

Stability and folding properties of a model β -sheet protein, *Escherichia coli* CspA

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Abstract

Although β -sheets represent a sizable fraction of the secondary structure found in proteins, the forces guiding the formation of β -sheets are still not well understood. Here we examine the folding of a small, all β -sheet protein, the *E. coli* major cold shock protein CspA, using both equilibrium and kinetic methods. The equilibrium denaturation of CspA is reversible and displays a single transition between folded and unfolded states. The kinetic traces of the unfolding and refolding of CspA studied by stopped-flow fluorescence spectroscopy are monoexponential and thus also consistent with a two-state model. In the absence of denaturant, CspA refolds very fast with a time constant of 5 ms. The unfolding of CspA is also rapid, and at urea concentrations above the denaturation midpoint, the rate of unfolding is largely independent of urea concentration. This suggests that the transition state ensemble more closely resembles the native state in terms of solvent accessibility than the denatured state. Based on the model of a compact transition state and on an unusual structural feature of CspA, a solvent-exposed cluster of aromatic side chains, we propose a novel folding mechanism for CspA. We have also investigated the possible complications that may arise from attaching polyhistidine affinity tags to the carboxy and amino termini of CspA.

Keywords: cold shock; fast protein folding; polyhistidine tags; protein stability; stopped-flow

The challenge to understanding β -sheet structure is to decipher how residues, which may be distant from one another along the polypeptide chain, locate one another specifically and in a reasonable amount of time. To help elucidate the mechanism of sheet formation, we have characterized the folding and stability of the all β -sheet protein, CspA, the major cold shock protein of *E. coli*. CspA is an ideal model for β -sheet folding studies not only because, as shown here, it is well-behaved biophysically, but also because the protein's structure represents a general single-stranded nucleic acid-binding motif. Having a firm grasp of the structure–function relationship in this protein should aid in future protein design endeavors that could lead to the development of novel therapeutics and other molecular tools.

Progress in understanding β -structure has, in part, been hampered by the difficulty in developing peptide models for sheets. Initial attempts to make isolated β -hairpins resulted in peptide aggregation because of the competition between identical intramolecular and inter-molecular interactions, and successful design of hairpins has only recently been reported (Blanco et al., 1994; Searle et al., 1995; Nesloney & Kelly, 1996; Ramirez-Alvarado et al., 1996).

The folding of several small β -sheet-containing proteins have been studied by kinetic methods. Although in general it appears

that smaller proteins are more likely to fold rapidly without detectable intermediates, the spectrum of folding behaviors for this group of proteins is broad. For example, the immunoglobulin-binding domain of streptococcal protein G (56 amino acids) folds rapidly—with (time constant (τ) = 5 ms) (Alexander et al., 1992), as does the *Bacillus subtilis* cold shock protein CspB (67 amino acids; τ = 1 ms) (Schindler et al., 1995). The SH3 domain of spectrin (62 amino acids) also folds without detectable intermediates, though more slowly (τ = 350 ms) (Viguera et al., 1994). Conversely, ubiquitin (76 amino acids) folds very rapidly (in under 3 ms), but through an intermediate (Khorasanizadeh et al., 1996). The related “ β clam” proteins (131 amino acids), cellular retinoic acid binding protein, and intestinal fatty acid binding protein (IFABP) acquire native structure on the second time scale via folding intermediates (Ropson et al., 1990; Clark et al., 1996). Beta conformation is detected early in interleukin 1 β (153 amino acids), but stable inter-strand hydrogen bonds do not appear until later (Varley et al., 1993). For the majority of molecules (Kiefhaber, 1995), the predominantly helical domain of hen egg white lysozyme (129 amino acids) folds within 200 ms, while the mostly β -sheet β -domain takes more than one second to fold (Dobson et al., 1994). Most curiously, the folding of the mostly β -sheet protein, β -lactoglobulin (162 amino acids), proceeds via an α -helical burst-phase intermediate (Hamada et al., 1996). In summary, it does not appear that β -sheet proteins generally require a folding intermediate or fold more slowly than α -helical proteins as initially postulated (Rudolph et al., 1990; Ropson & Frieden, 1992; Varley

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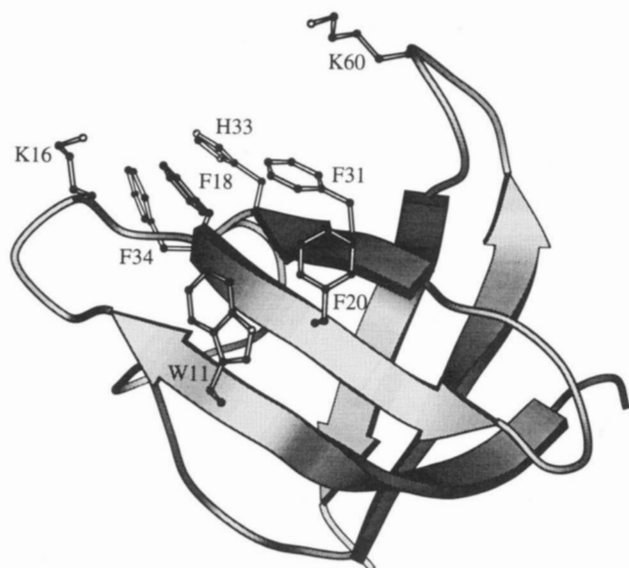


Fig. 1. Ribbon drawing of the cold shock protein CspA from *E. coli*. Side chains comprising the nucleic acid binding site are shown. This site includes an unusual solvent-exposed aromatic cluster. This representation is from MOLSCRIPT (Kraulis, 1991) based on the coordinates of Schindelin et al. (Schindelin et al., 1994).

et al., 1993; Liu et al., 1994). Rapid acquisition of β -structure is achievable and, therefore, the question of how β -sheet proteins fold remains open and all the more enticing. Furthermore, determining how β -sheets assemble could shed light on the protein folding problem in general.

