The effects of disulfide bonds on the denatured state of barnase

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Abstract

The effects of engineered disulfide bonds on protein stability are poorly understood because they can influence the structure, dynamics, and energetics of both the native and denatured states. To explore the effects of two engineered disulfide bonds on the stability of barnase, we have conducted a combined molecular dynamics and NMR study of the denatured state of the two mutants. As expected, the disulfide bonds constrain the denatured state. However, specific extended β -sheet structure can also be detected in one of the mutant proteins. This mutant is also more stable than would be predicted. Our study suggests a possible cause of the very high stability conferred by this disulfide bond: the wild-type denatured ensemble is stabilized by a nonnative hydrophobic cluster, which is constrained from occurring in the mutant due to the formation of secondary structure.

Keywords: barnase; denatured states; disulfide bonds; molecular dynamics; NMR

Efforts to increase protein stability by the introduction of disulfide bonds have met with mixed success (Betz, 1993), which underscores our incomplete understanding of the role of these covalent cross-links on protein structure and stability. It has long been predicted that disulfide bonds will exert their major effect through entropic destabilization of the denatured state in a manner that can be predicted from the size of the loop enclosed by the cross-link (Flory, 1956; Poland & Scheraga, 1965; Pace et al., 1988). Also, disulfide bonds can affect native-state entropy (Tidor & Karplus, 1994). In addition, for engineered disulfide bonds, the effects of mutation and induced “strain” can destabilize the native state (Katz & Kossiakoff, 1986; Villafranca et al., 1987; Katz & Kossiakoff, 1990; Jacobson et al., 1992; Clarke et al., 1995a, 1995b; Betz et al., 1996; Hinck et al., 1996). Thus, one must consider the balance of such interactions on rationalizing the effects of disulfide bonds. To

study this balance, we have undertaken a systematic study of the effects of introducing disulfide bonds into barnase, a small globular protein lacking natural Cys residues. Thus far, we have determined the effects on the thermodynamics of denaturation (Johnson et al., 1997), the kinetics of folding (Clarke & Fersht, 1993), and on the structure and stability of the native state (Clarke et al., 1995a, 1995b).

We focus on two mutants with disulfide bonds between residues 43 and 80 ([43–80]_{SS}) and residues 85 and 102 ([85–102]_{SS}) (Fig. 1). The 43–80 disulfide bond connects a small α -helix (α_3) to a loop between strands 2 and 3 of the five-stranded β -sheet. This mutant is stabilized by 2.0 kcal mol^{−1} relative to wild-type and 3.2 kcal mol^{−1} relative to its dithiol form (Clarke et al., 1995a). This degree of stabilization is close to that predicted from purely entropic effects within the denatured state (3.8 kcal mol^{−1}, Poland & Scheraga, 1965; Pace et al., 1988). The 85–102 disulfide bond encloses a β -hairpin comprising β -strands 3 and 4, which are joined by a tight turn between residues 91–94. This mutant is stabilized by 4.3 kcal mol^{−1} relative to wild-type and 4.8 kcal mol^{−1} relative to its dithiol form (Clarke et al., 1995a). This disulfide bond stabilizes the protein more than would be predicted (3.2 kcal mol^{−1}; Pace et al., 1988). The crystal structures of [43–80]_{SS} and [85–102]_{SS} reveal that their folded structures are very similar to wild-type with some local re-organization in the region of the cross-link in [43–80]_{SS} (Clarke et al., 1995a). Hydrogen exchange experiments reflect local destabilization induced by the 43–80 disulfide bond, whereas the 85–102 disulfide bond stabilizes the structure near the cross-link (Clarke et al., 1995b). De-

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Abbreviations: MD; molecular dynamics; [43–80]_{SS}, [85–102]_{SS}; barnase mutants with disulfide bonds between residues 43 and 80 and 85 and 102, respectively; GdmCl, guanidinium chloride; GSH and GSSH, oxidized and reduced glutathione; HSQC, heteronuclear single-quantum correlation; R_1 , longitudinal relaxation rate; R_2 , transverse relaxation rate; ppm, parts per million; RMS, root-mean-square; CPMG, Carl-Purcell-Meiboom-Gill; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.

Fig. 1. The secondary structure of wild-type barnase showing the position of the disulfide bonds between residues 43 and 80 ([43–80]_{SS}) and positions 85 and 102 ([85–102]_{SS}). The disulfide bond 43–80 joins a small helical region to a loop. The bond encloses β -strands 1, 2 of the antiparallel β -sheet, which are connected by a long loop region (residues 57–69). The 85–102 disulfide encloses β -strands 3 and 4, which are joined by a tight β -turn (residues 91–94).

tailed thermodynamic studies using calorimetry do not provide information on the relative effects on entropy and enthalpy in these disulfide mutants because of the large contributions from entropy/enthalpy compensation (Johnson et al., 1997). Here we describe molecular dynamics (MD) simulations and NMR experiments aimed at characterizing the dynamic and structural effects of the disulfide bonds on the denatured state of barnase.

