*Articles*

# Relationship between Equilibrium Amide Proton Exchange Behavior and the Folding Pathway of Barnase

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ABSTRACT: We describe a three-part strategy for analyzing the relationship between equilibrium amide proton exchange behavior of barnase and its folding/unfolding pathway. First, the effects of mutation on stability and kinetics are compared to reveal which residues exchange by local breathing, which by local unfolding, and which by a mixture of the two mechanisms. Second, is to detect any change of mechanism between EX2 and EX1 from the pH dependence of exchange and its relationship to structure and kinetics. The third is to determine from which state exchange takes place for residues that nominally exchange by a global process: the fully unfolded state or the folding intermediate. Experiments were performed at values of pH and temperature around physiological and close to conditions under which the folding pathway of barnase has been studied in detail. A set of residues was found for which the rate constants for exchange change on mutation by exactly the same factor as does the equilibrium constant for unfolding. Further, the protection factor against exchange for these residues in wild-type barnase is very similar to the equilibrium constant for overall folding measured by differential scanning calorimetry and extrapolated to the identical reaction conditions. These residues clearly exchange by a global unfolding mechanism, and the protection factors are consistent with the denatured state of barnase being largely as unprotected as model peptides. The rate constants for exchange of a second set of residues are unaffected by distant mutations, and so these exchange by local breathing. The logarithms of the rate constants (log $k_{ex}$) increase linearly with pH for the locally exchanging residues, consistent with the kinetics of the EX2 mechanism at these values of pH. The pH dependence for the globally exchanging residues, however, indicates a switch away from EX2 between pH 6.7 and 7.9 at 37 °C. The state from which "global" exchange occurs was probed also by using mutants in which the folded state of each is destabilized by the same amount by mutation relative to the unfolded state but the destabilization of the folding intermediate varies considerably. Under EX2 conditions, the changes in $k_{ex}$ for all these residues follow the overall destabilization, confirming that exchange occurs from the fully unfolded state, not from the folding intermediate. The common characteristic of the residues that exchange by global unfolding is that they are all buried within the protein.

Amide protons in proteins exchange with those from solvent. The rate constant for exchange, $k_{ex}$, can be much lower than the intrinsic rate, $k_{int}$, determined for the exchange of a chemically equivalent proton in a small peptide (Molday *et al.*, 1972; Bai *et al.*, 1993). Protection against exchange in a protein may result from intramolecular hydrogen bonding interactions or by burial. Exchange of protected protons may be mediated by local unfolding motions, termed local breathing, or by global unfolding (Wagner & Wüthrich, 1979; Woodward *et al.*, 1982; Kim *et al.*, 1993). It is possible to identify those protons that exchange through global unfolding by comparing exchange in wild-type and mutant proteins (Wagner & Wüthrich, 1979; Clarke *et al.*, 1993; Kim & Woodward, 1993). The change in activation energy, $\Delta\Delta G_{ex}$, for each proton is determined from

$$\Delta\Delta G_{ex} = -RT \ln\left(\frac{k_{ex}^{wt}}{k_{ex}^{mut}}\right) \quad (1)$$

where $k_{ex}^{wt}$ and $k_{ex}^{mut}$ are the observed exchange rate constants in the wild-type and mutant proteins, respectively. Protons that exchange by global unfolding will have $\Delta\Delta G_{ex} = \Delta\Delta G_{U\text{-}F}$, where $\Delta\Delta G_{U\text{-}F}$ is the change in free energy of unfolding on mutation (measured by calorimetry, or denaturant titration). Exchange by local "breathing" should give values of $\Delta\Delta G_{ex}$ close to zero in regions away from the site of the mutation. Intermediate values imply a mixture of the two mechanisms. Exchange is generally analyzed by eq 2 in which there is an equilibrium between the protected, closed form of the protein (C) and the unprotected, open form (O), and exchange occurs from the open form with rate constant $k_{int}$ (Linderstrøm-Lang, 1955; Hvidt & Nielsen, 1966; Roder *et al.*, 1985).

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$$C^H \underset{k_c}{\overset{k_o}{\rightleftharpoons}} O^H \xrightarrow[D_2O]{k_{int}} O^D \underset{k_o}{\overset{k_c}{\rightleftharpoons}} C^D \quad (2)$$

$k_o$ is the first-order rate constant for the opening of the folded protein, $k_c$ for its closing, and $k_{int}$ the intrinsic rate constant for exchange under the reaction conditions.

Since, under folding conditions, $k_c \gg k_o$,

$$k_{ex} = \frac{k_o k_{int}}{k_c + k_{int}} \quad (3)$$

and, when $k_c \gg k_{int}$,

$$k_{ex} = K k_{int} \quad (4)$$

where $K = k_o/k_c$. This is the EX2 mechanism.

At the other extreme, termed EX1 conditions, where $k_c \ll k_{int}$, then

$$k_{ex} = k_o \quad (5)$$

Exchange under EX1 conditions has been observed only over a narrow range of high pH and temperature for the slowest exchanging residues in BPTI[1] (Roder *et al.*, 1985). The EX2 mechanism is by far the dominant mechanism for most proteins.

The equilibrium constant $K$ (eq 4), under EX2 conditions and for residues that exchange by global unfolding, reflects the stability of the protein. Hence, $\Delta\Delta G_{ex}$ calculated under these conditions reflects the relative stabilities of wild type and mutant, i.e., $\Delta\Delta G_{U\text{-}F}$. However, outside the EX2 limit, $k_{ex}$ is not directly related to the stability of the protein. Values of $\Delta\Delta G_{ex}$ that are equal to the full value of $\Delta\Delta G_{U\text{-}F}$ will only be observed, therefore, under EX2 conditions.

It has been suggested that the slowest exchanging protons of a protein at equilibrium may represent the early folding core (Kim *et al.*, 1993). We have demonstrated that this is not the case for barnase where there is independent evidence about the order of events in folding (Clarke *et al.*, 1993; Fersht, 1993). Some parts of barnase that fold early exchange rapidly (through local unfolding) whereas other buried residues, which fold late, exchange slowly (through global unfolding).

The folding pathway of barnase, like most proteins, involves a folding intermediate. The folding intermediate of barnase is more stable than the unfolded state in water below about 45 °C and around neutral pH (M. Oliveberg and A.R.F., unpublished results) and is thus populated to a greater extent (Figure 1). This complicates the analysis of exchange since it may occur from the intermediate as well as the unfolded state (Figure 2). By examination of a series of mutant proteins with different relative stabilities of the intermediate and unfolded states, it should be possible to determine whether exchange is from the folding intermediate or from a more unfolded state.

In this study, the exchange behavior of wild-type barnase and five different mutants, three of which are destabilized and two stabilized, has been studied, each at two different

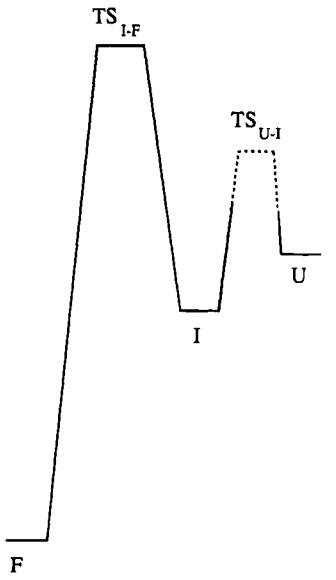


FIGURE 1: Energy level diagram for the folding pathway of barnase. The rate-determining transition state (TS) for folding is between the intermediate (I) and the folded state (F). The rate of refolding from the unfolded state (U) to I has not been measured. It appears to be complete within the dead-time of stopped-flow experiments ($t_{1/2}$ < 1 ms).

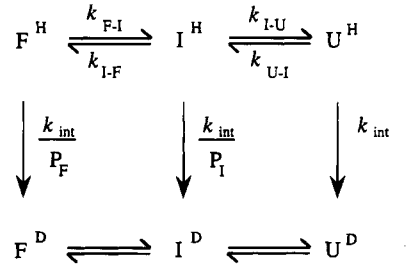


FIGURE 2: Reaction scheme for exchange of amide protons in barnase under native conditions. Exchange could occur from the folded state (F), the intermediate (I), or the unfolded state (U). However, exchange from F or I will be slowed by protection due to the presence of structure. Protection factors ($P$) for the folded state are expected to be high where there is hydrogen bonding or hydrophobic burial.

values of p$^2$H.[2] The folding kinetics and stability of the mutants have been previously analyzed (Matouschek *et al.*, 1992; Clarke & Fersht, 1993). We demonstrate that global exchange at equilibrium takes place from the unfolded protein, and there is no detectable exchange from the folding intermediate. We observe deviation from the EX2 mechanism for wild-type protein at 37 °C and high pH (p$^2$H = 7.9) and for destabilized mutants at lower temperature and pH. The critical rate constant that determines the exchange mechanism in barnase is not the rate-determining step seen in stopped-flow refolding experiments (which is the conversion of the folding intermediate to the native state).

## MATERIALS AND METHODS

*Materials.* Deuterated chemicals, imidazole ($^2$HCl, $^2H_2O$, [$^2$H]Tris and [$^{15}$N]ammonium chloride) were obtained from Aldrich Chemicals, C. K. Gas Products, Ltd., and MSD

[1] Abbreviations: BPTI, basic pancreatic trypsin inhibitor; NMR, nuclear magnetic resonance spectroscopy; HSQC, heteronuclear single-quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; $T_m$, the temperature at the midpoint of the thermal unfolding transition; TOCSY, total correlation spectroscopy; DSC, differential scanning calorimetry.

[2] The p$^2$H values stated in this paper were measured with a glass electrode at the temperature of the experiment. They have all been corrected to take account of the isotope effect: p$^2$H = $pH_{read}$ + 0.4 (Glasoe & Long, 1960).

Isotopes. Other chemicals and reagents were obtained from BDH, Fisons, or Sigma.