Here we report on both the equilibrium and kinetic folding of CspA, a small all- β protein. This protein is expressed at high levels when bacteria are chilled to $<15^\circ\text{C}$ (Goldstein et al., 1990) and is proposed to function as an RNA chaperone (Jiang et al., 1997). CspA is an ideal simple model system for studying protein folding for many reasons. The three-dimensional structure of CspA (Fig. 1) has been solved by both X-ray crystallography (Schindelin et al., 1994) and nuclear magnetic resonance spectroscopy (Newkirk et al., 1994). CspA contains an unusual cluster of solvent-exposed aromatic residues that are postulated to participate in nucleic acid binding (Newkirk et al., 1994). Among these residues is a single tryptophan (Trp 11), which can serve as a fluorescent probe for monitoring the folding spectroscopically. Furthermore, CspA has no disulfide bonds, *cis* peptide bonds, co-factors, or post-translational modifications that may complicate folding. It is also soluble in the folded and the unfolded state and monomeric. By contrast, *B. subtilis* CspB, whose folding has been studied in detail (Schindler et al., 1995; Schindler & Schmid, 1996) dimerizes under certain conditions (Schindelin et al., 1993; Makhatadze & Maratheil, 1994). Therefore, CspA is potentially a simpler model for investigating folding than CspB.

We have examined the stability of CspA to urea and GdnHCl denaturation at 25°C , as well as at 10°C , the temperature at which CspA functions *in vivo*. We have also monitored its stability to thermal denaturation by circular dichroism (CD) and fluorescence spectroscopies. The folding kinetics of CspA were examined using stopped-flow fluorescence. Because of the common use of poly-histidine tags for affinity purification of proteins, we have compared wild-type CspA to two variants: CspA, with six histidine

residues at the carboxy terminus (CspA-H6), and CspA, with six histidines at the amino terminus followed by a Factor Xa protease cleavage site (H6-Xa-CspA).

Although it is often expected that homologous proteins fold by similar pathways and on similar time scales (Hooke et al., 1994; Kragelund et al., 1996), it cannot be assumed. Case in point: a single-point mutation can dramatically alter the folding or refolding rates of a protein (Matouschek et al., 1995; Milla et al., 1995). Comparison of our data to a recent transition state theory-based analysis of the folding of *B. subtilis* CspB (Schindler & Schmid, 1996) indicates that these proteins, which are 58% identical, *do* behave similarly. Although the folding of CspB is faster, both proteins fold via a transition state that closely resembles the native state. We propose that a nucleation site comprised of conserved, solvent-exposed aromatic amino acids stabilizes the cold-shock proteins in native-like transition states, enabling the β -strands to align productively and for the proteins to proceed quickly to their native states. Alignment of aromatic residues may guide the folding of other small β -sheet proteins as well.

Results

Equilibrium denaturation followed by fluorescence

Although the single tryptophan (Trp 11) in CspA is partially exposed to solvent on the surface of the protein (Fig. 1) (Newkirk et al., 1994; Schindelin et al., 1994), its intrinsic fluorescence emission decreases approximately 50% from the folded to the unfolded state. The fluorescence emission spectra for the histidine-tagged CspA variants are identical (data not shown). The emission maximum of Trp 11 shifts slightly from 349 to 353 nm upon unfolding as expected for a partially exposed tryptophan, and confirming a previous report (Chatterjee et al., 1993).

Figure 2A shows the changes in fluorescence intensity at 349 nm of the three CspA variants upon titration with urea at 25°C . In each case, a single transition between the initial and final states was observed. The three variants exhibited a similar dependence of free energy of unfolding on urea concentration ($m_{eq} \approx 0.7$; Table 1). Linear extrapolation of the data results in the free energy of unfolding in the absence of urea ($\Delta G_{\text{H}_2\text{O}}$) as described by Equation 1. In Table 1, the values for $\Delta G_{\text{H}_2\text{O}}$, m_{eq} , and $[\text{urea}]_{1/2}$ (C_m) are reported for the urea unfolding of the CspA variants followed by fluorescence at 10 and 25°C . The unfolding free energy of all three variants at both temperatures is close to 3.0 kcal/mol.

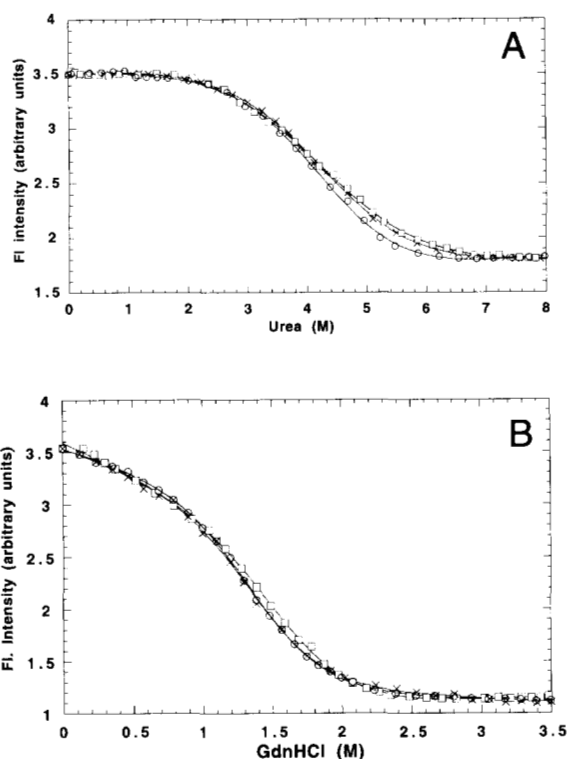
We also determined the change in fluorescence intensity at 349 nm of the CspA variants upon titration with GdnHCl at 25°C and 10°C . Again, a single transition was observed between the initial and final states for the variants at both temperatures. The values for $\Delta G_{\text{H}_2\text{O}}$, m_{eq} , and $[\text{GdnHCl}]_{1/2}$ are listed in Table 2. All three variants had similar GdnHCl unfolding curves at 25°C (Fig. 2B).

