, thermal unfolding clearly occurred in two stages, like urea-induced unfolding. In a typical experiment (Fig. 3A), an intermediate, retaining about 40% of the initial ellipticity, was obtained in the temperature range of 55 to 70 °C, approximately.

It proved possible to examine the state of aggregation of the CS subunits as a function of temperature by the method of ESI-

Fig. 3. (A) Thermal denaturation of wild-type *E. coli* CS as monitored by CD. (B) ESI-TOFMS of CS at 60 °C. CS (10 μ M, subunit concentration) in 5 mM ammonium bicarbonate, pH 7.5, was heated to 60 °C and sprayed directly into the instrument. Charge envelopes assigned to monomer, dimer, trimer, tetramer, pentamer, hexamer, and octamer are labeled, as are selected peaks with the measured m/z ratios and/or assigned charge states.

TOFMS, already shown to give valuable information about the dimer–hexamer equilibrium of native *E. coli* CS (Ayed et al., 1998). TFBA-modified CS, which does not form hexamers at low concentrations, was used for this experiment. The mass spectrum of this protein, taken on a solution heated to 60 °C and sprayed directly into the instrument, showed a series of well-resolved charge envelopes, which could be assigned to monomer, dimer, trimer, tetramer, pentamer, hexamer, and octamer (Fig. 3B). That is, heating to 60 °C, a temperature that produces an intermediate form as measured by CD, leads to a mixture of aggregates.

Exposure of new hydrophobic surfaces during urea unfolding

We considered the possibility that the intermediate form produced by urea and/or heat has some similarity to a classical molten globule, that is, a partly unfolded form with retained secondary structure but no regular tertiary structure, in which extensive hydrophobic surfaces are exposed to solvent (Ptitsyn, 1992, 1995; Christensen & Pain, 1994; Kuwajima, 1996). To test this, we added 10 μ M ANS to the 2 μ M-CS solutions, and measured ANS-associated fluorescence after equilibration with different urea concentrations. ANS fluorescence is considerably enhanced by binding to native *E. coli* CS (Talgoy et al., 1979); control experiments also showed that urea by itself has very little effect on ANS fluorescence in the absence of CS. The experiment (Fig. 4) showed that fluorescence of the ANS–CS complex falls somewhat as urea is added up to about 1 M, climbs to a new value with a maximum at about 2.5 M urea, then starts to decline again, until eventually, by 8 M urea, it reaches the same value as if CS were not present. Figure 4 compares the ANS fluorescence profile, as a function of urea concentration, with the CD-monitored denaturation profile, from which it can be seen that the region of high ANS fluorescence does not simply coincide with that in which the folding intermediate is stable. Thus, while new hydrophobic ANS-binding surfaces are exposed in the course of the first transition, the surfaces progressively disappear as urea concentrations are increased further through a range in which little change in CS folding occurs as measured by ellipticity or tryptophan fluorescence.

Fig. 4. Effect of urea on ANS-CS fluorescence. Fluorescence of the ANS–CS complex (2.09 μ M CS subunits, 10 μ M ANS) equilibrated with different urea concentrations (●). Excitation was at 380 nm, and emission (in arbitrary units, left-hand axis) at 470 nm. For reference, a smoothed curve showing ellipticity as a function of urea concentration is also shown (scale on the right-hand axis).

ANS fluorescence was also used to probe for exposed hydrophobic surfaces during thermal denaturation of CS (data not shown). ANS fluorescence reached a maximum at about 50 °C, the midpoint of the first folding transition as monitored by CD, and then decreased gradually as the temperature was raised further. This response of ANS fluorescence to thermal denaturation is similar to that seen in the urea unfolding experiment.

While selective binding of ANS to some components of an equilibrium mixture of CS unfolding intermediates should affect the composition of the mixture, the effect will be small as long as the ANS concentration is kept low. This is because the maximum effect of ANS on any equilibrium constant is given by:

$$K_{obs} = K_{eq}(1 + [\text{ANS}]/K_{d,ans}),$$

where K_{obs} is the apparent equilibrium constant in the presence of a given amount of ANS, and K_{eq} is the value of that constant in the absence of ANS, while $K_{d,ans}$ is the dissociation constant for a CS-ANS complex whose formation inhibits the equilibrium represented by K_{eq} . As long as the ratio $[\text{ANS}]/K_{d,ans}$ is kept low, K_{obs} will remain close to K_{eq} . For native CS, which binds two molecules of ANS per subunit, $K_{d,ans}$ is about 200 μM (Talgoy et al., 1979). As unfolding proceeds, the number of ANS sites probably increases, but it is unlikely that $K_{d,ans}$ decreases—this would imply that unfolding increases the *specificity* of the binding. The present experiments were done with 10 μM ANS, so the ratio $[\text{ANS}]/K_{d,ans}$ is probably small, and thus it is valid to compare experiments in which urea was used to unfold CS with and without ANS present.

