# Engineered Disulfide Bonds as Probes of the Folding Pathway of Barnase: Increasing the Stability of Proteins against the Rate of Denaturation

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ABSTRACT: Disulfide bridges have been introduced into barnase to act as probes of folding. One disulfide (between residues 85 and 102) links two loops known to pack together early in the folding pathway. A second disulfide bond (between residues 43 and 80) links two elements of secondary structure known to pack together only after the rate-determining step of folding. The disulfide-bridged proteins are more stable than wild-type by 4.1 and 1.2 kcal $mol^{-1}$, respectively. The kinetics of unfolding and refolding of the mutant proteins has been measured, and a comparison of the disulfide proteins and their corresponding dithiol forms has been made by use of thermodynamic cycles. These data have been used to construct folding profiles of the disulfide proteins. The disulfide bond engineered into the part of the protein that folds early confers stability upon the intermediate and transition states of folding. The protein with a disulfide bond connecting parts of the protein that fold late is not stabilized until the protein reaches its final folded form. Conversely, in the unfolding pathway, the rate of unfolding of this mutant is lowered considerably. This points to a method of decreasing the rate of denaturation of proteins that are used in medical and biotechnological applications: elements of structure that unfold in or before the rate-determining step of overall unfolding may be stabilized and so slow down the overall unfolding process. The barnase mutant linked between Cys 43 and Cys 80, for example, unfolds 20 times slower than wild-type and 170 times slower than the reduced protein.

In order to understand the folding pathway of a protein, it is necessary to characterize intermediates along the folding pathway. Since folding is generally a very rapid process, these intermediates are short lived and so have to be observed using rapid measurement techniques or by the trapping of disulfide-linked intermediates. Barnase, the extracellular ribonuclease of *Bacillus amyloquefaciens*, is a small protein (110 residues) with no cysteine residues and no *cis*-prolines. Its folding pathway is being elucidated by protein engineering techniques (Fersht et al., 1992; Matouschek et al., 1992a; Serrano et al., 1992a,b), and by pulse-labeled hydrogen exchange nuclear magnetic resonance spectroscopy (Matouschek et al., 1992b). The structures of its folding intermediate and transition states have been characterized. In the study reported here, disulfide bonds have been introduced into barnase and used as specific probes of protein folding.

Proteins with natural disulfide bonds are stabilized by those bonds, and, in many cases, reduction of the disulfide bonds will cause the protein to unfold (Creighton & Goldenberg, 1984; Oobatake et al., 1979). This characteristic has been exploited in the study of the oxidative refolding of bovine pancreatic trypsin inhibitor by trapping disulfide-linked intermediates along the folding pathway (Creighton, 1992; Weissman & Kim, 1991). Disulfide bonds have been engineered into proteins in an attempt to increase their stability. This has met with limited success; it has been possible to stabilize some proteins to a considerable extent, whereas other proteins have been destabilized by the engineered disulfide bond (Matsumura et al., 1989; Mitchinson & Wells, 1989; Wetzel, 1987). In almost all cases, however, the disulfide protein is more stable than the corresponding dithiol form. The stabilization of proteins by cross-links such as disulfide bonds is widely accepted to result largely from the entropic destabilization of the unfolded state (Poland & Scheraga, 1965; Lin et al., 1984; Pace et al., 1988; Matsumura & Matthews, 1991; Cooper et al., 1992), although some recent work has suggested that enthalpic factors may also be important (Doig & Williams, 1991; Kuroki et al., 1992). Balanced against this entropic effect is the destabilization due to mutation and conformational strain introduced by the covalent disulfide bond.

In this study, the engineered disulfide bond is used in a different way. Here, the primary aim of the introduction of disulfide bonds was not to stabilize the structure, but to report on the folding of specific regions of the protein and to use the results to design mutants that slow down the rate of denaturation. The disulfide bond is not required for the protein to fold, and so the kinetics of folding of the disulfide and the corresponding dithiol proteins can be compared. Since the disulfide proteins are more stable than their dithiol equivalents, the folding pathway can be analyzed to establish when, during the folding process, the protein achieves the stability conferred by the disulfide bond. This is a method of reporting on the folding of the region of the protein that is stabilized by an introduced disulfide. A disulfide bond in a region of the protein that folds early is shown to be stabilized early in the folding process, whereas a disulfide connecting two structures that fold together late does not confer stability until the final folded form is reached. The results of this study indicate that engineered disulfide bonds can be used as specific probes to follow the folding together of elements of secondary structure, where there may not be interactions which can be easily probed by previously described protein engineering or NMR techniques. The kinetic experiments described here point to a method of increasing the kinetic stability in proteins with biotechnological or medical applications, by cross-linking areas of the protein that unfold early. This is shown to decrease the rate of unfolding considerably.

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## MATERIALS AND METHODS

*Materials.* The urea and guanidinium chloride used in denaturation experiments were ultra pure enzyme grade from BRL. The buffer used was 2-(*N*-morpholino)ethanesulfonic acid (MES) from Sigma. The MES stock is a 1.0 M solution containing 387 mM acid form and 613 mM sodium salt, which gives a pH of 6.3 at 25 °C on dilution to 50 mM. Radiochemicals were obtained from Amersham International. SP-Trisacryl was from IBF and molecular biology grade dithiothreitol (DTT) from Sigma. Oxidized DTT was obtained from Calbiochem and purified as described by Creighton (1984). Other chemicals and reagents were obtained from Sigma, BDH, or Fisons.

*Selection of Suitable Sites for SS Bonds.* The X-ray crystal coordinates of barnase (Mauguen et al., 1982; A. Cameron and K. Henrick, unpublished results) were examined to select suitable sites for the introduction of a disulfide bond. The sites were selected according to the following criteria. (1) On the basis of previous studies (Matouschek et al., 1992a,b; Serrano et al., 1992b), the aim was to cross-link regions of the protein that are known either to be largely folded in the transition state or to be largely unfolded in the transition state. (2) Within these sites, pairs of residues which were within the following ranges, C$\alpha$–C$\alpha$, 4.4–6.8 Å, C$\beta$–C$\beta$, 3.45–4.5 Å (Hazes & Dijkstra, 1988; Richardson, 1981; Thornton, 1981) were identified. (3) The removal of stabilizing interactions from the structure when replacing a side chain (hydrogen bonds, hydrophobic interactions) was avoided if possible. (4) If possible, adverse steric effects were avoided by replacing a residue with a side chain of a similar size to Cys.