Equilibrium denaturation followed by circular dichroism

The CspA variants exhibited similar far UV-CD spectra at high and low temperatures. The spectrum of wild type CspA is shown in Figure 3. CspA exhibits an unusual far UV-CD spectrum for a predominantly β -sheet protein. The positive ellipticity in the far-UV region is attributed to the aromatic chromophores positioned on the surface of β -strands 1, 2, and 3 (Chatterjee et al., 1993) (Fig. 1).

Table 1. Midpoint of denaturation, m_{eq} , and ΔG_{H_2O} values for urea denaturation followed by fluorescence at 10 and 25°C

Temperature (°C)	[urea] _{1/2} (M)		ΔG_{H_2O} (kcal/mol)		m_{eq} (kcal/mol * M)	
	10	25	10	25	10	25
Wild-type CspA	4.9 ± 0.1	4.2 ± 0.1	2.9 ± 0.1	3.0 ± 0.1	0.59 ± 0.05	0.71 ± 0.04
H6-Xa-CspA	5.0 ± 0.2	4.2 ± 0.1	3.3 ± 0.2	2.9 ± 0.1	0.66 ± 0.04	0.69 ± 0.05
CspA-H6	4.5 ± 0.2	4.2 ± 0.1	2.8 ± 0.2	3.2 ± 0.2	0.64 ± 0.05	0.78 ± 0.06

**Fig. 2.** A: Urea-induced denaturation of CspA variants followed by fluorescence at 25°C. excitation, 280 nm; excitation slit, 2.5 nm; emission 349 nm; emission slit, 2.5 nm. Protein concentration was 7.0 μ M in 50 mM potassium phosphate, 100 mM KCl, pH 7.0 (Buffer B). (x) Wild-type CspA; (square) H6-Xa-CspA; (circle) CspA-H6. B: GdnHCl-induced denaturation of CspA variants followed by fluorescence at 25°C; (x) Wild-type CspA; (square) H6-Xa-CspA; (circle) CspA-H6. Protein concentration was 7.0 μ M in Buffer B.

These chromophores mask the peptide backbone optical activity typically seen for β -sheet proteins. The aromatic side chains have been shown to play a major role in the far-UV region of several other proteins including avidin (Green & Melamed, 1966), soybean and limbean trypsin inhibitor (Woody, 1994), cobra toxin (Dufton & Hider, 1983), and CspB (Schindler et al., 1995). The same mean residue ellipticity was observed for CspA at a concentration of 5 μ M (not shown) as at 50 μ M, implying that there is no structural change in this concentration range as might be caused by protein oligomerization.

To determine the reversibility of thermal denaturation, the three CspA variants were denatured by equilibration at 84°C for 30 min. CD spectra were compared at 10°C before and after denaturation. Figure 3 shows the far UV-CD spectra of wild-type CspA before and after thermal denaturation. Identical spectra were observed for the histidine tagged variants. In all three cases, at least 98% of the CD signal was recovered after thermal denaturation, demonstrating that the refolding of CspA is reversible and that the histidine tags do not affect this reversibility. Furthermore, the folding reaction remained reversible when the protein concentration was varied from 17 to 60 μ M, suggesting that aggregation does not play a role in this range.

The thermal stability of the CspA variants was determined by monitoring the change in ellipticity at 222 nm as a function of temperature in the range from 2 to 90°C (Fig. 4). The denaturation midpoint (T_m) was equivalent for all three variants ($T_m = 58.6 \pm 1.0^\circ\text{C}$). This is 4° lower than observed previously (Chatterjee et al., 1993). A single transition was observed between the folded and unfolded states for the three variants.

One criterion for a two-state folding mechanism is identical denaturation behavior observed by multiple techniques (Eftink, 1995). To this end, we have used circular dichroism spectroscopy to study the equilibrium unfolding of CspA as a function of urea (Table 3)

Table 2. Midpoint of denaturation, ΔG_{H_2O} , and m_{eq} values for GdnHCl denaturation followed by fluorescence at 10 and 25°C

Temperature (°C)	[GdnCl] _{1/2} (M)		ΔG_{H_2O} (kcal/mol)		m_{eq} (kcal/mol * M)	
	10	25	10	25	10	25
Wild-type CspA	1.8 ± 0.2	1.4 ± 0.1	3.1 ± 0.2	3.1 ± 0.1	1.7 ± 0.2	2.2 ± 0.1
H6-Xa-CspA	1.6 ± 0.1	1.6 ± 0.1	3.8 ± 0.2	3.2 ± 0.2	2.4 ± 0.2	2.0 ± 0.1
CspA-H6	1.6 ± 0.1	1.4 ± 0.1	3.3 ± 0.1	3.0 ± 0.1	2.1 ± 0.1	2.2 ± 0.1

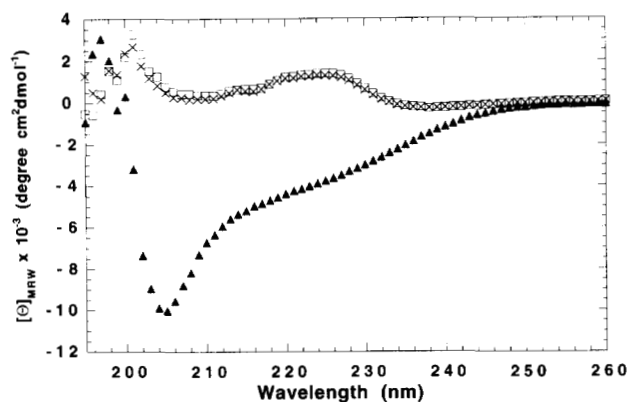


Fig. 3. Far UV-CD spectra of wild-type CspA at different temperatures; (square) 10°C; (triangle) 84°C; (×) 10°C after exposure to 84°C for 30 min. The path length of the cuvette was 1 mm and the protein concentration was 50 μ M in Buffer B.

and GdnHCl (Table 4) concentration to complement the fluorescence experiments. Because of the weak signal at 222 nm and manual mixing, the urea and GdnHCl titrations had a larger experimental error than observed for the titrations followed by fluorescence, which underwent constant stirring (Tables 1 and 2). Despite these technical difficulties, urea and GdnHCl denaturation followed by CD were consistent with our data from fluorescence at 25°C.