Transverse urea gradient gel electrophoresis

The technique of transverse urea gradient gel electrophoresis can be used to obtain information about the hydrodynamic properties of a protein as it unfolds, as a continuous function of urea concentration. In the simplest two-state mechanism of unfolding, the compact native structure will move through the gel more quickly, while unfolded state, being more extended, will move more slowly. Figure 5 shows a much more complex situation for *E. coli* CS. A number of CS protein bands appear in the region of the gel corresponding to 3 to 6 M urea, that is, the concentration range over which the folding intermediate is stable. Because all bands are formed of the same subunits, the basis of separation must be size, and therefore, the intermediate state observed by fluorescence and CD around 4 M urea is a collection of aggregates. At least eight bands can be distinguished in these gels, which presumably represent species ranging from a monomer to an octamer at least. In a separate experiment using urea concentrations between 0 and 1 M, it was found that the characteristic dimer-hexamer mixture observed upon gel electrophoresis of native CS (Molgat et al., 1992) is converted to subunits well below 1 M urea (data not shown). This is consistent with the observation that 0.6 M urea is sufficient to abolish CS activity, and it confirms that the enhanced ANS binding at 2.5 M urea is not the result of subunit dissociation alone.

The finding that the unfolding intermediate at 2.5 to 5.5 M urea is a collection of aggregates is consistent with the observation that the intermediate of thermal unfolding, observed at 60 °C, is also a collection of aggregates as demonstrated by ESI-TOFMS (see above). It appears that either urea or heat can induce the same

Fig. 5. Urea transverse gradient electrophoresis of *E. coli* CS. The concentration of urea increases linearly from about 0.5 to 9 M in the direction of the arrow. A sample of 0.3 mg protein, dissolved in 20 mM Tris-Cl, 1 mM EDTA, pH 7.8, was run through the slab gel for 3.5 h at 150 V (25 mA). The gel was then stained with Coomassie blue. The bands in the middle of the gradient are interpreted as CS aggregates with different numbers of subunits.

unfolding intermediate: a mixture of aggregates of subunits in which about 40% of the secondary structure is retained.

A structural hypothesis for the nature of the intermediate

As a first step toward understanding what parts of the CS subunits retain their structure in the unfolding intermediate, we took advantage of the existence of CS chimeras previously constructed by Molgat et al. (1992). These chimeras were formed by engineering the structural genes for the CS proteins of two Gram-negative bacteria, *E. coli* and *Acinetobacter anitratum*, by a domain swapping procedure in which DNA encoding the large domain from one of the proteins was combined with DNA encoding the small domain from the other (Molgat et al., 1992). The two proteins, designated ACI::eco (for a chimera with the large domain from *A. anitratum* CS and the small domain from the *E. coli* protein) and ECO::aci (for the other chimera), were originally used to study regulatory properties, but it was noted that the ACI::eco protein, like *A. anitratum* CS, was much more resistant to urea inactivation than the *E. coli* enzyme and the ECO::aci chimera (Molgat et al., 1992). We studied the urea unfolding of the two native CS proteins and the two chimeras, and found that the *A. anitratum* CS itself and the ACI::eco protein both unfold in a single transition, with a midpoint at about 5 M urea, while the ECO::aci protein, like the *E. coli* CS, unfolded in two stages, with an intermediate that retained about 40% of the native ellipticity (Fig. 6). The stability range of the ECO::aci intermediate was a bit narrower than that for *E. coli* CS, about 3.5 to 5 M urea, and the transitions between states appeared to be less cooperative. The striking qualitative conclusion from these studies is that an unfolding intermediate is found for CS proteins whose large domain is derived from the *E. coli* CS protein. This led us to the hypothesis that, for the *E. coli* protein, the first transition (involving the loss of 60% of the ellipticity) is simply the unfolding of the large domains, while the second transition (with loss of a further 30% of the ellipticity) is the unfolding of the small domains. Arguments can be offered both for and against this hypothesis, and the evidence will be discussed later, but we decided to test it by making a series of amino acid

Fig. 6. Urea denaturation curves for *A. anitratum* CS and chimeras as monitored by CD. (A) *A. anitratum* CS (○) and the ACI::eco CS chimera (●). (B) *E. coli* CS (○, similar data set to Fig. 1) and the ECO::aci CS chimera (●). For an explanation of the chimeras, see the text.

replacements in *E. coli* CS that would destabilize the large or the small domains selectively.