*Mutagenesis, Expression, and Purification of Mutant Proteins.* Single-stranded DNA from the modified pTZ18U harboring the wild-type barnase gene (Serrano et al., 1990) was obtained by infection of TG2 cells with the helper phage M13K07 (Pharmacia). Site-directed mutagenesis was carried out using the method of Sayers et al. (1988) with a kit supplied by Amersham. Two mutagenic oligonucleotides were used for each mutagenesis reaction, which yielded a mixture of single Cys mutants and double mutants. Mutants were identified directly by sequencing by the dideoxy chain termination method using a kit supplied by USB. Mutant DNA was transformed into *Escherichia coli* BL21DE3 pLysS (a generous gift to this laboratory of Dr. F. W. Studier), and proteins were expressed as has been previously described (Serrano et al., 1990). The double mutant proteins were purified in the absence of any reducing agent. Following purification, the proteins were extensively dialyzed against deionized water, flash frozen, and stored in aliquots in liquid nitrogen.

*Assay for the Reduction State of Disulfide Bonds.* The disulfide content of the proteins was assessed by two standard methods, Ellman's assay, using DTNB (Ellman, 1959), and nonreducing acid–urea gel electrophoresis. Both disulfide mutants were shown by these methods to be completely oxidized following purification and dialysis (results not shown). Incubation for 30 min at 37 °C with 10 mM DTT was shown by gel electrophoresis and reverse-phase HPLC to be sufficient to maintain the thiols in a reduced state (results not shown).

*Equilibrium Denaturation.* Unfolding was monitored by fluorescence spectroscopy (Hartley, 1975), with excitation at 290 nm and emission being measured at 315 nm. The intrinsic fluorescence of barnase decreases by about 80% on unfolding (Kellis et al., 1989). For each data point collected, 100 $\mu$L of a solution of ~9 $\mu$M barnase in 450 mM MES, pH 6.3, was added to an eppendorf tube containing 800 $\mu$L of the appropriate denaturant solution (urea or guanidinium chloride). The tube was incubated at 25 °C for at least 2 h. To obtain reduced protein, the 9 $\mu$M protein solution was made 180 mM with DTT and incubated for 30 min at 37 °C before being added to the denaturant solution (final [DTT] = 20 mM). For these experiments only, oxidized proteins were treated with 9 mM oxidized DTT for 30 min before being added to denaturant (final [oxidized DTT] = 1 mM). Unfolding has been demonstrated to be completely reversible, since dilution of denatured samples leads to full recovery of native fluorescence (Kellis et al., 1989). Fluorescence was measured in thermostated cuvette holders at 25 °C, with temperature monitored by a thermocouple in the cuvette held above the light beam.

*Unfolding Experiments.* Unfolding was initiated by rapidly mixing one volume of protein (~40 $\mu$M) with 10 volumes of a concentrated urea solution at 25 °C. Both solutions contained 50 mM MES, pH 6.3. Reduced proteins were incubated for 30 min at 37 °C in 110 mM DTT before the experiment was carried out (final [DTT] = 10 mM). The rate constant of unfolding can be measured by stop-flow fluorimetry (Matouschek et al. 1989). Urea solutions were made gravimetrically. The reactions were monitored in a Perkin-Elmer fluorescence spectrophotometer, MPF55B, which was equipped with a rapid mixing device. Two Hamilton syringes, a 1-mL syringe filled with a urea solution, and a 100-$\mu$L syringe containing the protein solution are fitted to three-way valves. These connect to the rapid mixing chamber. When the plungers are driven in manually, the protein and urea solutions mix in the ratio 1:10. To ensure complete mixing, a 30-ms delay loop is inserted between the mixing device and the flow cell. Fluorescence changes within this cell were measured, and the data were collected with a Tandon Target microcomputer. The data from four experiments were averaged and were fitted to the following equation by nonlinear least-squares analysis using the general curve fit option of the KaleidaGraph program:

$$F_{(t)} = m_1 + F_o e^{-k_u t} - m_4 t \quad (1)$$

where $F_{(t)}$ is the observed fluorescence, $F_o$ is the fluorescence at time, $t = 0$, $k_u$ is the rate constant of unfolding, $m_1$ is the offset for the final fluorescence, and $m_4 t$ is a term allowing for baseline instability. The deviation of the experimental data from the theoretical curves was plotted to check for any systematic deviation. For long time courses, the baseline instability required fitting of the data to a double exponential (Matouschek & Fersht, 1991).

*Refolding Experiments.* Refolding was studied by rapidly mixing one volume of acid denatured protein with one volume of refolding buffer at 25 °C. The rate constant of refolding can be measured by stop-flow fluorimetry, as described by Matouschek et al (1992a). Protein solutions were made to a concentration of ~24 $\mu$M. This was mixed with two volumes of HCl to a final pH of 1.5, to denature the protein. To obtain reduced proteins, the samples were preincubated with 60 mM DTT at 37 °C for 30 min. The refolding MES buffer was 7 mM acid form and 93 mM base, which gives a 50 mM MES solution, pH 6.3, upon mixing with an equal volume of acid denaturated protein solution. In order to calculate the dependence of the rate constant of refolding upon urea concentration, urea was added to both the protein solution and the refolding buffers to concentrations between 0 and 2 M. The rapid mixing experiments were performed in an Applied Photophysics SF.17MV stopped-flow apparatus. The refolding was followed by the increase of intrinsic fluorescence of barnase upon folding. Excitation was at 290 nm and

emission was monitored at wavelengths greater than 305 nm. The data from at least four experiments were averaged and analyzed on an Archimedes microcomputer, using Applied Photophysics software, or using the general curve fit option of the KaleidaGraph program on a Macintosh computer.

The refolding fluorescence traces can be fitted to a triple exponential:

$$F_{(t)} = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \exp(k_3 t) + C \quad (2)$$

where $F_{(t)}$ is the fluorescence at time $t$, $A_1$, $A_2$, and $A_3$ are the amplitudes of the three phases of the folding reaction, $k_1$, $k_2$, and $k_3$ are the rate constants, and $C$ is the offset. The triple exponential represents a major fast phase (~80% of the amplitude), which represents the folding of the bulk of the unfolded protein, and two slower phases which have been associated with proline isomerization (Matouschek et al., 1990). The rate constants derived from the fast phase are those used to analyze the energetics of refolding.

*Analysis of Kinetic Data.* (*i*) *Unfolding.* The rate of unfolding is a measure of the difference in energy between the folded state and the transition state. The rate constant for unfolding, $k_u$, is found to increase with increasing urea concentration, as shown in

$$\log k_u = \log k_u^{H_2O} + m_{k_u}[\text{urea}] \quad (3)$$

where $k_u^{H_2O}$ is the rate constant for unfolding in the absence of denaturant. The value of $k_u^{H_2O}$ is calculated by extrapolation of the data to 0 M urea. The activation energy of unfolding $\Delta G_{‡\text{-}F}$, is defined as the difference in energy between the folded protein and the transition state of the protein. By use of thermodynamic cycles, the kinetic data can be used to compare the activation energy of mutant proteins with wild-type proteins (Fersht et al., 1992). The difference in activation energy upon mutation, $\Delta\Delta G_{‡\text{-}F}$, is defined as

$$\Delta\Delta G_{‡\text{-}F} = \Delta G_{‡\text{-}F} - \Delta G'_{‡\text{-}F} \quad (4)$$

where $G'_{‡\text{-}F}$ is the activation energy of unfolding of the mutant protein. Experimentally,

$$\Delta\Delta G_{‡\text{-}F} = -RT \ln(k_u/k'_u) \quad (5)$$

where $k'_u$ is the rate of unfolding of the mutant protein.

