The Refolding of *cis*- and *trans*-Peptidylprolyl Isomers of Barstar†

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ABSTRACT: Barstar, a small globular protein which undergoes reversible unfolding, is a good candidate for studies on protein folding. It possesses two cysteine residues that complicate folding studies by forming a variable mixture of disulfide-bridged forms. We have constructed and analyzed, therefore, a double mutant Cys40→Ala,Cys82→Ala. Equilibrium unfolding with urea follows a simple two-step mechanism. The midpoint for unfolding ($[U]_{1/2}$) is 3.87 ± 0.03 M urea, with $m(\partial\Delta G/\partial[\text{urea}]) = 1.25 \pm 0.04$ kcal/mol^2. The free energy of unfolding, $\Delta G_{U-F}^{H_2O}$, is 4.84 ± 0.18 kcal/mol. Identical results were found on monitoring the intrinsic tryptophan fluorescence or the circular dichroism signal at 221 nm, showing that the transition is due to the global denaturation of the protein. Barstar contains two proline residues, one of which (Pro48) has a *cis N*-aminoacyl bond conformation in the folded state. A transiently generated form of the unfolded protein, which contains the proline residues in their native conformations, has a rate constant for refolding (31 s^{-1}) similar to that for refolding of the equilibrium-unfolded protein, which results in a "misfolded" form of the protein (32 s^{-1}). The two refolded states are different: the free energies of unfolding measured from kinetic constants for the native and misfolded variants are 5.4 ± 0.3 and 2.85 ± 0.1 kcal/mol, respectively. The rate constant for the unfolding in water of the misfolded protein is 0.87 s^{-1}, compared with 0.068 s^{-1} for the unfolding of the native protein. This difference can be explained by a nonnative *trans* peptidyl–proline bond at position 48 in the misfolded protein. The transiently misfolded form inhibits barnase, showing that the structure is native-like. There is a *trans* to *cis* isomerization in the misfolded protein with a rate constant of 0.01 s^{-1}, after which the protein has all the thermodynamic properties of native barstar. There is at least one further intermediate (I) on the folding pathway. ΔG_{U-I} is 0.74 kcal/mol for the folding of equilibrated unfolded protein and 1.9 kcal/mol when folding from the transiently generated unfolded protein. Thus, the folding of equilibrated unfolded barstar gives a native-like active structure, with a *trans* proline at position 48, which then isomerizes on a much slower time basis. The unfolding reaction goes rapidly from the folded to the unfolded state, after which the *cis* peptidyl–prolyl bond in the unfolded protein isomerizes to equilibrium. The last step accounts for the discrepancy between the 4.84 kcal/mol measured at equilibrium and the 5.4 kcal/mol measured from the kinetics for the free energy of unfolding, the *cis* nature of the peptidyl–proline bond in the native protein being a source of thermodynamic instability.

Investigation of the folding pathway of small monomeric proteins provides basic information for understanding the rules that govern this process. Barstar is the intracellular inhibitor of barnase, an extracellular RNase of *Bacillus amyloliquefaciens* (Hartley, 1989; Schreiber & Fersht, 1993). It consists of a single chain of 89 amino acids of M_r 10 211 (Hartley, 1989). The structure of the protein in solution is currently being solved by this laboratory (Lubienski et al., 1993). The wild-type protein contains two Cys residues at positions 40 and 82 that can form a disulfide bridge (Hartley, 1989). These two residues were mutated to Ala in this study to overcome the additional complications of a Cys–Cys bridge. All experiments described were performed on this mutant. The thermodynamic stability of this mutant resembles that of reduced wild-type protein, but is lower than that of oxidized barstar, where the Cys–Cys bridge is intact (Hartley, 1993). There are two proline residues. The *N*-aminoacyl bond of Pro27 is in the *trans* conformation, and the one at position 48 is in a *cis* conformation (Lubienski et al., 1993).

Proline residues having a *cis* conformation in the native state tend to complicate the kinetic analysis of protein folding (Brandts et al., 1975; Garel & Baldwin, 1976; Creighton, 1978; Schmid & Baldwin, 1978; Kiefhaber et al., 1992; Kiefhaber & Schmid, 1992). The basic two-state model for protein folding, $U \rightleftharpoons N$, is an oversimplification and must be replaced by a three-state model, $U_T \rightleftharpoons U_C \rightleftharpoons N$, where U_C represents the unfolded protein with the X-Pro in the native conformation, and U_T represents the nonnative conformation. The ratio of *trans* to *cis* isomers in the unfolded form depends on the residue preceding the proline (Grathwohl & Wüthrich, 1976), but it can be estimated from other proteins to be at least 5:1 (Matouschek et al., 1990; Jackson & Fersht, 1991a; Kiefhaber et al., 1992). The effect of a nonnative *cis–trans* isomer on the folding pathway is variable. In the refolding of the barley CI2 inhibitor, the slowest of the three folding rates is explained by the inability of the protein to fold with one of the X-Pro residues in the nonnative conformation, while the next slowest phase results from the ability of the protein to fold to an intermediate-like conformation in which a second X-Pro residue is in a nonnative conformation (Jackson & Fersht, 1991b). In ribonuclease T_1, the protein can fold to an intermediate-like conformation with secondary structure intact, even when one of the two proline residues with a native *cis* conformation (residues 39 or 55) is *trans* (Kiefhaber et al., 1990a,b; Kiefhaber & Schmid, 1992; Mollins et al., 1993) Staphylococcal nuclease and ribonuclease A will fold to a native-like conformation, with tertiary structure elements close to native even with a nonnative X-Pro isomer, and the conversion from *trans* to *cis* X-Pro will occur only at the final

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stage of the folding reaction (Schmid & Blaschek, 1981; Evans et al., 1989; Nakano et al., 1993).

In this article, we present the basic kinetic mechanism for the folding of barstar, as well as initial investigations of the influence of the different proline isomers on the folding pathway.

MATERIALS AND METHODS

Protein Expression and Purification. Site-directed mutagenesis of barstar was performed by the method of Sayers (1988). The oligonucleotide used to introduce an alanine codon in place of a cysteine at position 40 was TTA TGG GAT GCT CTG ACC GGA, and at position 82 it was GCG GAA GGC GCC GAC ATC ACC. Mutant plasmids were identified by direct sequencing. Cys40→Ala,Cys82→Ala barstar was purified from BL21(pLysE)(pML2bs) cells. A cell culture was grown in 2× TY medium supplemented with chloramphenicol (34 μg/mL) and ampicillin (100 mg/mL) to the stationary phase before harvesting. The pellet was resuspended in TE + 1 mM PMSF (phenylmethanesulfonyl fluoride) and stored at −70 °C. The solution was spun down in a Sorval SS34 rotor at 17 000 rpm for 30 min. Barstar is found in the 40–80% ammonium sulfate fraction, which was used for further purification. Purification was done in 50 mM Tris Cl buffer (pH 8) and 100 mM NaCl by subjecting the protein solution to gel filtration on a Superdex 75 column (Pharmacia). Final purification was on a Q Sepharose anion exchange column (Pharmacia), where barstar elutes at 300 mM NaCl. One liter of culture results in 60 mg of pure protein. Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 22 690 M^{-1} cm^{-1} estimated by the method of Gill and von Hippel (1989).