*Expression and Purification of Mutant Proteins.* $^{15}N$-labeled wild-type barnase and barnase mutants with Gly substituted for Glu at position 29 (EG29), Ala substituted for Asn at position 58 (NA58), Ala substituted for Ser at position 91 (SA91), and two disulfide mutants with Cys residues substituted for Ala and Ser at positions 43 and 80 (C43-C80) and with Cys substituted for Ser and His at 85 and 102 (C85-C102) were produced and purified. The cloning, mutagenesis, and method of expression and purification of proteins, both unlabeled and uniformly $^{15}N$-labeled, have been described previously (Mossakowska *et al.*, 1989; Serrano *et al.*, 1990; Clarke *et al.*, 1993). The disulfide proteins were shown by Ellman's (1959) assay to contain no free thiols following purification, and no dimers were detectable by gel electrophoresis (results not shown). Wild type, C43-C80, and C85-C102 were shown to be >95% pure, by gel electrophoresis, upon purification from ion exchange resin and so were not purified further. EG29, NA58, and SA91 were purified by FPLC, as described previously. The purified proteins were dialyzed against several changes of deionized water and lyophilized.

*NMR $^1H/^2H$ Exchange Experiments.* The NMR exchange experiments at 37 °C, using wild type and C43-C80 have been reported previously (Clarke *et al.*, 1993). For assignment of mutant proteins, 20 mg of lyophilized $^{15}N$-labeled protein was dissolved in the appropriate buffer containing 90% $^1H_2O$ and 10% $^2H_2O$. The exchange buffer was 20 mM deuterated imidazole, p$^2$H 6.7, or 50 mM deuterated Tris, p$^2$H 7.9, containing 0.05% sodium azide, dissolved in $^2H_2O$. Approximately 20 mg of lyophilized, uniformly $^{15}N$-labeled protein was dissolved in the exchange buffer (final concentration ~3 mM), centrifuged, transferred to an NMR tube, and allowed to equilibrate in the magnet for 10 min before the start of the first NMR experiment. The first spectrum was recorded approximately 20 min after the solution of the protein. The sample was kept at 33 or 37 °C, in the NMR tubes, throughout the study, which lasted up to 3 months. The final p$^2$H was measured using a glass electrode at the temperature of the experiment.

$^1H-^{15}N$ HSQC spectra (Bax *et al.*, 1990) were acquired on a Bruker AMX500 spectrometer, utilizing 2K complex data points over 4000 Hz in the $^1H$ dimension and 256 increments over 3000 Hz in the $^{15}N$ dimension. Both the $^1H$ and $^{15}N$ chemical shifts of the backbone amide groups were assigned by comparison with $^1H$ chemical shifts of wild-type barnase (Jones *et al.*, 1993). Where ambiguities arose, these were resolved by 2D X-filtered TOCSY and NOESY spectra using a $^{15}N$-labeled sample.

$^1H-^{15}N$ HSQC spectra were acquired at regular intervals. The initial 10 spectra acquired had two scans per increment, followed by six spectra with four scans per increment, and, thereafter, spectra with a total acquisition time of 40 min (eight scans per increment) were acquired with increasing delays until exchange was complete. The time point for each experiment was taken to be halfway through the acquisition time of the experiment. A total of 30−40 experiments were performed in the first 24 h. Using experiments with eight scans per increment from the start did not affect the final rate constant obtained. However, the fastest rates could not then be determined.

The spectra were zero-filled to 2K × 1K real points and were processed with a mild Gaussian window function in both dimensions. The volume integrals of the cross-peaks were calculated for each spectrum using the Bruker program UXNMR. The data were transferred to a Macintosh computer, and the decays were fitted to a single exponential, using the program KaleidaGraph (Abelbeck Software). Where two cross-peaks were overlapping in the HSQC spectrum, the decay was fitted to a double exponential, and a series of 2D X-filtered TOCSY spectra were run to ascertain which peak was decaying more rapidly. This allowed the assignment of a particular exchange rate to a specific amide proton. The first 10 and following six data points were omitted when fitting slow decays as they were relatively noisy data points, with only two and four scans per increment, respectively. This allowed a more accurate calculation of the rate constant of decay. In cases where the rate of exchange was very slow and the decay could not be fitted to an exponential, but where an initial rate could be determined, a rate constant was calculated from the ratio of the initial rate to the estimated amplitude of the decay. The same method was used for certain of the samples where spectrometer instability lead to inconsistent later data points. Where both methods could be used, there was good agreement between rate constants determined by the two methods.

*Calorimetry Experiments.* Differential scanning calorimetry was performed as described previously (Matouschek *et al.*, 1994) on wild-type protein in the exchange buffer (20 mM deuterated imidazole, p$^2$H 6.7, in $^2H_2O$).

*Analysis of Data: Identification of Residues Exchanging by Global Unfolding.* To determine which residues exchange through a global unfolding mechanism, the change in free energy of activation ($\Delta\Delta G_{ex}$) was determined by direct comparison of the exchange rate constants of the mutant with those of wild type at the same p$^2$H, using eq 1. Where the p$^2$H values of the wild-type and mutant proteins were slightly different, however, the value of $\Delta\Delta G_{ex}$ was determined by comparison of the protection factors of wild-type and mutant proteins. The sequence-specific intrinsic rate constants of exchange of each amide proton, $k_{int}$, for each protein, were calculated by the method of Bai *et al.* (1993), and the protection factor was determined using eq 6, using the value of $k_{int}$ calculated at the temperature and p$^2$H of the sample:

$$P = \frac{k_{int}}{k_{ex}} \tag{6}$$

where $P$ is the protection factor.

Assuming that any errors in calculating the intrinsic rates are the same in wild type and mutant, $\Delta\Delta G_{ex}$ was calculated using

$$\Delta\Delta G_{ex} = -RT \ln\left(\frac{P^{mut}}{P^{wt}}\right) \tag{7}$$

where $P^{wt}$ is the protection factor in the wild-type protein and $P^{mut}$ is the protection factor in the mutant.

*Identification of the Mechanism of Exchange from Its pH Dependence.* Amide proton exchange is catalyzed by acid, base, and water. However, the relative sizes of the rate constants for these processes means that, under the conditions studied here, only the base-catalyzed mechanism of exchange contributes significantly to $k_{int}$ (Jeng & Englander, 1991;

Table 1: Experimental Conditions for Measurement of Exchange Rates

| protein | exchange temperature (°C) | $p^2H_1$[a,c] | $p^2H_2$[b,c] |
|---|---|---|---|
| wild type | 33 | 6.5 | 7.6 |
| | 37 | 6.7 | 7.9 |
| EG29 | 33 | 6.5 | 7.6 |
| NA58 | 33 | 6.6 | 7.8 |
| SA91 | 33 | 6.1 | 7.3 |
| C43-C80 | 37 | 6.7 | 7.9 |
| C85-C102 | 37 | 6.7 | 7.9 |

[a] Protein dissolved in 20 mM imidazole. [b] Protein dissolved in 50 mM Tris. [c] Final measured $p^2H$ of sample: $p^2H = pH_{read} + 0.4$.

Connelly *et al.*, 1993; Gregory *et al.*, 1983), and so $k_{int}$ increases linearly with pH, i.e., $k_{int} = k^o_{ex}[OH^-]$ (where $k^o_{ex}$ is the component of the intrinsic rate that depends on the temperature and surrounding amino acid sequence, but not on pH). Thus, exchange by an EX2 mechanism will have a component in $k_{ex}$ that is proportional to $[OH^-]$ (eq 4). On the other hand, the value of $k_{int}$ is not part of the equation for exchange by an EX1 mechanism (eq 5), and so a simple pH dependence should not occur. Measurement of exchange rates at two different pH values and subsequent plotting of log $k_{ex}$ for individual residues at one pH ($pH_1$) against the value at another pH ($pH_2$) can provide insight into the mechanism of exchange (Skelton *et al.*, 1992). A straight line with slope 1 is diagnostic of EX2: from eq 4 describing the EX2 limit,

$$\log k_{ex}^{pH_1} = \log K^{pH_1} + \log k_{int}[OH^-]_1 \quad (8)$$

hence,

$$\log k_{ex}^{pH_1} = \log k_{ex}^{pH_2} + c \quad (9)$$

(In the case where the stability (global or local as appropriate) is unaffected by the change in pH, the intercept will be simply $pH_2 - pH_1$). Deviation from a slope of one will indicate deviation from an EX2 mechanism at the higher pH. An EX1 mechanism at the higher pH should give a slope of zero since $k_{ex}$ is simply $k_o$ (eq 5) and so will be a constant value for the globally exchanging residues at that pH. Between these limits, exchange is described by eq 3, and an intermediate slope will be obtained. The values of log $k_{ex}$ at two values of pH were compared for each protein in the study.

## RESULTS

The proteins used in these experiments and the conditions for exchange are described in Tables 1 and 2. Three mutants are destabilized by 2 kcal mol$^{-1}$, and the disulfide mutants are stabilized by 2 and 4 kcal mol$^{-1}$.

Exchange rate constants (Tables 3, 4, and 5) could be measured for the amide protons of approximately half of the 110 residues. Most of these protected protons are involved in hydrogen bonding interactions that are observed in the crystal structure of wild-type barnase (Mauguen *et al.*, 1982). The measured exchange rate constants vary over 5 orders of magnitude. In general, the exchange rates reflect the stability of the protein. Exchange is faster at high pH than at low pH, and faster at 37 than at 33 °C. Exchange rates for some of the slowest exchanging residues in C85-C102 could not be measured at $p^2H$ 6.7, there being no appreciable decrease in proton occupancy after 3 months, reflecting the greatly increased stability of this protein.

Calculated values of $\Delta\Delta G_{ex}/\Delta\Delta G_{U\text{-}F}$ determined at the lower values of pH for all the mutant proteins are in Table 6. A core of residues that have $\Delta\Delta G_{ex} = \Delta\Delta G_{U\text{-}F}$ can be identified and are shown in bold in the table. These are referred to as the globally exchanging residues. The mean value of $\Delta\Delta G_{ex}$ of the globally exchanging residues was determined for each protein and is very close to the value determined by equilibrium denaturation[3] (Table 2).