Folding followed by stopped flow fluorescence

The folding kinetics of CspA were followed by monitoring the change in fluorescence of Trp 11. Unfolding and refolding were initiated by a 10- or 11-fold dilution of the native and unfolded proteins, respectively, to the desired final urea concentration. Under all the conditions observed, the unfolding and refolding were well modeled by monoexponential time courses (Fig. 5). Fitting the kinetic data to a biphasic exponential time course did not result in a better fit, as the rate constant obtained for the second phase

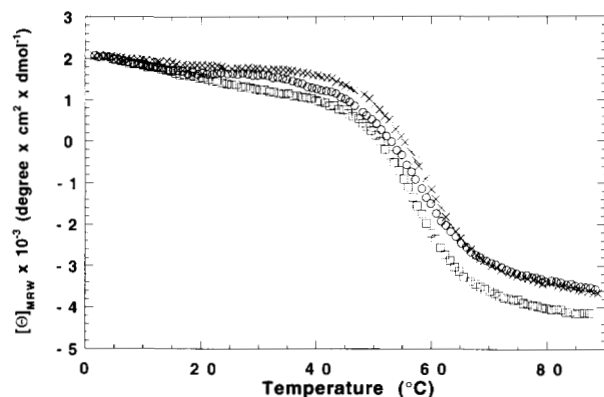


Fig. 4. Thermal stability of *E. coli* CspA variants in buffer B followed by far UV-CD; mean residue weight ellipticity at 222 nm as a function of temperature. (×) Wild-type CspA; (square) H6-Xa-CspA; (circle) CspA-H6. Temperature was increased from 2 to 90°C at a rate of 0.33°C/min. Protein concentration was 50.0 μ M in buffer containing 50 mM potassium phosphate, 100 mM KCl, pH 7.0.

Table 3. Midpoint of denaturation, m_{eq} , and ΔG_{H_2O} , values for urea denaturation followed by circular dichroism spectroscopy at 25°C

	[urea] _{1/2} (M)	ΔG_{H_2O} (kcal/mol)	m_{eq} (kcal/mol/M)
Wild-type CspA	4.0 \pm 1.1	2.6 \pm 0.5	0.6 \pm 0.1
H6-Xa-CspA	4.5 \pm 1.1	2.6 \pm 0.5	0.6 \pm 0.1
CspA-H6	4.1 \pm 0.9	3.1 \pm 0.4	0.7 \pm 0.1

was negative. The dependence of the measured rate constants (λ) as a function of urea concentration at 25°C for wild-type CspA are shown in Figure 6. Typically, the apparent rate constants (λ) follow a V-shaped dependence on denaturant concentration, the apex being the midpoint of denaturation (Tanford, 1970; Matthews & Hurle, 1987). However, the apparent rate constants for CspA unfolding showed virtually no dependence on urea concentration at concentrations above 4 M, causing the chevron plot to resemble a “lazy L” rather than the characteristic “V” shape. The same phenomenon was also observed for the *B. subtilis* homologue, CspB (Schindler et al., 1995; Schindler & Schmid, 1996).

All three CspA variants exhibited rapid unfolding and refolding behavior (Table 5). Wild-type and H6-Xa-CspA had comparable rates of refolding and unfolding at 10 and 25°C. CspA-H6, however, is predicted to re-fold slightly more rapidly (271 s^{-1}) than wild-type CspA (199 s^{-1}) in the absence of denaturant.

The kinetic data are consistent with a two-state folding mechanism. Unfolding and refolding were reversible at 10 and 25°C between 0.7 and 7 M urea based on the result that the same rate constants were observed at 4.0 M urea (Fig. 6), which is close to the equilibrium transition midpoint of 4.2 \pm 0.1 M urea. Also, there was no loss in fluorescence amplitude for unfolding or refolding.

Discussion

Effect of histidine affinity tags on the biophysical properties of CspA

It is often assumed that affinity tags added to proteins are not structured in solution. Because of the frequent use of such tags, we examined their effects on the equilibrium stability and folding properties of CspA. Milla and co-workers have studied the effects of polyhistidine affinity tags on the properties of Arc repressor

Table 4. Midpoint of denaturation, m_{eq} , and ΔG_{H_2O} values for GdnHCl denaturation followed by circular dichroism spectroscopy at 25°C

	[GdnCl] _{1/2} (M)	ΔG_{H_2O} (kcal/mol)	m_{eq} (kcal/mol/M)
Wild-type CspA	1.5 \pm 0.4	2.4 \pm 0.5	1.7 \pm 0.3
H6-Xa-CspA	1.3 \pm 0.2	3.7 \pm 0.5	2.9 \pm 0.4
CspA-H6	1.6 \pm 0.3	3.3 \pm 0.4	2.1 \pm 0.3