Equilibrium Studies. Equilibrium denaturation of barstar in urea was performed in 50 mM Tris Cl from 0 to 8.0 M urea. The change in fluorescence was recorded at 25 °C on a Aminco-Bowman Series 2 luminescence spectrometer. Excitation was at 280 nm (bandpass: 2 nm), and emission was monitored at 317 nm (bandpass: 8 nm). Circular dichroism spectra were recorded on a Jasco J720 spectropolarimeter using cells of path lengths 0.1 and 1.0 cm and a buffer of 20 mM potassium phosphate (pH 8) at 25 °C. Spectra were acquired at a scan speed of 50 nm/min with a 1-nm slit and 1-s response time. The values for $[U]_{1/2}$ and the free energy of unfolding were calculated from (Clarke & Fersht, 1993)

$$\Delta G = \Delta G^{H_2O} - m[\text{urea}] \quad (1)$$

$$K_{U-F} = (F_F - F)/(F - F_U) \quad (2)$$

where m is the slope of the plot, F_F is the fluorescence of the folded state, F_U is the fluorescence of the unfolded form, and F is the fluorescence at a given [denaturant].

Kinetic Experiments. All experiments were performed at 25 °C in 50 mM Tris Cl buffer (pH 8). Rapid mixing experiments were performed using an Applied Photophysics Bio Sequential DX-17MV stopped-flow apparatus with excitation at 280 nm. Emission was monitored above 315 or 335 nm using a cutoff filter. Protein unfolding was performed by mixing 1 vol of the protein solution with 5–25 vol of urea, with a final protein concentration of 2 μM. For protein refolding, barstar was unfolded for at least 1 h in 7.5 M urea before diluting the urea by a factor of 5–25. The folding process was monitored by the change in the fluorescence of barstar for up to 20 min. Kinetic folding data were fit to a double exponential, which corresponds to the fast refolding reaction and the *cis/trans* isomerization step.

Kinetic measurements of protein refolding after only transient unfolding were performed by double jump (Brandts et al., 1975), where barstar was unfolded for 5 s in 7.5 M urea and refolded by dilution. The experiment was performed in the sequential mode of the stopped-flow apparatus. Unfolding for 5 s results in at least 95% of the molecules being unfolded, with only minimal changes in the native *cis/trans* isomerization state of the prolines (which convert from *cis* to *trans* at a rate constant of about 0.02 s^{-1}).

Kinetic measurements were made of barstar "re-unfolding", after transient folding of an equilibrium-unfolded protein. Barstar was unfolded to equilibrium in 7.5 M urea and then refolded by diluting the urea to 1.0 M (unless stated otherwise) for 2–1800 s. The transiently refolded protein was re-unfolded in different urea concentrations and the unfolding rates and amplitudes were monitored as changes in the intrinsic fluorescence of the protein. Experiments with short delay times (for up to 50 s) were performed in the sequential mode of the stopped-flow apparatus, while for long delay times the initial refolding step was done by manual mixing.

Assay of Barstar Inhibition of Barnase Transesterification Activity Using the Oligonucleotide GpUp. Barstar acts by binding to and inhibiting the RNase activity of barnase. The ability of barstar to inhibit barnase was measured by mixing barstar in 7.5 M urea plus GpUp with barnase (in 50 mM MOPS, pH 7) to give final concentrations of 1.2 M urea and 0.6 μM barnase and barstar during refolding (GpUp and barnase do not seem to interfere with barstar refolding). The reaction of GpUp (100–200 μM final in 50 mM MOPS buffer, pH 7) was measured as a change in absorbance at 275 nm, either in the stopped-flow apparatus or in a 0.2-cm cell in a Cary 3 spectrophotometer (Day et al., 1992).

RESULTS

Mutant versus Wild-Type Barstar. The double mutant, Cys40→Ala,Cys82→Ala, exhibits normal barnase inhibitory activity. A comparison of the circular dichroism spectrum of the mutant protein with that of the wild type shows only small differences between the two which might be attributed to the replacement of cysteine with alanine. The structural similarity between the double mutant and the wild-type proteins was confirmed by 2D NMR (M. Lubienski & M. Bycroft, data not shown).

Urea Denaturation. Figure 1A shows the change in the fluorescence of Cys40→Ala,Cys82→Ala barstar upon titration with urea. The data fit a two-state transition. The entire set of data was fit (Clarke & Fersht, 1993) to an unfolding transition curve based on eqs 1 and 2. The midpoint for unfolding ($[U]_{1/2}$) is 3.87 ± 0.03 M urea with $m(\partial\Delta G/\partial[\text{urea}])$ = 1.25 ± 0.04 kcal/mol^2. The free energy of unfolding in water, $\Delta G_{U-F}^{H_2O}$ is 4.84 ± 0.18 kcal/mol. In order to ascertain that the transition monitored by fluorescence is due to the global denaturation of the protein rather than to a partial unfolding event, we monitored the urea unfolding using far-UV circular dichroism. A urea denaturation curve monitored at 221 nm, which mainly probes the helical structure of the protein, fits the same m and $[U]_{1/2}$ values as those found from the fluorescence measurements (Figure 1B).

Unfolding and Refolding Kinetics. Unfolding and refolding of barstar were monitored by the change in fluorescence that accompanies this reaction. The rate constants for unfolding and refolding at different [urea] are shown in Figure 2A. Unfolding was initiated by mixing the protein in a 50 mM

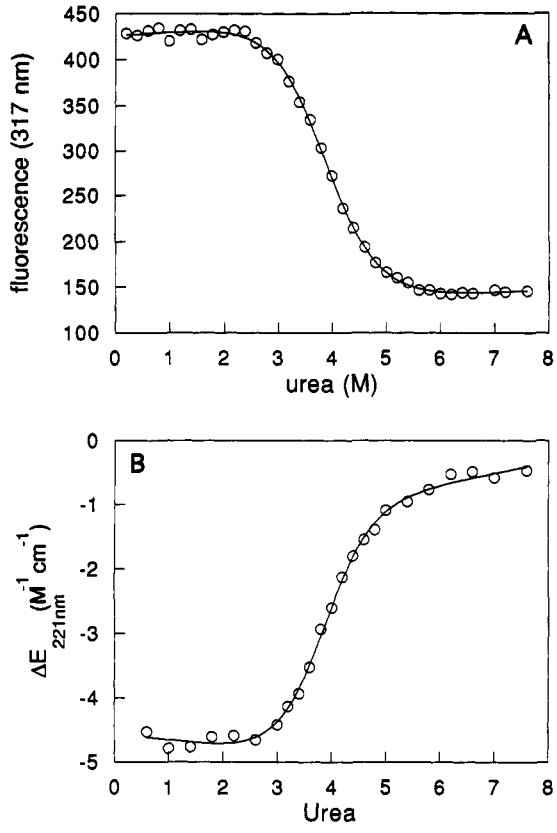


FIGURE 1: Urea-induced denaturation of barstar at pH 8 and 25 °C. (A) The intrinsic fluorescence was monitored at 317 nm, with an excitation wavelength of 280 nm, as a function of [urea]. (B) Far-UV circular dichroism spectrum at 221 nm as function of [urea]. The entire set of data was fit (Clarke & Fersht, 1993) to an unfolding transition curve based on eqs 1 and 2. The solid line is the theoretical curve for a two-state transition with m = 1.25 kcal/mol^2, $[U]_{1/2}$ = 3.87 M, and ΔG_U = 4.84 kcal/mol.

