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Importance of Two Buried Salt Bridges in the Stability and Folding Pathway of Barnase[†]

A. C. Tissot,[‡] S. Vuilleumier,[§] and A. R. Fersht*

Cambridge Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, England

Received December 12, 1995; Revised Manuscript Received March 18, 1996[©]

ABSTRACT: The importance of two buried salt bridges in barnase in the stability of its folded state, the major transition state for unfolding, and a folding intermediate has been analyzed by protein engineering, kinetic, and thermodynamic studies. The aspartate residues in the bridges Arg69–Asp93 and Arg83–Asp75 were replaced by the isosteric analogue asparagine, while various replacements were probed for the positively charged arginine partners. The mutations are very destabilizing, lowering stability by up to 5.4 kcal/mol. A value of 3.0–3.5 kcal/mol was derived for the coupling energy between Arg and Asp from a double mutant cycle analysis. Despite the radical nature of these mutations, they do not appear to alter the pathway of folding. The interaction between Arg69 and Asp93, located in a relatively conserved region among ribonucleases, is predominantly formed in the major transition state along the folding pathway, as found previously from an analysis of more benign mutations; the value of Φ_F for all mutations at positions 69 and 93 are 0.8–0.9 in the major transition state for folding (where $\Phi_F = 0$ = fully unfolded and $\Phi_F = 1$ = fully folded interaction energies). In contrast, the interaction between Arg83 and Asp75 in the active site of barnase is formed only in the native state of the protein. The analysis of folding pathways and the structure of folding intermediates by making kinetic and thermodynamic measurements on mutants appears even more robust than expected.

Structures on the folding pathway of the small ribonuclease barnase from *Bacillus amyloliquefaciens* are being characterized at the level of individual residues by kinetic and equilibrium measurements on engineered mutants, complemented by NMR methods (Fersht, 1993, 1995a). In general, the mutagenesis studies have concentrated on making small very conservative deletions of side chains, such as removing methylene groups from buried hydrophobic side chains or making larger changes in residues that are highly solvent-exposed, because these are the least prone to cause artifacts in the analysis (Fersht *et al.*, 1992; Fersht, 1995b). The importance of buried salt bridges in the native structure of barnase in the stability and the folding pathway of the protein has not been studied so far because this entails making more radical mutations. Such interactions are very intricate since an unpaired charge buried in the core of the protein is potentially very destabilizing (Fersht *et al.*, 1985; Dao-pin *et al.*, 1991) and is best counteracted by an interaction with a charge of the opposite sign (Ward *et al.*, 1987; Langsetmo *et al.*, 1991; Yang & Honig, 1993) in which all complementary charge–charge or charge–polar interactions are made (Fersht *et al.*, 1985; Ward *et al.*, 1987); each hydrogen bond that is not made with a charged atom loses up to ~3.5–4.5 kcal/mol of binding energy (Fersht *et al.*, 1985). Several empirical measurements of the importance of these interactions are available, ranging from 2.9 kcal/mol (Fersht, 1972) in chymotrypsin to 3–5 kcal/mol (Anderson *et al.*, 1990) in

T4 lysozyme. In the latter case, the interaction seems to provide a net stabilization of the protein. In general, it is still not clear what contribution such buried salt bridge interactions per se make to the stability of proteins (Mrabet *et al.*, 1992; Yang & Honig, 1993; Borders *et al.*, 1994; Marqusee & Sauer, 1994; Waldburger *et al.*, 1995).

A further aspect of buried salt bridge interactions is their specificity, as illustrated in hemoglobin (Perutz, 1989) and phosphofructokinase (Schirmer & Evans, 1990) where buried salt bridge interactions modulate the allosteric behavior of the protein. This raises the question of the possible role of such interactions in directing the folding of the polypeptide chain, for example by stabilizing a substructure early on the folding pathway. The accumulated knowledge on both the intermediate and the major transition state occurring on the folding pathway of barnase makes it a suitable system for addressing these questions by protein engineering techniques. The nature of the interactions requires the use of double mutant cycles (Carter *et al.*, 1984; Horovitz & Fersht, 1990, 1992; Horovitz *et al.*, 1990; Serrano *et al.*, 1990; Li Cata & Ackers, 1995) in which the free energies of the various states in wild type, two single mutants, and a double mutant are compared. This is necessary to estimate the effective contribution of the interaction to stability, since the destabilization of the protein arising from the presence of an unpaired charge as a result of mutation could be much greater than the actual interaction between the two charged residues in the wild-type protein, and will lead to an overestimate of their interaction energy. The use of the double mutant cycle procedure also avoids many sources of error linked with the use of single mutations in folding studies (Serrano *et al.*, 1990; Fersht *et al.*, 1992). We study here two buried salt bridges in barnase that involve the interaction between an aspartate and an arginine side chain. The first involves

[†] S.V. is a Postdoctoral Fellow, Swiss Foundation for Scientific Research, Grant 823A-031993.

* To whom correspondence should be addressed.

[‡] Present address: Biochemisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland.

[§] Present address: Mikrobiologisches Institut, ETH Zürich, CH-8092 Zürich, Switzerland.

[©] Abstract published in *Advance ACS Abstracts*, May 1, 1996.

Arg69 and Asp93, an interaction between loop 3 and the β -turn between strands 3 and 4, while the second interaction is between Arg83 and Asp75, at the active site of barnase.

MATERIALS AND METHODS

Materials. [35 S]dATP used for sequencing was from Amersham International. SP-Trisacryl was from IBF. Urea (ultrapure enzyme grade) and the agarose used for the native gel buffer strips (ultrapure electrophoresis grade) were obtained from Bethesda Research Laboratories. 2-(*N*-Morpholino)ethanesulfonic acid (MES) was from Sigma. Other chemicals and reagents were analytical grade and were purchased from Sigma, BDH, or Fisons.

Mutagenesis, Expression, and Purification of Mutant Proteins. The single-stranded DNA template from the modified plasmid pTZ18U containing the wild-type barnase gene (Serrano *et al.*, 1990) was obtained by infecting *Escherichia coli* TG2 cells with the helper phage M13KO7 (Pharmacia). Site-directed mutagenesis (Sayers *et al.*, 1988) was carried out with a kit supplied by Amersham. The mutants were identified by direct sequencing by the dideoxy chain termination method using the Sequenase Kit of USB. Mutant DNA was transformed into *E. coli* BL21DE3 pLyS (a generous gift to this laboratory of Dr. F. W. Studier). Barnase mutants were expressed and purified as previously described (Serrano *et al.*, 1990). An additional elution step with a 7 M urea high salt buffer was performed to increase the yield of protein eluted from the trisacryl resin. The protein was then renatured by dialysis. No difference in the properties of the renatured protein could be detected in comparison to those of the native protein isolated by the standard procedure (Sanz & Fersht, 1993; Vuilleumier & Fersht, 1994). Purification of the D75N, R83K, R83K/D75N, and R69S/D93N mutants was best achieved by chromatographic separation on a Mono-S FPLC column (Pharmacia) using the standard acetate/sodium chloride salt gradient, but containing 7 M urea in both buffers. Purified protein solutions (typically 10–20 mL) were dialyzed against 3×5 L of double-distilled water. The protein concentration was determined spectrophotometrically using an extinction coefficient of $27\,411\text{ M}^{-1}\text{ cm}^{-1}$ at 280 nm determined for the wild-type protein (Loewenthal *et al.*, 1991). The protein solution was flash frozen in liquid nitrogen for storage.