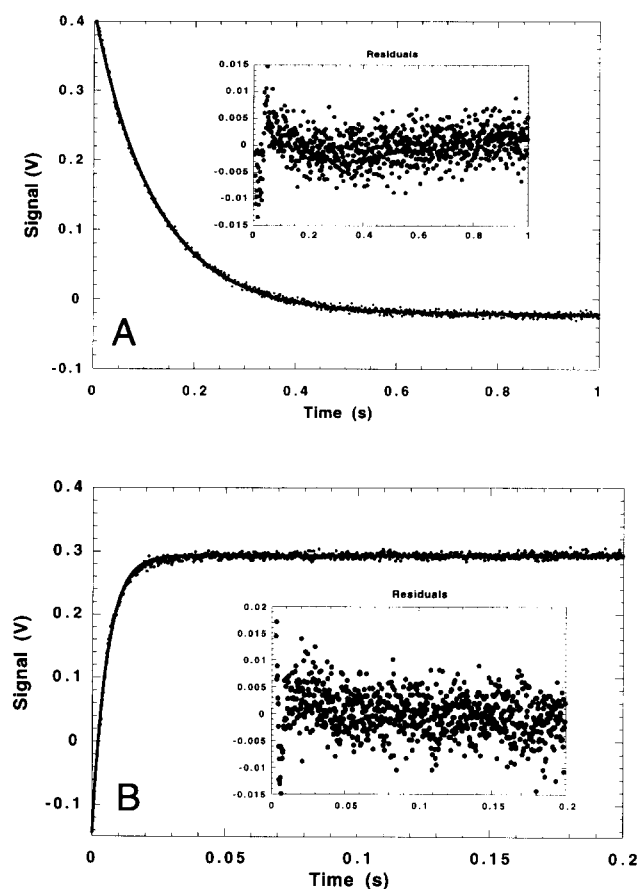


Fig. 5. Kinetic traces for unfolding (A) and refolding (B) of wild-type CspA. Residuals of the signal are shown in the insets. Unfolding was initiated by an 11-fold dilution of CspA in buffer B to a final urea concentration of 6.4 M. Refolding was initiated by an 11-fold dilution of CspA in 7 M urea to a final urea concentration of 0.64 M. The final protein concentration for both experiments was 5.1 μM . The observed amplitude and an apparent rate constant (λ) were 0.43 V and 8.1 s^{-1} for unfolding and 0.44 V, and 182 s^{-1} for refolding.

(Milla et al., 1993, 1995). The tags were found to have little effect on the stability or folding of this protein. It is not known, however, whether this result is general. It may be the case that a protein like CspA, which binds single-stranded nucleic acids, may be more

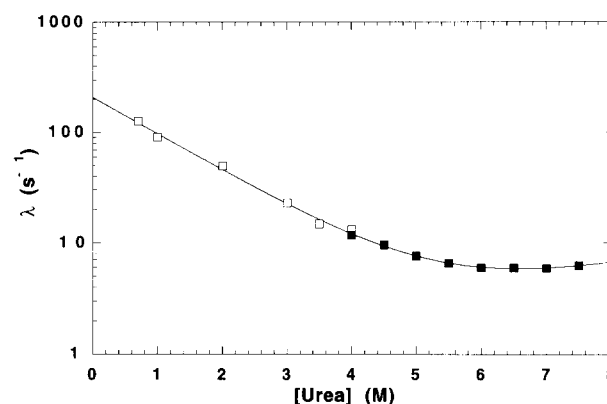


Fig. 6. The dependence of the apparent rate constant (λ) on urea concentration for wild type CspA; unfolding (filled symbol); refolding (open symbol). Final CspA concentrations after 10-fold dilution were 5.0 μM . The kinetics were monitored by the change in fluorescence above 325 nm in buffer B; excitation was 280 nm. The apparent rate constants are the average of at least 12 kinetic traces. The unfolding and refolding observed rate constants (λ) versus urea concentration were fit to the sum of Equations 5a and 5b.

sensitive to polyhistidine tags because they resemble the ligand. The affinity tags comprise a significant percentage of the CspA sequence: almost 9% of the residues of CspA-H6 and 14% of the residues of H6-Xa-CspA. The tags may alter either the folding behavior or spectroscopic properties of the protein by interacting with the wild-type portion of CspA or by adopting a defined structure.

All three CspA variants are comparable in terms of stability measured by thermal, guanidine hydrochloride, and urea denaturation at 25 $^{\circ}\text{C}$. Although the stabilities were similar, CD analysis of the thermal denaturation of the three proteins revealed that the N-terminal tag is not structurally silent, but may contribute a small amount of additional structure, as evidenced by the larger overall change in ellipticity upon unfolding (Fig. 4). One would expect that the addition of 10 residues (six histidines plus four residues comprising the Factor Xa site) at the amino terminus of a protein would be unstructured. If these 10 residues contained no structure in the native protein, we would expect that the overall change in mean residue ellipticity would decrease slightly. The observed increase may indicate that some of these are in a helical or β -sheet

Table 5. Kinetics of CspA unfolding and refolding in urea^a

Temperature ($^{\circ}\text{C}$)	Wild-type CspA		H6-Xa-CspA		CspA-H6		Error
	10	25	10	25	10	25	
k_u (H_2O) (s^{-1})	3.3	4.2	3.0	4.9	2.6	3.2	± 7
k_f (H_2O) (s^{-1})	188	199	170	224	189	271	± 14
m_u	0.07	0.05	0.06	0.02	0.03	0.19	± 0.07
m_f	-0.69	-0.72	-0.92	-0.82	-0.91	-0.72	± 0.1
m_{eq}	0.43	0.45	0.55	0.50	0.53	0.54	± 0.20
α^{\ddagger}	0.10	0.07	0.06	0.03	0.03	0.22	± 0.13

^aValues were obtained from the kinetic two-state analysis (Equations 5a and 5b) of the chevron plots. m_u and m_f values were obtained by extrapolation to 0 M denaturant. m_{eq} and α^{\ddagger} values were obtained from Equations 6 and 7, respectively.

conformation. However, this additional structure does not affect the thermal denaturation midpoint of the protein. Kinetic analysis repeatedly demonstrated that CspA-H6 refolds slightly faster than the other two variants at 25 °C ($k_f = 271 \text{ s}^{-1}$). This faster refolding rate was not observed at 10 °C. Although this increased rate may not be significant, it may indicate that the histidines on the C-terminal end act as an intra-molecular catalyst that facilitates the folding process.

The equilibrium and kinetic studies presented here show that the affinity tags affect the biophysical properties of CspA only modestly, even though they comprise a significant portion of the whole protein. Therefore, studies on variants of tagged CspA should be comparable with those on analogous wild-type CspA mutants.

CspA folding is consistent with a two-state mechanism

All kinetic and equilibrium experiments performed on CspA suggest that it folds by a two-state process where only the native and the denatured states are significantly populated. Our equilibrium denaturation experiments show an unfolding curve with a single transition. The same unfolding transition was obtained by two independent methods, fluorescence and far UV-CD. Although the CD spectrum of CspA is dominated in the far-UV region by the aromatic cluster that includes fluorescent Trp 11, a loss or retention of secondary structure non-concomitantly with the dissolution of the aromatic cluster may be observed as either a non-cooperative transition, or as a much broader transition leading to inconsistent values of $[\text{urea}]_{1/2}$ and/or $\Delta G_{\text{H}_2\text{O}}$. However, both spectroscopic methods gave comparable free energy values ($\Delta G_{\text{H}_2\text{O}} = 3.0 \text{ kcal/mol}$) and $[\text{urea}]_{1/2}$ values (4.2 M). The single unfolding curve was observed independent of the denaturant or denaturation method, i.e., urea, GdnHCl, or heat. Furthermore, the thermal denaturation of CspA is at least 98% reversible. Taken together, the equilibrium unfolding data obtained by the two different spectroscopic methods are consistent with a two state model of unfolding, at least at steady state (Eftink, 1995).