Tris Cl solution with urea at different concentrations in the stopped-flow fluorimeter. For refolding experiments, barstar was unfolded for at least 1 h in 7.5 M urea, before the urea was diluted by mixing in the stopped-flow fluorimeter. Unfolding barstar at concentrations of urea above 6 M results in a monophasic unfolding reaction. The rate constant for unfolding, k_u, increases with increasing final [urea], according to the commonly found equation

$$\ln k_u = \ln k_u^{H_2O} + m_{ku}[\text{urea}] \qquad (3)$$

where $k_u^{H_2O}$ is the rate constant for unfolding in water and m_{k_u} is mole^{-1}. $k_u^{H_2O}$ is 0.068 s^{-1}.

The refolding rates could be measured only down to a final urea concentration of 0.288 M, at which the refolding rate constant is 29 s^{-1}. It is possible, however, to estimate the refolding rate in water by making a short linear extrapolation from the refolding data between 1 and 0.288 M urea. This extrapolation results in a value for $k_f^{H_2O}$ of 32 s^{-1}. pH jumps, which were used before for measuring the folding rate in water in other proteins (Matouschek et al., 1990; Jackson & Fersht, 1991a), could not be applied for barstar because it seems that pH denaturation in barstar does not result in a fully unfolded protein (data not shown).

The slow phases for unfolding and refolding were found to be significant only when the reaction was carried out in urea concentrations where the protein was in the transition region (Figure 2B,C). The reduced amplitude of the slow refolding rate constants peaks at 3 M urea, while the reduced amplitude for the slow unfolding rate constants peaks at around 4.5 M

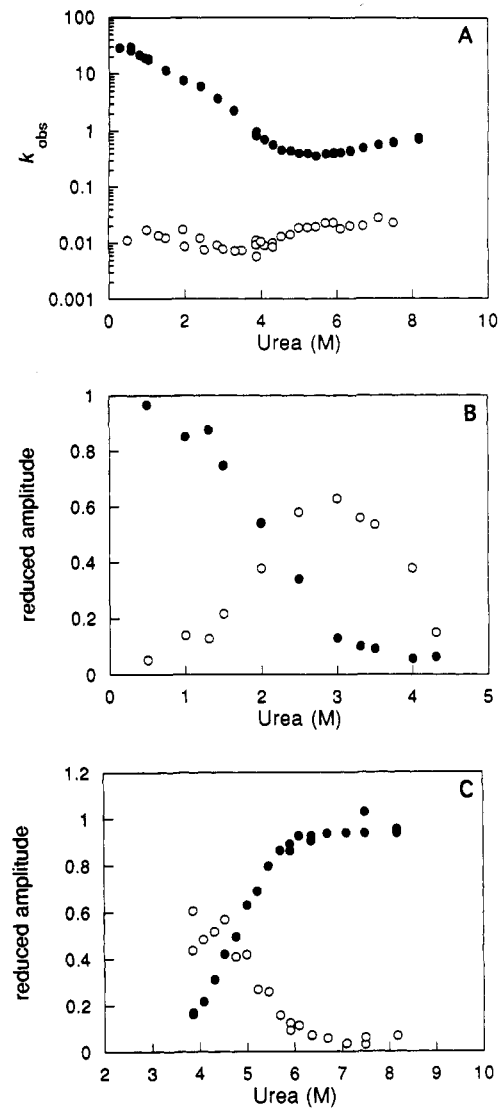


FIGURE 2: Rates and amplitudes of barstar unfolding and refolding. Unfolding was at final urea concentrations of 3.87–7.5 M. Refolding was initiated by dilution of the urea-unfolded protein (in 7.5 M urea for at least 1 h) to final concentrations of 0.28–4.3 M urea. Mixing was in a stopped-flow apparatus at ratios of 1:5 to 1:25. Unfolding and refolding were monitored by the change in the intrinsic fluorescence, with excitation at 280 nm and emission above 335 nm. All experiments were performed in 50 mM Tris Cl (pH 8) with a final protein concentration of 2 μM. ● represents the fast rate constants and their amplitudes. ○ represents slow rate constants and their amplitudes. (A) k_{obs} for unfolding and refolding. (B) Reduced amplitudes for refolding. The reduced amplitudes represent the specific change in fluorescence divided by the total change in fluorescence between native and unfolded proteins. (C) Reduced amplitudes of unfolding.

urea. [The reduced amplitude is defined as the change in amplitude of the signal for unfolding or refolding at a specific urea concentration divided by the total change in amplitude (slow plus fast phases) between the folded and unfolded states of the protein.] The reduced amplitudes of the slow phases of unfolding or refolding can reach up to 60% of the total change in amplitude of the folded versus unfolded form in barstar. The increase in the amplitudes of the slow phases is accompanied by a parallel decrease in the amplitudes of the fast phases, which almost completely diminish when the amplitudes of the slow phases peak. When barstar is unfolded in 3.87 M urea, about 75% of the unfolding molecules do so at the slow unfolding rate constant, and when folded to 3.5 M urea, 85% of the folding molecules do so according to the

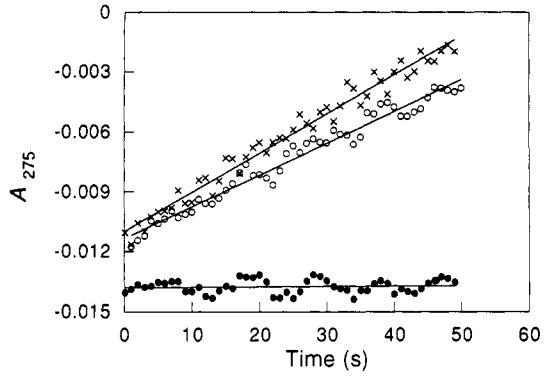


FIGURE 3: Inhibition by barstar of the barnase-catalyzed reaction of GpUp. The ability of refolded barstar to prevent barnase transesterification activity was measured by mixing unfolded barstar in 7.5 M urea and GpUp with barnase to a final concentration of 1.2 M urea. GpUp degradation (80 μM final in 50 mM MOPS buffer, pH 7) was measured as a change in absorbance at 275 nm. Conditions: ●, 0.6 μM barstar was refolded in 1.2 M urea in the presence of 0.5 μM of barnase (no degradation activity was observed); ×, 0.5 μM of barnase, no barstar (GpUp was degraded at a rate of 1.37 μM s^{-1}); ○, 0.6 μM barstar was refolded in 1.2 M urea in the presence of 1 μM of barnase (GpUp was degraded at a rate of 1.03 μM s^{-1}).

Table I: Catalysis of Folding of the Fast and Slow Refolding Species of Barstar by Peptidyl-prolyl Isomerase[a]

PPI (μM)	k_1 (s^{-1})	% of amplitude (1)	k_2 (s^{-1})	% of amplitude (2)
	7.35	74	0.011	26
1	6.54	71	0.069	29
2	6.46	70	0.121	30

[a] All rate constants were measured for refolding of 2 μM (final) barstar from 7.5 to 2 M urea in 50 mM Tris Cl (pH 8) at 25 °C.

slow rate constant for refolding. The slow refolding rate constant at ca. 3 M urea was found be about 0.0075 s^{-1}, while the slow unfolding rate constant at 5.5 M urea is about 0.02 s^{-1}.

Refolded Barstar Regains Its Inhibitory Activity. The binding of barstar to barnase was measured from the inhibition of GpUp degradation by barnase. Barstar was unfolded in 7.5 M urea for 1 h before it was diluted to 1.2 M urea to allow refolding (in the presence of GpUp and barnase). Barstar was found to regain at least 90% of its inhibitory activity within the first 2 s after refolding, which is the shortest time for which we could measure GpUp degradation by barnase (Figure 3). This result implies that barstar regains activity on a time scale similar to that of the major fast refolding step, which was seen by the change in fluorescence of the refolding protein.