The amino acid replacements to be made were chosen on the basis of the well-tested expectation that internal cavities are created when well-buried, bulky hydrophobic side chains such as those of phenylalanine, leucine, isoleucine, valine, or methionine are replaced by the methyl group of alanine. In one well-studied series of cavity mutants of T4 lysozyme, Matthews' group has shown that the size of the cavity correlates well with the effect on stability (Eriksson et al., 1992; Matthews, 1996). Our candidates for replacement were chosen by aligning the known CS sequences, identifying bulky hydrophobic residues that are strongly conserved throughout the series, and then checking that each residue was really buried in the known three-dimensional structure of pig heart CS. A total of 16 amino acid replacements were studied: 12 alanine replacements of strongly conserved, buried Phe, Leu, Met, and Val residues; alanine replacements of the three tryptophans; and one active site Arg→Leu replacement that was known (Pereira et al., 1994) to have a severe effect on activity. The mutant proteins are listed in Table 1.

All but two of these mutant proteins could be purified to homogeneity by our standard methods. The W391A mutant protein was also purified extensively, but the final preparation still contained contaminating bands. Although a band of the correct mobility for CS was seen in crude extracts of cells containing plasmid for the W395A mutant gene, little of this survived the purification procedure, and what did was heavily contaminated with other proteins. Some properties of the purified mutant proteins are listed in Table 1. Many retained a significant part of wild-type enzymatic activity, but with a few, such as M131A, L259A, L275A, and F383A, the activity was severely reduced. This effect was investigated further by steady-state kinetics (Table 2). Some comments on the effects of the mutations on activity will be made in the Discussion.

Table 1. Properties of mutant CS proteins used in this study

Protein	Location of residue; usual residue here in prokaryotes (P) and eukaryotes (E)	MRE ₂₂₂ (deg·cm ² ·dmol ⁻¹)	Specific activity (units/mg)
Wild-type CS	—	-16,700	70
TFBA CS	—	-15,600	30
F121A	Helix F; Phe (P); Leu/Ile (E)	-17,900	12
M131A	Helix G; Met (P, E)	-16,600	1.0
L141A	Helix G; Leu (P, E)	-18,000	30
L164A	Helix I; Leu (P, E)	-17,100	90
L259A	Helix M; Leu (P, E)	-15,900	3.0
W260A	Helix M; Trp (P); Gly (E)	-14,200	10
M274A	Helix N; Met (P); Trp (E)	-18,600	5.0
L275A	Helix N; Leu (P); Leu/Ile (E)	-18,500	0.06
L328A	Helix P; Leu (P); Leu/Phe (E)	-17,300	70
L336A	Helix Q; Leu (P); Phe/Ala (E)	-17,300	30
V338A	Helix Q; Val/Leu (P); Leu (E)	-15,900	30
L369A	Helix R; Leu (P, E)	-16,700	20
F383A	Helix S; Phe (P, E)	-17,200	0.03
R387A	Helix S; Arg (P, E)	-16,300	0.002
W391A	Helix S; Trp (P); Val/Ile (E)	-16,100	1.0
W395A	Helix S; Trp (P); Leu (E)	Not isolated	Not done

Table 2. Steady-state kinetic parameters of the CS reaction as catalyzed by selected mutants

Citrate synthase	K_{AcCoA} (μM)	K_{oa} (μM)	Comments
Wild type	120 ± 20	26 ± 5	Anderson and Duckworth (1988)
M131A	360 ± 175	160 ± 60	This paper
L164A	68 ± 11	26 ± 4	This paper
L259A	840 ± 180	11 ± 1	This paper
L275A	$1,000^a$	380 ± 90	Sigmoid AcCoA saturation; this paper
L369A	690 ± 140	19 ± 1	This paper
F383A	$2,000 \pm 100^a$	47 ± 4	Sigmoid AcCoA saturation; Pereira et al. (1994)

^aBecause the saturation curve was sigmoid, the substrate concentration needed for half-maximal activity was taken.