It is not uncommon, however, for proteins that exhibit two state unfolding behavior at equilibrium, to have kinetically identifiable intermediates (Jennings & Wright, 1993; Khorasanizadeh et al., 1996). CspA does not have any detectable kinetic intermediates on the millisecond or greater time scale. Both the unfolding and refolding of CspA monitored by stopped-flow fluorescence are monophasic and well described by monoexponential time dependences. The rate constants for unfolding and refolding are linearly dependent on the concentration of denaturant (Fig. 6). Another important criterion for the two-state argument is close agreement of the m -values obtained from equilibrium and kinetic experiments (Equation 6). CspA exhibits an atypical dependence on urea for unfolding. The m_u values observed from kinetic experiments are small. Although inconsistency in the m -values from kinetic and equilibrium experiments usually suggests the presence of intermediates, in the case of CspA, the small m_u values and the error associated with fitting a curve to a small slope complicates comparison of m_{eq} values for kinetic and equilibrium experiments. The equilibrium and kinetic m values ($\text{kcal mol}^{-1} \text{ M}^{-1}$) at 25 °C are as follows: 0.71 and 0.45 for wild-type CspA; 0.78 and 0.54 for CspA-H6; 0.69 and 0.50 for H6-Xa-CspA. However, the urea denaturation midpoint ($[\text{urea}]_{1/2}$) of wild-type CspA is in excellent agreement with kinetic experiments. Finally, the amplitudes of the kinetic traces account for the entire fluorescence change expected (Fig. 5): no burst phase was observed.

The above points are strong evidence for a two-state mechanism. However, inherent limitations in kinetic measurements detect only intermediates with stabilities greater than 10% of the native state (Jackson & Fersht, 1991). Therefore, highly unstable intermediates may still be present on the folding pathway. Also, transient intermediates that may be present within the dead time of the experiments (2 ms) would not be detected.

CspA folds rapidly without intermediates

CspA is a β -sheet protein that folds rapidly with no detectable intermediates. This observation is interesting from two standpoints. First, because of the long chain separation between residues in β -sheets, proteins containing this type of secondary structure have been expected to fold more slowly than helical proteins. Our observations of CspA do not fulfill this expectation. CspA folds very rapidly—its extrapolated refolding rate in the absence of denaturant at 25 °C is 199 s^{-1} . This rate is comparable to only two other β -sheet proteins studied thus far: *B. subtilis* CspB (Schindler et al., 1995) and ubiquitin (Khorasanizadeh et al., 1996). Although it may turn out that the upper limit for fast folding is intrinsically higher for all-helical proteins than for all-sheet proteins (Burton et al., 1996), millisecond folding is clearly possible for β -sheet proteins. Second, CspA folds fast in the absence of intermediates. CspB folds even more rapidly without detectable intermediates (Schindler et al., 1995; Schindler & Schmid, 1996). This lack of intermediates along the folding pathways of the cold shock proteins is not entirely surprising, because they have only one domain and no disulfide bonds. It does, however, contradict the hypothesis that intermediates are necessary to direct a protein to the final folded conformation (Kuwajima et al., 1987; Kim & Baldwin, 1990) and that intermediates are required for rapid β -sheet folding (Rudolph et al., 1990; Ropson & Frieden, 1992; Varley et al., 1993; Liu et al., 1994).

CspA has a compact transition state

Although CspA appears to fold without intermediates, it is still possible to envision its folding pathway by studying the transition state of folding. The solvent accessibility of the transition state (α^\ddagger) may be inferred from the dependence of the folding and refolding rate constants on urea concentration as reflected in Equation 7 (Tanford, 1970; Chen et al., 1989). The low value of α^\ddagger calculated for CspA suggests that its ensemble of transition state structures resembles the native state much more closely than the denatured state. For wild-type CspA, $\alpha^\ddagger = 0.07$, implying that the transition state is 93% native-like. Although Burton and co-workers have noted the lack of a relationship between the folding rate constant and the compactness of the transition state (Burton et al., 1996), we believe that in this case, the unusually compact transition state is correlated with the observed rapid folding of CspA and its homologue CspB. For both of these proteins, there is essentially no dependence on urea concentration above 4.0 M [$m_u = 0.05 \text{ kcal mol}^{-1} \text{ M}^{-1}$ for CspA and $0.03 \text{ kcal mol}^{-1} \text{ M}^{-1}$ for CspB (Schindler & Schmid, 1996)] indicating that the native state and the activated states of folding interact similarly with the denaturant.

An unusual structural feature of CspA is the large, solvent accessible cluster of aromatic residues on β -strands one, two, and three. Six aromatic residues contribute to this cluster, which to-

gether with other nonpolar atoms, create a continuous nonpolar patch of 790 \AA^2 , comprising 19% of the total surface area of CspA. This is unusual, particularly for such a small protein, because nonpolar patches greater than 350 \AA^2 are highly uncommon (Lijnzaad et al., 1996). Although this aromatic patch is undoubtedly involved in nucleic acid binding (Newkirk et al., 1994), it may also direct folding. Tisi and Evans have observed that smaller solvent exposed hydrophobic clusters are often seen in proteins with β -barrel architectures and postulate that these clusters may assist in the correct alignment of β -strands (Tisi & Evans, 1995). We speculate that coalescence of the surface aromatic residues occurs in the folding transition of CspA. The conserved aromatic cluster in the cold shock proteins may therefore serve a dual purpose in ligand binding as well as rapid folding. It is worth noting that the SH3 domain of spectrin is another small β -barrel protein that has a cluster of aromatic amino acids on its surface (mostly tyrosines) that are involved in polyproline peptide binding. This protein also re-folds rapidly and via a compact transition state (Viguera et al., 1994).