The Slow Refolding Rate of Barstar Results from X-Pro Trans to Cis Isomerization. Barstar has proline residues at positions 27 and 48. In the native Cys40→Ala,Cys82→Ala protein, the *N*-aminoacyl bond of Pro27 is in the *trans* conformation, while that of Pro48 is in the *cis* conformation (Lubienski et al., 1993). Peptidyl-prolyl isomerase (PPI) has been found to catalyze the *cis*/*trans* isomerization of peptidyl–proline bonds (Lang et al., 1987; Lin et al., 1988). Table I shows the effect of PPI on the slow and fast folding species. While the relative amplitudes of the fast and slow refolding species are not affected, the rate constant for the slow refolding reaction increases 7-fold with 1 μM PPI and 12-fold with 2 μM PPI. This result implies that the slow refolding rates are a consequence of *trans* to *cis* isomerization of an X-Pro residue. The basic folding scheme of a protein, taking into account the slow *cis* to *trans* isomerization step, leads to a linear three-

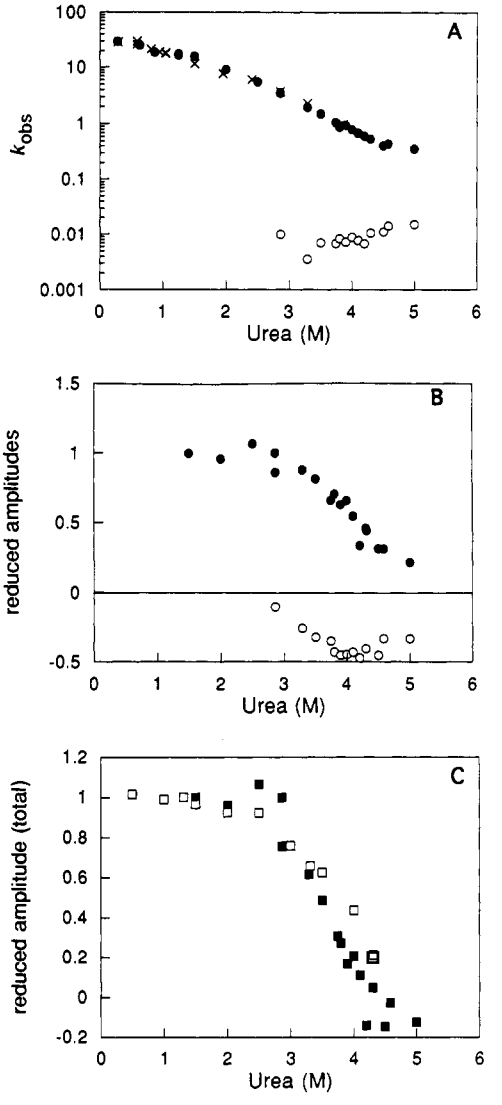


FIGURE 4: Rates and amplitudes of barstar refolding after transient unfolding for 5 s. Barstar was unfolded for 5 s in 7.5 M urea before refolding was initiated by dilution to final urea concentrations of between 0.28 and 5 M, in 50 mM Tris Cl (pH 8). After 5 s of unfolding, at least 95% of the molecules are unfolded, but the X-Pro isomerization state should be the same as in native molecules. Mixing was done in the sequential stopped-flow apparatus. Symbols: ●, fast rate constants and their amplitudes; ○, slow rate constants and their amplitudes; ×, refolding data from Figure 2 (refolding from equilibrated unfolded protein). (A) k_{obs} for refolding. (B) Reduced amplitudes for refolding. Negative amplitude values show that the change in fluorescence of the slow folding species goes in the opposite direction from the fast folding species, in other words, the slow folding species is actually an unfolding event. (C) Total reduced amplitudes of refolding (fast + slow species) for equilibrium-unfolded molecules (□) and for transiently unfolded molecules (■).

state model:

$$U_T \rightleftharpoons U_C \rightleftharpoons N \qquad (4)$$

Measurement of Barstar Refolding Rates Starting from U_C Molecules. U_C molecules have the X-Pro peptide bond in the same conformation as the native protein, whereas the nonnative isomer is present in the U_T species. The refolding rates starting from U_C molecules can be measured by a double-jump experiment, where the protein is unfolded for only a short time before it is refolded. This avoids the complexities from the *cis*/*trans* isomerization step (Brandts et al., 1975).

The fast and slow rate constants of refolding, as well as their reduced amplitudesas measured in a double-jump experiment, are shown in Figure 4. Barstar was unfolded in 7.5

M urea for 5 s (which should lead to the unfolding of more than 95% of the molecules), and then the concentration of urea in the protein solution was rediluted to allow refolding. The refolding reaction was monitored by the change in the fluorescence of the solution. The rate constants for refolding, measured in the double-jump experiment (where refolding starts from U_C molecules), are similar to those obtained from refolding barstar that had been unfolded to equilibrium (where refolding starts from U_T molecules) (Figure 4A). From the refolding rate constant in low urea concentrations, we can estimate the refolding rate in water as $k_f^{H_2O} = 31\ s^{-1}$. The reduced amplitude (the change in amplitude in refolding or unfolding in a specific [urea] divided by the total amplitude of folded versus unfolded) of the fast refolding species starting from U_C molecules reaches 50% at 4.1 M urea (Figure 4B), compared with a $[U]_{1/2}$ of 2.27 M urea when refolding begins from U_T molecules. A second major difference between refolding U_T and U_C molecules is in the sign of the slow refolding phases. When refolding starts from U_T molecules, the changes in fluorescence for the slow and fast refolding species have the same sign, meaning that both monitor refolding (Figure 2B). On the other hand, when refolding is initiated from U_C molecules (as in the double-jump experiment), the change in fluorescence for the slow refolding phase is in the opposite direction from the fast one (Figure 4B), suggesting that the slow phase is an unfolding phase. The explanation for this phenomenon is that, when there are initially mainly U_C molecules, the equilibrium between U_C and N (governed by the fast refolding rate) is rapid and leads to the accumulation of folded molecules. The native folded and U_C states are in transient equilibrium. However, the equilibrium is slightly displaced toward unfolding as the U_C slowly converts to the U_T state (Kiefhaber et al., 1992). Despite these differences between refolding U_T or U_C molecules, the total change in amplitude of slow plus fast phases is the same when starting from U_T or U_C molecules, once the system has come to equilibrium (Figure 4C). This is not surprising because the change between U_C and U_T does not involve a change in the fluorescence of the protein. In other words, addition of the negative amplitudes of refolding U_C molecules (in the slow phase) has the effect of correcting the overshot in amplitude of the U_C fast refolding phase due to the $U_C \rightleftharpoons U_T$ equilibrium.

Barstar Refolding Is at Least a Three-Step Process. A simple two-state model for protein folding involves the reversible transition between just two states, E_F and E_U:

$$E_U \underset{k_u}{\overset{k_f}{\rightleftharpoons}} E_F \qquad (5)$$

In the case of barstar, which has one native *cis*-proline a three-state model is required to allow for the *trans* to *cis* conversion of the proline residue:

$$U_T \underset{k_{cis\rightarrow trans}}{\overset{k_{trans\rightarrow cis}}{\rightleftharpoons}} U_C \underset{k_u}{\overset{k_f}{\rightleftharpoons}} N \qquad (6)$$

The complexity of the three-state model may be reduced to that of a two-state model by separating the *cis/trans* isomerization reaction from the unfolding reaction by unfolding the protein for only a short time before refolding, so that the prolines will remain in their native conformation.