We show that the effect of a disulfide bond on the denatured state need not be limited to relatively nonspecific entropic effects. NMR provides some clues as to the position and extent of the specific interactions occurring in the denatured states of wild-type and disulfide-bonded mutant forms of barnase. But, owing to the dynamic, unstructured, and heterogeneous nature of denatured proteins, it is not possible to transform the results into a comprehensive description of the structural properties of the denatured state. MD complements NMR by providing detailed structural models for the denatured state with and without the disulfide bonds (see Wong et al., 2000). This is particularly important here since the β -sheet structure stabilized in [85–102]_{SS} is especially difficult to distinguish from random coil in the highly mobile denatured state by NMR. A cross-link can induce nonspecific changes in mobility that are difficult to distinguish from specific structural effects. In the case of [43–80]_{SS}, the disulfide bond does not induce structure, but it causes a drop in the mobility of the chain immediately around the disulfide bond. However, with [85–102]_{SS}, the disulfide bond stabilizes a native-like β -hairpin in the denatured state. Paradoxically, this structure destabilizes the protein by limiting the configurations sampled and by preventing the formation of non-native hydrophobic clusters, which have been shown to be important in the wild-type denatured state (Bond et al., 1997; Wong et al., 2000).

Results

The effect of a disulfide bond on the relative stability of the denatured state can be determined from the equilibrium between

oxidized and reduced forms (K_{eq}^D) in the presence of redox reagents (Creighton, 1986) (Equation 1):

$$K_{eq}^D = \frac{[P_{SS}][\text{GSH}]^2}{[P_{(SH)_2}][\text{GSSG}]} \quad (1)$$

where $[P_{SS}]$ and $[P_{(SH)_2}]$ are the concentrations of oxidized and reduced proteins, respectively, and $[\text{GSH}]$ and $[\text{GSSG}]$ are equilibrium concentrations of reduced and oxidized glutathione, respectively. Figure 2 shows the effect of guanidinium hydrochloride (GdmCl) on the relative stability of the disulfide and dithiol forms of [43–80]_{SS} and [85–102]_{SS} under conditions where the protein is fully denatured, as judged by fluorescence (Clarke & Fersht, 1993). GdmCl concentration has no effect on the relative stability of the disulfide and dithiol crosslinked forms of [43–80]_{SS}, as has previously been observed for other proteins (Creighton, 1986; Lin & Kim, 1989). However, increasing [denaturant] decreases the relative stability of the disulfide form of [85–102]_{SS} between 4 and 6 M GdmCl. This suggests that there is residual structure in [85–102]_{SS} in the presence of 4 M GdmCl that is destabilized upon increasing the denaturant concentration, such that the reduced, noncross-linked form becomes increasingly favored. There is no evidence for such residual structure in [43–80]_{SS}. These results are the basis for both the NMR and MD studies described below, designed to investigate the nature of the residual structure in denatured [85–102]_{SS}.

NMR studies

Here we focus on wild-type and [85–102]_{SS} proteins at pH 6.3 in 4 M GdmCl at 298 K. Some of the NMR experiments were also performed under the same conditions on [43–80]_{SS} as a control, to distinguish specific structural effects from nonspecific effects of cross-linking.

Fig. 2. The effect of [GdmCl] on the relative stability of the disulfide and dithiol forms of [43–80]_{SS} and [85–102]_{SS}. The concentrations of denaturant used are those where the protein appears fully denatured by fluorescence. ([GdmCl]_{50%} = 2.5 M and 3 M for [43–80]_{SS} and [85–102]_{SS}, respectively.) K_{eq}^U was determined using Equation 1. A decrease in K_{eq}^D represents a decrease in the stability of the disulfide form relative to the dithiol form.

HSQC spectra

The HSQC spectra for all three proteins are very similar, and characteristic of denatured proteins with little dispersion and no detectable folded peaks. Assignment of many of the cross peaks was possible by direct comparison to the wild-type spectra obtained under other denaturing conditions (Arcus et al., 1994, 1995). Comparisons of HN and ^{15}N chemical shifts of the mutants relative to wild-type are shown in Figure 3. In [85–102]_{SS}, there are significant changes in chemical shift encompassing the entire region enclosed by the disulfide bond. In [43–80]_{SS}, however, the chemical shift differences are localized to the sites of mutation.