Urea-induced unfolding of mutant CS proteins

Like wild-type CS, each of the mutant proteins clearly showed biphasic urea unfolding patterns when monitored by CD. Examples of the data obtained for some mutant proteins are shown in

Figure 7. The fractions of native ellipticity lost in each of the two phases were similar for all mutants, while the urea concentrations needed to reach the midpoint of each phase were usually different. For each protein studied, Table 3 lists two key descriptive parameters for each of the two phases—the $[\text{urea}]_{1/2}$ value and the frac-

Fig. 7. Examples of urea denaturation curves for CS mutants. In all cases the data for the mutant are shown (●), and the fine solid line is the best fit to Equation 4. For reference, a smoothed curve (broken line) representing the wild-type CS data from Figure 1 is also shown. (A) F121A mutant; (B) M131A mutant; (C) L164A mutant; (D) L336A mutant. Data from these and other curves were fitted to denaturation curves, as explained in the Appendix, to give the parameters in Table 3.

Table 3. Fitted parameters for urea denaturation of wild-type and mutant citrate synthase (for details see text)

Protein	First phase		Second phase	
	[urea] _{1/2} (M)	Fraction of ellipticity lost	[urea] _{1/2} (M)	Fraction of ellipticity lost
wtCS	1.79 ± 0.06	0.61 ± 0.02	6.57 ± 0.06	0.32 ± 0.02
F121A	1.58 ± 0.06	0.65 ± 0.03	5.94 ± 0.12	0.29 ± 0.03
M131A	2.27 ± 0.08	0.60 ± 0.03	6.23 ± 0.10	0.32 ± 0.03
L141A	1.08 ± 0.07	0.53 ± 0.04	5.97 ± 0.12	0.35 ± 0.03
L164A	1.40 ± 0.08	0.57 ± 0.04	6.25 ± 0.09	0.32 ± 0.03
L259A	2.06 ± 0.08	0.61 ± 0.03	6.13 ± 0.09	0.30 ± 0.03
W260A	1.68 ± 0.04	0.60 ± 0.02	6.21 ± 0.06	0.26 ± 0.02
M274A	1.76 ± 0.04	0.57 ± 0.02	6.09 ± 0.08	0.35 ± 0.02
L275A	1.31 ± 0.05	0.55 ± 0.03	6.23 ± 0.17	0.38 ± 0.04
L326A	1.85 ± 0.10	0.67 ± 0.03	6.36 ± 0.07	0.36 ± 0.07
L336A	2.43 ± 0.07	0.60 ± 0.03	5.95 ± 0.13	0.28 ± 0.03
V338A	2.30 ± 0.07	0.58 ± 0.03	6.14 ± 0.10	0.37 ± 0.03
L369A	2.35 ± 0.05	0.57 ± 0.02	6.16 ± 0.04	0.29 ± 0.02
F383A	1.79 ± 0.05	0.50 ± 0.03	5.41 ± 0.10	0.40 ± 0.02
R387L	2.62 ± 0.04	0.59 ± 0.03	7.15 ± 0.36	0.40 ± 0.08
W391A	2.32 ± 0.22	0.66 ± 0.08	5.63 ± 0.14	0.17 ± 0.05

tion of native ellipticity lost. The curve-fitting procedure used to extract these four parameters from the data is explained in the Appendix.

The [urea]_{1/2} values are proportional to the standard free energies of stabilization of the native state, for a series of mutants of a given protein, provided that the parameter *m* is the same for all the mutants in the series. This parameter *m* is the absolute value of the slope of a line that relates the observed ΔG for the unfolding process to the urea concentration (Pace, 1986). In general, *m* should not change as a result of single mutations, because this parameter is usually interpreted as a measure of the difference between the numbers of urea interaction sites in the native and the denatured state, and the mutants studied differ at only one or two amino acids. We have calculated *m* values for both transitions, for all the proteins studied, and most of them cluster around 1.0 kcal·L·mol⁻² (data not shown). In the analysis that follows, we will assume that the [urea]_{1/2} values for the two transitions are a reliable indication of the relative stabilities of the two states that the transitions link.

The mutants we studied had been designed to destabilize the folded state or states of CS, and so we expected that the unfolding profile of the mutant proteins would be shifted toward lower urea concentrations. For four of the mutants—F121A, L141A, L164A, and L275A—the first phase of the unfolding does occur at a lower urea concentration, and for all but one, R387L, the second phase is also shifted downward to different extents. An intriguing and unexpected finding, however, is that seven of the mutants—M131A, L259A, L336A, V338A, L369A, R387L, and W391A—actually show a *stabilized* first unfolding phase. Three of these amino acid changes occur in the large domain, three are in the small domain, and one (R387L) is in the active site, at or near the domain interface. This apparent stabilization of the native state cannot be explained by a model in which the large domain of each subunit unfolds in the first phase, and the small domain not until the second.