(*ii*) *Refolding.* The rate of refolding is a measure of the difference in energy between the intermediate and transition state of folding. The rate constant for refolding, $k_{-u}$, is calculated at 0 M urea. The energy change of the intermediate relative to the folded state can be calculated from the rate constants for its reaction and its formation, using

$$\Delta\Delta G_{I\text{-}F} = -RT \ln[(k_u^{H_2O}/k_{-u}^{H_2O})(k'_{-u}{}^{H_2O}/k'^{H_2O}_u)] \quad (6)$$

## RESULTS

*Selection of Sites for Disulfide Bonds.* On the basis of the criteria given, two sites were chosen to introduce disulfide bonds, 43–80 and 85–102; these positions in wild-type barnase are shown in Figure 1.

*Ala43–Ser80 (43–80).* A disulfide at this site connects a short, single turn $\alpha$-helix (residues 41–45) to the loop (residues 76–85) between the second and third strands of the central $\beta$-sheet. An examination of the X-ray crystal structure reveals that, in the folded wild-type protein, these two elements of secondary structure are packed upon one another, with Leu 42, in the helix, making hydrophobic interactions with the loop. There are no hydrogen bonds connecting the two structures. The 41–45 helix is packed onto the end of the second major helix (residues 26–34). No hydrogen bonds are displaced when Ala 43 and Ser 80 are replaced by Cys and

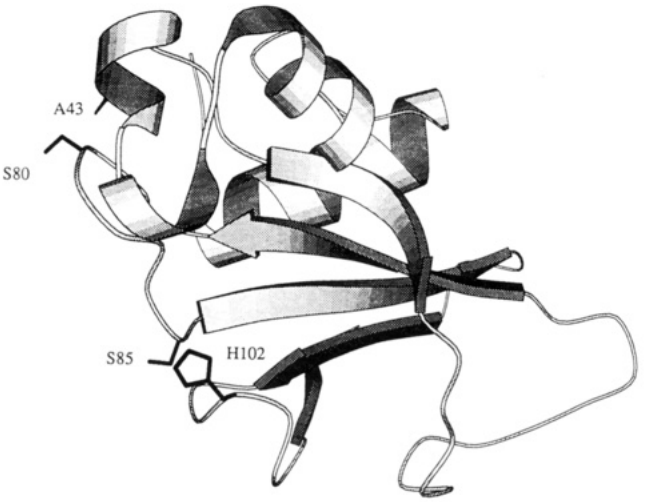



FIGURE 1: Wild-type barnase showing the site of introduction of disulfide bonds 43–80 and 85–102.

both side chains are exposed to solvent [calculated by the method of Hubbard and Blundell (1987)]. This region has been probed during unfolding and refolding by protein engineering and NMR (Matouschek et al., 1992a,b; Serrano et al., 1992b). NMR experiments demonstrate that protons within the 41–45 helix are protected early in the folding pathway, consistent with the view that this element of secondary structure is formed early during refolding. The protein engineering studies show that the hydrogen-bonding interactions of residues within the loop 76–85 are not formed until the protein is fully folded. Both studies show that interactions between the 41–45 helix and the second major helix are not present until after the rate-determining step of folding. A disulfide in this region, therefore, will bring together two regions of the protein that apparently do not associate until the protein is fully formed. There is no other tertiary probe of this region.

*Ser85–His102 (85–102).* This disulfide bond connects the residue preceding the first residue of the third $\beta$-strand (residues 86–91) with the loop (residues 100–105) which follows the fourth strand. Both of these side chains are exposed to the solvent (Hubbard & Blundell, 1987), and neither is involved in hydrogen bonding. The backbone carbonyl oxygen of Ser 85 is hydrogen bonded to the backbone amide hydrogen of His 102. Ser 85 is at the end of a long loop (76–85) between $\beta$-strands 2 and 3. The NMR solution structure of wild-type barnase (Bycroft et al., 1991) shows this loop to be flexible in solution. An examination of the crystal structure shows that the side chains of residues 84 and 86 are involved in hydrogen-bonding interactions as well as the main chain hydrogen-bonding patterns, suggesting that the end part of this loop is ordered. Residues 99–102 are involved in an extensive network of hydrogen bonds. $\beta$-Strands 3 and 4 are connected by a tight turn (residues 92–93). Protein engineering has shown that interactions within the $\beta$-sheet central strands are largely made in the intermediate and fully made in the transition state (Matouschek et al., 1992a; Serrano et al., 1992b). The $\beta$-turn 91–94 is partly formed in the intermediate and more completely formed in the transition state of folding. The loop immediately preceding strand 3 (residues 77–85) is formed only late in the folding pathway. The loop following strand 4 (residues 100–105) is partially formed during the transition state of folding (Sancho et al., 1991). NMR studies show early folding of the third and fourth $\beta$-strands upon each other and partial formation of the 100–105 loop early in folding (Matouschek et al., 1992b).

The evidence suggests that the structure in the region of 85–102 is at least partially formed early in the folding process.

*Analysis of Equilibrium Denaturation Data. (i) Calculation of $[D]_{50\%}$ and m.* We use in this study an equation for analyzing the relative stability of wild-type and mutant proteins that is different from that used previously. The equilibrium constant ($K_{U\text{-}F}$) for unfolding at a given denaturant concentration is given in

$$K_{U\text{-}F} = (F_F - F)/(F - F_U) \quad (7)$$

where $F_F$ is the fluorescence of the folded form, $F_U$ is the fluorescence of the unfolded form, and $F$ is the fluorescence at the given [denaturant]. $F_F$ and $F_U$ are linearly dependent upon denaturant concentration.

It has been shown experimentally that the free energy of unfolded proteins in the presence of denaturant ($\Delta G_{U\text{-}F}{}^{D}$) is linearly related to the concentration of denaturant (Pace, 1986), so

$$\Delta G_{U\text{-}F}{}^{D} = \Delta G_{U\text{-}F}{}^{H_2O} - m[D] \quad (8)$$

where $\Delta G_{U\text{-}F}{}^{H_2O}$ is the value of the apparent free energy of unfolding in the absence of denaturant, [D] is the concentration of the denaturant, and $m$ is the slope of the transition. Since $\Delta G_{U\text{-}F}{}^{H_2O}$ and $m$ can be calculated from eq 9, which is derived from eqs 7 and 8, assuming that both $F_F$ and $F_U$ are linearly dependent upon denaturant concentration (Horovitz et al., 1992):

$$F = [(\alpha_F + \beta_F[D]) + (\alpha_U + \beta_U[D])\exp\{(m[D] - \Delta G_{U\text{-}F}{}^{H_2O})/RT\}]/[1 + \exp\{(m[D] - \Delta G_{U\text{-}F}{}^{H_2O})/RT\}] \quad (9)$$

where $\alpha_F$ and $\alpha_U$ are the intercepts and $\beta_F$ and $\beta_U$ are the slopes of the baselines at low (F) and high (U) denaturant concentrations.