In a two-state folding model, k_{obs}, the observed rate constant for equilibration, equals $k_u + k_f$. This argument can be put to test by calculating the theoretical value for k_{obs} from the kinetic data for urea unfolding and the equilibrium denaturation data (Matouschek et al., 1990; Jackson & Fersht 1991a). Since k_{obs} is calculated by starting from only U_C molecules, ΔG will be equal to

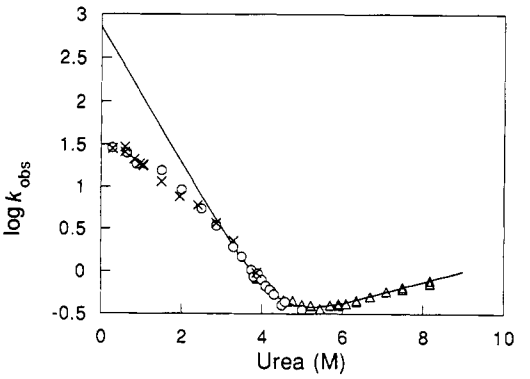


FIGURE 5: Observed unfolding and refolding rate constants versus a theoretical calculated curve representing a two-state model. Unfolding rate constants (Δ) and refolding rate constants from equilibrated unfolded protein (O) are taken from Figure 2A. Refolding rate constants from the transiently unfolded protein are from Figure 4A (×). The solid curve is the calculated k_{obs} value from equilibrium denaturation data (Figure 1) and kinetic unfolding data for a two-state model, where $k_{obs} = k_f + k_u$ (Matouschek et al., 1990).

$$\Delta G_{U_C\text{-}N} = \Delta G_{U_T\text{-}N} - \Delta G_{trans\rightarrow cis} \qquad (7)$$

The value of ΔG for the *trans* to *cis* isomerization may be calculated from

$$\Delta G_{trans\rightarrow cis} = -RT \ln (k_{cis\rightarrow trans}/k_{trans\rightarrow cis}) \qquad (8)$$

where the value for $k_{cis\rightarrow trans}$ is taken from the slow rate constant of unfolding, which is 0.022 s^{-1}, and the value for $k_{trans\rightarrow cis}$ is taken from the slow refolding rate in the transition region, which is 0.0075 s^{-1}. The rate constants for *cis/trans* isomerization of an unfolded protein are independent of urea concentrations (Garel & Baldwin, 1976; Schmid & Baldwin, 1978; Kiefhaber et al., 1992), so that we can assume that this rate constant will be the same in water. From eq 8, $\Delta G_{trans\rightarrow cis}$ = –0.635 kcal/mol, so that $\Delta G_{U_C\text{-}N}$ is 5.47 kcal/mol. k_f may be calculated from

$$\log k_f = \log k_f^{H_2O} - m_{k_f}[\text{urea}] \qquad (9)$$

where $\log k_f^{H_2O}$ is calculated from eq 10 and m_{k_f} from $m_{k_f} = m - m_{k_u}$.

$$k_f^{H_2O} = k_u^{H_2O}/\exp(-\Delta G_{N\text{-}U_C}/RT) \qquad (10)$$

The comparison between the calculated k_{obs} (which equals $k_u + k_f$) and our data is shown in Figure 5. The experimental data follow the calculated data up to about 3 M urea, where the experimental rate constants for folding become slower than the calculated ones. We can conclude from this comparison that the folding process of barstar does not fit to a simple two-state model, but there is at least one additional intermediate (Matouschek et al., 1990). The midpoint for transition is around 5.4 M urea in the calculated, as well as in the experimental, data.

The Unfolding Rate Constant of Transiently Refolded Barstar Is Much Higher Than the Rate Constant Starting from Native Protein. Do U_C and U_T molecules fold to the same folded conformation? To answer this question, a solution of barstar, which had been unfolded to equilibrium was refolded for 5 s in 1.25 M urea, after which it was re-unfolded at various urea concentrations. The folding rate constant of barstar at 1.25 M urea is about 13 s^{-1}, so that after 5 s nearly all molecules should be in their native-like conformations. The unfolding rate constants of transient refolded molecules

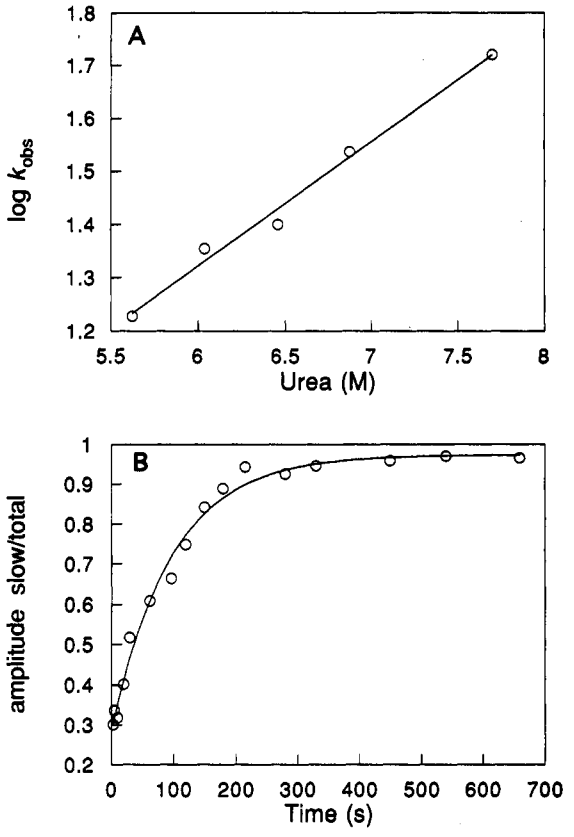


FIGURE 6: Re-unfolding of transiently refolded (misfolded) barstar. (A) Barstar was unfolded in 7.5 M urea to equilibrium before it was refolded for 5 s in 1.25 M urea. Re-unfolding was done at different urea concentrations, and the rate of unfolding was measured as the change in the intrinsic fluorescence of the protein. Under these conditions of unfolding, about 75% of the amplitude changes at a fast rate, which is presented in the figure. The additional 25% of the amplitude changes at a rate which is equal to the unfolding rate of the native protein. (B) Barstar was unfolded as in A, but refolded at 1 M urea for different times before it was re-unfolded in urea. The ratio of amplitudes of the slow unfolding species and the total change in fluorescence (fast + slow unfolding species) was plotted versus the delay time before re-unfolding and fit to a single exponential. The change between the populations of the two species is at a rate constant of 0.01 s^{-1}. This is the rate constant of misfolded to native barstar.

are much faster than the unfolding rate constants of native protein (compare Figure 6A with Figure 2A). The unfolding rate constants extrapolated to $k'_u{}^{H_2O}$ = 0.87 s^{-1}, compared with 0.068 s^{-1} which is the extrapolated unfolding rate constant of native protein in water. This observation implies the existence of an intermediate unstable folded state, when U_T molecules are folded.