There are more weak peaks in the HSQC spectrum of [85–102]_{SS} than in the wild-type and [43–80]_{SS} spectra. The intensity of the cross peaks was estimated using both peak height and integrated peak volume (Fig. 4). There is a significant decrease in intensity of the HSQC peaks extending through and beyond the region connecting residues 85 and 102 in [85–102]_{SS}. This trend is not observed in wild-type or [43–80]_{SS}. We cannot assume any

Fig. 3. Changes in (A, C) HN and (B, D) ^{15}N chemical shifts for amide protons in (A, B) [85–102]_{SS} and (C, D) [43–80]_{SS} relative to wild-type (● ppm, wild-type—mutant).

Fig. 4. Peak intensities of HSQC cross-peaks for (A) wild-type, (B) [85–102]_{SS}, and (C) [43–80]_{SS}. There is a significant decrease in peak intensity through and beyond the region of the disulfide in [85–102]_{SS}.

specific mechanism, but these data are consistent with conformational exchange in the region enclosed by the 85–102 disulfide bond.

Relaxation studies

Heteronuclear relaxation studies have previously been used to identify possible regions of residual structure in denatured proteins (Freund et al., 1996; Farrow et al., 1997; Wong et al., 2000). Relaxation studies of the proteins investigated here indicate that the covalent cross-links lead to a local decrease in mobility. While the longitudinal relaxation rates R_1 are unaffected by mutation (data not shown), the transverse relaxation rates R_2 show an increase in the region of the disulfide bond in both mutants (Fig. 5). This increase is of similar magnitude in both mutants. Any specific structural effects in [85–102]_{SS} cannot be distinguished by the dynamic data.

Main-chain NOE data

Normalized $dNN(i, i + 1)$ NOEs (σ_{NN}) could be assigned for the majority of protons in both wild-type and [85–102]_{SS}. Increased σ_{NN} values are observed in the helical and β -sheet regions of the wild-type protein (Fig. 6), which correlates with decreased chain mobility associated with residual structure (Wong et al., 2000). In [85–102]_{SS}, the data for the helical, N-terminal half of the protein are similar to wild-type; however, there is a significant increase in σ_{NN} values including, and extending well beyond, the region enclosed by the disulfide bond. These data are consistent with resid-

Fig. 5. R_2 measurements in (A) wild-type (●), [85–102]_{SS} (○), and (B) wild-type (●), [43–80]_{SS} (○). In both mutants there is a significant decrease in mobility associated with the introduced crosslink. Arrows show the position of the Cys residues.

ual structure in the region enclosed by the cross-link, but again, it is not possible to rule out restriction in motion due solely to restriction of the polypeptide chain by the cross-link, or some other effect that is not directly linked to structure. We also note that nonsequential dNN NOEs cannot be detected in this region.

Fig. 6. Normalized $dNN(i, i + 1)$ NOEs (σ_{NN}), for wild-type and [85–102]_{SS}. The fluctuations in wild-type are present in the mutant. The σ_{NN} values are increased in the whole region enclosed by the disulfide bond.

The NMR data thus support the idea that a disulfide bond can affect and induce further residual structure in the denatured state, but they do not provide structural detail. Parameters such as chemical shift deviations indicate that in [43–80]_{SS} the specific effects are strictly localized, but that in [85–102]_{SS} there are concerted effects across the entire region enclosed by the disulfide bond. These results are consistent with the initial observations of denaturant dependence on K_{eq}^D (Fig. 2). Unfortunately, however, the NMR data do not distinguish specific details of residual structure from the general dynamic restraints of the covalent cross-link, and so quantitative and definitive analysis of the structure and dynamics of these denatured states are not possible. Therefore, to obtain more structural information, MD simulations were performed.