When data are fitted to this equation, the values of $\Delta G_{U\text{-}F}{}^{H_2O}$ and $m$ are obtained, with their standard errors. However, when comparing the stability of wild-type and mutant proteins, it is often important to know the accuracy of $[D]_{50\%}$, the concentration of denaturant at which 50% of the protein is unfolded, and the accuracy of estimates of $\Delta G_{U\text{-}F}{}^{D}$ at various values of [D]. At $[D]_{50\%}$ it is apparent from eq 8 that $\Delta G_{U\text{-}F}{}^{H_2O} = m[D]_{50\%}$ and thus

$$\Delta G_{U\text{-}F}{}^{D} = m([D]_{50\%} - [D]) \quad (10)$$

So, from eqs 9 and 10

$$F = [(\alpha_F + \beta_F[D]) + (\alpha_U + \beta_U[D])\exp\{(m[D] - [D]_{50\%})/RT\}]/[1 + \exp\{(m[D] - [D]_{50\%})/RT\}] \quad (11)$$

The data were fitted to this equation by nonlinear least-squares analysis using the general curve fit option of the KaleidaGraph (Albeck Software) program, which gives the calculated standard errors for individual experimental measurements of $m$ and $[D]_{50\%}$.

*(ii) Calculation of $\Delta\Delta G_{U\text{-}F}$.* It has been shown that the measurement of $[D]_{50\%}$ is very reproducible (Kellis et al., 1989). The value of $\Delta\Delta G_{U\text{-}F}{}^{D_{50\%}}$, the difference in stability between wild-type and a mutant protein at the denaturant concentration $[D] = 0.5([D]_{50\%} - [D]'_{50\%})$, can be obtained accurately by applying

$$\Delta\Delta G_{U\text{-}F}{}^{D_{50\%}} = \langle m\rangle \Delta[D]_{50\%} \quad (12)$$

where $\langle m\rangle$ is the average value of $m$ for the mutant and wild-type proteins (Kellis et al., 1989; Serrano & Fersht, 1989; Serrano et al., 1992a), and $\Delta[D]_{50\%} = [D]_{50\%} - [D]'_{50\%}$ (the difference in the value of $[D]_{50\%}$ between wild-type and mutant proteins). The value of $m$ reflects the cooperativity of the transition and the exposure of the denatured state to solvent relative to the native state. For mutants of barnase studied thus far, the $m$ values for mutants and wild-type are the same, within experimental error, and we have used a value of $\langle m\rangle$ obtained from measurements on a large number of mutant enzymes and repetative runs on wild-type (Serrano et al., 1990, 1992a). This use of a mean value of $m$ obtained from a very large number of different experimental measurements allows calculation of $\Delta\Delta G_{U\text{-}F}{}^{D_{50\%}}$ with low standard error.

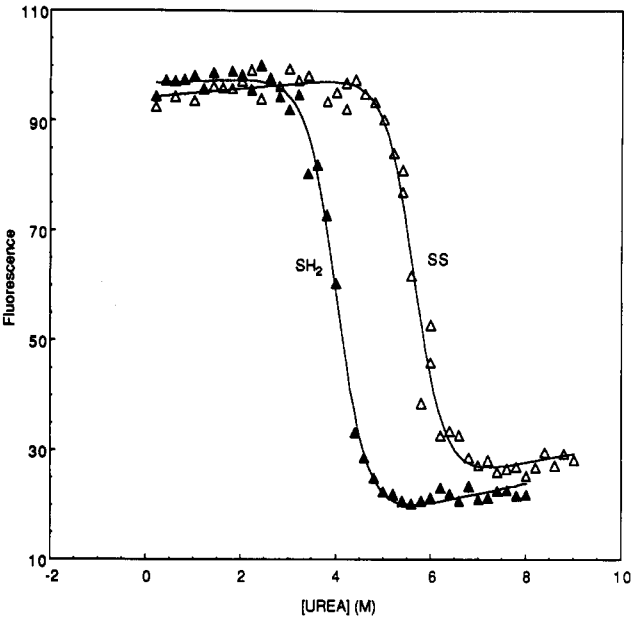



FIGURE 2: Urea equilibrium denaturation of barnase mutant 43–80. Fluorescence intensities of disulfide (SS) and dithiol ($SH_2$) forms of 43–80 at given concentrations of urea. The data were fitted to eq 11, assuming that the fluorescence of the folded and unfolded states varied linearly with urea concentration.

For disulfide mutants of barnase where there may be some residual structure in the denatured state affecting the relative exposure to solvent, there may well be a lowering of $m$, and so the differences in free energy will vary with [D]. The value of $\Delta G_{U\text{-}F}{}^{H_2O}$ from extrapolation to 0 M denaturant should be used in calculation of the $\Delta\Delta G_{U\text{-}F}{}^{H_2O}$ as shown in

$$\Delta\Delta G_{U\text{-}F}{}^{H_2O} = \Delta G_{U\text{-}F}{}^{H_2O} - \Delta G'_{U\text{-}F}{}^{H_2O} \quad (13)$$

All calculations of $\Delta\Delta G_{U\text{-}F}{}^{H_2O}$ reported in this study were obtained by application of eq 13. This method, however, introduces more error. The value and error in $\Delta G_{U\text{-}F}$ at any concentration of denaturant D is calculated from

$$\Delta G_{U\text{-}F}{}^{D} = m([D]_{50\%} - [D]) \pm \sqrt{se_m{}^2([D]_{50\%} - [D])^2 + m^2 se_D{}^2} \quad (14)$$

where $se_m$ and $se_D$ are the standard errors in $m$ and $D_{50\%}$, respectively. The difference free energies between any two proteins at any denaturant concentration D is given by

$$\Delta\Delta G_D = m([D]_{50\%} - [D]) - m'([D']_{50\%} - [D]) \quad (15)$$

and the standard error, $se_{\Delta\Delta G_D}$ is

$$\pm\sqrt{se_m{}^2([D]_{50\%} - [D])^2 + m^2 se_D{}^2 + se'_{m'}{}^2([D']_{50\%} - [D])^2 + m'^2 se'_{D'}{}^2} \quad (16)$$

It is apparent that errors are larger the further [D] is from $[D]_{50\%}$.