Activity Measurements. Activity of the mutant proteins R69S, D93N, and R69S/D93N was assayed as described previously (Day *et al.*, 1992).

Equilibrium Denaturation with Urea Monitored by Fluorescence. Urea solutions were prepared by weighing both the amount of urea and water, the urea concentration being calculated from the formula $d/d_0 = 1 + 0.2658W + 0.0330W^2$ (Kawahara & Tanford, 1966), where W is the weight fraction of urea in the solution, d is the density of the solution, and d_0 is the density of water at the particular temperature. Equilibrium unfolding by urea was monitored by recording the intrinsic fluorescence of barnase at 315 nm, upon excitation at 290 nm (Serrano *et al.*, 1990), and analyzed in terms of a two-state transition as described previously (Serrano *et al.*, 1992a; Vuilleumier & Fersht, 1994). The fluorescence measurements were performed in a thermostatted cuvette holder at 25 °C, the temperature being controlled with a thermocouple fitted above the light beam or in an adjacent cuvette. The spectrofluorimeter was a series 2 Aminco Luminescence spectrophotometer (American Instruments Co.).

Kinetic Experiments. Unfolding studies were performed as described (Matouschek & Fersht, 1991; Serrano *et al.*, 1992b). Unfolding was initiated by mixing 1 volume of protein solution (~ 30 – $40\text{ }\mu\text{M}$), 50 mM MES (pH 6.3), with 10 volumes of a 50 mM MES (pH 6.3) concentrated urea solution in a rapid mixing device. The unfolding was monitored by following the intrinsic fluorescence of barnase with excitation at 290 nm and detection of the emission at 315 nm at 25 °C using a Perkin-Elmer MPF 44 B fluorescence spectrophotometer. The slit width was 10 nm for the excitation and 15 nm for the emission wavelengths, respectively. The unfolding rate constant in water and at various urea concentrations was determined by fitting the measured unfolding rate constants to the equation

$$\log k_u = \log k_u^{\text{H}_2\text{O}} + m k_u[\text{urea}] - 0.014[\text{urea}]^2 \quad (1)$$

as described (Matouschek *et al.*, 1994).

Refolding experiments were performed as described (Matouschek *et al.*, 1992). Refolding was initiated by mixing 1 volume of acid-denatured protein (0.2 mg/mL) at pH 1.5 (32 mM HCl) with 1 volume of refolding buffer. The refolding buffer was 100 mM MES, calculated to give a final pH of 6.3 upon mixing with the acid-denatured protein. The measurements were performed in an Applied Photophysics SF 17MV stopped-flow apparatus. Refolding was followed by monitoring the change in intrinsic fluorescence of barnase above 315 nm using a cutoff filter, the excitation being at 290 nm. The slit width for excitation was 2 nm. The refolding is usually characterized by three phases and was fitted to a triple exponential with offset. The fastest phase (70–80%) corresponds to the refolding of the intermediate to the folded state, whereas the two slower phases correspond to peptidylproline isomerization events (Matouschek *et al.*, 1992). The mutants at positions 83 and 75 showed a biphasic refolding profile in which a fast proline phase could not be resolved. The curves were fitted to a double exponential with offset using the software supplied by the manufacturer.

Thermodynamic Analysis. The change in free energy of unfolding upon mutation was calculated as described (Serrano *et al.*, 1992a). The standard error was determined as described previously (Clarke & Fersht, 1993). $\Delta\Delta G_{\text{A,int}}$, the coupling energy in a particular state A of a protein with reference to the unfolded state is given by

$$\Delta G_{\text{A,int}} = \Delta\Delta G_{\text{EXY} \rightarrow \text{EY}} - \Delta\Delta G_{\text{EX} \rightarrow \text{E}} \quad (2)$$

where the coupling energy in the particular state A, $\Delta\Delta G_{\text{A,int}}$, is the free energy difference for removing the side chain X in the wild-type protein in the presence of Y ($=\Delta\Delta G_{\text{EXY} \rightarrow \text{EY}}$) minus the free energy difference for removing the side chain X in the absence of Y ($=\Delta\Delta G_{\text{EX} \rightarrow \text{E}}$). Equation 2 is usually rearranged to

$$\Delta G_{\text{A,int}} = \Delta\Delta G_{\text{EXY} \rightarrow \text{EX}} + \Delta\Delta G_{\text{EXY} \rightarrow \text{EY}} - \Delta\Delta G_{\text{EXY} \rightarrow \text{E}} \quad (3)$$

where X and Y are the side chains forming the interaction and E stands for the enzyme without X and Y (Fersht *et al.*, 1992).

The difference in free energy between the transition state and the folded state upon mutation is given by

$$\Delta G_{\ddagger \rightarrow \text{F}} = -RT \ln(k_u/k'_u) \quad (4)$$

where k_u and k'_u are the rate of unfolding of wild type and mutant (Serrano *et al.*, 1992b).

The difference in free energy between the intermediate and the folded state upon mutation is described by the equation

$$\Delta G_{I-F} = -RT \ln[(k_u^{\text{H}_2\text{O}}/k_{-u}^{\text{H}_2\text{O}})/(k'_u^{\text{H}_2\text{O}}/k'_{-u}^{\text{H}_2\text{O}})] \quad (5)$$

where $k_{-u}^{\text{H}_2\text{O}}$ is the rate of refolding in water and $k_u^{\text{H}_2\text{O}}$ is the rate of unfolding that is extrapolated to water and calculated from eq 1 (Matouschek *et al.*, 1992).

The Φ values for the mutants in the transition state and the intermediate state are calculated according to

$$\Phi_{\ddagger} = 1 - \Delta\Delta G_{\ddagger-F}/\Delta\Delta G_{U-F} \quad (6)$$

$$\Phi_I = 1 - \Delta\Delta G_{I-F}/\Delta\Delta G_{U-F} \quad (7)$$

as described (Matouschek *et al.*, 1992).

The Φ values for the salt bridge interactions, Φ_{int} , in the transition and intermediate state are calculated from

$$\Phi_{A,\text{int}} = \Delta\Delta G_{A,\text{int}}/\Delta\Delta G_{F,\text{int}} \quad (8)$$

where A is either the transition or the intermediate state (Fersht *et al.*, 1992).

RESULTS

Design of Mutations. Several strategies were considered for the replacement of residues involved in the buried salt bridge interactions between Arg69 and Asp93 and between Arg83 and Asp75 in barnase since it was expected that replacements may in some instances significantly destabilize the protein. However, the study of an interaction such as a buried salt bridge by protein engineering requires that the interaction be removed by the mutation without causing significant structural rearrangements. Finding suitable conservative replacements for arginine residues is particularly difficult, since this amino acid has both hydrophobic character by virtue of its aliphatic chain and hydrophilic character due to its guanidinium group. The properties of arginine residues in protein interiors have been investigated in detail recently (Borders *et al.*, 1994). Amino acid substitutions for arginine residues can take into account the hydrophobic or hydrogen-bonding potential of arginine to various extents; replacement of an arginine residue by lysine is a relatively conservative replacement with respect to both the charge and the length of the aliphatic chain but, importantly, has a different set of hydrogen-bonding interactions. This means that it will not be possible, in general, to find hydrogen-bonding partners for each of its NH atoms, with there being a loss of some 4 kcal/mol or so for each absent hydrogen bond. The methionine side chain may mimic the length and aliphatic character of the arginine side chain.