Molecular dynamics simulations

Thermal unfolding simulations of wild-type (D-1, D-2, and D-3 of Wong et al., 2000) and the [85–102]_{SS} and [43–80]_{SS} disulfide-bonded forms of barnase were performed at 498 K in water for 3 ns to generate denatured ensembles for comparison with the experimental data. As a control, we include [43–80]_{SS}, for which no residual structure can be inferred from the equilibrium data (Fig. 2). By comparing the different forms, we can address whether MD simulations can distinguish specific residual structure from non-specific cross-linking effects. Identification of the denatured “state” was based on a variety of criteria, as described previously for the wild-type protein (Bond et al., 1997; Wong et al., 2000). Similar to the denaturation of wild-type barnase, structural changes associated with the thermal denaturation of [85–102]_{SS} and [43–80]_{SS} reached a plateau by ~ 2 ns. The α -carbon RMS deviation (RMSD) from the starting structure increased rapidly in the [43–80]_{SS} simulation to 6 Å over the initial 0.5 ns. This rapid initial period was followed by a gradual increase to 10 Å by ~ 2 ns. [85–102]_{SS} was similar, reaching a final value of ~ 11 Å. The final values and time-dependent behavior were similar to that of the wild-type protein (Table 1; Wong et al., 2000). Maximal tryptophan solvent accessibility was reached by 2 ns. All the denatured structures were compact, with radii of gyration of ~ 15 – 16 Å (Table 1) compared with an estimated value of 34 Å for a random coil of 110 residues (Miller & Goebel, 1968). Overall, these and other average properties of the denatured ensembles were similar (Table 1). However, detailed analysis reveals specific structural differences. In the present study, the denatured ensemble is defined as the collection of structures from 2–3 ns (5,000 structures from each simulation).

Tertiary contacts

Heavy atom contacts are shown in Figure 7 for the reference, wild-type NMR structure and the three denatured ensembles. Both native (above the diagonal) and nonnative (below the diagonal) contacts are present in the denatured form of all three proteins. All of these contacts persist in $>25\%$ of the structures, and a significant number of these are made in at least 80% percent of the structures (blue). Detailed analysis of the residual structure in the wild-type denatured state, and comparison with experiment have been reported previously (Bond et al., 1997; Wong et al., 2000). Here, we compare the residual structure observed in the disulfide bonded proteins with the wild-type.

Persistent contacts are made in each of the disulfide mutants simulated. As in the wild-type protein, helical contacts are present in $\alpha 1$, as evidenced by the sequential contacts along the diagonal

Table 1. Overall properties of the wild-type and mutant denatured ensembles^a

Property	WT	[43–80] _{SS}	[85–102] _{SS}
Radius of gyration (Å) ^b	15.9 (0.7)	15.4 (0.3)	15.5 (0.6)
⟨pairwise RMSD⟩ (Å) ^c	5.7 (1.4)	4.9 (1.0)	5.1 (1.2)
% α -Content ^d	12 (4)	8 (2)	4 (2)
% β -Content ^d	5 (3)	6 (3)	2 (2)
⟨SASA⟩ (Å ²) ^e	9,513 (370)	9,327 (243)	10,027 (281)
⟨Trp35 SASA⟩ (Å ²) ^e	162 (35)	189 (35)	223 (19)
⟨Trp71 SASA⟩ (Å ²) ^e	142 (37)	191 (30)	114 (28)
⟨Trp94 SASA⟩ (Å ²) ^e	94 (41)	167 (31)	185 (27)

^aThe values for the denatured ensembles were calculated for the 2–3 ns time period of each simulation. Values for the WT D-1 simulation are provided. Standard deviations are given in parentheses. The brackets, ⟨ ⟩, denote a time average.

^bOnly the α -carbons were used for the calculation of the radius of gyration.

^cThe average pairwise C α RMSD is calculated by comparing all structures in the denatured ensemble in a pairwise manner.

^dThe secondary structure content is the fraction of residues (out of a total of 107) that adopted repeating secondary structure (three or more consecutive residues) based on whether the (ϕ, ψ) values fall in the α - or β -region of conformational space (Daggett et al., 1991).

^eThe solvent accessible surface area was calculated using the program of NACCESS (Hubbard & Thornton, 1993) using a 1.4 Å probe radius. The total SASA is given for the full protein and individual Trp residues.

(Fig. 7). In contrast, $\alpha 2$ is weakened considerably in both of the mutants, but $\alpha 2$ is also lost in the D-2 wild-type simulation (Wong et al., 2000). Native contacts between the loop preceding $\alpha 2$ and the N-terminal portion of $\beta 1$ (labeled pinch in Fig. 7A) are preserved, but are not as strong as those made in the wild-type ensemble. In all three simulations, dynamic nonnative contacts are made throughout the molecule. As expected, the most persistent contacts are centered about the site of the disulfide linkage. In [43–80]_{SS} native contacts are made between residues in $\beta 1$ and $\beta 2$, and some nonnative contacts are formed between the residues in the intervening loop (Fig. 7D). Contacts in [85–102]_{SS} extend throughout the loop enclosed by the disulfide bond, making both native and non-native contacts (Fig. 7C). [43–80]_{SS} also makes some contacts in this region, but they are weak and distorted (Fig. 7D). In addition, in [85–102]_{SS} there are native contacts between $\beta 2$ and $\beta 3$, although they are much less persistent than the $\beta(3-4)$ contacts. The majority of the off-diagonal contacts in the β -sheet region are very weak in the wild-type protein (Fig. 7B).