To test whether the aromatic cluster aids in nucleating the folding of CspA, we examined the refolding of wild-type CspA from 7 to 0.64 M urea in the presence of a 15-mer deoxyoligonucleotide ($K_D = 6 \mu\text{M}$; Hillier et al., in press). We hypothesized that folding would be accelerated because the ligand would stabilize a transition state in which the aromatic cluster is formed. We observed a faster rate of folding in the presence of the 15-mer ($\lambda = 230 \text{ s}^{-1}$) compared to an apparent rate constant of 131 s^{-1} under the same conditions in the absence of the oligonucleotide. Additionally, of 14 aromatic cluster mutants investigated thus far, all are destabilizing (Hillier et al., in press), implying that the residues responsible for the protein's function are also necessary for stability. Although these preliminary results support our hypothesis, additional experiments, including more extensive ligand binding studies together with an analysis of the effects of aromatic cluster mutations on the transition state, are necessary to determine whether the aromatic cluster is indeed critical to the rapid folding of CspA.

Materials and methods

Materials

Guanidine hydrochloride (GdnHCl) 8.0 M sequenal grade was obtained from Pierce. Urea analytical grade was obtained from ICN. The components of all other buffers and purification reagents were of analytical grade and purchased from Fisher. Ni-NTA agarose used to purify the histidine-tagged CspA was purchased from Qiagen (Germany). Factor Xa protease used to cleave the affinity tag was purchased from New England Biolabs. Protein concentration was determined using a UV-visible Hewlett-Packard 8452A diode array spectrophotometer. The extinction coefficient was calculated to be $8,437 \text{ M}^{-1}$ (Pace et al., 1995).

Cloning of CspA variants

The gene coding for CspA with six histidines at the carboxy terminus (CspA-H6) was created synthetically and subsequently ligated into the vector pAD100 (Davidson & Sauer, 1994). Expression of CspA-H6 protein is controlled by a hybrid trp/lac repressor-based trc promoter. The plasmid (pLG200) was introduced into the *E. coli* strain X90 by electroporation. The gene sequence was

verified using the dideoxy chain termination method (Sanger et al., 1977). The gene for CspA with N-terminal histidines and the Factor Xa cleavage site (H6-Xa-CspA) was created by PCR using the above-described C-terminal six histidine construct as a template. The nonannealing 5' end of the primer for the beginning of the new gene was designed to code for the amino acids M(H)₆IEGR. The primer for the 3' end was designed to omit the C-terminal H₆ tag. The PCR product was cloned into the polylinker region of the plasmid pAED4 (Doerring, 1992), contains a T7 polymerase-based promoter. This plasmid (pBH1) was transformed into the *E. coli* strain BL21(DE3). The gene for the C-terminal histidine-tagged CspA was also subsequently cloned into the vector pAED4 using a similar PCR method.

Protein purification

Cells with the plasmid containing the *cspA* gene of interest were grown to an OD_{600} of 0.6 then induced with 1 mM IPTG for 3 h. From an original culture volume of 6 L, the cell pellets were resuspended in 40 mL of Buffer A (6 M GdnHCl, 0.1 M NaH_2PO_4 , 10 mM Tris; pH 8.0) and stirred at room temperature for 1 h. The lysate was cleared by centrifugation at $15,000 \times g$, and the supernatant loaded on a 1-mL Ni-NTA column equilibrated with Buffer A. The column was washed with 100 mL of Buffer A. The protein was eluted with 15 mL of Buffer F (6 M GdnHCl, 0.2 M acetic acid) and dialyzed against 4 L of Buffer B (50 mM potassium phosphate, 100 mM KCl, pH 7.0) with one exchange. Wild-type CspA was obtained by cleaving the histidine tag with Factor Xa protease at a 1:100 (w/w) ratio for 15 h at room temperature and then separated via a G-75 (Pharmacia) size exclusion column. The molecular mass of CspA-H6 was determined using a MicroMass Quattro II electrospray mass spectrometer. The mass obtained (8094 amu) was consistent with complete processing of the N-terminal methionine.

GdnHCl and urea-induced denaturation followed by fluorescence

Fluorescence measurements were performed using a Spex Fluorolog 2 or Spex Fluoromax-2 by exciting the single tryptophan of CspA (Trp 11) at 280 nm and monitoring its emission at 349 nm (wavelength-corrected spectra). A 2.5-mm slit width was used for the excitation and emission beams. A 1-mm sample containing $7.0 \mu\text{M}$ protein in Buffer B was placed in a 1-mL fluorescence quartz cuvette. The sample was continually mixed throughout the experiment using a magnetic stir bar (Labcraft). A thermostatted cuvette holder was used to maintain a temperature of 10 or 25 °C. GdnHCl and urea titrations were performed by removing an aliquot of protein solution and replacing the same volume with a solution containing identical protein concentration in 6 M GdnHCl or 9 M urea. The folding equilibrium was reached rapidly as determined with stopped-flow fluorescence experiments. The urea and GdnHCl stock solutions were prepared fresh daily and the concentrations were calculated by index of refraction (Nozaki, 1972) using a Bausch & Lomb refractometer. To obtain values for $\Delta G_{\text{H}_2\text{O}}$ and m_{vq} (slope of the transitional region), a two-state transition was assumed and the relative changes in fluorescence intensity with increasing denaturant were calculated using the Pace method (Pace, 1986) by a six-parameter fit to the following equation using Kaleidagraph for the Macintosh (variable parameters are italicized in the text):

$$I = \frac{(m_f \cdot [D] + y_f) + (m_u \cdot [D] + y_u) \cdot \exp\left(\frac{-\Delta G_{H_2O} + m_{eq} \cdot [D]}{RT}\right)}{1 + \exp\left(\frac{-\Delta G_{H_2O} + m_{eq} \cdot [D]}{RT}\right)} \quad (1)$$