The characteristics of the two buried salt bridges between aspartate and arginine residues in barnase are quite different with respect to their location and environment in the protein structure. In the salt bridge interaction between Arg69 and Asp93 in wild type (Figure 1A, left), the N^εH of the arginine is completely buried and makes a strong H bond with the O^{δ1} of Asp93, which is also completely shielded from the solvent. N^{η1}H₂ of Arg69 makes two H bonds, with O^{δ1} and O^{δ2} of Asp93. Both the O^{δ2} of Asp93 (9 Å²) and the N^{η1}H₂ of Arg69 (4 Å²) have very limited surface area accessible to

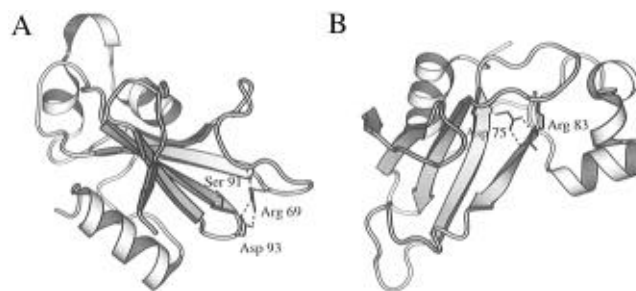


FIGURE 1: Cartoon representations of the location of buried aspartate-arginine salt bridges in barnase, generated using the program Molscript (Kraulis, 1991) using the structure of wild-type barnase at pH 6.5 (Cameron and Henrick, unpublished): (right) salt bridge between Arg69 and Asp93 and (left) salt bridge between Arg83 and Asp75. Hydrogen bonds between Asp93 and Arg69, Asp93 and Ser91, Arg69 and Pro64, and Arg83 and Asp75 are indicated as dashed lines.

solvent, as calculated by the program DSSP (Kabsch & Sander, 1983). The N^{η2}H₂ of Arg69 (15 Å² accessible to solvent) makes an additional hydrogen bond with the peptide carbonyl of Pro64 and the O^{δ1} of Asp93 another hydrogen bond with the side chain of Ser91. Removal of the guanidinium moiety of Arg69 will break the salt bridge interaction with Asp93 but will also remove the hydrogen-bonding interaction with Pro64. Therefore, the replacement of arginine by lysine is expected to remove two hydrogen bonds, since lysine has only one hydrogen bond donor and arginine three, but this will depend on the conformation adopted by the lysine residue in the mutant. Mutation of Asp93 to asparagine will break at least one hydrogen bond with Arg69 but may also remove that with the side chain of Ser91.

In the salt bridge interaction between Arg83 and Asp75 in the wild-type protein, Arg83 makes two hydrogen bonds with Asp75 (Figure 1B). The N^εH of Arg83 makes a hydrogen bond with O^{δ2} of Asp75, and N^{η2}H₂ makes a hydrogen bond with O^{δ1} of Asp75. All four atomic groups involved are inaccessible to solvent. While the salt bridge interaction itself is buried, the degree of exposure of some individual atoms of Arg83 to solvent varies widely. For instance, 20 Å² of the solvent accessible area of N^{η1}H₂ of Arg83 is exposed. Mutation of Arg83 to lysine, in this case, should remove at least one hydrogen bond, whereas a mutation to glutamine would remove at least one hydrogen bond and weaken the remaining one by removing its ionic character. Mutation of Asp75 to asparagine is also expected to remove one hydrogen bond and to weaken the remaining one.

Mutants of barnase, R69M, R69S, R69K, D93N, R69S/D93N, R83Q, R83K, R83K/D75N, and D75N, were made in this study. Glutamine was considered as a replacement for arginine at position 83 since it may maintain the hydrogen bond formed by the N^εH of arginine with Asp75. A serine residue was also chosen for replacement of Arg69 since a serine occupies the position corresponding to Arg69 of barnase in the structurally related ribonuclease T1, whereas Asp93 in barnase has an asparagine equivalent in ribonuclease T1 (Hill *et al.*, 1983).

Characterization of Mutants. While the majority of the mutants could be purified as previously described (Serrano *et al.*, 1990), mutants R83K, R83K/D75N, D75N, and R69S/D93N required addition of 7 M urea to the buffers used in the ion-exchange chromatography step, in order to release

these mutants from the chromatography column matrix (Sanz & Fersht, 1993; Vuilleumier *et al.*, 1993; Vuilleumier & Fersht, 1994). All mutants behave in native gel electrophoresis as expected for mutants from which charged residues have been removed (data not shown; A. C. Tissot, Diploma Thesis, ETH Zürich). The three mutants R69S, D93N, and R69S/D93N showed activity similar to that of the wild-type enzyme (86, 92, and 67% of the wild-type activity, respectively) when measured under the same conditions (Day *et al.*, 1992), confirming that no substantial structural rearrangements have occurred upon mutation. However, for mutants of residues involved in the salt bridge between Arg83 and Asp75, the removal of Arg83, which is part of the active site (Meiering *et al.*, 1993), significantly decreases the activity of the enzyme. In the case of mutant R83M, the mutation mainly affects k_{cat} (0.07 s^{-1} , versus 53.1 s^{-1} for the wild-type enzyme), leaving K_m largely unaffected (19.4 mM, versus 19.9 mM for the wild-type enzyme; Day *et al.*, 1992).

Unfolding of barnase wild type and mutants was previously shown to follow a reversible two-state transition at equilibrium by absorbance (Kellis *et al.*, 1988), fluorescence (Kellis *et al.*, 1989), circular dichroism (Vuilleumier *et al.*, 1993), and calorimetry (Matouschek *et al.*, 1994; Johnson & Fersht, 1995) measurements. In particular, calorimetric measurements of the free energy of unfolding of the mutants R69M and R83Q also show a two-state transition (Matouschek *et al.*, 1994). No aggregation could be detected in equilibrium, unfolding, and refolding experiments for any of the mutants investigated in the present study. Aggregation phenomena have been observed for barnase under conditions of low pH, high salt, and high protein concentrations (Oliveberg *et al.*, 1994). As shown in the accompanying paper (Oliveberg & Fersht, 1996), the ratio of the rates of refolding and unfolding at low pH of the fairly destabilized mutant D93N exactly follows the equilibrium constant for unfolding measured under the same conditions, ruling out aggregation phenomena.