In the wild-type denatured ensemble, a dynamic hydrophobic cluster is present in which Trp94 figures prominently (Bond et al., 1997; Wong et al., 2000; see Fig. 7B). The packing of these residues aids in maintenance of a turn from approximately residues 90–94 between $\beta 3$ and $\beta 4$ (see Figs. 4, 5 of Wong et al., 2000). But, the turn does not necessarily propagate to form a hairpin, as demonstrated by the lack of the appropriate contacts in Figure 7B. [We note, however, that three wild-type simulations are presented by Wong et al., and in one of these (D-3) the hairpin is partially populated in the denatured state.] In [85–102]_{SS}, the $\beta(3-4)$ hairpin structure is present throughout the simulation. This hairpin makes the majority of the native contacts in addition to less stable nonnative contacts (Fig. 7C). Overall, the β -hairpin is similar to the one in the native NMR structure but the backbone conformation is distorted (Fig. 8), and it does not adopt the appropriate

(ϕ, ψ) angles. Most of the distortions represent slight, short-lived shifts along the sequence that put the side chains out of register. In [85–102]_{SS}, both native and nonnative hydrogen bonds are distributed throughout the loop enclosed by the cross-link (Fig. 8). The nonnative hydrogen bonds are due to a change in the orientation of the carbonyl oxygen and amide hydrogen of Leu89 and Ile96—they become inverted (Fig. 8). To verify that this behavior is not an aberration, another independent simulation of [85–102]_{SS} was performed, and the $\beta(3-4)$ hairpin was indeed retained (Fig. 9). The properties of its denatured ensemble are consistent with those of the [85–102]_{SS} simulation discussed above (data not presented). Interstrand hydrogen bonding among main-chain atoms is less extensive in the loop enclosed by the 43–80 disulfide; the only persistent hydrogen bonds are between the main-chain atoms of Ser50–Asn77 or Tyr78, and Ile51–Asp75 or Ile76. Few main-chain hydrogen bonds are formed in the rest of the β -sheet region.

Although some side-chain contacts in the [85–102]_{SS} disulfide mutant are consistent with native β -structure, the main-chain geometries are disrupted. For example, only residues 88–89 and 98–99 in [85–102]_{SS} maintain proper β -dihedral angles. Similarly, in the [43–80]_{SS} ensemble only residues 47, 50, 51, 73, 74 adopt the appropriate dihedral angles for β -structure. In both cases, the number of sequential residues encompassed by the disulfide with extended β -like geometry is never greater than 2; that is, the observed β -structure is a local measure of (ϕ, ψ) preference, but true extended β -strands or sheets do not form.

Internal motion

As would be expected, the backbone motion is reduced at the Cys residues relative to the wild-type protein (Fig. 10) and less conformations are sampled as a result (see the pairwise C α RMSDs in Table 1). The decrease in mobility is not localized entirely to the site of the disulfide. In [85–102]_{SS}, the decreased internal main-chain motion extends throughout the β -sheet region from residues 40–110, although the values are not continuous, uniform, nor large (Fig. 10B). Nonetheless, there is a distinct trend toward lower chain mobility in the $\beta(3-4)$ hairpin in [85–102]_{SS} that is not maintained in wild-type or [43–80]_{SS}. In contrast, in [43–80]_{SS} there are no strong trends of either heightened or decreased mobility relative to wild-type, but the average fluctuations are lower, and mobility is decreased at the disulfide bond (Fig. 10C).

Representative snapshots from the simulations illustrate the dynamic nature of these denatured species (Fig. 9B). In particular, the chain is locally restricted about the cross-link in [43–80]_{SS}, but quickly diverges both within and away from the enclosed loop. In both [85–102]_{SS} simulations, there is significant restriction of the main chain through the disulfide-bonded loop and a smaller number of conformations compared with [43–80]_{SS} (compare the colored regions of the structures in the two disulfide-bonded forms). While the overall properties of all three proteins are similar (Table 1), the ensembles are distinct (Fig. 9).

Discussion

The denaturant dependence of the relative stability of the disulfide and dithiol forms of [85–102]_{SS} provides clear evidence of residual structure induced by this disulfide bond, which is not apparent in [43–80]_{SS}. By using both NMR and MD, we are able to examine the possible nature of this residual structure. Comparison of the mutants with the wild-type protein identifies the effects of the

Fig. 7. Heavy atom contact maps for (A) the NMR structure; (B) the wild-type denatured ensemble (D-1, 2–3 ns); (C) [85–102]_{SS}, 2–3 ns; and (D) [43–80]_{SS}, 2–3 ns. Contacts are defined as heavy-atom distances between nonneighboring residues of ≤ 5.4 Å for aliphatic carbons and 4.6 Å for all others. Except for A, native contacts are given above the diagonal and nonnative contacts are below. The contacts are colored as follows: blue, intact 80–100% of the time period indicated; red, intact 60–80% of the time; and green, intact 10–60%.

disulfide bonds. Comparisons between [43–80]_{SS} and [85–102]_{SS} allow us to distinguish between nonspecific cross-linking effects and specific structural details.