*Stability of Mutants.* Urea denaturation curves for the 43–80 proteins are shown in Figure 2. The $[urea]_{50\%}$ for the disulfide form of the 85–102 protein was found to be 7.4 M. This high value makes it difficult to get accurate $m$ values

Table I: Changes in the Free Energies of Unfolding upon Mutation, Determined by Equilibrium Denaturation[a]

| protein | [denaturant]$_{50\%}$[b] (M) | $m$ (kcal mol$^{-2}$) | $\Delta G_{U\text{-}F}^{H_2O}$ (kcal mol$^{-1}$) | $\Delta\Delta G_{U\text{-}F}^{D_{50\%}}$ (relative to wild-type)[c] (kcal mol$^{-1}$) | $\Delta\Delta G_{U\text{-}F}^{H_2O}$ (relative to wild-type)[c] (kcal mol$^{-1}$) | $\Delta\Delta G_{U\text{-}F}^{D_{50\%}}$ (relative to dithiol)[c] (kcal mol$^{-1}$) | $\Delta\Delta G_{U\text{-}F}^{H_2O}$ (relative to dithiol)[c] (kcal mol$^{-1}$) |
|---|---|---|---|---|---|---|---|
| wild-type (urea)[d] | 4.58 (±0.01) | 1.92 (±0.03) | 8.79 (±0.14) | | | | |
| 43–80 SS | 5.77 (±0.02) | 1.73 (±0.13) | 10.0 (±0.8) | −2.1 (±0.2) | −1.2 (±0.8) | −3.2 (±0.2) | −2.3 (±0.9) |
| 43–80 $(SH)_2$ | 4.00 (±0.03) | 1.92 (±0.14) | 7.7 (±0.6) | 1.1 (±0.1) | 1.1 (±0.6) | | |
| wild-type (GdmCl) | 2.00 (±0.01) | 4.40 (±0.25) | 8.8 (±0.5) | | | | |
| 85–102 SS | 3.00 (±0.01) | 4.30 (±0.20) | 12.9 (±0.6) | −4.3 (±0.2) | −4.1 (±0.8) | −4.8 (±0.2) | −4.5 (±0.7) |
| 85–102 $(SH)_2$ | 1.89 (±0.01) | 4.47 (±0.21) | 8.4 (±0.4) | 0.5 (±0.1) | 0.4 (±0.6) | | |

[a] The standard errors of the data are given in parentheses. [b] Denaturation of 43–80 was carried out in the presence of urea. For 85–102, the denaturant was guanidinium chloride (GdmCl) (see Results). [c] $\Delta\Delta G_{U\text{-}F}$ values for were calculated using the formula $\Delta\Delta G_{U\text{-}F} = \Delta G_{U\text{-}F} - \Delta G'_{U\text{-}F}$ (as described under Materials and Methods) and have been calculated in 0 M urea ($\Delta\Delta G_{U\text{-}F}^{H_2O}$) and at the concentration of denaturant at which 50% of the wild-type protein is unfolded ($\Delta\Delta G_{U\text{-}F}^{D_{50\%}}$) (4.58 M urea or 3.00 M GdmCl). [d] Wild-type values taken from six different measurements of different preparations of wild-type protein from this laboratory.

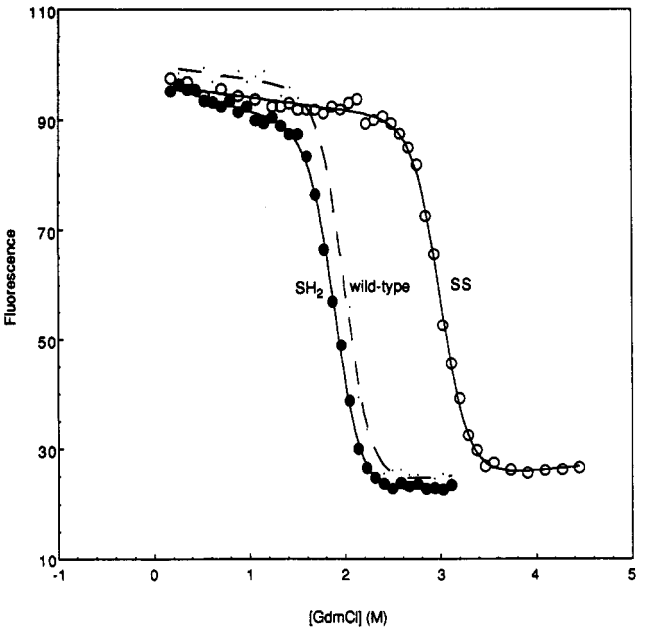



FIGURE 3: Guanidinium chloride denaturation of 85–102 and wild-type barnase. Fluorescence intensities of disulfide (SS) and dithiol ($SH_2$) forms of 85–102 and wild-type barnase at given concentrations of guanidinium chloride (GdmCl). The data were fitted to eq 11, assuming that the fluorescence of the folded and unfolded states varies linearly with GdmCl concentration.

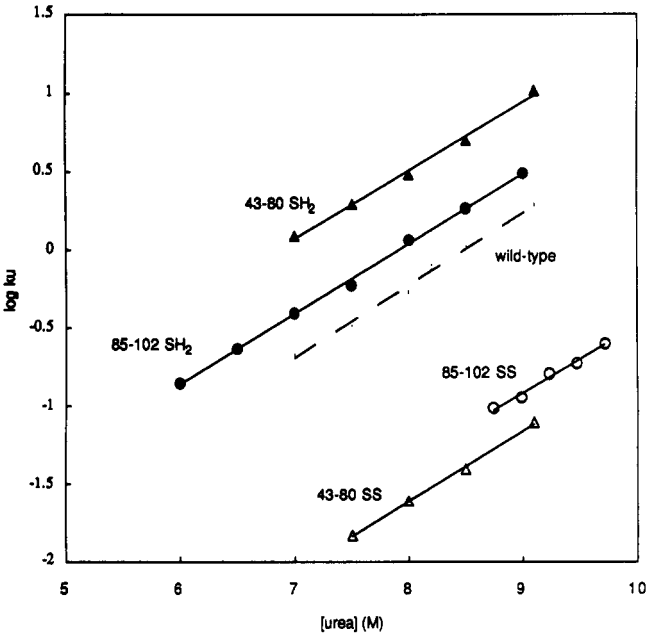



FIGURE 4: Plots of the log of the unfolding rate constants versus urea concentration. The log $k_u$ ($s^{-1}$) of the disulfide (SS) and dithiol ($SH_2$) mutant proteins and wild-type barnase are plotted at different urea concentrations.

from a denaturation curve, since a final baseline cannot be accurately measured, so guanidinium chloride was used as a denaturant for 85–102 (Figure 3). Values of $m$, $[D]_{50\%}$, $\Delta G_{U\text{-}F}$ at 0 M denaturant and at the $[D]_{50\%}$ for wild-type and the difference free energies at 0 M denaturant and at $[D]_{50\%}$ for wild-type ($\Delta\Delta G_{U\text{-}F}^{H_2O}$ and $\Delta\Delta G_{U\text{-}F}^{D50\%}$) are given in Table I. The values of $m$ of all four proteins fall within the range found for repetitive measurements on wild-type preparations of barnase and are within the range found for random error. The $\Delta\Delta G_{U\text{-}F}^{H_2O}$ values for the proteins in this study were calculated by extrapolation of the data to 0 M denaturant. The error values quoted of approximately ± 0.8 kcal mol$^{-1}$ reflect the error introduced. The values of $\Delta\Delta G_{U\text{-}F}^{D_{50\%}}$ are similar to those extrapolated to water but have less error. It is interesting to note that, had the standard equation of $\Delta\Delta G_{U\text{-}F}^{H_2O} = \langle m \rangle \Delta[D]_{50\%}$ been applied, the $\Delta\Delta G_{U\text{-}F}^{H_2O}$ values would have fallen well within the error quoted, and there would have been no qualitative difference to the final analysis.