Characterization of the Stability and Interaction Energies in the Folded State. Mutation of a partner in a buried salt bridge interaction is expected to destabilize a protein considerably, since it not only removes a stabilizing interaction between two residues but also leaves an unmatched charge inside the core of the protein (Fersht *et al.*, 1985). The stabilities of the different mutants of Arg69 were measured from urea denaturation curves, and the changes in stability around the midpoint of the folding transition, $\Delta\Delta G_{\text{U-F}}^{\text{U50\%}}$, were used as the most reliable indices of these energetics (Serrano *et al.*, 1992a). The methionine mutant is more stable than the serine mutant by 0.6 kcal/mol and the serine mutant more stable than the lysine mutant by 0.4 kcal/mol (Table 1). Two compensating effects may contribute to the difference in stability between the methionine and the serine mutant: a greater ability to form van der Waals contacts by the methionine side chain and stabilization of the unfolded state for the serine side chain. The three most destabilized mutants (D75N, D93N, and the double mutant R83K/D75N) are found for mutations where the negatively charged salt bridge partner has been modified, creating an unpaired positive charge (Table 1). They are less stable by more than 1 kcal/mol than the mutants where a negative charge is left unpaired. Interestingly, removal of positively charged residues from the active site of barnase that interact

Table 1: Stability of Mutants Measured from Equilibrium Urea Denaturation^a

protein	[urea] _{50%} (M)	m (kcal mol ⁻²)	$\Delta\Delta G_{\text{U-F}}^{\text{U50\%}}$ ^b (kcal mol ⁻¹)
wt ^c	4.57 (±0.01)	1.93 (±0.03)	
R69S	3.16 (±0.03)	1.86 (±0.11)	2.72 (±0.07)
D93N	2.44 (±0.04)	1.99 (±0.20)	4.11 (±0.10)
R69S/D93N	2.76 (±0.02)	2.07 (±0.13)	3.49 (±0.07)
R69K	2.94 (±0.04)	1.89 (±0.19)	3.13 (±0.09)
R69M	3.47 (±0.02)	2.14 (±0.15)	2.12 (±0.05)
R83K	2.43 (±0.05)	1.68 (±0.17)	4.13 (±0.12)
R83Q	3.51 (±0.02)	2.28 (±0.15)	2.05 (±0.05)
D75N	2.08 (±0.03)	1.69 (±0.10)	4.80 (±0.09)
R83K/D75N	1.77 (±0.07)	1.78 (±0.15)	5.40 (±0.16)

^a Data were obtained from equilibrium urea denaturation experiments monitored by fluorescence spectroscopy except for R69K, where the data were obtained from CD urea denaturation measurements (Sanz & Fersht, 1993; Vuilleumier *et al.*, 1993) conducted under the same conditions of buffer concentration and pH as for the fluorescence experiment. The data were fitted to the equation $\Delta G_{\text{U-F}} = \Delta G_{\text{U-F}}^{\text{H}_2\text{O}} - m[\text{urea}]$, modified as described by Clarke and Fersht (1993), and the values of [urea]_{50%} and m as described. ^b The change in free energy of unfolding at the mean of the values of [urea]_{50%} for wild type and mutant (Serrano *et al.*, 1992a), calculated according to Clarke and Fersht (1993). The slight curvatures in the plots of $\Delta G_{\text{U-F}}$ versus [urea] (Johnson & Fersht, 1995) cancel when calculating $\Delta\Delta G_{\text{U-F}}^{\text{U50\%}}$ using the equation $\Delta\Delta G_{\text{U-F}}^{\text{U50\%}} = m[[\text{urea}]_{50\%}(\text{wt}) - [\text{urea}]_{50\%}(\text{mut})]$ (Matouschek *et al.*, 1994). ^c Wild-type data are the average of six different experiments with different batches of wild-type protein.

with substrate was shown to increase the stability of the protein (Meiering *et al.*, 1992). In particular, the mutation of Arg83, which also interacts with substrate in the active site, to glutamine is less destabilizing than mutation to lysine and far less destabilizing than the mutation of the salt bridge partner at position 75 (Table 1).

Strikingly, lysine does not seem to be a good substitute for arginine in buried salt bridges. R69K is the most destabilized of the mutants at position 69 investigated. Similarly, in the case of arginine at position 83, mutation of arginine to lysine is much more destabilizing than mutation to glutamine (Table 1). The charged ammonium group of the side chain of lysine requires its own specific hydrogen-bonding interactions and has much more specific steric requirements for burial than a neutral analogue.

Energies of Salt Bridge Interactions. The coupling energies obtained for the two interactions studied are in the range obtained previously by Fersht (1972), who found a value of 2.9 kcal/mol for a buried salt bridge interaction in chymotrypsin, by Fersht *et al.* (1985), who found a range of values around 3 kcal/mol in complexes of the tyrosyl-tRNA synthetase with its substrates, by Anderson *et al.* (1990), who found a value between 3 and 5 kcal/mol for a buried salt bridge interaction in T4 lysozyme, and by Waldburger *et al.* (1995), who found values of 1.7 and 4.7 kcal/mol for two buried salt bridges in the Arc repressor. The magnitude of experimentally determined coupling energies depends also on the nature of the reorganization in the structure upon mutation. For the Arc repressor (Waldburger *et al.*, 1995), the structure of a triple mutant lacking the salt bridge interactions shows only minimal reorganization.

In the double mutant cycle of the interaction between Arg69 and Asp93, arginine is mutated to serine and aspartate to asparagine. If the structural rearrangement energy terms are more important in the single than in the double mutants, it is seen from eqs 3, 10, and 11 (see Appendix) that the coupling energy will be underestimated. There are no other

charged residues in direct vicinity, and therefore, we do not expect the double mutant to be more affected than the single mutants, assuming that long-range electrostatic interactions are not significant (Russell *et al.*, 1987; Loewenthal *et al.*, 1993). In other words, any occurring structural rearrangements not accounted for in our analysis will not cause the obtained values for the energy of this buried salt bridge interaction to be an overestimate of the true interaction energy.

While mutation of arginine to serine probably causes the side chain of Asp93 to be solvated by water, mutation of aspartate to asparagine may maintain at least part of the hydrogen-bonding network of wild-type barnase in this region of the protein, with the hydrogen bond of the N^ε of Arg69 with the carbonyl oxygen of Pro64 helping to maintain a conformation close to the situation in the native state. This would prevent extensive solvation of the unpaired arginine residue and provide in this case an attractive explanation for the difference in stability of the residues left with an unmatched positive or negative charge. A crystal structure of the mutant S91A, which forms a hydrogen bond with Asp93, has indeed shown that the structure is rather rigid in this region of the protein, and the cavity left by the removed hydroxyl group was not filled by other protein groups or water in the mutant (Chen *et al.*, 1993). Thus, the obtained coupling energy for the Arg69–Asp93 salt bridge is most likely an underestimate of the true coupling energy between Arg69 and Asp93.

For the interaction of Arg83 with Asp75, both a single and the double mutant have an additional positive charge compared with wild type, whereas the other single mutant has the same charge balance as wild type. In addition, several positively charged residues in the direct vicinity of the site of mutation complicate the interpretations and make a prediction of the effects of structural rearrangements on the value of the interaction energy very difficult. For example, a similar structural rearrangement occurring in the one single and the double mutant would cancel out in the cycle. Therefore, the effects of removing a negative charge in a region of the protein crowded with positively charged residues in the mutant D75N could be compensated by similar effects in the mutant R83K/D75N. In contrast, however, a rearrangement in the structure occurring in all three mutants forming the cycle or in only one of them would not cancel out. Mutation of Arg83 to lysine may maintain some interactions with Asp75, although it is not clear to what extent. In any case, the mutant R83K retaining Asp75 is less destabilized than mutants D75N and R83K/D75N. Thus, in this case also, the true interaction energy of the salt bridge could be underestimated by the mutations used in the present work.