Fluorescence properties of the W260A mutant protein

As already mentioned, attempts were made to produce mutant proteins in which one of the three tryptophans was replaced by alanine. The W260A mutant was isolated in good yield and purity without trouble, and a small amount of partly purified W391A protein was also isolated. We were unable to obtain purified samples of the third potential mutant, W395A, and this protein was not studied further. Urea denaturation of W260A protein was monitored not only by CD but also by tryptophan fluorescence, in the hope of gaining insight about the environment of Trp260 itself in the wild-type protein. The fluorescence emission spectrum of the W260A protein was clearly different from the wild-type protein (Fig. 8A)—it lacked a shoulder at 360–370 nm. The simplest interpretation of these data is that fluorescence of Trp260 is the source of the shoulder on the red side of the fluorescence emission spectrum. The position of this shoulder, in fact, is significantly

Fig. 8. Urea denaturation of the CS W260A mutant as monitored by tryptophan fluorescence; excitation was at 280 nm and protein subunit concentration was 2.1 μ M. **A:** Fluorescence emission spectra for wild-type CS (solid line) and the W260A mutant (broken line). **B:** Fluorescence (relative to native state) of proteins equilibrated with different concentrations of urea; (●) wild-type CS; (○) W260A mutant. The lines show the trends of the data and have no theoretical significance.

more toward the red than the fluorescence emission peak shown by tryptophan itself in small peptides, 356 nm, so it seems that Trp260 must be in an unusually polar environment in the native state. The other two tryptophans, Trp391 and Trp395, are more deeply buried. Urea denaturation of W260A produces an intermediate species, as usual, but whereas with wild-type protein this retained about 65% as much fluorescence as the native state, with W260A only about 45% of the native fluorescence was retained in the intermediate (Fig. 8B). This result shows that the environment of Trp260, as well as that of Trp391 and Trp395, retains some structure in the intermediate state.

Discussion

Nature of the urea unfolding pathway for wild-type CS

About 60% of the secondary structure of wild-type *E. coli* CS is lost in an event favored by urea concentrations in excess of 2 M. The resulting *I* or intermediate state resists further unfolding until the urea concentration is raised to about 6 M. This *I* state must display new exposed hydrophobic surfaces, because it enhances the fluorescence of low concentrations of ANS more than the native or *N* state does, but these surfaces become progressively buried because of aggregation of the subunits. Aggregation of the *I* state subunits leads to species at least as high as an octamer, at the protein concentrations we studied. Thermal denaturation of wild-type *E. coli* CS likewise proceeds through a well-defined intermediate, which may well be structurally equivalent to the *I* state seen in urea unfolding, because it also loses about 60% of the secondary structure and also aggregates to form species containing at least eight subunits. Aggregation of the *I* state is very likely the reason why the *I* state is stable over a moderate range of urea concentrations or temperatures. All three tryptophan residues in the sequence are partly buried in the *N* state, with Trp260 in an unusually polar environment; all three become more solvent exposed in the *I* state, although they are not fully exposed until the most completely unfolded or *D* state is formed. The *D* state seems to have some residual structure. It still has about 10% of the ellipticity of the native protein at 222 nm, and the urea transient gradient experiment shows two different oligomeric forms at high urea concentration (Figs. 1, 5).

Interpretation of unfolding data for amino acid substitution mutant proteins

As already noted, our simple hypothesis that the first or *N* to *I* transition is the unfolding of the large domains, while the *I* to *D* transition is the unfolding of the small ones, is inconsistent with the results from the mutant proteins. Nonetheless, the data obtained from the mutants do provide much detailed information about the nature of the *I* state. Fourteen mutations, located at different points within the subunit structure, destabilize the second transition—they shift the $[\text{urea}]_{1/2}$ value for the second transition downward by amounts between 0.3 and 1.2 M (Table 3). Four of these mutations have no effect on the first transition, within the errors of the measurements, and three destabilize it.