In defining residual structure in denatured proteins, several kinds of NMR evidence may be considered. Deviation of chemical shift and other parameters (such as σ/N NOEs and coupling constants) from standard random coil values and changes in dynamic characteristics, determined using heteronuclear relaxation studies, may

be general indicators of residual structure, which is defined as nonrandom sampling of conformational states by the denatured polypeptide. Specific structural details can be observed from inter-residue NOEs, such as $i \rightarrow i + 3$, and $i \rightarrow i + 4$ NOEs characteristic of turn or helical structures, and side-chain NOEs have been observed in clusters of hydrophobic residues. This study highlights a problem in using NMR to probe residual structure in cross-linked denatured proteins. By their very nature, the disulfide

Fig. 8. A stereoview comparison of residues 85–102 in the [85–102]_{SS} crystal structure and a snapshot after 3 ns. Main-chain hydrogen bonds (<2.6 Å between donor and acceptor atoms) are given by dashed lines. Various hydrogen bonds in the β -hairpin and their lifetimes (the percentage of time formed from 2–3 ns) are given below the structures.

cross-links will restrict the dynamics of the enclosed loop. Nonetheless, the NMR data provide indications that there is residual structure in [85–102]_{SS}, which extends through the entire region enclosed by the cross-link. Changes in chemical shift and peak intensity are consistent with a concerted region of structure induced by the bond. However, NMR alone provides insufficient data to discern the nature of this structure, and the NMR data are

not good enough for definitive and quantitative analysis. The MD simulations, on the other hand, provide evidence for heightened residual structure in the denatured state of [85–102]_{SS} compared with the wild-type. The MD-generated structural models are used here to complement experiment and aid in interpretation of the NMR data.

Comparison of the residual structure in the wild-type MD ensemble has previously been shown to be consistent with the wild-type NMR data (Bond et al., 1997; Wong et al., 2000). Figure 9A shows individual, but representative, structures taken from the three wild-type, [43–80]_{SS} and the two [85–102]_{SS} denatured ensembles. It must be emphasized that the structures are fluctuating significantly (Fig. 9B), but the major features can be discerned. In all three forms of the protein, at least the C-terminal portion of α_1 and parts of α_2 persist. In the wild-type ensemble, there is no evidence for residual structure in the region enclosed by the 43–80 cross-link (colored cyan in Fig. 9), but there is a nonnative hydrophobic cluster in the region enclosed by the 85–102 disulfide, near the turn. The cluster persists in [43–80]_{SS} but is not present in [85–102]_{SS}. In [43–80]_{SS}, the MD simulations also reveal restricted motion in the residues adjacent to the disulfide bond. The behavior of [43–80]_{SS} is generally consistent with the standard model of denatured state destabilization by a loss of entropy, which is balanced by folded state effects (Clarke et al., 1995a, 1995b). The cross-link induces very local interactions that are mainly nonnative.

The effects are not localized to the disulfide bond in the case of [85–102]_{SS}. In the ensemble of denatured structures for the [85–102]_{SS} disulfide mutant, a persistent β -hairpin is observed between residues 86–99 (Figs. 7C, 8, 9). The β -structure that remains is distorted, but the general topology remains as in the wild-type, with both native and nonnative hydrogen bonding patterns and some shifts in register. The strongest side chain contacts across the sheet are primarily native, but nonnative interactions are also prevalent (Fig. 7D, 8). The nonnative hydrophobic cluster observed in the wild-type denatured state is not present, because the residual structure prevents such cluster formation. The result of this structure is to increase the exposed hydrophobic surface area in the region relative to the wild-type.

The experimentally determined ΔC_p and m -values of the disulfide-bonded proteins are not significantly different from the wild-type (Clarke et al., 1995a; Johnson et al., 1997). This is consistent with there being no large overall difference in solvent exposed surface area in the denatured states of the cross-linked proteins (Table 1).