*Comparison of the Free Energy of Unfolding, $\Delta G_{U\text{-}F}$, with the Free Energy of Protection, $\Delta G_{ex}$.* The value of $\Delta G_{ex}$, determined from the exchange experiments, was compared to the value of $\Delta G_{U\text{-}F}$ obtained by differential scanning calorimetry (DSC). $\Delta G_{ex}$ was determined from the rate constants for exchange for each of the globally exchanging residues in wild type, at 37 °C, $p^2H$ 6.7, using

$$\Delta G_{ex} = -RT \ln\left(\frac{k_{int}}{k_{ex}}\right) \quad (10)$$

where $k_{int}$ is the value determined as described by Bai *et al.* (1993). The mean value of $\Delta G_{ex}$ is $-8.26 \pm 0.07$ kcal mol$^{-1}$. The $\Delta G_{U\text{-}F}$ determined from DSC for wild-type protein in the exchange buffer is $-8.3$ kcal mol$^{-1}$ at 37 °C. It is of interest that the $T_m$ for wild-type barnase in the exchange buffer ($^2H_2O$, 20 mM imidazole, $p^2H$ 6.7) is at least 3 °C higher than that determined in water (50 mM MES, pH 6.3). The stability of barnase is approximately constant over the range pH 5−7 (Pace *et al.*, 1992). The simplified equation relating change in free energy of unfolding to the change in $T_m$, $\Delta\Delta G_{U\text{-}F}(T_m) \approx \Delta T_m \Delta S_{U\text{-}F}(T_m)$ (Becktel & Schellman, 1987), has been shown to be reliable for barnase, particularly over small changes in $T_m$ (Matouschek *et al.*, 1994). Accordingly a change of 3 °C in the $T_m$ corresponds to a change in $\Delta G_{U\text{-}F}$ of 1.4 kcal mol$^{-1}$, i.e., barnase is significantly more stable in $^2H_2O$ than in water, as has been noted for a number of other proteins (Maybury & Katz, 1956; Hermans & Scheraga, 1959). This effect is most likely due to the stabilization of hydrophobic interactions in $^2H_2O$, although an isotope effect on hydrogen bonding may also contribute significantly (Kresheck *et al.*, 1965; Lee & Berns, 1968; Baghurst *et al.*, 1972). The agreement between $\Delta G$ determined from the exchange data and from DSC indicates that the protons are exchanging from a state which is essentially unstructured. This is consistent with the unprotected state being the unfolded state.

## DISCUSSION

Our strategy for analyzing the $^1H/^2H$ exchange has three parts. The first is to determine which residues exchange by local breathing, which by global unfolding, and which by a mixture of the two. The second is to determine which residues exchange by an EX2 mechanism and which by EX1, and to detect any changes in mechanism from one mutant

[3] For barnase the values of $\Delta G_{U\text{-}F}$ from calorimetry are significantly larger than those obtained from urea denaturation, when both are measured in $^1H_2O$. However the changes in stability on mutation, $\Delta\Delta G_{U\text{-}F}$, determined by the two methods are identical (Matouschek *et al.*, 1994). Unpublished data from this laboratory show that the calorimetric value of $\Delta G_{U\text{-}F}$ is correct for barnase (C. Johnson and A.R.F.).

Table 2: Properties of Proteins Used in This Study[a]

| protein | $\Delta\Delta G_{U\text{-}F}$[b] (kcal mol$^{-1}$) | $\Delta\Delta G_{U\text{-}I}$[c] (kcal mol$^{-1}$) | $\Delta\Delta G_{U\text{-}TS}$[d] (kcal mol$^{-1}$) | $\Delta\Delta G_{ex}$[e] (kcal mol$^{-1}$) | $k_{I\rightarrow F}$[f] (s$^{-1}$) | $k_{int}$[g] (s$^{-1}$) | $T_m$[i] (°C) |
|---|---|---|---|---|---|---|---|
| wild type | | | | | 12.7 | 9−178[h] | 57.5 |
| EG29 | 1.9 | −0.2 | −0.2 | 2.0 | 12.9 | 9−178 | ~53 |
| NA58 | 2.2 | 1.9 | 2.0 | 2.0 | 10.6 | 16−32 | ~52 |
| SA91 | 1.9 | 1.1 | 1.8 | 1.9 | 3.8 | 5−90 | ~53 |
| C43-C80 | −2.0 | −0.6 | −0.2 | −2.0 | 7.5 | 38−753 | ~63 |
| C85-C102 | −4.1 | −1.8 | −2.9 | −3.9 | 80.1 | 38−753 | ~68 |

[a] Data, other than $\Delta\Delta G_{ex}$, $k_{int}$, and $T_m$, are taken from Matouschek *et al.* (1992) and Clarke and Fersht (1993). [b] $\Delta\Delta G_{U\text{-}F}$, measured by urea or guanidinium chloride denaturation ($\Delta G_{U\text{-}F}$ for wild-type barnase in $^2H_2O$ is 11.8 kcal mol$^{-1}$ at 25 °C and 8.3 kcal mol$^{-1}$ at 37 °C under the exchange conditions). [c] $\Delta\Delta G_{U\text{-}I}$, change in free energy of intermediate relative to the unfolded state on mutation. [d] $\Delta\Delta G_{U\text{-}TS}$, change in free energy of transition state relative to the unfolded state on mutation. [e] $\Delta\Delta G_{ex}$ was taken to be the mean $\Delta\Delta G_{ex}$ of all globally exchanging residues at the lower pH (Tables 4, 5 and 6). [f] Observed rate constant for refolding of intermediate to folded state, measured at 25 °C at pH 6.3 in 50 mM MES buffer, in $^1H_2O$. [g] Range of $k_{int}$ for the globally exchanging residues, calculated at the temperature and p$^2$H of the higher pH experiment (see Table 1). [h] Wild-type values at 33 °C. [i] Value of $T_m$ for mutant proteins under the exchange conditions estimated from $\Delta\Delta G_{U\text{-}F}$ and the measured $T_m$ for wild type, using the formula $\Delta\Delta G_{U\text{-}F} = \Delta S_{U\text{-}F}\Delta T_m$.

Table 3: Rate Constants for Exchange ($k_{ex}$, min$^{-1}$) and Protection Factors ($P$)[a] for Wild-Type Barnase under Different Conditions of Temperature and p$^2$H[b]

| | p$^2$H 6.5, 33 °C | | p$^2$H 7.6, 33 °C | | p$^2$H 6.7, 37 °C | | p$^2$H 7.9, 37 °C | | p$^2$H 8.5, 37 °C | |
|---|---|---|---|---|---|---|---|---|---|---|
| residue | $k_{ex} \times 10^4$ | $P/10^4$ | $k_{ex} \times 10^4$ | $P/10^4$ | $k_{ex} \times 10^4$ | $P/10^4$ | $k_{ex} \times 10^4$ | $P/10^4$ | $k_{ex} \times 10^4$ | $P/10^4$ |
| 10 | 27 | 3.3 | 350 | 2.9 | 99 | 2.8 | [c] | | [c] | |
| 11 | 2.0 | 110 | 32 | 77 | 14 | 48 | 230 | 46 | [c] | |
| 12 | 12 | 13 | 160 | 11 | 40 | 12 | 500 | 15 | [c] | |
| 13 | 1.4 | 75 | 19 | 64 | 8.8 | 37 | 140 | 37 | [c] | |
| 14 | 0.082 | 1100 | 1.7 | 590 | 4.8 | 57 | 76 | 57 | 96 | 180 |
| 15 | 0.52 | 410 | 7.8 | 310 | 9.6 | 68 | 100 | 100 | 200 | 210 |
| 16 | 25 | 16 | 400 | 12 | 83 | 15 | [c] | | [c] | |
| 17 | 7.6 | 34 | 95 | 31 | 31 | 25 | 400 | 31 | [c] | |
| 25 | 0.069 | 930 | 1.6 | 450 | 3.4 | 57 | 78 | 39 | 91 | 130 |
| 26 | 3.8 | 40 | 40 | 43 | 23 | 20 | 240 | 30 | [c] | |
| 30 | 0.19 | 1100 | 4.3 | 560 | 9.5 | 69 | 100 | 100 | 140 | 290 |
| 31 | 6.2 | 56 | 120 | 33 | 35 | 30 | 530 | 32 | [c] | |
| 33 | 1.2 | 65 | 17 | 53 | 7.9 | 31 | 160 | 24 | [c] | |
| 35 | 8.1 | 22 | [c] | | 43 | 12 | 690 | 12 | [c] | |
| 36 | 64 | 0.73 | [c] | | 250 | 0.57 | [c] | | [c] | |
| 44 | 68 | 2.3 | 560 | 3.0 | 220 | 2.1 | [c] | | [c] | |
| 45 | 11 | 3.7 | 210 | 2.1 | 41 | 3.0 | [c] | | [c] | |
| 46 | 0.23 | 960 | 6.3 | 390 | 6.7 | 100 | 110 | 96 | 170 | 250 |
| 49 | 0.64 | 640 | 5.3 | 870 | 18 | 69 | 120 | 160 | 160 | 490 |
| 50 | 0.70 | 1400 | 6.1 | 1700 | 25 | 110 | 130 | 350 | 180 | 1000 |
| 51 | 0.34 | 330 | 5.8 | 220 | 14 | 24 | 120 | 45 | 150 | 140 |
| 52 | 0.29 | 1200 | 3.4 | 1100 | 17 | 60 | 110 | 150 | 130 | 490 |
| 53 | 1.2 | 730 | 14 | 670 | 22 | 120 | 150 | 270 | [c] | |
| 56 | 0.36 | 290 | 6.0 | 190 | 10 | 31 | 110 | 45 | 160 | 120 |
| 71 | 19 | 9.8 | 330 | 6.4 | 95 | 6.0 | [c] | | [c] | |
| 72 | 0.21 | 1400 | 3.8 | 840 | 120 | 72 | 87 | 160 | 91 | 600 |
| 73 | [c] | | 3.5 | 500 | 13 | 36 | 96 | 78 | 98 | 300 |
| 74 | 0.082 | 2600 | 1.7 | 1400 | 4.8 | 140 | 76 | 140 | [c] | |
| 75 | 0.18 | 870 | 3.2 | 530 | 11 | 42 | 96 | 76 | 130 | 220 |
| 76 | 0.17 | 220 | 2.9 | 140 | 5.4 | 21 | 82 | 22 | 120 | 60 |
| 87 | 2.4 | 100 | 19 | 140 | 18 | 41 | 130 | 89 | 240 | 190 |
| 88 | 0.11 | 860 | 2.2 | 480 | 5.4 | 53 | 81 | 56 | 110 | 160 |
| 89 | 0.074 | 630 | 1.8 | 290 | 2.7 | 53 | 72 | 32 | 78 | 120 |
| 90 | 0.076 | 1300 | 1.7 | 660 | 6.2 | 49 | 83 | 58 | 85 | 230 |
| 91 | 0.23 | 3500 | 4.0 | 2200 | 18 | 140 | 100 | 380 | [c] | |
| 94 | 23 | 3.4 | 460 | 1.9 | 85 | 2.8 | [c] | | [c] | |
| 95 | 0.84 | 74 | 15 | 46 | 5.2 | 36 | 120 | 25 | 250 | 48 |
| 97 | 0.063 | 1500 | 1.6 | 670 | 3.1 | 94 | 78 | 59 | 77 | 240 |
| 98 | 0.22 | 1400 | 3.9 | 900 | 11 | 86 | 87 | 170 | 110 | 540 |
| 99 | 0.73 | 470 | 6.0 | 640 | 21 | 49 | 110 | 150 | 120 | 540 |
| 107 | 10 | 28 | 210 | 16 | 79 | 8.3 | [c] | | [c] | |

[a] Protection factor, $P = k_{int}/k_{ex}$, where $k_{int}$ is calculated by the method of Bai *et al.* (1993). [b] Final measured p$^2$H of sample: p$^2$H = pH$_{read}$ + 0.4. [c] Accurate exchange rates could not be measured for these protons.

to another. The basis for this part of the analysis is the pH dependence of exchange. The third part of the strategy is to use mutants with different stabilities of the folded and intermediate states to determine from which state exchange occurs.