Unfolding Kinetics and Transition State. The values of the logarithm of the rate constant for unfolding extrapolated to water and to 4 M urea are listed in Table 2. The dependence of the rate constant of unfolding on urea for the mutants of the two salt bridge interactions is shown in Figure 2. The values of $\Delta\Delta G_{\ddagger-F}$ and Φ_{\ddagger} and the Φ value for the transition state of the mutants studied are listed in Table 3. They are calculated at 4 M urea to minimize the errors associated with extrapolations over a wide range of urea concentrations (Serrano *et al.*, 1992b). Φ values of the mutants in the transition state in water and at a 4 M urea concentration differ only slightly [within 0.1, Table 3 legend and Serrano *et al.* 1992b)].

Table 2: Rate Constants of Unfolding (k_u) and Refolding (k_{-u})^a

protein	k_{-u} (s ⁻¹)	$\log k_u$ (H ₂ O) ^c (s ⁻¹)	$\log k_u$ (4 M) ^d (s ⁻¹)
wt ^b	12.7 ± 0.07	-4.59 ± 0.02	-2.21 ± 0.01
R69M	4.95 ± 0.05	-4.07 ± 0.05	-1.86 ± 0.02
R69S	3.90 ± 0.03	-3.99 ± 0.09	-1.76 ± 0.04
D93N	0.78 ± 0.03	-4.07 ± 0.07	-1.78 ± 0.03
R69S/D93N	1.28 ± 0.03	-4.28 ± 0.05	-1.82 ± 0.02
R83K	12.1 ± 0.08	-1.35 ± 0.04	0.42 ± 0.01
D75N	15.1 ± 0.08	-0.78 ± 0.14	1.29 ± 0.03
R83K/D75N	14.0 ± 0.2	-0.95 ± 0.10	1.46 ± 0.03

^a Rate constants of refolding (k_{-u}) and of unfolding (k_u) as a function of [urea] were obtained as described in Materials and Methods and have units of s⁻¹. ^b A. Matouschek, J. Matthews, and A. R. Fersht, unpublished results, and Matouschek and Fersht (1993). The value of k_{-u} was taken from Matouschek *et al.* (1990). ^c Value extrapolated to water. ^d Value extrapolated to 4 M urea.

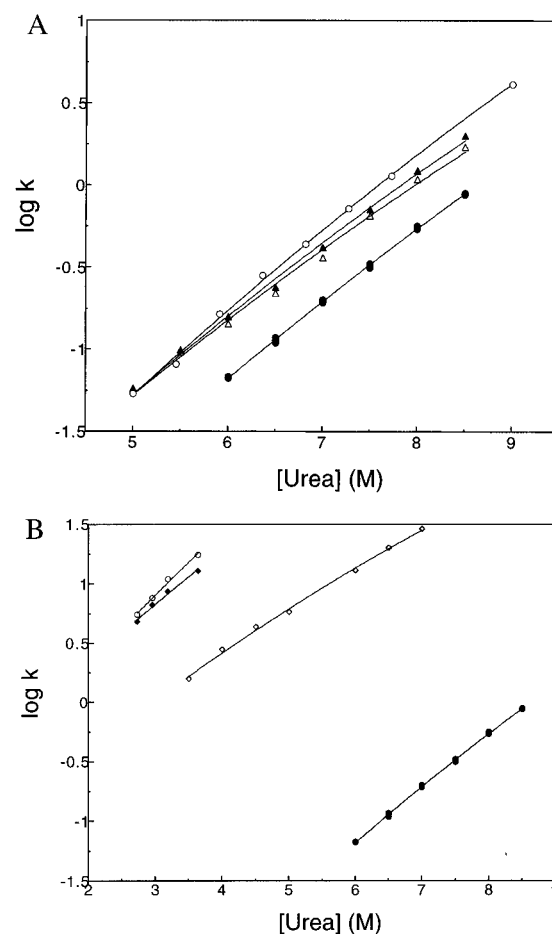


FIGURE 2: Dependence of the rate of unfolding on urea concentration for (A) the mutants R69S (open triangles) and D93N (filled triangles) and the double mutant R69S/D93N (open circles) and (B) the mutants R83K (open diamonds) and D75N (filled diamonds) and the double mutant R83K/D75N (open circles), compared to that of wild-type barnase (filled circles). Wild-type data are from four independent experiments. The curves were obtained by fitting the experimental data using eq 1.

For the mutants R69S, R69M, D93N, and R69S/D93N, the Φ_{\ddagger} values are 0.8, 0.8, 0.9, and 0.9, respectively, remarkably similar and close to 1. A Φ value of 1, or close to 1, can be interpreted as the studied interaction being fully formed (Fersht *et al.*, 1992). In contrast, the mutants R83K, D75N, and R83K/D75N have Φ_{\ddagger} values of 0.1, 0.0, and 0.1, respectively, the standard error for the determination of these values being about 0.1. A Φ value of 0 or close to 0 is interpreted as showing that the interaction is not formed in the transition state (Fersht, 1995b). Interpretation of Φ values

Table 3: Difference Free Energies and Φ Values for Salt Bridge Mutants in the Intermediate and Transition States on the Folding Pathway

protein	$\Delta\Delta G_{I-F}^{H_2O}$ (kcal mol ⁻¹)	$\Delta\Delta G_{\ddagger-F}^{H_2O}$ (kcal mol ⁻¹)	$\Delta\Delta G_{\ddagger-F}^{4M}$ (kcal mol ⁻¹)	$\Delta\Delta G_{U-F}^{U50\%}$ (kcal mol ⁻¹)	Φ_{\ddagger} (4 M urea)	Φ_I
R69M	1.26 ± 0.09	0.71 ± 0.07	0.46 ± 0.03	2.12 ± 0.06	0.8	0.6
R69S	1.50 ± 0.13	0.81 ± 0.12	0.59 ± 0.06	2.72 ± 0.07	0.8	0.4
D93N	2.35 ± 0.11	0.71 ± 0.09	0.58 ± 0.05	4.11 ± 0.10	0.9	0.4
R69S/D93N	1.77 ± 0.09	0.42 ± 0.07	0.52 ± 0.04	3.49 ± 0.07	0.9	0.5
R83K	4.43 ± 0.09	4.40 ± 0.05	3.57 ± 0.02	4.13 ± 0.12	0.1	-0.1
D75N	5.07 ± 0.20	5.18 ± 0.19	4.75 ± 0.04	4.80 ± 0.09	0.0	-0.1
R83K/D75N	4.89 ± 0.19	4.94 ± 0.13	4.98 ± 0.04	5.40 ± 0.16	0.1	0.0

^a $\Delta\Delta G_{I-F}^{H_2O}$, $\Delta\Delta G_{\ddagger-F}^{H_2O}$, $\Delta\Delta G_{\ddagger-F}^{4M}$, and $\Delta\Delta G_{U-F}^{U50\%}$ are the difference free energies defined in the text. U50% is the urea concentration at which the protein is half-unfolded at equilibrium. The Φ value of the transition state Φ_{\ddagger} is given at 4 M urea; the value in water is either identical or lower by 0.1 unit. The Φ value of the intermediate Φ_I is determined in water.