*43–80.* The reduced, dithiol mutant protein is less stable than wild-type, in water, by 1.1 kcal mol$^{-1}$, but the disulfide mutant is 1.2 kcal mol$^{-1}$ more stable than wild-type and 2.3 kcal mol$^{-1}$ more stable than its own dithiol form.

*85–102.* The dithiol form is 0.4 kcal mol$^{-1}$ less stable than wild-type in 0 M denaturant, while the disulfide mutant is 4.1 kcal mol$^{-1}$ more stable than wild-type and 4.5 kcal mol$^{-1}$ more stable than its own dithiol form.

*Unfolding Experiments.* The urea dependence of log $k_u$, the logarithm of the rate constants of unfolding, is shown in Figure 4. The rate constants of unfolding and $\Delta\Delta G_{\ddagger\text{-}F}^{H_2O}$ are given in Table II. Inspection of the data shows that formation of a disulfide bond slows the unfolding of both proteins. Both disulfide proteins unfold more slowly than wild-type and considerably more slowly than the corresponding dithiol forms. Both dithiol proteins unfold more rapidly than wild-type. 85–102 unfolds approximately 17 times more slowly when a disulfide bond is present, and 43–80 unfolds approximately 170 times more slowly when the disulfide bond is formed.

*Refolding Experiments.* The rate constants of refolding and $\Delta\Delta G_{I\text{-}F}^{H_2O}$ are given in Table III. Both dithiol mutants refold at a rate similar to that of wild-type. The disulfide proteins show different refolding kinetics. 85–102 refolds more rapidly than the other proteins, whereas 43–80 refolds more slowly than all the proteins in this study. Formation of a disulfide bond at 85–102 causes the protein to refold 5 times more rapidly, whereas when a disulfide bond is formed at 43–80, refolding is 2 times slower.

Table II: Rate Constants for Unfolding and Difference Energies for the Transition State[a]

| protein | log $k_u$ ($s^{-1}$) | $m_{k_u}$ ($M^{-1}$) | $k_u$[b] ($s^{-1}$) | $\Delta\Delta G_{‡\text{-}F}^{H_2O}$ (wt–mutant) (kcal $mol^{-1}$) | $\Delta\Delta G_{‡\text{-}F}^{H_2O}$ [$(SH)_2$–SS] (kcal $mol^{-1}$) |
|---|---|---|---|---|---|
| wild-type | −3.93 (±0.10) | 0.462 (±0.012) | 1.19 (±0.07) × $10^{-4}$ | | |
| 43–80 SS | −5.21 (±0.11) | 0.450 (±0.013) | 6.15 (±0.29) × $10^{-6}$ | −1.8 (±0.2) | −3.0 (±0.2) |
| 43–80 $(SH)_2$ | −2.98 (±0.14) | 0.436 (±0.012) | 1.04 (±0.11) × $10^{-3}$ | 1.3 (±0.2) | |
| 85–102 SS | −4.79 (±0.17) | 0.430 (±0.018) | 1.64 (±0.13) × $10^{-5}$ | −1.2 (±0.3) | −1.7 (±0.2) |
| 85–102 $(SH)_2$ | −3.55 (±0.06) | 0.449 (±0.008) | 2.81 (±0.11) × $10^{-4}$ | 0.5 (±0.2) | |

[a] The standard errors of the data are given in parentheses. [b] Values of $k_u$ were taken from the extrapolation of the logarithm of the rate constants to 0 M urea (Figure 4).

Table III: Rate Constants for Refolding and Difference Energies for the Intermediate[a]

| protein | $k_{-u}$ ($s^{-1}$) | $\Delta\Delta G_{I\text{-}F}^{H_2O}$ (wt–mutant) (kcal $mol^{-1}$) | $\Delta\Delta G_{I\text{-}F}^{H_2O}$ [$(SH)_2$–SS] (kcal $mol^{-1}$) |
|---|---|---|---|
| wild-type | 12.9 (±0.1) | | |
| 43–80 SS | 7.5 (±0.1) | −1.4 (±0.2) | −2.7 (±0.2) |
| 43–80 $(SH)_2$ | 13.6 (±0.1) | 1.3 (±0.2) | |
| 85–102 SS | 80.1 (±1.5) | −2.3 (±0.3) | −2.7 (±0.2) |
| 85–102 $(SH)_2$ | 14.4 (±0.2) | 0.5 (±0.2) | |

[a] The standard errors of the data are given in parentheses.

## DISCUSSION

*Stability of Mutant Proteins.* Substitution of the wild-type side chains for Cys is destabilizing in all cases. Since the side chains are not involved in any packing or hydrogen-bonding interactions, it is possible that this is the result of replacing side chains which are relatively solvent exposed with a relatively hydrophobic side chain. All the single Cys mutants in these positions dimerize to a considerable extent (J. Clarke and A. R. Fersht, unpublished results), which is indicative of their exposed position. Both disulfide mutants are more stable than their dithiol form. Although the mutations were not made to stabilize the proteins specifically, it is important that the proteins are more stable than their own dithiol form, since destabilization of the protein on formation of a disulfide bond would indicate that the final form of the protein was strained and structural changes would be indicated (Katz & Kossiakoff, 1990). It is generally accepted that the stability of a disulfide protein can be attributed principally to the destabilization of the unfolded form by the loss in conformational entropy imposed by the cross-link (Poland & Scheraga, 1965; Lin et al., 1984; Pace et al., 1988). Balanced against this is the strain energy imposed upon the folded protein by the disulfide bond. 43–80 has a $\Delta\Delta G_{U\text{-}F}$ of −2.3 kcal $mol^{-1}$, and 85–102 is stabilized by 4.5 kcal $mol^{-1}$. These values, when compared with other engineered disulfides (Kanaya et al., 1991; Matsumura et al., 1989; Villafranca et al., 1987), indicate that the disulfide bonds are formed with little strain. 85–102 has been stabilized to a considerable degree; structural and thermodynamic studies are currently underway to examine this stability.