*Global vs Local Exchange*

Barnase has three α-helices and a five-stranded β-sheet. The first helix packs onto the β-sheet to form the major hydrophobic core of the protein. A smaller hydrophobic core

Table 4: Rate Constants for Exchange ($k_{ex}$),[a] Protection Factors ($P$),[b] and $\Delta\Delta G_{ex}$[c] Values for Each of the Destabilized Mutants in the Study Measured at 33 °C

| | EG29 | | | | | | NA58 | | | | | | SA91 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH 6.5 | | | pH 7.6 | | | pH 6.6 | | | pH 7.8 | | | pH 6.1 | | | pH 7.3 | | |
| residue | $k_{ex}$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex}$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex}$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex}$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex}$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex}$ | $P/10^4$ | $\Delta\Delta G_{ex}$ |
| 10 | 27 | 3.3 | 0.0 | *d* | | | 40 | 3.0 | 0.0 | *d* | | | 7.6 | 4.7 | −0.2 | 220 | 2.4 | 0.1 |
| 11 | 4.1 | 54 | 0.4 | 67 | 37 | 0.4 | 6.7 | 44 | 0.6 | 110 | 40 | 0.4 | 1.2 | 73 | 0.3 | 27 | 47 | 0.3 |
| 12 | 9.0 | 17 | −0.2 | 210 | 8.1 | 0.2 | 15 | 14 | 0.0 | *d* | | | 7.1 | 8.6 | 0.3 | 100 | 8.8 | 0.1 |
| 13 | 3.6 | 30 | 0.6 | 48 | 25 | 0.6 | 5.0 | 29 | 0.6 | 71 | 30 | 0.5 | 0.90 | 48 | 0.3 | 19 | 33 | 0.4 |
| 14 | 2.3 | 39 | 2.0 | 22 | 46 | 1.5 | 3.1 | 39 | 2.0 | 45 | 40 | 1.6 | 0.44 | 81 | 1.6 | 9.7 | 53 | 1.5 |
| 15 | 3.6 | 60 | 1.2 | 50 | 48 | 1.1 | 5.3 | 55 | 1.2 | 69 | 62 | 1.0 | 0.81 | 110 | 0.8 | 15 | 83 | 0.8 |
| 16 | 34.0 | 12 | 0.2 | *d* | | | 42 | 13 | 0.1 | *d* | | | 9.6 | 17 | 0.0 | *d* | | |
| 17 | 14 | 18 | 0.4 | 140 | 21 | 0.2 | 14 | 25 | 0.2 | 240 | 22 | 0.2 | 3.7 | 28 | 0.1 | 86 | 17 | 0.3 |
| 25 | 2.2 | 29 | 2.1 | 27 | 26 | 1.7 | 1.7 | 50 | 1.8 | 40 | 32 | 1.6 | 0.31 | 82 | 1.5 | 8.3 | 44 | 1.4 |
| 26 | *d* | | | *d* | | | 10 | 21 | 0.4 | 110 | 28 | 0.3 | 3.1 | 20 | 0.4 | 35 | 25 | 0.3 |
| 30 | *d* | | | *d* | | | 5.9 | 49 | 1.9 | 45 | 96 | 1.8 | 1.3 | 66 | 1.7 | 12 | 100 | 1.0 |
| 31 | 58 | 6.0 | 1.4 | *d* | | | 13 | 36 | 0.3 | 180 | 39 | 0.1 | 2.8 | 50 | 0.1 | 63 | 32 | 0.0 |
| 32 | *d* | | | *d* | | | *d* | | | *d* | | | *d* | | | *d* | | |
| 33 | 10 | 8.0 | 1.3 | 150 | 6.0 | 1.3 | 4.4 | 24 | 0.6 | 76 | 21 | 0.2 | 0.78 | 41 | 0.3 | 16 | 29 | 0.4 |
| 35 | 24 | 7.3 | 0.7 | *d* | | | 12 | 20 | 0.1 | *d* | | | 3.7 | 19 | 0.1 | 65 | 16 | *e* |
| 36 | 110 | 0.43 | 0.3 | *d* | | | 86 | 0.74 | 0.0 | *d* | | | 19 | 0.99 | −0.2 | 100 | 2.7 | *e* |
| 44 | 92 | 1.7 | 0.2 | *d* | | | 90 | 2.3 | 0.0 | *d* | | | 29 | 2.1 | 0.0 | *d* | | |
| 45 | 17 | 2.4 | 0.3 | *d* | | | 14 | 3.9 | 0.0 | *d* | | | 4.3 | 3.7 | 0.0 | 100 | 2.3 | 0.0 |
| 46 | 3.0 | 74 | 1.6 | 37 | 67 | 1.1 | 3.3 | 90 | 1.4 | 48 | 92 | 0.9 | 0.58 | 150 | 1.1 | 12 | 110 | 0.8 |
| 49 | 9.1 | 45 | 1.6 | 66 | 70 | 1.5 | 12 | 46 | 1.6 | 61 | 130 | 1.4 | 3.6 | 45 | 1.6 | 14 | 170 | 1.0 |
| 50 | 22 | 43 | 2.1 | 94 | 110 | 1.6 | 20 | 63 | 1.9 | 76 | 250 | 1.6 | 7.4 | 51 | 2.0 | 16 | 340 | 1.0 |
| 51 | 7.9 | 14 | 1.9 | 62 | 20 | 1.4 | 9.2 | 17 | 1.8 | 63 | 36 | −0.1 | 2.5 | 18 | 1.8 | 16 | 41 | 1.0 |
| 52 | 12 | 28 | 2.3 | 69 | 54 | 1.8 | 14 | 32 | 2.2 | 84 | 79 | 2.1 | 3.4 | 39 | 2.1 | 13 | 150 | 1.2 |
| 53 | 15 | 56 | 1.6 | 77 | 120 | 1.0 | 14 | 81 | 1.3 | 68 | 250 | 1.3 | 3.9 | 86 | 1.3 | 20 | 240 | 0.6 |
| 56 | 4.8 | 22 | 1.6 | 46 | 25 | 1.2 | 16 | 8.7 | 2.1 | *d* | | | 1.8 | 23 | 1.5 | 13 | 46 | 0.9 |
| 71 | 27 | 7.0 | 0.2 | 250 | 8.4 | −0.2 | *d* | | | *d* | | | 7.7 | 9.7 | 0.0 | 210 | 5.1 | 0.1 |
| 72 | 5.5 | 52 | 2.0 | 48 | 66 | 1.5 | 7.3 | 52 | 2.0 | 72 | 79 | 1.4 | 2.0 | 56 | 1.9 | 14 | 120 | 1.2 |
| 73 | 6.1 | 26 | *e* | 54 | 32 | 1.6 | *d* | | | *d* | | | 2.6 | 24 | *e* | 14 | 64 | 1.3 |
| 74 | 2.3 | 94 | 2.0 | *d* | | | 3.1 | 94 | 2.0 | *d* | | | 7.6 | 11 | 3.3 | 9.7 | 130 | 1.5 |
| 75 | 4.0 | 38 | 1.9 | 40 | 43 | 1.5 | 7.1 | 29 | 2.1 | 58 | 52 | 1.4 | 1.4 | 43 | 1.8 | 13 | 68 | 1.3 |
| 76 | 1.8 | 21 | 1.4 | 24 | 18 | 1.3 | 2.7 | 19 | 1.5 | 58 | 13 | 1.5 | 0.65 | 23 | 1.4 | 9.1 | 24 | 1.1 |
| 87 | 4.8 | 50 | 0.4 | 54 | 50 | 0.6 | 21 | 16 | 1.1 | 200 | 24 | 1.1 | 1.4 | 69 | 0.2 | 20 | 70 | 0.4 |
| 88 | 0.36 | 260 | 0.7 | 28 | 38 | 1.5 | 2.6 | 49 | 1.7 | 35 | 54 | 1.8 | 0.56 | 67 | 1.6 | 10 | 54 | 1.3 |
| 89 | 1.7 | 28 | 1.9 | 24 | 22 | 1.6 | 2.2 | 29 | 1.9 | 43 | 22 | 1.0 | 0.89 | 21 | 2.1 | 8.9 | 30 | 1.4 |
| 90 | 2.6 | 39 | 2.2 | 31 | 36 | 1.7 | 3.1 | 44 | 2.1 | 44 | 46 | 2.1 | 0.45 | 210 | 1.1 | 8.4 | 160 | 0.8 |
| 91 | 11 | 73 | 2.4 | 55 | 160 | 1.6 | 14 | 77 | 2.3 | 61 | 260 | 2.4 | 1.8 | 76 | 2.3 | 14 | 140 | 1.7 |
| 93 | 3.6 | 84 | 1.1 | *d* | | | *d* | | | *d* | | | *d* | | | *d* | | |
| 94 | 27 | 2.9 | 0.1 | *d* | | | 30 | 3.5 | 0.0 | *d* | | | *d* | | | *d* | | |
| 95 | 2.2 | 28 | 0.6 | 32 | 22 | 0.5 | 2.6 | 32 | 0.5 | 57 | 22 | 0.5 | *d* | | | *d* | | |
| 97 | 1.7 | 57 | 2.0 | 22 | 49 | 1.6 | 2.0 | 65 | 1.9 | 39 | 49 | 1.6 | 0.36 | 110 | 1.6 | 9.1 | 61 | 1.5 |
| 98 | 5.9 | 53 | 2.0 | 36 | 97 | 1.3 | 8.5 | 49 | 2.1 | 62 | 100 | 1.3 | 2.0 | 62 | 1.9 | 14 | 130 | 1.2 |
| 99 | 12 | 28 | 1.7 | 51 | 75 | 1.3 | 18 | 26 | 1.8 | 88 | 77 | 1.3 | 5.5 | 25 | 1.8 | 17 | 120 | 1.0 |
| 107 | 20 | 15 | 0.4 | *d* | | | *d* | | | *d* | | | 9.8 | 12 | 0.5 | 110 | 16 | 0.0 |