Table 4: Coupling Energies and Values of Φ_{int} for the Buried Salt Bridges Arg69–Asp93 and Arg83–Asp75 as Obtained from Double Mutant Cycles^a

salt bridge interaction	$\Delta\Delta G_{F,int}$ (kcal mol ⁻¹)	$\Delta\Delta G_{\ddagger,int}$ (4 M urea) (kcal mol ⁻¹)	$\Delta\Delta G_{I,int}$ (H ₂ O) (kcal mol ⁻¹)	$\Phi_{\ddagger,int}$ (4 M urea)	$\Phi_{\ddagger,int}$ (H ₂ O)	$\Phi_{I,int}$ (H ₂ O)
R69–D93	-3.34 ± 0.15	-2.69 ± 0.17	-1.26 ± 0.24	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
R83–D75	-3.53 ± 0.22	-0.16 ± 0.23	1.08 ± 0.58	0.1 ± 0.2	-0.3 ± 0.2	-0.3 ± 0.2

^a $\Delta\Delta G_{I,int}$, $\Delta\Delta G_{\ddagger,int}$, and $\Delta\Delta G_{F,int}$ are the coupling energies in the intermediate, transition, and folded state, respectively, as defined in the text. $\Delta\Delta G_{F,int}$ is determined at a urea concentration intermediate for the mutants of the cycle. $\Phi_{I,int}$ and $\Phi_{\ddagger,int}$ are the Φ values in the intermediate and transition state, respectively, as described in the text. The value of $\Phi_{I,int}$ is determined in water, whereas the value of $\Phi_{\ddagger,int}$ can be more reliably determined at 4 M urea, although the value obtained in water is the same within experimental error. The standard errors are calculated from standard formulas for propagation of the errors in experimental data.

is of course safest in the case of nondisruptive mutations, as analyzed previously in detail (Fersht *et al.*, 1992). For more radical mutations such as some of those investigated here, factors such as changes in solvation relative to wild type in only one state of the folding pathway or formation of new interactions in the transition state may obscure the interpretation. However, our interpretation is vindicated by the Φ values obtained for the salt bridge interactions themselves by the double mutant cycle procedure (see below).

Refolding Kinetics and Intermediate State. The rate constants of refolding in water are shown in Table 2. The values of $\Delta\Delta G_{I-F}$ and Φ_I are listed in Table 3. The Φ values obtained for the mutants at positions 69 and 93 vary between 0.4 and 0.6. Φ values far from the extremes of 0 or 1 are difficult to interpret, and an estimate of the energy term accounting for the studied interaction can only be arrived at by considering several additional parameters (Fersht *et al.*, 1992). A destabilization of the intermediate state of the three mutants R69S, D93N, and R69S/D93N of comparable magnitude to that observed in the native state by equilibrium denaturation may cause a significant proportion of the population of the mutant proteins to exist as unfolded or earlier intermediate species in a fast preequilibrium during the refolding experiment. This would lead to an apparent decrease in the population of the intermediate at any given time and would result in an underestimation of Φ_I (Oliveberg & Fersht, 1996a; Matouschek *et al.*, 1992). Thus, the extent of formation of the interaction may be greater than indicated by the Φ_I values (between 0.4 and 0.5) observed in these mutants. The fact that the Φ_I value of the less destabilized mutant R69M is higher (0.6) than for the other mutants at positions 69 and 93 also supports this interpretation. For the mutants at positions 83 and 75, however, as expected for the case of an interaction not formed in the transition state, the rates of refolding are comparable to the wild-type value and the Φ values in the intermediate are equal to 0 within experimental error.

The mutants D75N, R83K, and R83K/D75N show a decrease in fluorescence upon refolding. This has been previously observed for another barnase mutant, V36T (Matouschek *et al.*, 1992). The side chain of Val36 is in contact with Trp35, the main determinant of fluorescence in barnase (Loewenthal *et al.*, 1991), and it has been proposed that the reversal of the refolding amplitude for this mutant results from the change in the local environment of the tryptophan residue (Matouschek *et al.*, 1992). Arg83 is in loop 4 which packs against core 2, which contains Trp35. An effect of mutation on the fluorescence of Trp35 might thus also provide here an explanation for the decrease in fluorescence upon refolding. In contrast to the V36T mutant, however, the fast proline-related phase is absent, and the refolding traces were fitted to double exponentials with offset. The proline-related phases have been previously found to be affected in the mutant Y24F (Matouschek *et al.*, 1992); Tyr24 packs against the ring of Pro21, and its hydroxyl group is hydrogen-bonded to the backbone CO of Asp75. The mutations at positions 75 and 83 could thus affect the isomerization of Pro21.

Coupling Energies for the Buried Salt Bridges Arg69–Asp93 and Arg83–Asp75. Single mutants of the components of a salt bridge cannot give a satisfactory measure of salt bridge interactions because the major effect on stability will be the presence of a possibly poorly solvated unpaired charge of the remaining partner in the salt bridge interaction (Fersht *et al.*, 1992). However, double mutant cycles involving single and double mutants of the residues involved in a given interaction give the merits of having both partners present relative to their absence (Horovitz & Fersht, 1990; Horovitz & Fersht, 1992). The values of the coupling energies for buried salt bridge interactions in barnase, computed from the data for single and double mutants (Table 1), are given in Table 4. Coupling energies at equilibrium are 3.34 and 3.53 kcal/mol for the interaction of Arg69 and Asp93 and Arg83 and Asp75, respectively.

The extent of formation of salt bridge interactions in the transition state is described by the $\Phi_{\ddagger,\text{int}}$ value computed from the values of $\Delta\Delta G_{\ddagger,\text{int}}$ and $\Delta\Delta G_{\text{F},\text{int}}$ of Table 4. The value of 0.8 found for the salt bridge interaction between Arg69 and Asp93 is consistent with the results of the single mutants. $\Phi_{\ddagger,\text{int}}$ is not subjected to the same reservations as Φ_{\ddagger} , as analyzed previously (Fersht *et al.*, 1992), and it can be assumed that the salt bridge interaction between Arg69 and Asp93 is essentially fully formed in the major transition state on the folding pathway of barnase. In the case of the interaction between Arg83 and Asp75, the result obtained through the double mutant cycle procedure is in line with the values found for the single mutants (Table 3). It is concluded that the interaction between Arg83 and Asp75 is formed only after the major transition state on the folding pathway of barnase, as Φ values of 0 indicate that the studied interaction is not formed in the particular state (Fersht *et al.*, 1992).

The value of $\Phi_{\text{I},\text{int}}$ obtained for the coupling between Arg69 and Asp93 in the intermediate state is 0.4 (Table 4), suggesting that the interaction is partially formed in the intermediate. As already stated for the values of Φ_{I} of the individual mutants, this value may well represent an underestimate for the extent of formation of the interaction in the wild-type intermediate. Any suitable correction would bring it even closer to the Φ values of 0.7 and 0.6 found for the mutants S91A and S92A, respectively (Matouschek *et al.*, 1992). In wild type, Ser92 makes a hydrogen bond with Thr70, the first residue of β -strand 3, and Ser91 makes a hydrogen bond with Asp93. Fractional values of Φ are more difficult to interpret but show that the two residues are already in close contact in that state (Fersht *et al.*, 1992; Fersht, 1995b). For the interaction between Arg83 and Asp75, errors in the extrapolations required in the determination of Φ are likely to have led to the observed negative value. In any event, the fact that this interaction is not formed in the transition state makes it likely that it is not formed in the intermediate state either. Such an assumption is supported by previous results on interactions in the same region (loop 4) of the protein (Matouschek *et al.*, 1992).