*Kinetic Stability of Proteins.* A disulfide bond, as well as increasing the thermodynamic stability of a protein, may well increase the kinetic stability, by decreasing the rate of unfolding of a protein. Both disulfide mutants unfold more slowly than wild-type barnase and more slowly than their corresponding dithiol form. The effect of placing a disulfide bond to cross-link parts of a protein which normally unfold early is dramatic. 43–80 unfolds 19 times more slowly than wild-type barnase and 170 times more slowly than its dithiol form. Proteins used in biotechnology become degraded during the course of reaction or on recycling. In some cases, it is the rate of unfolding that is important. This study points to a way of stabilizing such proteins. The structure should be examined to determine which regions unfold early, using either the kinetic procedures developed on barnase (Matouschek et al., 1989; Fersht et al., 1992) or other biophysical or computational methods. These early-unfolding elements of structure may then be stabilized by disulfide cross-links.

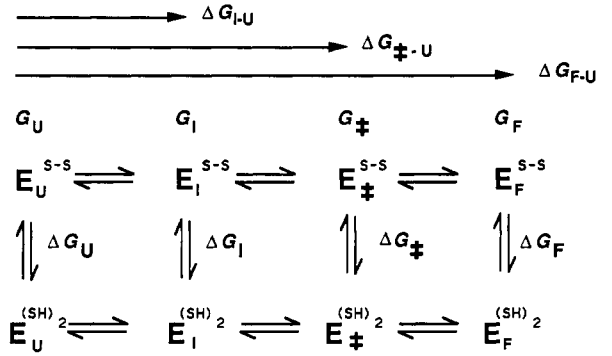



FIGURE 5: Thermodynamic cycles relating a disulfide and a dithiol protein. In this analysis, a disulfide protein (SS) is compared with its corresponding dithiol ($SH_2$) form. This allows the effects of replacing a wild-type residue with Cys to be ignored. The apparent energy difference between the two proteins in any state, A, is defined using the cycles as $\Delta\Delta G_{A\text{-}U} = \Delta G_{A\text{-}U}^{SS} - \Delta G_{A\text{-}U}^{(SH)_2}$. These apparent energy differences are related through the cycle to true energy differences between states by the relationship $\Delta\Delta G_{A\text{-}U} = \Delta G_A - \Delta G_U$.

*Folding Profiles.* Carefully constructed mutants, with specific interactions within the folded proteins removed, have previously been constructed to analyze the folding of barnase by use of thermodynamic cycles. In this case, however, since the mutated side chains chosen do not participate in specific interactions within the folded protein, the dithiol proteins will not report upon the folding of the protein. It is possible that the dithiol proteins are destabilized at least partly due to solvation changes in the folded state. It is also possible that introduction of the Cys side chains introduces local unfavorable interactions into the protein. In the following analysis the disulfide mutant has been compared to the reduced dithiol form of the protein, so any changes are due entirely to the formation of the disulfide bond, uncomplicated by the effects of the mutation. The minimal kinetic scheme for the refolding of barnase is U → I → TS‡ → F, where TS‡ is the major transition state. There are certainly more intermediates on the folding pathway (Matouschek et al., 1990). This minimal scheme is the basis of Figure 5, which shows the thermodynamic cycles used to compare the disulfide and dithiol forms of the protein.

Values of $\Delta\Delta G_{‡\text{-}U}$ and $\Delta\Delta G_{I\text{-}U}$ can be calculated from the kinetic data (Matouschek et al., 1990) since $\Delta\Delta G_{‡\text{-}U} = \Delta\Delta G_{‡\text{-}F} + \Delta\Delta G_{F\text{-}U}$ and $\Delta\Delta G_{I\text{-}U} = \Delta\Delta G_{I\text{-}F} + \Delta\Delta G_{F\text{-}U}$. From these cycles, it is apparent that the experimentally determined difference energies are related to true differences between the two proteins as follows (Matouschek et al., 1990; Fersht et

al., 1992):

$$\Delta\Delta G_{F\text{-}U} = \Delta G_F - \Delta G_U$$
$$\Delta\Delta G_{‡\text{-}U} = \Delta G_‡ - \Delta G_U$$
$$\Delta\Delta G_{I\text{-}U} = \Delta G_I - \Delta G_U$$

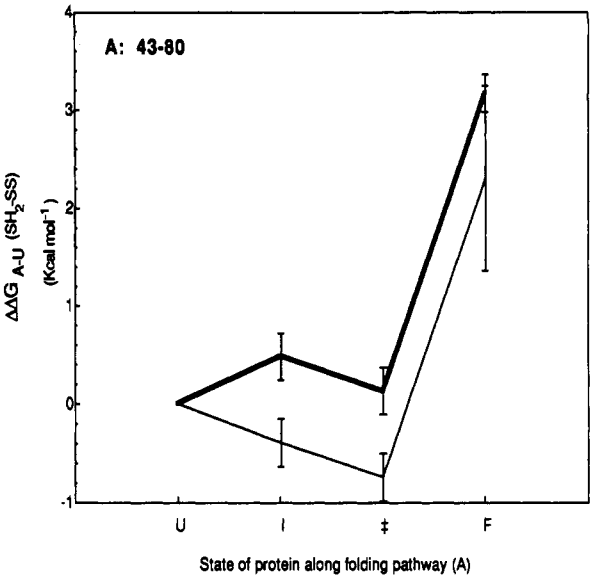
In the analysis of the folding of the disulfide protein, a folding profile is constructed from the difference in free energies between the dithiol and the disulfide proteins. Difference free energies for each state A ($\Delta\Delta G_{A\text{-}U}$) are displayed on the folding profile. Taking the difference in free energies of the unfolded proteins, $\Delta G_U$, as a reference point, this shows when, during the folding of a disulfide protein, stability is conferred upon the protein by the disulfide bond. If we accept the assumption that the stability conferred by the disulfide bond is due principally to decreased stability of the unfolded state, it follows that when the part of the protein which is destabilized by the disulfide folds, the protein will be stabilized, relative to its unfolded state. There are two extreme cases we could predict using disulfide bonds to probe protein folding.

*Case 1.* If the disulfide bond connects two parts of a protein that fold together very early, before the rate-determining step of folding, and the structure concerned is completely formed early, then the instability conferred upon the unfolded state by the disulfide bond will be lost early. This will mean that both the intermediate and transition state of folding will be stabilized relative to the unfolded state. In the extreme case, both the intermediate and the transition state will be stabilized to the same extent as the folded state, $\Delta\Delta G_{F\text{-}U} = \Delta\Delta G_{I\text{-}U} = \Delta\Delta G_{‡\text{-}U}$. In practice, we might expect this extreme case to occur only rarely, since we might expect only disulfide bonds within elements of secondary structure to satisfy these criteria, and disulfides within helices or between strands of sheets rarely occur (Thornton, 1981). In less extreme cases, any increase of stability of the intermediate and transition state over the unfolded state will be indicative of folding within the region destabilized in the unfolded state by the disulfide bond.

*Case 2.* If the disulfide bond connects parts of the protein which do not dock together until the final folded form of the protein is reached, then the disulfide bond will destabilize the intermediate and transition state in the same way as the unfolded state, i.e., $\Delta G_U = \Delta G_I = \Delta G_‡$. Stability will not be conferred by the disulfide bond until the protein is completely folded.