Why is the [85–102]_{SS} disulfide mutant so stable? It has been assumed that the stabilizing effect of a disulfide bond results from entropic destabilization of the denatured state (Flory, 1956; Poland & Scheraga, 1965; Pace et al., 1988; Betz, 1993). The disulfide mutants do indeed show lower mobility than the wild-type. However, there is also evidence for specific residual structure in the denatured state of [85–102]_{SS}, which might imply that the denatured state is actually stabilized by the cross-link (Fig. 2). The MD data, however, suggest that this may not be so. Both MD and NMR studies show that there is a nonnative, hydrophobic cluster, centered around Trp94 in the denatured state of the wild-type protein (Arcus et al., 1994, 1995; Freund et al., 1996; Bond et al., 1997; Wong et al., 2000). MD shows that this cluster does not form in [85–102]_{SS} and, consequently, the nonpolar surface area is greater, presumably destabilizing the denatured state. Thus, the 85–102 disulfide bond affects the stability of both the native (Clarke et al., 1995b) and denatured states in a manner that could not be predicted from standard polymer theory.

Fig. 9. Representative structures from the wild-type and disulfide-bonded mutant simulations. **A:** 3 ns snapshots from the three wild-type, [43-80]_{SS}, and two [85-102]_{SS} unfolding trajectories. The N-terminus is colored green in each case. **B:** Snapshots (every 0.2 ns) from the [43-80]_{SS} and [85-102]_{SS} denatured ensembles depicting the range of motion of the main chain. Structures were aligned at the Cys residues. Residues 43 to 80 are cyan and residues 85 to 102 are red. Cys residues are in yellow.

Conclusions

Residual structure has been observed both in NMR and MD studies of wild-type barnase (Arcus et al., 1994, 1995; Freund et al., 1996; Bond et al., 1997; Wong et al., 2000). The NMR and MD simulations presented here indicate that both the [43-80]_{SS} and the [85-102]_{SS} disulfide bonds alter the denatured state ensemble. In [43-80]_{SS}, the disulfide bond induces minor native and nonnative clusters of residual structure that are principally localized to the site of the crosslink. In [85-102]_{SS}, however, the structural effects extend throughout the region enclosed by the disulfide bond, stabilizing a distorted, but native-like β -hairpin. The effects of disulfide bonds on the denatured state are clearly hard to predict, as they can be specific and are not restricted to changes in chain entropy. Evidence from both experiment and simulation may be needed to build a detailed model of the effect of disulfide bonds on the denatured states of proteins.

Materials and methods

Protein purification

The method of production and purification of both wild-type and mutant proteins, labeled and unlabeled, has been described previously (Clarke & Fersht, 1993; Clarke et al., 1993; Arcus et al., 1994).

Measurement of K_{eq}^D between disulfide and dithiol proteins

The equilibrium constants between disulfide and dithiol forms of the protein, K_{eq}^D , were measured under conditions favoring unfolding, at [GdmCl] concentrations where the protein appears fully denatured by fluorescence (Clarke et al., 1993). The stock solutions of reduced and oxidized glutathione (GSH and GSSG) were prepared gravimetrically, and their concentrations were determined

Fig. 10. Differences in the RMS fluctuations of the structures in the denatured ensemble about the mean structure between 2–3 ns. In each case, the fluctuations are given as a difference from the wild-type D-1 ensemble. The variations in the wild-type denatured state of two different simulations is shown in **A** for comparison. Values greater than 0 indicate that WT is more mobile, or in other words that the disulfide bonds constrain the main chain (**B** and **C**).

spectrophotometrically (Cleland, 1964; Huyghues-Despointes & Nelson, 1992). Water, 1 M MES pH 6.3, and glutathione stock solutions were degassed by extensive bubbling with nitrogen. Reaction mixtures (100 μ L), in 0.5 mL microfuge tubes, each con-

taining 40 μ M protein, 50 mM MES pH 6.3, 0–20 mM GSH and 0–20 mM GSSG were individually overlaid with argon and incubated at 25 °C overnight, to allow equilibration. A 50 μ L aliquot of the equilibrium mixture was withdrawn, mixed with 5 μ L 0.5 M HCl to bring the pH to $\sim$ 2, to quench the exchange reaction (Weissman & Kim, 1991), and the sample was immediately loaded onto a previously equilibrated Dynamax C8 300 Å (10 $\times$ 250 mm) reversed-phase high-performance liquid chromatography (HPLC) column. Disulfide and dithiol proteins were separated by HPLC and separated and analyzed as described previously, for folded proteins (Clarke et al., 1995a).

NMR studies

All NMR experiments were carried out on a Bruker AMX500, equipped with either a 5 mm broadband probe or a 5 mm inverse triple resonance (^1H , ^{15}N , ^{13}C) probe, both fitted with single-axis gradients. Gradient-selected sensitivity enhanced ^1H , ^{15}N HSQC spectra (Kay et al., 1992), and 3D HSQC-NOESY-HSQC (Frenkiel et al., 1990), with mixing times of 250 ms were collected at 25 °C, using uniformly ^{15}N -labeled samples (3 mM in 4 M GdmCl, 10% $^2\text{H}_2\text{O}$ pH 6.3).