At this stage, we wanted to measure the rate constant at which this intermediate folds to a native conformation. This could not be accomplished by following the change in the fluorescence of the molecule or the regain of barstar activity, because these two parameters already show native-like properties at this intermediate state. The approach chosen was to unfold transiently refolded protein as before, but this time to increase the delay time between the refolding reaction and the re-unfolding reaction. The parameter that correlates to the final folding reaction between the native-like (but *trans*-Pro48) and the native structures (*cis*-Pro48) of barstar is the amplitude of the fast unfolding species (which corresponds to the unfolding of the native-like structure) relative to the slow unfolding species (which corresponds to the unfolding of a native molecule). Unfolded barstar was refolded in 1 M urea for different times before it was re-unfolded in urea. The changes in amplitudes of the two unfolding phases with increasing delay times between refolding and re-unfolding can be fit to a single exponential curve, with a rate constant of 0.01 s^{-1} (Figure 6B). If, as expected, this rate constant reflects the accumulation of native barstar, the unfolding rate of this species should be the same as for unfolding native barstar, which is actually the case here. The unfolding of barstar in 7.6 M urea has a rate constant of about 0.7 s^{-1}, which is the same as that found for the slower of the two re-unfolding species. Even when delay times before re-unfolding were only a few seconds, we still could observe 25% of the slower unfolding species. This percentage is in agreement with the proportion of *cis*-prolines in the unfolded protein. This experiment shows that the folding of U_T molecules results in the transient accumulation of mainly native-like protein (in which the *cis*-proline is in the "wrong" conformation, with a *trans* to *cis* isomerization step with a rate constant of 0.01 s^{-1}).

DISCUSSION

The aim in this work was to analyze the folding pathway of barstar, using the Cys40→Ala,Cys82→Ala double mutant as our "pseudo-wild-type" to avoid the complexity of a partly formed Cys–Cys bridge in the wild type. The minimal kinetic folding pathway of C40→A,C82→A barstar is summarized in Scheme I. Barstar can fold by two separate pathways, depending on the concentration of urea at which folding occurs. When folding is initiated in water, the folding process is a three-step one. The first step is folding to an intermediate (I_{1T}). This step is very fast and cannot be separated by direct kinetic or equilibrium folding measurements. In the second step of refolding, barstar folds to a native-like structure (I_{2T}). The rate constant for this reaction is 32 s^{-1} in water (k_1). The third step, which follows a very slow rate constant, is a *trans* to *cis* isomerization step, after which the protein is fully folded (N). When the refolding reaction is monitored by the change in the intrinsic fluorescence of the protein and the protein refolds at low urea concentrations, it is mainly the fast refolding step that is observed. Folding from I_{2T} to N can be monitored only through indirect experiments. Our observations on the stability of I_{2T} and the folding rate to N are based on the ability to isolate (for a short time) the protein in the I_{2T} form by transiently refolding U_T molecules to I_{2T} and then measuring the unfolding rate constants for I_{2T} molecules. This process gives the rate constant for unfolding I_{2T}, which is 0.87 s^{-1} extrapolated to water (k_2). The rate constant for transforming I_{2T} to N was measured by monitoring the change in the population of very fast unfolding molecules (for I_{2T}) relative to those that will unfold from the native form of the protein (which is a simple exponential process).

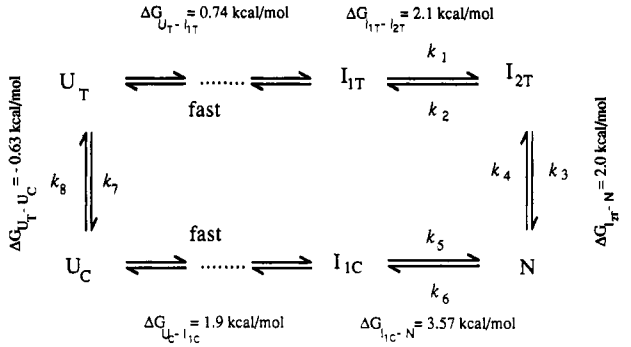
In 7.5 M urea, I_{2T} molecules unfold about 50 times faster than N molecules. The fact that all of the I_{2T} molecules will eventually be transformed to native molecules (by measuring their unfolding rate constants) shows the ability of barstar to refold. In this way, we found that the proline isomerization rate constant, which is probably the rate-limiting event in folding I_{2T} to N, is about 0.01 s^{-1} when the protein is folded in 1 M urea. This rate constant is similar to that for *trans* to *cis* isomerization of the unfolded protein, which shows that the reaction from I_{2T} to N does not go through the complete unfolding of the protein, in which case the rate for folding I_{2T} to N would be the isomerization rate of U_T to U_C multiplied by K for I_{2T} to U_T which will be, in 1 M urea, about 0.075, so that the rate for folding I_{2T} to N would be at least 10 times slower than that observed. Interestingly, it seems that the isomerization rate of proline 48 from *trans* to *cis* is about the same in the folded and the unfolded proteins. This is not

always the case. In RNase A, the rate of *trans*→*cis* isomerization was found to be accelerated by the formation of the structure (Schmid, 1986), while in RNase T_1 the isomerization rate seemed to be dramatically slowed down by structure formation (Kiefhaber & Schmid, 1992). The fast refolding reaction from U_T to I_{2T} is sufficient for barstar to regain activity, as measured by its ability to inhibit GpUp degradation by barnase. This shows that the structure of I_{2T} should indeed be quite similar to that of N.

When barstar is refolded at a concentration of urea around the folding transition, a slow refolding reaction is predominantly observed. The rate constant for this refolding reaction can be enhanced by the addition of peptidyl-prolyl isomerase (PPI), which is known to catalyze the *cis*/*trans* isomerization step of X-Pro residues (Lang et al., 1987; Lin et al., 1988). Because the slow rate constant dominates the refolding reaction in the region of concentrations of urea around the unfolding transition, we can assume that the rate-limiting proline isomerization step (at least for the larger part of the refolding amplitude) is *trans* to *cis*, because in the unfolded form most of the proline residues will have a *trans* conformation (Grathwohl & Wüthrich, 1976; Jackson & Fersht, 1991b; Kiefhaber et al., 1992). There are two proline residues in barstar, at positions 27 and 48. NMR studies show that Pro48 is the *cis*-X-Pro residue, while Pro27 has a *trans* conformation in the native structure (Lubienski et al., 1993). Since the *trans* to *cis* isomerization step is much slower than the folding reaction starting from U_C molecules (in which X-Pro residues have a native-like conformation), it will be the rate-determining step in folding the protein in the region of concentrations of urea around the unfolding transition ($U_C \rightarrow I_{1C} \rightarrow N$).

The slow isomerization step, $U_T \rightarrow U_C$ (proline residues in U_T molecules will be in their equilibrated form in an unfolded protein which is mainly *trans*), can be avoided by starting the refolding reaction from the transiently unfolded protein, in which the proline residues will remain in their native conformation (U_C). This allows the protein to refold via the lower pathway in Scheme I ($U_C \rightarrow I_{1C} \rightarrow N$), but with $I_{1C} \rightarrow N$ as the rate-determining step (since $U_C \rightarrow I_{1C}$ is very fast). On refolding U_C molecules, the amplitude and rate constant of the fast refolding step are identical with the macroscopic rate constant for folding in the absence of proline isomerization. The rate constant for refolding U_C molecules in water (k_5) is similar to k_1 (31 s^{-1}). Despite the similar rate constants for refolding U_T and U_C molecules, there are two major differences between the two reactions (Figure 2B and 4B). One is the shift in the $[U]_{1/2}$ of the amplitudes of the fast refolding species from 2.27, starting from U_T molecules, to 4.12 M urea, starting from U_C molecules. The second difference is the sign of the amplitude of the slow refolding phases compared with the fast phases. When refolding starts from U_T, the slow refolding phases have the same sign as the fast refolding one, which means that both are folding reactions. When refolding starts with U_C molecules, the slow refolding phase has a sign opposite that of the fast folding phase, meaning that the species with the slow rate constant is actually unfolding. The explanation for this phenomenon is that the equilibrium between U_C and N (governed by the fast folding reaction) is rapid and leads to a transient accumulation of N molecules. However, this is followed by the slow $U_C \rightleftharpoons U_T$ equilibration that displaces the reaction somewhat in the direction of unfolding. This gives a slow decrease in amplitude. This kind of behavior has been reported before in proteins with a *cis*-proline in their native form (Kiefhaber et al., 1992; Kiefhaber & Schmid, 1992).