where I is the signal intensity, m_f and m_u are the slopes of the folded and unfolded baselines, y_f and y_u are the intercepts of the folded and unfolded baselines, $[D]$ is the denaturant concentration, K is the equilibrium constant, f_u is the unfolded fraction of molecules, ΔG_u is the free energy of unfolding, ΔG_{H_2O} is the extrapolated free energy of unfolding in water (in the absence of denaturant) and m_{eq} , the “ m value,” is the dependence of ΔG_u on urea. The concentration at the midpoint of denaturation, [denaturant]_{1/2}, also known as the C_m , is given by

$$[\text{denaturant}]_{1/2} = \frac{\Delta G_{H_2O}}{m_{eq}} \quad (2)$$

Circular dichroism studies

Far UV-circular dichroism (CD) denaturation experiments were performed on an Aviv Associates (Lakewood, NJ) model 60DS instrument. Urea and GdnHCl denaturation assays were performed at 25°C. A 1-mm path length cuvette was used to minimize absorbance by the denaturant. A similar titration method as employed for fluorescence was used for denaturation unfolding followed by far UV-CD except the CD cuvette was manually inverted several times to ensure mixing. The protein concentrations were between 35 and 50 μM in buffer B, and kept constant for a given titration. The change in ellipticity at 222 nm was monitored as a function of denaturant concentration. Each titration point was the average of 60 data points recorded every second using a 1.5-nm bandwidth.

The thermal denaturation of CspA variants were determined by measuring the change in ellipticity at 222 nm with increasing temperature. The temperature was increased at a constant rate of 0.33°C/min using a programmable water bath. The spectrometer was equipped with a temperature probe that was calibrated to an external probe ($\pm 0.1^\circ\text{C}$). Temperature and ellipticity at 222 nm were recorded every 3 min from 2 to 90°C, and each of the data points were averaged over 35 s. Thermal denaturation curves were fit using a nonlinear least-squares method using the Pace method (Pace et al., 1989).

Far UV-CD spectra were recorded from 190 to 260 nm. Data were collected every 1 nm, with a 5 s averaging time and 1.5-nm bandwidth. Five wavelength scans were averaged to obtain each spectrum. Equilibrium unfolding experiments using fluorescence and far UV-CD were performed at least twice; averaged values are reported.

Stopped flow fluorescence

Folding and unfolding reactions were followed using a Biologic SFM-2 stopped-flow apparatus employing Biokine 3.0 software (Claix, France) or a DX.17MV sequential mixing stopped-flow from Applied Photophysics (Leatherhead, UK). The dead time of both stopped-flow instruments was determined to be approximately 2 ms. Using the Biologic SFM-2, unfolding of CspA was initiated by a rapid 10-fold dilution with different urea concentra-

tions in buffer B to a final protein concentration between 4 and 6 μM . Final urea concentrations for unfolding after dilution were 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0 M. The fluorescence emission increase associated with CspA refolding was monitored using 50 μM CspA in 7 M urea stock solution. Re-folding was initiated upon a 10-fold dilution giving a final protein concentration of 5 μM and final urea concentrations of 0.7, 2, 3, 3.5, and 4 M. Each kinetic measurement consisted of a 100 μL sample containing 10 μL of the protein stock and 90 μL of the urea stock solution. The fastest mixing time of 6 $\mu\text{L}/\text{ms}$ was used in all kinetic experiments. Experiments using Applied Photophysics stopped-flow were performed in a similar manner except an 11-fold dilution was used, resulting in final urea concentrations between 4–6.4 M and 0.64–4 M for unfolding and refolding, respectively. The rates of the unfolding and refolding reactions were determined by fitting the average of the data to a monoexponential curve using Kaleidagraph software. The fluorescence change was monitored using a 325 nm filter following excitation at 280 nm using a 2-mm slit width. The path length and the volume of the cuvettes were $2 \times 2 \times 10$ mm and 90 μL , respectively. All samples were filtered through a 0.45- μm filter and degassed for 30 min to reduce noise. The apparent rate constants (λ) are the average of at least 12 kinetic traces. Kinetic measurements were consistent for both stopped-flow instruments.

Refolding of CspA (50 μM) in the presence of single-stranded DNA (100 μM) was initiated upon an 11-fold dilution from 7 M to 0.64 M urea. Applied Photophysics stopped-flow parameters were the same as above. The sequence of the single-stranded oligonucleotide was 5'-CTTGAGGTTAATCCA-3'. Fluorescence binding studies (K. Reid, unpubl. results) revealed that this particular 15-mer binds CspA with higher affinity than its complement.

Analysis of the folding kinetics

The folding and unfolding reaction for CspA was modeled as a two-state reaction:



The apparent rate constant, λ , is equal to the sum of the microscopic rate constants for refolding, k_f , and unfolding k_u :

$$\lambda = k_u + k_f \quad (4)$$

The natural logarithms of k_f and k_u will vary linearly with the urea concentration. For a two-state folding mechanism, the relation for unfolding is

$$\ln k_u = \ln k_u(\text{H}_2\text{O}) + (m_u \cdot [\text{urea}]) \quad (5a)$$

and for folding

$$\ln k_f = \ln k_f(\text{H}_2\text{O}) + (m_f \cdot [\text{urea}]) \quad (5b)$$

The m_u and m_f values describe the denaturant dependence of the folding or unfolding reactions and are thought to be proportional to the change in buried surface area that occurs between the folded or unfolded state and the transition state. The m_u and m_f values were obtained by the sum of Equations 5a and 5b, excluding the points

in the transition region (Chen et al., 1992; Viguera et al., 1995). If the folding and unfolding of a protein follow a two-state mechanism, then the m values obtained from equilibrium and kinetic data should be equal within experimental error:

$$m_{eq} = (m_u - m_f) \cdot RT \quad (6)$$

The solvent accessibility of the transition state is determined by α^\ddagger :

$$\alpha^\ddagger = \frac{m_u}{m_u - m_f} = \frac{RTm_u}{m_{eq}} \quad (7)$$

which describes the fractional change in solvent upon moving from the native state to the transition state relative to the total change upon unfolding (Tanford, 1970; Chen et al., 1989).

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