2D ^1H - ^{15}N HSQC spectra (incorporating gradient selection and sensitivity enhancement) were used to measure heteronuclear R_1 , R_2 , and ^1H - ^{15}N NOE. T_1 relaxation delays were set to 50, 100, 200, 350, 500, 800, 1,500, and 3,000 ms. T_2 experiments utilized CPMG pulse trains with 900 μ s spin echo delays, and relaxation delays of 10, 17, 52, 87, 138, 208, and 346 ms. ^1H - ^{15}N NOE measurements were calculated as the ratio of peak heights from spectra obtained with and without presaturation of the amide resonances. Presaturation was achieved by a 120° pulse, 5 ms delay train for 3.1 s during the 5 s recycle delay.

HSQC and relaxation experiments were processed with Bruker software and peak volumes calculated from within the program. Single exponential decays were fitted to all the relaxation data using KaleidaGraph (Synergy software). The 3D HSQC-NOESY-HSQC spectra were processed with Felix2.30 (Biosym, San Diego, California) and peak heights measured from 1D vectors, to obtain the ratio of $dNN(i, i+1)$ NOE to diagonal signal intensities.

Molecular dynamics simulations

Molecular dynamics simulations were performed using an in-house version of the program ENCAD (Levitt, 1990). The potential energy function (Levitt et al., 1995, 1997) and MD protocols have been described elsewhere (Wong et al., 2000). Each molecular system simulated was described using an all atom representation of both the protein and solvent. Initial starting structures were derived from either crystal structures ([85–102]_{ss}—Protein Data Bank (PDB) accession code 1bng, and [43–80]_{ss}—PDB accession code 1bne; Clarke et al., 1995a) or the average NMR solution structures (PDB accession code 1bnr; Bycroft et al., 1991). Residual strain in the PDB structures was relieved using 500 ([85–102]_{ss}, [43–80]_{ss}) or 1,000 (WT NMR) steps of conjugate gradient minimization. Side-chain protonation states were chosen to mimic neutral pH (Lys and Arg were positively charged and Asp and Glu were negatively charged and His residues were neutral). The proteins were then solvated using a box extending at least 8 Å in all directions but no closer than 1.67 Å to a protein atom. For the second [85–102]_{ss} simulation, a 2 Å water exclusion distance was used to generate a different water starting system for MD. The

wild-type D-1, D-2, and D-3 simulations contained 3,665, 3,332, and 3,755 waters, respectively. The [43–80]_{SS} simulation contained 3,789 waters, the primary [85–102]_{SS} simulation contained 3,698 waters, and the second had 3,601. Periodic boundary conditions were employed to reduce edge effects and simulate a semidilute solvent environment.

The solvent was then subjected to 1,000 cycles of minimization, 2 ps of MD, and another 1,000 steps of minimization. This water preparation was followed by 1,000 steps of minimization of the protein in the field of the solvent and then 1,000 steps of minimization of the full protein–solvent system. The water density was set to the experimental value at 498 K of 0.829 g/mL (Kell, 1967; Levitt et al., 1997) to relieve excess pressure. This density corresponds to the lowest pressure (~26 atm) required for water to stay in the liquid phase at 498 (Haar et al., 1984). The lower density was obtained by expansion of the box to extend ~10 Å from any protein atom. After the preparatory steps described above, the systems were heated to 498 K. Initial atomic velocities were chosen using a Maxwellian distribution. Velocities were periodically scaled until the target temperature was reached, typically 2–3 ps. At this point, classical MD simulations were performed in the NVE microcanonical ensemble. The simulations were carried out for 3 ns each using a 2 fs integration time step (3,000 ps, or 1.5×10^6 iterations). An 8 Å nonbonded cutoff was used, and the nonbonded list was updated every 2–5 cycles. 15,000 structures were saved for analysis (5 per ps), of which the last 5,000 are used to represent the denatured state.

Control simulations at 298 K were performed for each of the structures. The protocol described above was used except the water density was 0.999 g/mL. The wild-type control has been presented elsewhere (Li & Daggett, 1998). The low-temperature simulations of the disulfide mutants yield C α RMSDs of the average structure over the 3 ns simulation to the pertinent starting structure of 2.5 and 3.1 Å for [43–80]_{SS} and [85–102]_{SS}, respectively, compared with 2.5 Å for the wild-type. The simulations were relatively well behaved such that the high-temperature simulations could be pursued.

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