Scheme I: Proposed Scheme for the Folding of Barstar[a]



[a] The upper part is a scheme for folding starting from U_T molecules, where the conformation of the proline residues is determined by their conformation in unfolded protein. In the lower part of the scheme, folding starts with the proline residues in their native conformation (U_C). The calculations for the different rate constants and ΔG values are explained in the text.

Table II: Kinetic Rate Constants for Unfolding and Refolding Barstar According to the Model Shown in Scheme I

$k_1^{H_2O}$	32 ± 3 s^{-1}	$k_5^{H_2O}$	31 ± 3 s^{-1}
$k_2^{H_2O}$	0.87 ± 0.1 s^{-1}	$k_6^{H_2O}$	0.068 ± 0.006 s^{-1}
$k_3^{(1.0\ M\ urea)}$	0.01 ± 0.002 s^{-1}	k_7	0.0075 ± 0.001 s^{-1}
k_4	NA	k_8	0.022 ± 0.003 s^{-1}

As stated before, there is at least one intermediate (I_{1C} or I_{1T} dependent on the folding pathway) between the unfolded and the native or native-like (I_{2T}) forms of the protein. This conclusion is supported by comparing the calculated k_{obs} from unfolding and equilibrium denaturation data (Matouschek et al., 1990; Jackson & Fersht, 1991a) with the experimental k_{obs} from folding transiently unfolded protein as well as equilibrium-unfolded protein. In a two-state folding model, which should be the simplest case for folding U_C molecules, we expect that $k_{obs} = k_u + k_f$. The value of k_u is taken from the experimental data since an intermediate does not exist in the unfolding reaction, and k_f was calculated from eqs 7 and 8. We found that the experimental data follow the calculated values down to about 3 M urea, where the experimental rate constants for folding begin to be slower than the calculated ones. For a two-state folding model, we would expect the experimental curve to follow the calculated curve, as in the case of CI2 (Jackson & Fersht, 1991a). This result indicates the existence of an intermediate in the folding pathway of barstar.

Calculation of the Thermodynamic and Kinetic Parameters on the Folding Pathway of Barstar. Most of the rate constants and all of the free energies on the folding pathway of barstar were calculated. On the upper pathway in Scheme I, we calculate the following (also see Table II): k_1 is the refolding rate constant starting with U_T molecules, $k_1^{H_2O} = 32\ s^{-1}$; k_2 is the unfolding rate constant of transiently refolded (misfolded) protein, $k_2^{H_2O} = 0.87\ s^{-1}$. These two (misfolded) rate constants were measured for the transformation of $U_T \rightleftharpoons I_{2T}$, but since the reaction of $U_T \rightleftharpoons I_{1T}$ is very fast and not rate-limiting, the observed rate constants are those for the reaction of the intermediate and not its formation (Matouschek et al., 1990; Matouschek & Fersht, 1991). Therefore, $\Delta G_{I_{1T}-I_{2T}} = -RT \ln (k_2/k_1) = 2.1$ kcal/mol.

$\Delta G_{U_T-I_{2T}}$ is estimated as follows. The fast and slow rate constants for folding from U_T molecules differ by more than 100-fold, so that we can actually separate these two steps. The reduced amplitudes of the fast refolding species can be fit separately to the same equation that was used for the equilibrium unfolding measurement (Figure 7A). The equa-

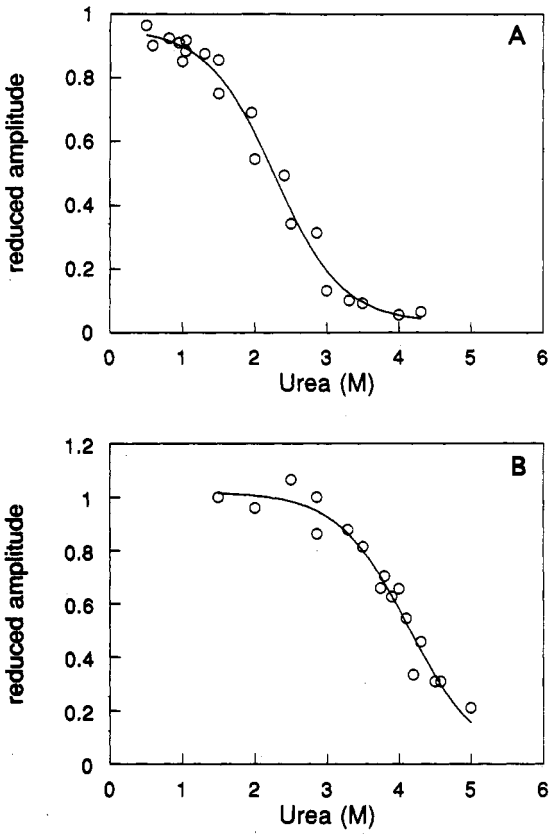


FIGURE 7: Reduced amplitudes (the change in amplitude in refolding or unfolding in a specific [urea] divided by the total amplitude of folded minus unfolded state) for the fast refolding species fit to an unfolding transition curve. The reduced amplitudes for the fast refolding species of equilibrium-unfolded barstar (A) are from Figure 2B, and those for refolding of transiently unfolded barstar (B) are from Figure 4B. The data were fit to an unfolding transition curve which is based on eq 2 (Clarke & Fersht, 1993). $[U]_{1/2}$ for A is 2.27 ± 0.07 M urea and for B is 4.125 ± 0.06 M urea.

tion from which the m and $[U]_{1/2}$ values were derived is based on eq 2. When we apply eq 2 to the reduced amplitudes of the fast refolding species, F_F is the fluorescence of the I_{2T} form. The m value derived from the fit is 1.25 kcal/mol[2] (which is similar to the m value from equilibrium denaturation data) and the $[U]_{1/2}$ is 2.27 ± 0.07 M urea. $\Delta G_{U_T-I_{2T}} = 2.84 \pm 0.1$ kcal/mol. From the data above, we can calculate $\Delta G_{U_T-I_{1T}}$ and $\Delta G_{I_{2T}-N}$: $\Delta G_{U_T-I_{1T}} = \Delta G_{U_T-I_{2T}} - \Delta G_{I_{1T}-I_{2T}} = 0.74$ kcal/mol, and $\Delta G_{I_{2T}-N} = \Delta G_{U_T-N} - \Delta G_{U_T-I_{2T}} = 2.0$ kcal/mol.

k_3 is the rate constant measured in Figure 6B and is the rate constant for the final refolding step, in which the *trans* proline is converted to the native *cis* form. The value of k_3 for refolding in 1.0 M urea and re-unfolding in 7.6 M urea is 0.01 s^{-1}.