[a] $k_{ex}$ is expressed in units of $\times 10^4$ min$^{-1}$. [b] Protection factor, $P = k_{int}/k_{ex}$, where $k_{int}$ is calculated by the method of Bai *et al.* (1993). [c] Calculated from eq 1 or eq 7 using wild-type data measured at the same temperature and in the same buffer. The conditions are shown in Table 1. [d] Accurate exchange rates could not be measured. [e] A value for $\Delta\Delta G_{ex}$ could not be calculated as no value of $k_{ex}$ for wild type is available (see note *c*).

is formed by packing of the second and third α-helices against the edge of the β-sheet. Protein engineering studies indicate that the final two-thirds of the first α-helix, the second half of the second α-helix, and the β-sheet form early in the folding pathway (Fersht, 1993). The major hydrophobic core, into which the first α-helix is anchored, begins to form early but is consolidated later. Formation of the second core, into which the second α-helix is anchored, is a late event.

Analysis of exchange data of a number of mutants allows the pattern of local and global exchange in the wild-type protein to be established (shown in Figure 3). Earlier experiments were performed on disulfide mutants (Clarke *et al.*, 1993) whereas this study includes the analysis of more conservative mutants. The same pattern of exchange is seen in all barnase mutants studied (other than at the actual site of mutation). The residues which exchange by global unfolding (Table 6, shown in bold; Figure 3, shown in black) are mostly in the β-sheet. Residues which exchange by local unfolding (Table 6, underlined; Figure 3, shown in white) are predominantly in the α-helices and loop regions. Residues 14 and 30 anchor the first and second α-helices into the hydrophobic cores (Figure 3). The extent of burial of these residues results in values of $\Delta\Delta G_{ex}$ that approach $\Delta\Delta G_{U\text{-}F}$. A symmetrical pattern of local exchange around the central, anchoring, highly protected residue is observed for both these helices.

This indicates that there is not a direct relationship between the folding pathway and the pattern of local vs global exchange: the final folded structure of the protein—specifically the extent of burial of the residues and the flexibility of surrounding secondary structure—determines the mechanism of amide proton exchange.

Table 5: Rate Constants for Exchange ($k_{ex}$, $min^{-1}$), Protection Factors ($P$),[a] and $\Delta\Delta G_{ex}$[b] Values for Each of the Stabilized Mutants in the Study Measured at 37 °C

| | C43-C80 | | | | | | C85-C102[c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH 6.7 | | | pH 7.9 | | | pH 6.7 | | | pH 7.9 | | |
| residue | $k_{ex} \times 10^4$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex} \times 10^4$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex} \times 10^4$ | $P/10^4$ | $\Delta\Delta G_{ex}$ | $k_{ex} \times 10^4$ | $P/10^4$ | $\Delta\Delta G_{ex}$ |
| 10 | 170 | 1.6 | 0.3 | *d* | | | 47 | 5.8 | −0.5 | *d* | | |
| 11 | 18 | 37 | 0.2 | 300 | 35 | 0.2 | 5.3 | 130 | −0.6 | 130 | 81 | −0.4 |
| 12 | 27 | 17 | −0.2 | 380 | 19 | −0.2 | 28 | 17 | −0.2 | 550 | 13 | 0.1 |
| 13 | 4.9 | 67 | −0.4 | 64 | 81 | −0.5 | 2.5 | 130 | −0.8 | 55 | 94 | −0.6 |
| 14 | 0.22 | 1200 | −1.9 | 2.9 | 1500 | −2.0 | *d* | | | 0.32 | 13000 | −3.4 |
| 15 | 1.2 | 540 | −1.3 | 16 | 650 | −1.1 | 0.91 | 720 | −1.4 | 25 | 410 | −0.9 |
| 16 | 54 | 23 | −0.3 | *d* | | | 72 | 17 | −0.1 | *d* | | |
| 17 | 19 | 41 | −0.3 | 240 | 52 | −0.3 | 22 | 36 | −0.2 | 360 | 35 | −0.1 |
| 25 | 0.18 | 1100 | −1.8 | 2.9 | 1100 | −2.0 | 0.0050 | 37000 | −4.0 | 0.36 | 8500 | −3.3 |
| 26 | 11 | 42 | −0.5 | 160 | 46 | −0.2 | 12 | 39 | −0.4 | 12 | 610 | −1.8 |
| 30 | 0.65 | 1000 | −1.6 | 9.2 | 1100 | −1.5 | 0.31 | 2100 | −2.1 | 13 | 800 | −1.3 |
| 31 | 17 | 62 | −0.4 | 430 | 39 | −0.1 | 20 | 53 | −0.3 | 510 | 33 | 0.0 |
| 33 | 3.3 | 74 | −0.5 | 61 | 63 | −0.6 | 3.3 | 74 | −0.5 | 85 | 45 | −0.4 |
| 35 | 40 | 13 | 0.0 | 750 | 11 | 0.1 | 41 | 13 | 0.0 | 580 | 15 | −0.1 |
| 36 | 400 | 0.36 | 0.3 | *d* | | | 270 | 0.53 | 0.0 | *d* | | |
| 44 | *d* | | | *d* | | | 200 | 2.3 | −0.1 | *d* | | |
| 45 | 260 | 0.47 | 1.1 | *d* | | | 45 | 2.7 | 0.1 | 100 | 19 | *e* |
| 46 | 1.3 | 510 | −1.0 | 25 | 420 | −0.9 | 1.0 | 670 | −1.2 | 34 | 310 | −0.7 |
| 49 | 1.7 | 730 | −1.5 | 20 | 990 | −1.1 | 0.48 | 2600 | −2.2 | 210 | 94 | 0.3 |
| 50 | 0.76 | 3800 | −2.1 | 7.6 | 5900 | −1.7 | 0.13 | 22000 | −3.2 | 5.1 | 8900 | −2.0 |
| 51 | 1.4 | 240 | −1.4 | 22 | 250 | −1.0 | 0.46 | 750 | −2.1 | 15 | 360 | −1.3 |
| 52 | 0.44 | 2300 | −2.2 | 4.2 | 3800 | −2.0 | 0.026 | 39000 | −4.0 | 2.0 | 8000 | −2.5 |
| 53 | 2.0 | 1300 | −1.5 | 25 | 1600 | −1.1 | 2.0 | 1300 | −1.5 | 50 | 800 | −0.7 |
| 56 | 0.93 | 340 | −1.5 | 10 | 500 | −1.5 | 0.68 | 460 | −1.7 | *d* | | |
| 71 | 73 | 7.8 | −0.2 | *d* | | | 78 | 7.3 | −0.1 | *d* | | |
| 72 | 0.40 | 2200 | −2.1 | 2.5 | 5500 | −2.2 | 0.019 | 45000 | −4.0 | 0.74 | 18000 | −2.9 |
| 73 | 0.42 | 1100 | −2.1 | 3.3 | 2300 | −2.1 | 0.0083 | 57000 | −4.5 | 0.63 | 12000 | −3.1 |
| 74 | 0.22 | 3000 | −1.9 | 2.9 | 3600 | −2.0 | *d* | | | 0.32 | 32000 | −3.4 |
| 75 | 0.37 | 1200 | −2.1 | 2.9 | 2500 | −2.2 | *d* | | | *d* | | |
| 76 | 0.85 | 130 | −1.1 | 13 | 140 | −1.1 | 0.10 | 1100 | −2.5 | 3 | 600 | −2.0 |
| 87 | 9.7 | 76 | −0.4 | 50 | 230 | −0.6 | *d* | | | *d* | | |
| 88 | 0.26 | 1100 | −1.9 | 2.2 | 2100 | −2.2 | *d* | | | 0.28 | 16000 | −3.5 |
| 89 | 0.15 | 950 | −1.8 | 1.8 | 1300 | −2.3 | *d* | | | 0.18 | 13000 | −3.7 |
| 90 | 0.16 | 1900 | −2.2 | 2.3 | 2100 | −2.2 | *d* | | | 0.28 | 17000 | −3.5 |
| 91 | 0.52 | 4700 | −2.2 | 5.3 | 7300 | −1.8 | 0.13 | 19000 | −3.0 | 4.2 | 9200 | −1.9 |
| 94 | 61 | 3.9 | −0.2 | *d* | | | 90 | 2.6 | 0.0 | *d* | | |
| 95 | 2.5 | 75 | −0.5 | 4.3 | 700 | −2.0 | 3.3 | 57 | −0.3 | 68 | 44 | −0.3 |
| 97 | 0.14 | 2100 | −1.9 | 1.9 | 2400 | −2.3 | *d* | | | 0.19 | 24000 | −3.7 |
| 98 | 0.54 | 1800 | −1.9 | 5.7 | 2600 | −1.7 | 0.019 | 50000 | −2.9 | 0.95 | 16000 | −2.8 |
| 99 | 1.7 | 610 | −1.5 | 13 | 1300 | −1.3 | 0.016 | 65000 | −4.4 | 0.91 | 18000 | −2.9 |
| 107 | 42 | 16 | −0.4 | 840 | 12 | *e* | 13 | 50 | −1.1 | 400 | 26 | *e* |

[a] Protection factor, $P = k_{int}/k_{ex}$, where $k_{int}$ is calculated by the method of Bai *et al.* (1993). [b] Calculated from eq 1 using wild-type data measured at the same temperature and in the same buffer. [c] C85-C102 has a lower $\Delta\Delta G_{U\text{-}F}$ at pH 7.5 than at pH 6.3, as measured by guanidinium chloride denaturation (J. Clarke, unpublished data). [d] Accurate exchange rates could not be measured. [e] A value for $\Delta\Delta G_{ex}$ could no be calculated as no value of $k_{ex}$ for wild type is available (see note *b*).