Unexpectedly, seven mutations *increase* the stability of the first transition—they shift the $[\text{urea}]_{1/2}$ value upward by 0.3 to 0.8 M (Table 3). This latter effect can be explained in two ways: either the *N* state is stabilized, or the *I* state is destabilized more than the

Table 4. *E. coli* CS mutants and their associated secondary structural elements

Category A— Transition 1 midpoint decreased		Category B— Transition 1 midpoint increased		Category C— Transition 1 midpoint unchanged	
Mutant	Location	Mutant	Location	Mutant	Location
F121A	Helix F	M131A	Helix G	W260A	Helix M
L141A	Helix G	L259A	Helix M	M274A	Helix N
L164A	Helix I	L336A	Helix Q	L326A	Helix P
L275A	Helix N	V338A	Helix Q	F383A	Helix S
		L369A	Helix R		
		R387A	Helix S		

N state is. The former possibility is unlikely, as the mutations were designed to destabilize the *N* state. The second possibility is more interesting, however, for it implies that any amino acid substitution that appears to stabilize the first transition must be in a part of the protein that is still folded in the *I* state. In Table 4 we place the 10 mutants that have an effect on the stability of the *N* to *I* transition into two categories, as measured by the $[\text{urea}]_{1/2}$ values for that transition (Table 3; the W391A mutant is omitted, but is discussed separately below). Category A mutants show a destabilized *N* to *I* transition, while for Category B that transition is destabilized. Category C mutants have no detectable effect on the stability of the transition. Also specified in Table 4, for each mutant protein, is the secondary structure element (using the helix nomenclature employed for pig CS; Remington et al., 1982) in which each substituted amino acid is expected to be found.

From the information in Table 4, it may be tentatively concluded that parts of helices G (N-terminal part), M, Q, R, and S remain folded in the *I* state. The single residues studied in helices F and I, the one residue near the C-terminus of helix G, and one of the two residues studied in helix N fall into Category A, however. This may mean that those parts of the protein unfold completely during the *N* to *I* transition. It is also possible, of course, that at least some of the regions defined by Category A mutants do remain folded in the *I* state, but that the mutations affect the stability of the *N* state more than that of the intermediate. Similarly, failure of the mutations in Category C to affect the *N* to *I* transition may be because they destabilize *N* and *I* to about the same extent, and the effects cancel each other.

At least three of the residues in Category C, in fact, probably are located within a structure that is retained in the intermediate. Mutants M274A (helix N) and F383A (helix S) from this category are probably in regions still folded in the *I* state, for the *I* to *D* transition occurs at significantly lower urea concentrations in these mutants (Table 3). As already noted, evidence from fluorescence monitoring of unfolding indicates that all three tryptophan residues, Trp260 (helix M), Trp391, and Trp395 (both helix S) are also in folded regions in the intermediate. In summary, we propose that the folded portion of the *I* state consists, at a minimum, of secondary structure elements derived from most of the small domain (helices N, Q, and R) plus three core helices of the large domain (helices G, M, and S). The proposed structure, shown in Figure 9, contains about 115 residues still in a folded state. The number of α -helical residues in this structure is about 34% of the α -helical

Fig. 9. Diagram of proposed CS unfolding intermediate. One subunit is shown, using the previously described model of the *E. coli* CS subunit (Duckworth et al., 1987). The six helices proposed to retain their structure in the intermediate or *I* state are depicted in gray and labeled according to Remington et al. (1982), while the rest of the molecule is shown as a string tracing the peptide backbone as it would appear in the native structure. This figure was prepared using the program MOLSCRIPT (Kraulis, 1991) and the Protein Data Bank structure file 1CTS (described by Remington et al., 1982).

residues in the *N* state—in excellent agreement with the amplitude of the second transition as measured by CD, $32 \pm 2\%$ of the total mean residue ellipticity observed for native CS. Modeling further suggests that the surface of this proposed *I* state should indeed be highly hydrophobic (details not shown).

It is interesting that mutation of either of Trp391 or Trp395 to alanine affected the stability of CS so profoundly. The fact that these residues are predicted to be on the same face of helix S, coupled with the evidence that they remain in an apolar environment in the *I* state, raises the possibility that they are important in initiating CS folding. Tryptophan residues in the fully unfolded yeast phosphoglycerate kinase, and other aromatic residues in barnase (Arcus et al., 1994), 434 repressor (Neri et al., 1992), and FK506 binding protein (Logan et al., 1994) have been shown to have residual structure, even under strongly denaturing conditions. These residues are thought to act as hydrophobic nucleation sites for the initiation of folding, and this may be the case with Trp391 and Trp395 of *E. coli* CS.

Significance of the *I* state for CS folding

The reversibility of the *I* to *D* transition, when urea concentration is decreased by simple dialysis, suggests that the *I* state may be the product of a key early step in the folding pathway of *E. coli* CS, an intermediate with considerable exposed hydrophobic surface, prone to aggregation. The fact that CS is one of the proteins that fails to accumulate in *E. coli* strains where the chaperonin GroE is not expressed (Horwich et al., 1993) leads us to speculate that this *I* state is the actual substrate for chaperonin binding. It is also pos-

sible that the *I* state is an off-pathway intermediate, not directly involved in CS folding. Experiments to test these possibilities are an obvious next step.