The refolding pathway of barstar in the region of concentrations of urea around the unfolding transition goes via the lower pathway in Scheme I. The rate of the refolding reaction is dominated by the *trans* to *cis* conversion of proline 48 (with a rate constant of about 0.0075 s^{-1}), while the amplitude of the fast refolding rates, which represents the upper pathway, diminishes. The following rate constants can be calculated on this pathway (also see Table II): k_7 is the *trans*- to *cis*-proline isomerization rate constant, which is derived from the slow refolding rate; $k_7 = 0.0075 \pm 0.001$ s^{-1}. k_8 is the *cis*- to *trans*-proline isomerization rate constant, which is derived from the slow unfolding rates in the transition zone. The reasoning behind this is that, in the unfolding reaction, there is initially a fast equilibration of $N \rightleftharpoons U_C$, after which there is a slow equilibration of $U_C \rightleftharpoons U_T$ which is dominated by the *cis* to *trans* isomerization rate constant and will feed back into the first half of the pathway. $k_8 = 0.022 \pm 0.003$ s^{-1}, so that $\Delta G_{U_T-U_C} = -0.63 \pm 0.16$ kcal/mol. k_5 is the refolding rate constant when refolding starts with mainly U_C molecules (Figure 4); $k_5^{H_2O} = 31$ s^{-1}. k_6 is the unfolding rate constant of the protein; $k_6^{H_2O} = 0.068$ s^{-1}. From k_5 and k_6 we calculate $\Delta G_{I_{1C}-N} = 3.57$ kcal/mol (for an explanation of why k_5 and k_6 represent the reaction I_{1C} to N, see the explanation for k_1 and k_2).

For calculating $\Delta G_{U_C-I_{1C}}$, we first have to calculate ΔG_{U_C-N}. This can be done in two ways. First, $\Delta G_{U_C-N} = \Delta G_{U_T-N} - \Delta G_{trans \rightarrow cis} = 5.47 \pm 0.34$ kcal/mol. A second approach for calculating ΔG_{U_C-N} is very similar to the method of calculating $\Delta G_{U_T-I_{2T}}$, but in this case we plot the amplitudes of the fast folding reaction starting from U_C molecules. In Figure 7B, we see that by fitting the data to the same equation that was used for the equilibrium unfolding measurement, $\Delta G_{U_C-N} = 5.16 \pm 0.01$ kcal/mol. The two results agree to within the error. $\Delta G_{U_C-I_{1C}} = \Delta G_{U_C-N} - \Delta G_{N-I_{1C}} = 1.9$ kcal/mol.

It is important to note here that our folding model, as well as the different calculated values, only takes into account the *trans/cis* isomerization reaction of the proline residue in the native *cis* conformation (Pro48). As stated before, barstar has a second proline residue, with a native *trans* conformation (27). Because in the unfolded state most of the molecules will also have this X-Pro as *trans*, the influence on the folding pathway of a *cis* to *trans* isomerization reaction of this residue will be small and was not detected in our experiments.

How are the two folding pathways of barstar related? The folding reaction in water clearly goes via the upper pathway, with a fast initial folding reaction which will bring the protein to a native-like conformation (I_{2T}) followed by a slow *trans* to *cis* isomerization step. In unfolding conditions, the unfolding reaction will go via the lower pathway, from N to U_C molecules. In these terms, this is a folding/unfolding cycle where the folding and unfolding reactions go through different pathways. At this stage, we do not know how close the I_{2T} and the native structures are in structural terms, as well as I_{1T} compared with I_{1C}, which are the folding intermediates of the two pathways. RNase A has been shown to refold to a native-like structure, with proline 93 in the nonnative conformation. This structure showed native-like activity, but in contrast to barstar, the final proline-dependent refolding reaction involved a major change in the fluorescence of the protein (Schmid & Blaschek, 1981). Staphylococcal nuclease, which also has a native-like structure when one of the proline residues is in the nonnative *trans* conformation, shows nonlocalized small changes in many parts of the protein (Evans et al., 1987, 1989). But, in staphylococcal nuclease, it seems that the two folded forms of the protein coexist at equilibrium, while in barstar it seems that the only folded form in equilibrium should be the *cis*-proline form of the protein. This is supported by the single kinetic unfolding rate constant when unfolded in high urea concentration, with no evidence for a very fast unfolding rate constant which will result from the unfolding of the native-like species (I_{2T}).

The significance of proline isomerization in protein folding has been the subject of considerable interest (Brandts et al., 1975; Garel & Baldwin, 1976; Creighton, 1978; Kiefhaber et al., 1992). In this article, we show that in the case of barstar, protein folding, as measured by the change in intrinsic fluorescence as well as by regaining activity, will commence even when one X-Pro residue is in the nonnative-like conformation. Further, the refolding rate constants starting from U_C or U_T molecules seem to be the same, suggesting a similar

transition-state barrier for the two different forms. Perhaps Pro48 is in a part of the protein that folds very late. The major difference between the folding reactions of U_C versus U_T molecules is in their different energy profiles and is seen in kinetic experiments. In spite of the complexity of the system, it has been possible not only to show the different kinetic relationships but also to determine rates for most of the interconversion processes.

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REFERENCES

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.
Clarke, J., & Fersht, A. R. (1993) *Biochemistry 32*, 4322–4329.
Creighton, T. E. (1978) *J. Mol. Biol. 125*, 401–406.
Day, A. G., Parsonage, D., Ebel, S., Brown, T., & Fersht, A. R. (1992) *Biochemistry 31*, 6390–6395.
Evans, P. A., Dobson, C. M., Hatfull, G., Kautz, R. A., & Fox, R. O. (1987) *Nature 329*, 266.
Evans, P. A., Kautz, R. A., Fox, R. O., & Dobson, C. M. (1989) *Biochemistry 28*, 362–370.
Garel, J. R., & Baldwin, R. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1853–7.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319–326.
Grathwohl, C., & Wüthrich, K. (1976) *Biopolymers 15*, 2025–2041.
Hartley, R. W. (1989) *Trends Biochem. Sci. 14*, 450–454.
Hartley, R. W. (1993) *Biochemistry 32*, 5978–5984.
Jackson, S. E., & Fersht, A. R. (1991a) *Biochemistry 30*, 10428–10435.
Jackson, S. E., & Fersht, A. R. (1991b) *Biochemistry 30*, 10428–10435.
Kiefhaber, T., & Schmid, F. X. (1992) *J. Mol. Biol. 224*, 231–240.
Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990a) *Biochemistry 29*, 3053–3061.
Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990b) *Biochemistry 29*, 3061–3070.
Kiefhaber, T., Kohler, H. H., & Schmid, F. X. (1992) *J. Mol. Biol. 224*, 217–229.
Lang, K., Schmid, F. K., & Fischer, G. (1987) *Nature 329*, 268–270.
Lin, L. N., Hasumi, H., & Brandts, J. F. (1988) *Biochim. Biophys. Acta 956*, 256–266.
Lubienski, M. J., Bycroft, M., Jones, D. N. M., & Fersht, A. R. (1993) *FEBS Lett.* (in press).
Matouschek, A., & Fersht, A. R. (1991) *Methods in Enzymol. 202*, 82–112.
Matouschek, A., Kellis, J. T., Jr., Serrano, L., Bycroft, M., & Fersht, A. R. (1990) *Nature 346*, 440–445.
Mollins, L. S., Pace, C. N., & Raushel, F. M. (1993) *Biochemistry 32*, 6152–6156.
Nakano, T., Antonino, L. C., Fox, R. O., & Fink, A. L. (1993) *Biochemistry 32*, 2534–2541.
Schmid, F. X. (1986) *FEBS Lett. 31*, 217–220.
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764–4768.
Schmid, F. X., & Blaschek, H. (1981) *Eur. J. Biochem. 114*, 111–117.
Schreiber, G., & Fersht, A. R. (1993) *Biochemistry 32*, 5145–5150.