Probing the folding pathway of an interaction using the analysis of Φ values is generally much less sensitive to structural rearrangements than the determination of the strength of an interaction at equilibrium. The use of the double mutant cycle procedure also removes the uncertainties linked with possible effects in the unfolded state, as previously discussed (Fersht *et al.*, 1992). Structural rearrangements upon mutation tend to reduce to second order effects in folding studies (Fersht *et al.*, 1992). The cost of burying a charge in the core of the protein can be very high (Fersht *et al.*, 1985; Langsetmo *et al.*, 1991; Dao-Pin *et al.*, 1991). Accordingly, significant structural rearrangements may occur in compensation. However, Blaber *et al.* (1995) have recently described a multiple mutant of T4 lysozyme in which modest collapse and repacking of the structure resulted in a stabilization of 0.5 kcal/mol at most. In our case, it seems most likely that the unmatched charge created upon mutation becomes at least partially accessible to solvent, thus limiting the magnitude of the destabilization and hence structural rearrangements. Nevertheless, because of the rather radical nature of the mutation investigated here, we have to consider the possible consequences of such rearrangements. We find that, for an interaction formed in the transition state, the effects of structural rearrangements

in the folded and transition states are expected to cancel out. This can be seen by inspection of eq 9 (see Appendix for the derivation):

$$\begin{aligned}\Phi_{\ddagger,\text{int}}^{\circ} &= \Delta\Delta G_{\ddagger,\text{int}}^{\circ} / \Delta\Delta G_{\text{F},\text{int}}^{\circ} \\ &= \Phi_{\ddagger,\text{int}} - (\Delta\Delta G_{\ddagger,\text{reorg}} - \Phi_{\ddagger,\text{int}} \Delta\Delta G_{\text{F},\text{reorg}}) / (\Delta\Delta G_{\text{F},\text{int}} - \Delta\Delta G_{\text{F},\text{reorg}}) \quad (9)\end{aligned}$$

where $\Phi_{\ddagger,\text{int}}^{\circ}$, $\Delta\Delta G_{\ddagger,\text{int}}^{\circ}$, and $\Delta\Delta G_{\text{F},\text{int}}^{\circ}$ are the values that would be obtained if no structural rearrangements would occur, $\Phi_{\ddagger,\text{int}}$ is the experimentally determined value, and $\Delta\Delta G_{\text{F},\text{reorg}}$ and $\Delta\Delta G_{\ddagger,\text{reorg}}$ are the reorganization energy terms for the folded and the transition state, respectively. It should be noted that these two values already represent differences between corresponding energy terms in the individual mutants.

DISCUSSION

We have measured and described the effects of a series of mutations on two buried salt bridges in barnase. These have been analyzed in terms of single mutations and of coupling energies from double mutant cycles. The structural basis of these data and precise interpretations have to await detailed structural information on the mutants and improvements in present computational procedures. However, we can use these data now, in terms of how these energies change as the reaction proceeds, to describe features of the transition and intermediate states of barnase as it folds and unfolds. Barnase folds via a folding intermediate. This may be a single intermediate or a series of intermediates, but for simplicity, we use the framework of a single intermediate. The rate-determining step in folding is the conversion of this intermediate via the major transition state to the final folded structure.

Previous studies from this laboratory have avoided mutations that can cause large changes in energy and buried charges because they are considered likely to cause artifacts from reorganization of the native structure and/or the transition state for the folding reaction. The mutations studied here are among the most radical made on barnase. The mutant D93N has the slowest refolding rate of all the barnase mutants studied so far. Nevertheless, we find a consistent pattern of behavior with the radical mutations. All the mutations of the salt bridge Arg69–Asp93 have Φ_{\ddagger} values of 0.8–0.9 (i.e., they destabilize the transition state by 80–90% of the 2–4 kcal/mol that they destabilize the native protein, Table 3). This is within experimental error of the ratio of coupling energies between the two residues ($\Phi_{\ddagger,\text{int}} = 0.8 \pm 0.1$, Table 4). Conversely, mutations of the bridge between Arg83 and Asp75 yield Φ values that are consistently close to 0.

Formation of Salt Bridge Interactions on the Folding Pathway

Salt Bridge between Arg69 and Asp93. Arg69 is the last residue of loop 3. Two structural probes in this loop have been analyzed previously (Serrano *et al.*, 1992a,b): the hydrogen bond between the side chain of Asn58 and the peptide O of Leu63 and the hydrogen bonds between the side chain of Lys62 and the peptide carbonyl oxygens of Tyr103 and Gln104 (loop 5). These interactions were found to be present in the intermediate and the transition state.

Asp93 is located on the β -turn between β -strands 3 and 4. The hydrogen bond between Asp93 and Ser91 appears also to be formed in the transition state (Serrano *et al.*, 1992b), indicating that interactions formed by neighboring residues are essentially native-like. A crystal structure of the S91A mutant protein has shown that the mutation is nondisruptive, supporting the interpretation of the Φ value (Chen *et al.*, 1993). Quite generally, this portion of the transition state of barnase has been shown to resemble the folded state (Serrano *et al.*, 1992c).

In the present example, a Φ_{int} value close to 1 has been found for the interaction between Arg69 and Asp93 in the transition state (Table 4, Figure 3), indicating that this salt bridge interaction is essentially formed at this stage. As seen from eqs 8 and 9, rearrangements in the structure which affect predominantly the transition state of folding, and the double mutant more significantly than the single mutants ($\Delta\Delta G_{\text{reorg}}$ is then positive in both the folded state and the transition state), could lead to an overestimate of Φ_{int} . However, for the reasons discussed above, we do not expect more significant structural rearrangements in the double mutants than in the single mutants.¹ Thus, the value of Φ_{int} obtained here for the salt bridge interaction between Arg69 and Asp93 is in keeping with previous findings. Similar results are also obtained when deriving the value of Φ_{int} from the variation of the interaction energy in the transition state with pH (see accompanying paper).

The extent of formation of this interaction in the intermediate state may not be given precisely by the values of Φ_{int} and Φ_{I} because of effects of radical mutations on the equilibrium constant between the unfolded state and the intermediate so that the intermediate no longer is fully formed (Oliveberg & Fersht, 1996a). However, the values found in this study, together with the additional evidence presented in the accompanying paper (Oliveberg & Fersht, 1996b), suggest that the two residues are already interacting in the intermediate state to a significant extent.

Salt Bridge between Arg83 and Asp75. Arg83 is in loop 4, which is formed only after the main transition state of folding (Serrano *et al.*, 1992a,b). Asp75 is in β -strand 3. The value of Φ_{int} found for the salt bridge interaction is 0.1 (Table 4, Figure 3). This value is consistent with the Φ_{I} values found for the other previously investigated interactions of loop 4 (Serrano *et al.*, 1992b) and confirms that this salt bridge interaction is not formed until after the main transition state on the folding pathway of barnase. As a confirmation for this, the dependence of the rate constant for unfolding on the pH for the mutant D75N (Oliveberg *et al.*, 1995) has been shown to be decreased relative to that of wild type. This is consistent with the fact that the interaction should not be formed in the transition state.