Folding profiles for both proteins are illustrated in Figure 6. The folding of the two proteins give clearly different folding profiles.

*43–80.* An examination of the folding profiles shows plainly that 43–80 is not stabilized relative to $\Delta G_U$ until after the major transition state of folding. $\Delta G_I \approx \Delta G_U$ and $\Delta G_‡ \approx \Delta G_U$. This is taken to mean that the instability imposed upon the unfolded state by the disulfide bond is still present in the intermediate and transition states. This part of the protein is not folded in either the intermediate or the transition state, since, once the protein folds, the destabilizing effect of loss of conformational entropy will be lost. All the stability is gained after the rate-determining step of folding. When we consider the unfolding of this protein, it is clear that all the stability conferred by the disulfide bond is lost before the transition state is reached. The effect of this is to destabilize the transition state relative to the folded state. This is reflected in the 170-fold decrease in the rate of unfolding relative to the dithiol form (Table II). Thus 43–80 satisfies the conditions laid down in case 2, above. This result is quite clear-cut in this case as the stabilization by the disulfide bridge plainly occurs after the major transition state for refolding.



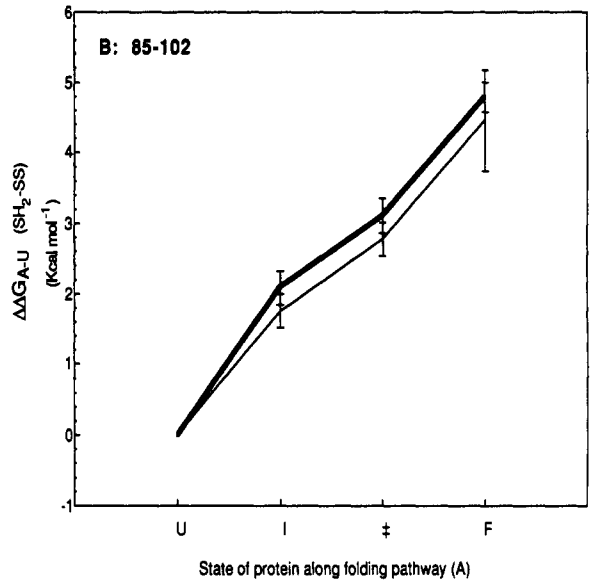




FIGURE 6: Folding profiles of disulfide proteins. (Top) 43–80. (Bottom) 85–102. The stability of the disulfide protein over the corresponding dithiol form at each state along the folding pathway, A, $\Delta\Delta G_{A\text{-}U}$, is shown on the ordinate. The unfolded (U), intermediate (I), transition state (‡), and folded (F) forms are represented on the abscissa. $\Delta\Delta G_{A\text{-}U}$ is calculated from the formula $\Delta\Delta G_{A\text{-}U} = \Delta\Delta G_{A\text{-}F} - \Delta\Delta G_{U\text{-}F}$. As described in the text, the value of $\Delta\Delta G_{U\text{-}F}{}^{H_2O}$ has a high error when calculated by extrapolation to 0 M denaturant, whereas there is less error in the calculated value of $\Delta\Delta G_{U\text{-}F}{}^{D50\%}$. For these folding profiles, both values of $\Delta\Delta G_{U\text{-}F}$ have been used to calculate the values of $\Delta\Delta G_{A\text{-}U}$ shown. The bold line shows the calculation using $\Delta\Delta G_{U\text{-}F}{}^{D50\%}$, and the thin line shows the calculation using $\Delta\Delta G_{U\text{-}F}{}^{H_2O}$. The profiles are the same, within error, and the differences clearly do not affect the interferences which can be drawn.

*85–102.* An examination of the folding profile of 85–102 shows a different folding pattern, corresponding to the less extreme case described in case 1, above. There is not a clear-cut all-or-none stabilization of a set of states. 85–102 has gained considerable stability, relative to $\Delta G_U$, in the intermediate, is more stable in the transition state, and gains full stability upon reaching the final folded form. The increased stabilization between the intermediate and transition state is reflected in the 5-fold increase in the rate of refolding (Table III). When unfolding is considered, the disulfide protein clearly loses some stability on going from the folded to the transition state. This is reflected in the 17-fold decrease in the rate of unfolding upon formation of the disulfide bond.

This analysis does not allow a quantitative assessment of the folding at each state along the folding pathway. In previous mutant studies where a single interaction has been considered, it has been possible to infer the partial formation of structure at the site of a mutation, but here the situation is more complex. The disulfide bond encloses a loop of 18 residues, and the conformational entropy of flanking residues will also be affected. These results give no quantitative information upon the amount of folding in the region, and, further, there is no information on the relative contributions of strain energy and conformational entropy to the stabilization of the different partially folded states. What is clear, however, is that there is some structure formed early in this region of the protein. There could, for example, be formation of structure in a part of the protein destabilized by the disulfide bond in the unfolded state and no structure in another part. Protein engineering and NMR experiments have shown that the central parts of $\beta$-strands 3 and 4 that are connected by this disulfide bond are folded in the intermediate, and the tight turn connecting the strands is completely formed in the transition state of folding. The 100–105 loop is partially formed early in folding, but not fully formed until the final step in folding. The 77–85 loop is not formed until after the rate-determining step of folding. This model of folding in the region of the protein destabilized by the 85–102 disulfide bond is entirely consistent with the folding profile obtained, showing considerable stabilization early, with full stability not being achieved until late in the folding pathway.

The folding of barnase has been extensively studied by protein engineering and NMR. It is possible to follow folding within elements of secondary structure by protein engineering to remove small stabilizing interactions within the structural elements (Matouschek et al., 1992a; Serrano et al., 1992b). Disulfide bonds would be inappropriate probes of folding within these elements since there are few such disulfide within helices or within $\beta$-sheets (Thornton, 1981). The results presented in this paper show that disulfide bonds engineered into proteins can be used as probes to monitor the folding together of elements of secondary structure within the protein. Disulfide bonds will be useful probes between elements of structure where there are often no specific interactions which can be probed by other methods. The insertion of disulfide bonds between elements of secondary structure which fold late is characterized by the gaining of stability late in the folding process, reflected in a marked decrease in the rate of unfolding, accompanied by little effect on the rate of refolding. The results presented in this study indicate that disulfide bonds can report successfully upon late folding. When inserted between elements of secondary structure that fold early, there is a gain of stability early in the folding pathway, accompanied by an increase in the rate of refolding. A disulfide such as that at 85–102 which indicates some early stabilization, but where complete stabilization is not reached until the protein is finally folded, could provide valuable information that there is only partial structure formed early. Protein engineering of specific interactions within the area stabilized by an early folding disulfide, such as 85–102, could be used to probe the folding of this region further to determine the relative contribution of different elements within the structure to the folding profile.

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