Enzymatic activities of CS mutants

Eight of the cavity-inducing mutants in Table 1 had specific enzyme activities within a factor of 10 of the wild-type value. These replacements are remote from the active site, and their small effects on activity may arise because *E. coli* CS is an allosteric enzyme, and the two conformational states implied by that fact might well be destabilized by a given mutation to different extents. The L164A mutant shows higher affinity for both substrates than wild-type CS does (Table 2), perhaps because this mutant protein is shifted toward the R or active state. The R329L mutation of *E. coli* CS (not included in this study) has this same effect on the allosteric equilibrium, according to a detailed kinetic analysis reported earlier (Anderson et al., 1991). On the other hand, the kinetic data for the L369A mutant suggest that this mutation has the opposite effect—the R state is destabilized more than the T state, so that the K_m for acetyl-CoA is increased about fivefold (Table 2).

The most striking effects on activity are seen with the mutants M131A, L259A, L275A, and F383A. The F383A mutation has a markedly weaker affinity for one substrate, acetyl-CoA, though its affinity for OAA is almost normal. The acetyl-CoA saturation curve for this mutant is markedly sigmoid, even in the presence of amounts of the allosteric activator, KCl, which convert acetyl-CoA saturation of wild-type CS to a hyperbolic form (Pereira et al., 1994). The phenyl group of Phe383 is located in the active site, where it makes an unusual edge-on interaction with the acetyl group of acetyl-CoA (Remington et al., 1982). We have argued that this phenyl ring is somehow critical for productive binding of acetyl-CoA, and that removal of the ring by the F383A mutation favors conversion of the enzyme to the non-acetyl-CoA binding conformational state (Pereira et al., 1994). Inspection of the known CS structures now shows that Leu259 and Met131 (which, like Phe383, are conserved in all known CS sequences) are parts of a hydrophobic network that may help maintain a catalytically favorable conformation for Phe383. Leu259 interacts sideways with the phenyl ring, while Met131 interacts with Leu259. The acetyl-CoA affinity of these three mutants decreases in the order M131A < L259A < F383A (Table 2); that is, the effect on acetyl-CoA binding becomes more severe as the mutation moves through the hydrophobic network toward the active. It is also interesting to note that Met131, Leu259, and Phe383 are all parts of the proposed *I* state; thus, this network probably assembles early in the CS folding pathway.

The mutants M274A and L275A, particularly the latter, exhibit greatly reduced specific activities, and in the latter case acetyl-CoA saturation is sigmoid even in the presence of the allosteric activator KCl (Table 2). This effect resembles that of the F383A mutation already mentioned, but in the case of L275A the affinity for the other substrate, OAA, is also lower. The homologues of M274A and L275A, in the known CS structures, interact with a hydrophobic side chain (Val314 in pig CS) in the acetyl-CoA-binding loop between helices O and P. The *E. coli* CS residue equivalent to Val314, Leu300, was not mutated in our studies, so its importance in acetyl-CoA binding is still untested. The strong effect of the L275A mutation on enzyme activity, including the weakening of affinity for both substrates, suggests that Leu275 is

critical for the maintenance of the architecture of the entire active site. Further studies will be needed before we know whether this is the case.

Methods

Wild-type and mutant CS proteins

Previously described procedures were used for expression, purification, assay, and kinetic characterization of wild-type and mutant CS proteins (Pereira et al., 1994). Mutant proteins were prepared by the modified Zoller and Smith method (Zoller & Smith, 1983; Kunkel et al., 1987), also as previously described (Anderson & Duckworth, 1988). Wild-type CS was alkylated with TFBA by the method of Donald et al. (1991).

Preparation of urea denaturation curves

Stock urea solutions (9 to 10 M) in 20 mM Tris, 1 mM EDTA, pH 7.8, were prepared fresh and used within three days. Twenty to 30 different urea concentrations were typically used to define each denaturation curve by mixing the appropriate volumes of urea, buffer, and CS solutions for a total solution volume of 1 mL. Unless otherwise stated, CS concentrations of 0.1 mg·mL⁻¹ (2.09 μM) were used for all denaturation curves, and were determined spectrophotometrically for each protein stock solution. Solutions were prepared and incubated overnight at room temperature before CD or fluorescence measurements.