The multiple hydrogen bonds to backbone carbonyl and side chain atoms made by Arg69 are typical of "structural arginines" (Mrabet *et al.*, 1992; Borders *et al.*, 1994), as opposed to functional arginines, of which Arg83 is a good

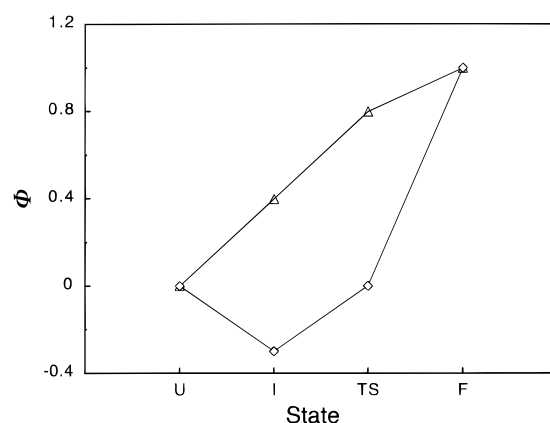


FIGURE 3: Φ values representing the extent of formation of the interaction between Arg69 and Asp93 (triangles) and Arg83 and Asp75 (diamonds) along the folding pathway of barnase.

example. In the salt bridge interaction between Arg69 and Asp93, both residues make additional hydrogen bonds with other residues. The interaction is found to be partially formed in the intermediate state, and mainly present in the transition state for folding. It is thus an early event on the folding pathway. In contrast, Arg83 and Asp75 make no hydrogen bonds with other residues. The salt bridge interaction is formed only late in the folding pathway. Arg83 is involved in the binding of the substrate and makes H bonds with phosphates P+1 and P+2 of the oligodeoxynucleotide CGAC in complex with barnase (Buckle & Fersht, 1994). Asp75 holds Arg83 in a conformation that allows binding to the substrate in a highly positive region of the protein. Comparison of the coupling energy of this salt bridge determined here, with the pK_a of Asp75 (3.1; Oliveberg *et al.*, 1995), suggests that this interaction may destabilize the protein overall.

A striking conclusion from this study is that the transition state for folding of the protein can withstand the great destabilization brought about by the presence of an unpaired charge, such as in the mutant D93N, so that the mutant protein can still proceed to the native state. The core of the β -sheet, which is formed early on the folding pathway and then consolidated (Serrano *et al.*, 1992c), is thus quite robust. Comparison of the coupling energy between Arg69 and Asp93 determined in this work, with the pK_a estimated for Asp93 (0.7; Oliveberg *et al.*, 1995), shows that the desolvation penalty for burying these two charges is likely to be matched by their interaction energy. It appears thus that the sum of the interactions made by Asp93 and by Arg69 should, at least, not destabilize the protein. The contribution of this interaction may however be more pronounced for the folding of the protein than its stability. This salt bridge is one of the interactions linking loop 3 with the tight turn between the third and fourth β -strand. Both are evolutionarily conserved and formed early on the folding pathway (Serrano *et al.*, 1992b). We propose thus that this very specific interaction may act early on the folding pathway by restricting the number of available conformations within the substructure it is embedded in and thus speed up the folding of the protein.

ACKNOWLEDGMENT

We thank Anthony Day for help with enzymatic measurements.

¹ In principle, a positive value of $\Delta\Delta G_{\text{F, reorg}}$ with $\Delta\Delta G_{\text{I, reorg}}$ equal to zero could cause Φ_{int} to be an overestimate of the true value. However, $\Delta\Delta G_{\text{F, reorg}}$ would have to be of the same order of magnitude as the interaction energy to be detectable in our analysis. The Φ_{I} values for the individual mutants, including a mutant not used in the cycle and Φ_{int} , were found to be very similar (Tables 3 and 4), showing that differential structural rearrangements between mutants on the one hand, and between the transition state and the folded state of each mutant on the other hand, are not significant and most likely cancel out.

APPENDIX

As previously discussed (Fersht *et al.*, 1992), rearrangements in the structure of the mutants composing a double mutant cycle may affect the value of $\Phi_{\ddagger,\text{int}}$. The effect of structural rearrangements on the value of $\Phi_{\ddagger,\text{int}}$ shown in eq 9 may be derived as follows.

We express explicitly the contribution of structural rearrangements to the stability of the individual mutants:

$$\Delta G_{\text{U-F(measured)}} = \Delta \Delta G_{\text{U-F}}^{\circ} + \Delta G_{\text{reorg}} \quad (10)$$

We assume that structural rearrangements occur when the structure can "relax" into a more stable conformation. ΔG_{reorg} , a positive number, is a free energy difference between the unrearranged and rearranged conformations. Substituting in eq 3 and repeating the procedure for the transition state, we obtain the following expressions for the interaction energies:

$$\Delta G_{\text{F,int}} = \Delta \Delta G_{\text{F,int}}^{\circ} + \Delta \Delta G_{\text{F,reorg}}; \quad \Delta \Delta G_{\ddagger,\text{int}} = \Delta \Delta G_{\ddagger,\text{int}}^{\circ} + \Delta \Delta G_{\ddagger,\text{reorg}} \quad (11)$$

where $\Delta \Delta G_{\text{F,int}}$ and $\Delta \Delta G_{\ddagger,\text{int}}$ are the experimental values and $\Delta \Delta G_{\text{F,int}}^{\circ}$ and $\Delta \Delta G_{\ddagger,\text{int}}^{\circ}$ are the values that would be obtained if no structural rearrangements occurred. Combining eq 11 with eq 8, we then obtain

$$\Phi_{\ddagger,\text{int}} = \frac{(\Delta \Delta G_{\ddagger,\text{int}}^{\circ} + \Delta \Delta G_{\ddagger,\text{reorg}})}{(\Delta \Delta G_{\text{F,int}}^{\circ} + \Delta \Delta G_{\text{F,reorg}})} \quad (12)$$

$\Phi_{\ddagger,\text{int}}^{\circ}$, the Φ value obtained in the absence of structural rearrangements, is defined as

$$\Phi_{\ddagger,\text{int}}^{\circ} = \Delta \Delta G_{\ddagger,\text{int}}^{\circ} / \Delta \Delta G_{\text{F,int}}^{\circ} \quad (13)$$

Equations 12 and 13 can then be rearranged to give

$$\Phi_{\ddagger,\text{int}}^{\circ} = \Delta \Delta G_{\ddagger,\text{int}}^{\circ} / \Delta \Delta G_{\text{F,int}}^{\circ} = \Phi_{\ddagger,\text{int}} - (\Delta \Delta G_{\ddagger,\text{reorg}} - \Phi_{\ddagger,\text{int}} \Delta \Delta G_{\text{F,reorg}}) / \Delta \Delta G_{\text{F,int}}^{\circ} \quad (14)$$

and substitution of eq 11 into eq 14 yields eq 9.

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