This is in accord with the finding that in lysozyme, for residues that are highly buried or near the surface of the protein, the observed amide proton exchange rate is related to the distance from solvent (Pedersen *et al.*, 1993). Residues of intermediate distance from the protein surface of lysozyme exchange with rates related to the type of secondary structure.

A detailed analysis of the kinetics of exchange may also be useful in determining the extent of disruption of structure on mutation. This method has been used as part of a comprehensive study of the effects of introduction of disulfide bonds on the structure and stability of barnase (J.C., A.M.H., & and A.R.F., unpublished results). In the case of the destabilized mutants studied here, the exchange pattern indicates that for NA58 and SA91 (located in a loop region and in a $\beta$-strand, respectively) the effect of the mutation is localized. However, in the case of EG29, the exchange pattern is altered throughout the helix in which the mutation is located.

*Detection of a Change in Exchange Mechanism from EX2*

The locally exchanging residues show EX2 behavior under all conditions studied (Figure 4). The globally exchanging residues deviate from EX2 when $k_{int}$ is sufficiently high, i.e., at sufficiently high temperature and pH.

*Wild-Type Barnase.* The exchange rate constants of wild-type protein at two different values of $p^2H$ at 33 °C show the same pH dependence for all residues, indicative of an EX2 mechanism of exchange (Figure 5A). At 37 °C, however, there is a change in mechanism for certain residues that exhibit greatly reduced pH dependence (Figure 5B). It is observed that it is the globally exchanging residues that deviate from an EX2 mechanism. Residues that exchange partly by local and partly through global unfolding (mixed exchange) deviate to a lesser extent, if at all, from EX2 behavior. Exchange rates were also obtained at $p^2H$ 8.5 and 37 °C (see Table 3) and the values plotted as in Figure 5 (plot not shown). No further decrease in the slope of the

Table 6: Ratio of $\Delta\Delta G_{ex}/\Delta\Delta G_{U\text{-}F}$ for Mutants at $p^2H$ 6.1−6.7[a]

| residue | location of residue | $\Delta\Delta G_{ex}/\Delta\Delta G_{U\text{-}F}$[a] EG29 | NA58 | SA91 | C43-C80 | C85-C102 |
|---|---|---|---|---|---|---|
| 10 | α-helix$_1$ | 0.0 | 0.0 | −0.1 | −0.2 | 0.1 |
| 11 | α-helix$_1$ | 0.3 | 0.3 | 0.1 | −0.1 | 0.1 |
| 12 | α-helix$_1$ | −0.1 | 0.0 | 0.1 | 0.1 | 0.0 |
| 13 | α-helix$_1$ | 0.3 | 0.3 | 0.1 | 0.2 | 0.2 |
| **14** | α-helix$_1$ | **1.1** | **0.9** | **0.8** | **0.9** | *c* |
| 15 | α-helix$_1$ | 0.7 | 0.6 | 0.4 | 0.6 | 0.3 |
| 16 | α-helix$_1$ | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 |
| 17 | α-helix$_1$ | 0.2 | 0.1 | 0.1 | 0.2 | 0.0 |
| **25** | loop$_1$ | **1.1** | **0.8** | **0.8** | **0.9** | **1.0** |
| 26 | α-helix$_2$ | *b* | 0.2 | 0.2 | 0.2 | 0.1 |
| 30 | α-helix$_2$ | *b* | 0.9 | 0.9 | 0.8 | 0.5 |
| 31 | α-helix$_2$ | *0.8* | 0.1 | 0.0 | 0.2 | 0.1 |
| 33 | α-helix$_2$ | 0.7 | 0.3 | 0.1 | 0.3 | 0.1 |
| 35 | loop$_2$ | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| 36 | loop$_2$ | 0.2 | 0.0 | −0.1 | −0.1 | 0.0 |
| 44 | α-helix$_3$ | 0.1 | 0.0 | 0.0 | *b* | 0.0 |
| 45 | α-helix$_3$ | 0.2 | 0.0 | 0.0 | *−0.6* | 0.0 |
| 46 | α-helix$_3$ | 0.9 | 0.7 | 0.6 | 0.5 | 0.3 |
| 49 | β-turn | 0.9 | 0.7 | 0.8 | 0.7 | 0.5 |
| **50** | β-strand$_1$ | **1.1** | **0.9** | **1.0** | **1.1** | **0.7** |
| 51 | β-strand$_1$ | 1.0 | 0.8 | 0.9 | 0.7 | 0.5 |
| **52** | β-strand$_1$ | **1.2** | **1.0** | **1.1** | **1.1** | **0.9** |
| 53 | β-strand$_1$ | 0.9 | 0.6 | 0.7 | 0.7 | 0.3 |
| 56 | loop$_3$ | 0.9 | *1.0* | 0.8 | 0.7 | 0.4 |
| 71 | β-strand$_2$ | 0.1 | *b* | 0.0 | 0.1 | 0.0 |
| **72** | β-strand$_2$ | **1.0** | **0.9** | **1.0** | **1.0** | **0.9** |
| **73** | β-strand$_2$ | *b* | *b* | *b* | **1.1** | **1.0** |
| **74** | β-strand$_2$ | **1.1** | **0.9** | *b* | **0.9** | *c* |
| **75** | β-strand$_2$ | **1.0** | **1.0** | **0.9** | **1.0** | *d* |
| 76 | β-strand$_3$ | 0.8 | 0.7 | 0.7 | 0.6 | 0.5 |
| 87 | β-strand$_3$ | 0.2 | 0.5 | 0.1 | 0.2 | *d* |
| 88 | β-strand$_3$ | 0.4 | 0.8 | 0.8 | 0.9 | *c* |
| **89** | β-strand$_3$ | **1.0** | **0.9** | **1.1** | **0.9** | *c* |
| 90 | β-strand$_3$ | **1.1** | **1.0** | *0.6* | **1.1** | *c* |
| **91** | β-strand$_3$ | **1.2** | **1.1** | *1.2* | **1.1** | **0.7** |
| 94 | β-strand$_4$ | 0.1 | 0.0 | *b* | 0.1 | 0.0 |
| 95 | β-strand$_4$ | 0.3 | 0.2 | *b* | 0.2 | 0.1 |
| **97** | β-strand$_4$ | **1.0** | **0.9** | **0.8** | **1.0** | *c* |
| **98** | β-strand$_4$ | **1.0** | **0.9** | **1.0** | **0.9** | **0.9** |
| **99** | β-strand$_4$ | **0.9** | **0.8** | **0.9** | **0.8** | *1.0* |
| 107 | β-strand$_5$ | 0.2 | *b* | 0.3 | 0.2 | 0.2 |

[a] $\Delta\Delta G_{ex}$ calculated according to eq 1 or 7, from data measured at $p^2H_1$ as shown in Table 1. $\Delta\Delta G_{U\text{-}F}$ as shown in Table 2. Residues with $\Delta\Delta G_{ex}/\Delta\Delta G_{U\text{-}F}$ close to 1 are shown in boldface. Those with $\Delta\Delta G_{ex}/\Delta\Delta G_{U\text{-}F}$ close to 0 are underlined. $\Delta\Delta G_{ex}/\Delta\Delta G_{U\text{-}F}$ values in italics describe exchange behavior close to the site of mutation, which has been ignored in the analysis. [b] Accurate exchange rates could not be determined because of very fast exchange. [c] Very slow exchange, no decrease in intensity of peak after 14 weeks. [d] In C85-C102 the cross-peaks of residues 75 and 87 could not be deconvoluted.

globally exchanging residues was obtained. This is the limit of conditions where barnase will remain soluble at NMR concentrations.

*Stabilized Mutants C43-C80 and C85-C102.* These mutants are stabilized with respect to wild-type by 2.0 and 4.1 kcal mol$^{-1}$, respectively. Unlike wild-type protein, no deviation from EX2 kinetics is observed at 37 °C (Figure 6).

*Destabilized Mutants SA91 and NA58.* SA91 and NA58 are destabilized by 1.9 and 2.2 kcal mol$^{-1}$, respectively. Both these mutants have a destabilized transition state ($TS_{I\to F}$) and intermediate. Whereas locally exchanging protons exchange by an EX2 mechanism, globally exchanging protons of both proteins display a deviation from EX2 behavior at 33 °C, unlike the behavior of wild-type protein (Figure 7).

*Destabilized Mutant EG29.* EG29 is destabilized by an extent similar to SA91 and NA58 (see Table 2), and, like SA91 and NA58, the globally exchanging residues are seen

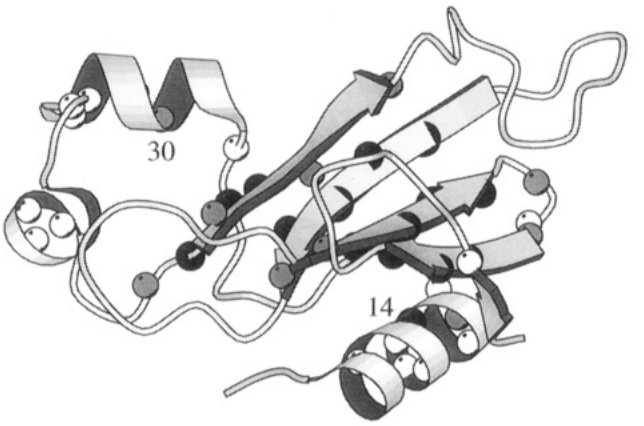


FIGURE 3: Structure of barnase. Values of $\Delta\Delta G_{ex}$ identify residues that exchange by local "breathing" (white), global unfolding (black), or a mixture of these mechanisms (gray). The site of residues (14 and 30) which anchor the helices into the hydrophobic cores are shown [figure prepared using the program Molscript (Kraulis, 1991)].