CD and fluorescence spectroscopies

A JASCO A500 spectropolarimeter was used for all CD measurements in a 0.1-cm quartz cell (Hellma Cells, Inc., Forest Hills, New York). Temperature was regulated by a Haake circulating water bath. To minimize the contribution of instrument drift during lengthy experiments, baseline measurements were taken at least every hour. The mean residue ellipticity at 222 nm (MRE₂₂₂) in units of deg·cm²·dmol⁻¹ was calculated for each point in the denaturation curve by the equation:

$$\text{MRE}_\lambda = \theta_\lambda M_{\text{avg}} / (10LC)$$

where θ_λ is the measured ellipticity in millidegrees at wavelength λ , M_{avg} is the average mass per amino acid of CS (112.4 Da), 10 is a scaling factor, L is the path length of the sample cell in centimeters, and C is the concentration of the protein in mg·mL⁻¹.

Fluorescence measurements were made on a Fluoro IV Fluorimeter, with excitation at 280 or 295 nm, in 1 cm quartz cuvettes. All buffers and stock solutions were filtered through a 0.2 μM cellulose acetate filter (Nalge Nunc International Corp., Rochester, New York).

Reversibility of CS denaturation

Samples preincubated at different urea concentrations were dialyzed against 0 and 4 M urea to test the reversibility of the two transitions in CS unfolding. CD and fluorescence measurements of the samples were carried out before and after dialysis. Because native extinction coefficients would not be useful under denaturing

conditions, the Bradford protein assay was used for protein determination (Bradford, 1976).

ANS fluorescence enhancement

ANS has been used extensively as a marker for exposed hydrophobic surfaces, a method originally introduced by Semisotnov et al. (1991). ANS, 10 μM, was included in each of the urea solutions in the CS denaturation curve to determine whether CS exhibited exposed hydrophobic surfaces. ANS emission was monitored at 470 nm with excitation at 380 nm; ANS fluorescence is not enhanced by urea alone.

Urea gradient gel electrophoresis

Urea gradient acrylamide gels were prepared by a method similar to that described (Goldenberg, 1988) where the urea concentration gradient is perpendicular to the direction of electrophoresis. Gels (15.7 × 18 cm and 0.1 cm thick) were cast from high to low urea concentration in the presence of 33 mM Tris-glycine, pH 7.5, pouring the polymerizing mixture with a gradient-forming device. Acrylamide concentrations were typically 5 to 10%, and a reverse acrylamide gradient (1–2% difference) was employed to minimize the reduced mobility of protein due to viscosity differences between high and low concentrations of urea. The success of the gradient formation was evaluated by including bromophenol blue in the concentrated urea solution and inspecting the color gradation. Typically, a sample of 0.1 to 0.3 mg CS in 20 mM Tris, 1 mM EDTA, pH 7.8 was loaded on prerun gels and was electrophoresed for 5 h at 200 V (~25 mA) in a 33 mM Tris-glycine buffer, pH 7.5. The gels were stained with Coomassie blue in a solution of 10% acetic acid and 25% methanol.

Data fitting

Plots of fraction of native ellipticity remaining after equilibration of CS proteins with different urea solutions were fitted to Equation 1 (see Appendix) using the Macintosh program Kaleidagraph v3.0.5 (Synergy Software, Reading, Pennsylvania).

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Appendix: Analysis of urea denaturation curves

Plots of CD values as a function of urea concentration, in a series of samples that had been allowed to equilibrate in different concentrations of urea, were fitted to an equation that was chosen to extract desired parameters as directly as possible. Equation A1 describes a given denaturation profile, y_{obs} vs. [urea], as the sum of two saturation functions, in each of which the urea concentration is raised to an empirical power n , and a certain fraction y of the ellipticity of the native protein is lost. The concentration of urea needed to reach the midpoint of each transition is designated C for [urea]_{1/2}. This approach is empirical and is analogous to the well-known Hill equation as a description of sigmoid saturation curves. It has the advantage that the two [urea]_{1/2} and y -values are extracted directly in the curve-fitting procedure. If the two phases are designated 1 and 2, we can write Equation A1:

$$y_{obs} = 1 - \frac{y_1 \cdot [\text{urea}]^{n_1}}{C_1^{n_1} + [\text{urea}]^{n_1}} - \frac{y_2 \cdot [\text{urea}]^{n_2}}{C_2^{n_2} + [\text{urea}]^{n_2}} \quad (\text{A1})$$

This equation has six independently adjustable parameters, of which the values of C_1 and C_2 (the [urea]_{1/2} concentrations for the two transitions) and y_1 and y_2 are of interest; these are reported in Table 3. The values of the urea exponents n_1 and n_2 , which are not listed in Table 3, simply reflect the steepness of the transitions between states as a function of urea concentration.