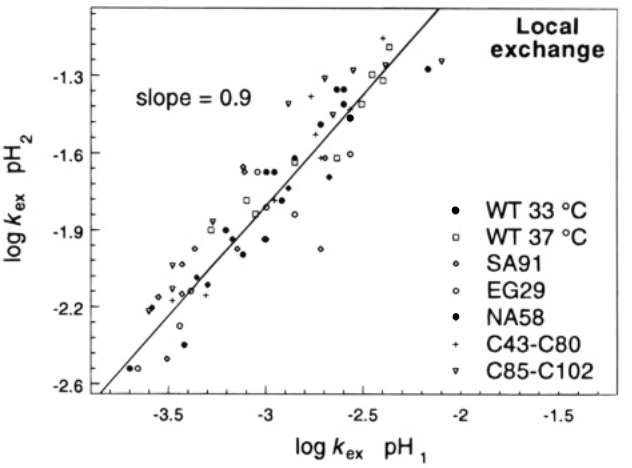


FIGURE 4: Plot of the pH dependence of exchange: locally exchanging residues for wild-type barnase and all mutants studied. For all residues here, log $k_{ex}$ varies linearly with pH under all conditions studied. The value of log $k_{ex}$ at $p^2H_1$ for each residue is plotted against log $k_{ex}$ at $p^2H_2$ for the same residue ($p^2H_1$ and $p^2H_2$ in each case are as shown in Table 1). A slope of 1 indicates exchange is within the EX2 limit. A slope of zero would indicate that the EX1 limit had been reached. Units of $k_{ex}$ are min$^{-1}$.

to deviate from EX2 at 33 °C (Figure 8). However, the deviation from EX2 is less pronounced for EG29. Where SA91 and NA58 have a destabilized intermediate and $TS_{I\to F}$, the intermediate of EG29 has stability close to that of wild type.

### *From Which State Does Exchange Occur?*

In the case of barnase, there are at least two states populated in solution from which global exchange might occur: the unfolded state (U), and the intermediate (I). Exchange from U will occur with the intrinsic rate constant. Exchange from I will be protected by any structure already formed in this state (Figure 2). The data are consistent with exchange taking place entirely from the unfolded state.

*$\Delta G_{ex}$ vs $\Delta G_{U\text{-}F}$.* The calculation of an absolute free energy of protection for a residue requires an accurate value of $k_{int}$ (eq 10). Apart from the approximations made to allow straightforward calculation of $k_{int}$ values, inaccuracies may also arise from the assumption that the unfolded state of a

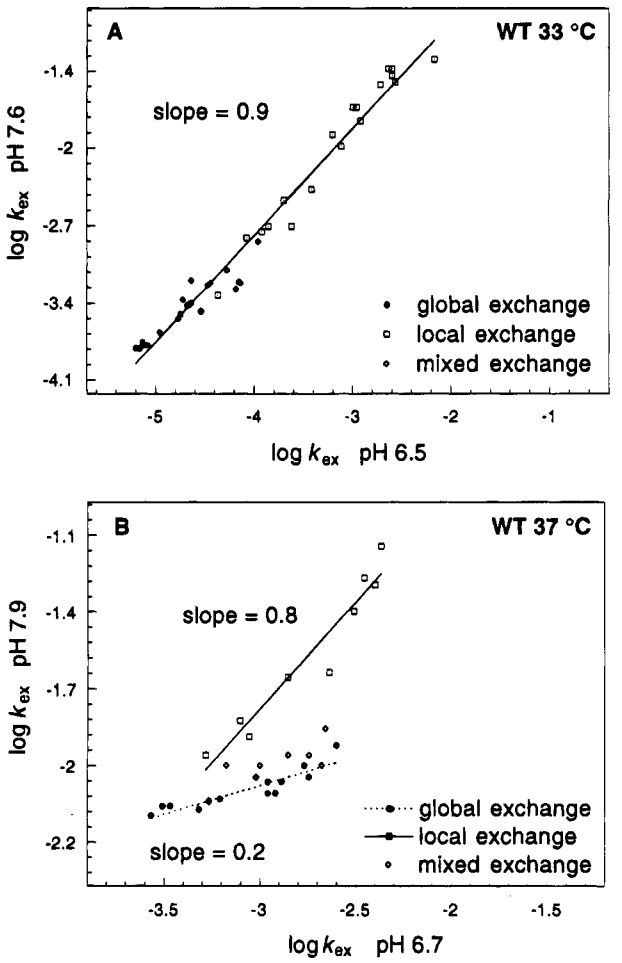


FIGURE 5: Plot of the pH dependence of exchange. (A) Wild-type barnase at 33 °C. No significant deviation from EX2 is observed. (B) Wild-type barnase at 37 °C. The globally exchanging residues show substantial deviation from EX2.

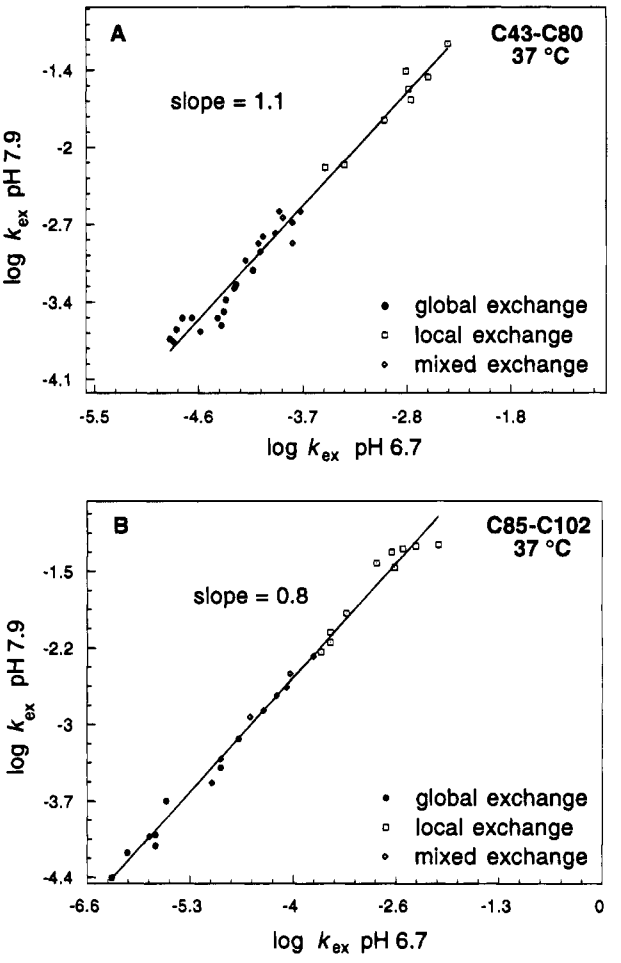


FIGURE 6: Plot of the pH dependence of exchange for stabilized mutants of barnase at 37 °C. (A) C43-C80. (B) C85-C102. No significant deviation from EX2 is observed.

protein can be compared to unstructured peptides: protection due to residual structure in the unfolded state of the protein will result in calculated $k_{int}$ values being too high.

For barnase, exchange studies have been performed on the pH denatured state (Arcus *et al.*, 1994). NOE values suggest that there are localized regions of residual structure present in the unfolded state. Thermodynamic studies on the acid denaturation of barnase and its dependence on ionic strength also suggest that the unfolded state likely to be present in water contains some residual structure or at least some degree of compactness (Oliveberg *et al.*, 1994). However, the protection factors obtained in that NMR study indicate that any residual structure is in rapid exchange with a more fully unfolded state, resulting in no residual protection being observed. The fact that >90% of protection factors obtained for the denatured state were between 0.7 and 3.5, and none greater than 7, indicates that the calculated values of $k_{int}$ are a true reflection of exchange rates from the unfolded state of barnase.

The result that the mean free energy of protection for the globally exchanging residues of wild-type barnase is the same as the free energy for unfolding as determined by calorimetry indicates that exchange of these residues is occurring from the fully unfolded state.

Values of $\Delta G_{op}$ ($\Delta G_{ex}$ for globally exchanging residues, eq 10) and $\Delta G_{den}$ ($\Delta G_{U\text{-}F}$ as determined from thermal denaturation in the exchange buffer) have been compared for other proteins. For BPTI, ribonuclease A, and cytochrome c (oxidized form), it was found that $\Delta G_{op}$ is greater than $\Delta G_{den}$ (Roder, 1989; Marmorino *et al.*, 1993; Mayo, 1993). In contrast, $\Delta G_{op}$ for staphylococcal nuclease is equal to $\Delta G_{den}$ within experimental error (Loh *et al.*, 1993). However the value of $\Delta G_{op}$ in the case of staphylococcal nuclease is obtained from three consecutive residues and so does not rule out the possibility of residual structure in other regions of the unfolded protein, whereas $\Delta G_{op}$ calculated for barnase is an average over 14 residues distributed across the length of the amino acid chain. This suggests that there is no significant residual protection in the unfolded state of barnase present at equilibrium in water, as was found for the acid denatured state.

*$\Delta\Delta G_{ex}$ vs $\Delta\Delta G_{U\text{-}F}$.* Under EX2 conditions, the values of $\Delta\Delta G_{ex}$ observed for all the global residues in all the proteins are close to the values of $\Delta\Delta G_{U\text{-}F}$ obtained from denaturation experiments (Table 2). This indicates that exchange is taking place from the same state in wild type and each of the mutants. SA91 and NA58 have destabilized intermediates compared with wild type, and C85-C102 has a stabilized intermediate. If a significant amount of exchange is occurring from the intermediate for any of the globally exchanging residues, we do not expect the value of $\Delta\Delta G_{ex}$ for that residue to be a simple reflection of the global stability of the proteins ($\Delta\Delta G_{U\text{-}F}$). Differentiation might be expected between residues protected by formation of the $\beta$-sheet, which forms early, and residues likely to be protected later: those

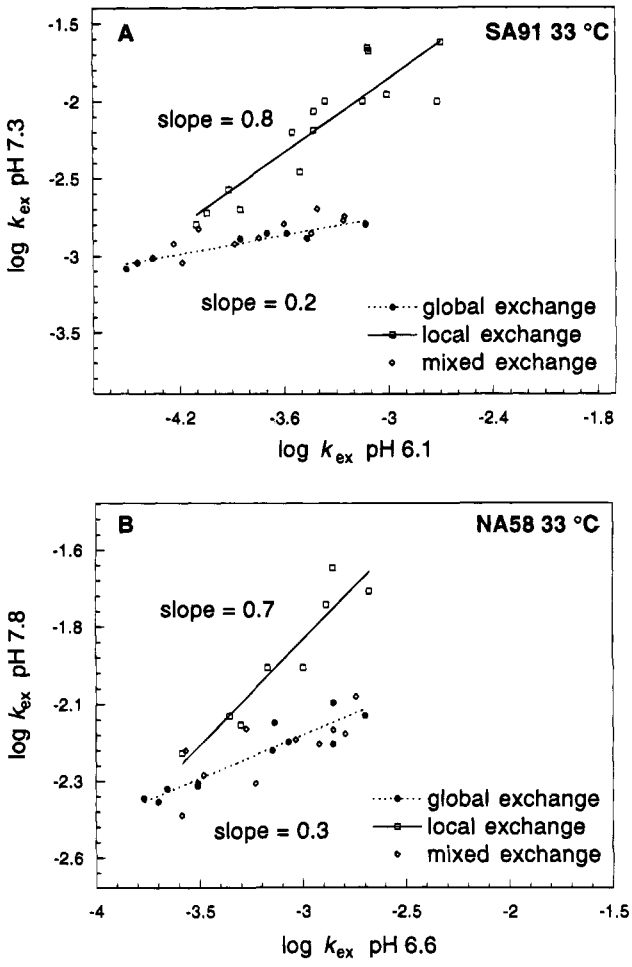


FIGURE 7: Plot of the pH dependence of exchange for destabilized mutants SA91 (A) and NA58 (B) at 33 °C. The globally exchanging residues show substantial deviation from EX2.

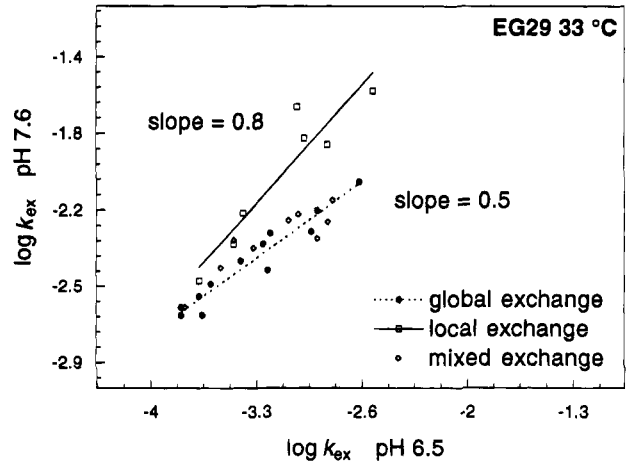


FIGURE 8: Plot of the pH dependence of exchange for destabilized mutant EG29 at 33 °C. The globally exchanging residues show deviation from EX2, but less than is observed for SA91 and NA58 (Figure 7).

protected by tertiary interactions (residues 25 and 50), by consolidation of the major hydrophobic core (residue 14), or by formation of the second hydrophobic core (residue 30). However, there is no differentiation between the different mutants in the behavior of any of these residues, indicating that global exchange is from the unfolded state. It appears that the intermediate may be a collapsed state, protecting these residues from exchange, although they are not at this stage involved in specific interactions.

*Conditions under Which Deviation from EX2 Occurs.* For barnase, the rate-determining step for folding is the transition from the intermediate state (I) to fully folded state (F). The rate constants for refolding ($k_{I\rightarrow F}$) for each of the mutants in this study are shown in Table 2. Deviation from EX2 will occur when the condition $k_c \gg k_{int}$ no longer holds (eq 2). Table 2 shows the range of calculated values of $k_{int}$ for the globally exchanging residues at the temperature and $p^2H$ of the higher pH experiment in each case (see Table 1).

The rate constant for refolding from intermediate to folded state, $k_{I\rightarrow F}$, for wild type, is 12 $s^{-1}$ at 33 °C and 10 $s^{-1}$ at 37 °C (M. Oliveberg and A.R.F., unpublished results). If exchange is occurring from I, i.e., $k_c \equiv k_{I\rightarrow F}$, then we would expect to see deviations from EX2 behavior at both 33 and 37 °C. That we do not suggests that the rate of reprotection is a faster process, involving the unfolded state refolding to a more protected state. The rate of refolding from the unfolded to the intermediate state could be this step. This rate constant has not been determined but is expected to be high since the process appears to be complete within the dead-time of stopped-flow experiments ($t_{1/2} < 1$ ms), unless it is the case that the probes used in the rapid reaction experiments (fluorescence, CD) are inadequate for observing the structural change U to I. Another possibility would be an alternative, faster folding pathway where the unfolded state refolds to the fully folded state without going via the folding intermediate.

*Is the Reprotection Step the Transition U→I?*

If the reprotection step is the transition U→I, and there is no temperature dependence of $\Delta\Delta G_{U\text{-}I}$, then C43-C80 and EG29 which have intermediates of similar stability to wild type would be expected to behave the same as wild type in terms of deviation from EX2 behavior of globally exchanging residues. What is observed is that EG29 shows deviation from EX2 under conditions where wild type shows none, and at higher temperature, where wild type shows deviation from EX2, the more stable C43-C80 remains within the EX2 limit. This suggests that EX2 behavior correlates not simply with the stability of the intermediate (or transition state) but also with the overall stability of the protein. Experiments are underway to obtain a more complete explanation of these results.

*Conclusions*

A series of mutants have been used to define which residues in barnase exchange by local fluctuations and which require global unfolding of the protein to exchange. Those residues that exchange by local "breathing" displayed EX2 kinetics in all mutants and conditions studied. Deviation from an EX2 mechanism could be observed for residues that exchange by global unfolding, when the temperature and pH were sufficiently high. EX1 conditions could not be unequivocally established for barnase. The extent of deviation from EX2 did not correlate to the overall rate of refolding of the protein, $k_{I\rightarrow F}$, but did seem to be related both to the stability of its intermediate and transition state ($TS_{I\rightarrow F}$) and to the overall stability of the protein.

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## REFERENCES


Arcus, V. L., Vuilleumier, S., Freund, S. M. V., Bycroft, M., & Fersht, A. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9412−9416.

Baghurst, P. A., Nichol, L. W., & Sawyer, W. H. (1972) *J. Biol. Chem. 10*, 3198−3204.

Bai, Y., Milne, J. S., Mayne, L., & Englander, S. W. (1993) *Proteins: Struct., Funct., Genet. 17*, 75−86.

Bax, A., Ikura, M., Kay, L. E., Torchia, D. A., & Tschudin, R. (1990) *J. Magn. Reson. 86*, 304−318.

Becktel, W. J., & Schellman, J. A. (1987) *Biopolymers 26*, 1859−1877.

Clarke, J., & Fersht, A. R. (1993) *Biochemistry 32*, 4322−4329.

Clarke, J., Hounslow, A. M., Bycroft, M., & Fersht, A. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9837−9841.

Connelly, G. P., Bai, Y., Jeng, M.-F., & Englander, S. W. (1993) *Proteins: Struct., Funct., Genet. 17*, 87−92.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Fersht, A. R. (1993) *FEBS Lett. 325*, 5−16.

Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188−190.

Gregory, R. B., Crabo, L., Percy, A. J., & Rosenberg, A. (1983) *Biochemistry 22*, 910−917.

Hermans, J., & Scheraga, H. A. (1959) *Biochim. Biophys. Acta 36*, 534−535.

Hvidt, A., & Nielsen, S. O. (1966) *Adv. Protein Chem. 21*, 287−386.

Jeng, M., & Englander, S. W. (1991) *J. Mol. Biol. 221*, 1045−1061.

Johnson, C. M., & Fersht, A. R. F. (1995) *Biochemistry 34*, 6795−6804.

Jones, D. N., Bycroft, M., Lubienski, M. J., & Fersht, A. R. (1993) *FEBS Lett. 331*, 165−172.

Kim, K.-S., & Woodward, C. (1993) *Biochemistry 32*, 9609−9613.

Kim, K.-S., Fuchs, J. A., & Woodward, C. K. (1993) *Biochemistry 32*, 9600−9608.

Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946−950.

Kresheck, G. C., Schneider, H., & Scheraga, H. A. (1965) *J. Phys. Chem. 69*, 3132−3144.

Lee, J. J., & Berns, D. S. (1968) *Biochem. J. 110*, 465−470.

Linderstrøm-Lang, K. (1955) *Chem. Soc. (London), Spec. Publ. 2*, 1−20.

Loh, S. N., Prehoda, K. E., Wang, J., & Markley, J. L. (1993) *Biochemistry 32*, 11022−11028.

Marmorino, J. L., Auld, D. S., Betz, S. F., Doyle, D. F., Young, G. B., & Pielak, G. J. (1993) *Protein Sci. 2*, 1966−1974.

Matouschek, A., Serrano, L., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 819−835.

Matouschek, A., Matthews, J. M., Johnson, C. M., & Fersht, A. R. (1994) *Protein Eng. 7*, 1089−1095.

Mauguen, Y., Hartley, R. W., Dodson, E. J., Dodson, G. G., Bricogne, G., Chothia, C., & Jack, A. (1982) *Nature 297*, 162−164.

Maybury, R. H., & Katz, J. J. (1956) *Nature 177*, 629−630.

Mayo, S. L. (1993) *Science 262*, 873−876.

Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150−158.

Mossakowska, D. E., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 3843−3850.

Oliveberg, M., Vuilleumier, S., & Fersht, A. R. (1994) *Biochemistry 33*, 8826−8832.

Pace, C. N., Laurents, D. V., & Erickson, R. E. (1992) *Biochemistry 31*, 2728−2734.

Pedersen, T. G., Thomsen, N. K., Andersen, K. V., Madsen, J. C., & Poulsen, F. M. (1993) *J. Mol. Biol. 230*, 651−660.

Roder, H. (1989) *Methods Enzymol. 176*, 446−473.

Roder, H., Wagner, G., & Wüthrich, K. (1985) *Biochemistry 24*, 7396−7407.

Serrano, L., Horovitz, A., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *Biochemistry 29*, 9343−9352.

Skelton, N. J., Kördel, J., Akke, M., & Chazin, W. J. (1992) *J. Mol. Biol. 227*, 1100−1117.

Wagner, G., & Wüthrich, K. (1979) *J. Mol. Biol. 130*, 31−37.

Woodward, C., Simon, I., & Tuechsen, E. (1982) *Mol. Cell. Biochem. 48*, 135−160.


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