Absence of stable intermediates on the folding pathway of barnase

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Barnase is one of the few protein models that has been studied extensively for protein folding. Previous studies led to the conclusion that barnase folds through a very stable submillisecond intermediate (~3 kcal/mol). The structure of this intermediate was characterized intensively by using a protein engineering approach. This intermediate has now been reexamined with three direct and independent methods. (i) Hydrogen exchange experiments show very small protection factors (~2) for the putative intermediate, indicating a stability of ~0.0 kcal/mol. (ii) Denaturant-dependent unfolding of the putative intermediate is noncooperative and indicates a stability less than 0.0 kcal/mol. (iii) The logarithm of the unfolding rate constant of native barnase vs. denaturant concentrations is not linear. Together with the measured rate ("app", calculated as

\[ \text{app} = \frac{k_u}{k_i} \]

this nonlinear behavior accounts for almost all of the protein stability, leaving only about 0.3 kcal/mol that could be attributed to the rapidly formed intermediate. Other observations previously interpreted to support the presence of an intermediate are now known to have alternative explanations. These results cast doubts on the previous conclusions on the nature of the early folding state in barnase and therefore should have important implications in understanding the early folding events of barnase and other proteins in general.

Barnase folds in multiple kinetic phases at pH 6.3 and 25°C (1). A submillisecond burst phase is observed (2). Approximately 80% of the molecules then fold in a fast single exponential phase with a folding rate constant \( k_i \) of ~13 s\(^{-1}\). The other 20% folds slowly because of cis–trans isomerization of prolines (1). In the earlier studies of the folding pathway of barnase, Fersht and coworkers (1) concluded that the major folding phase represented the folding from a stable submillisecond intermediate to the native state. This conclusion was based on the following experimental results. (i) There was a loss of CD signal within the submillisecond burst phase (2), consistent with a fast intermediate. (ii) The log \( k_i \) (folding rate constant) as a function of denaturant concentrations is not linear (1), as illustrated in the chevron plot in Fig. 1, consistent with an on-pathway intermediate. (iii) An H/D pulse-labeling experiment revealed strong protections for some amide protons within 6 ms, suggesting a very stable intermediate (3). (iv) The stability for the intermediate was calculated to be 3.2 kcal/mol, taken as the difference between the equilibrium unfolding free energy for native barnase (\( \Delta G_{\text{u}}^{\text{eq}} \)) and the free energy for the major folding phase, \( \Delta G_{\text{f}}^{\text{pp}} \), calculated as

\[ \Delta G_{\text{f}}^{\text{pp}} = -RT \ln(k_u/k_i) \]

Here, \( k_u \) is the unfolding rate constant extrapolated from the right limb of the chevron plot assuming a linear relationship between log \( k_u \) and denaturant concentrations (see illustrations in Fig. 1).

Recent progress in protein-folding studies questioned these conclusions. (i) The burst phase signal lost within submilliseconds may simply represent the conformational readjustment of the unfolded state to lower denaturant concentrations as suggested for cytochrome c and ribonuclease A (4, 5). (ii) Alternative interpretations such as movement of the transition state ensemble can explain curved chevron plots, as proposed for Arc repressor (6) and U1A (7). (iii) A more recent hydrogen-exchange (HX) pulse-labeling experiment could not reproduce the earlier barnase results and failed to detect any stable intermediate (8). (iv) A native-state HX experiment also failed to reveal the postulated intermediate (9). (v) The correct extrapolation of log \( k_u \) may curve downward at low denaturant concentrations, as has been shown for several proteins including Arc repressor (6), U1A (7), protein L (10), and CI2 (11). This nonlinear behavior would increase the calculated \( \Delta G_{\text{f}}^{\text{pp}} \) for the major folding phase and decrease the calculated stability left over for the putative intermediate. These alternative interpretations and new experimental results suggest that the earlier conclusion needs to be reexamined by using more direct methods. Such a reexamination is important, because barnase has been taken as a classical illustration of how proteins fold, and its intermediate has been characterized intensively with a protein-engineering approach (12). In particular, a definite conclusion on the postulated intermediate of barnase will help to understand the ability and limitations of both the protein-engineering and the native-state HX approaches (1, 13). This article reports a reexamination of these issues.

Materials and Methods

Materials. Proteins and buffer solutions were prepared as described (8).

Folding and HX Competition Experiments. The folding-HX competition experiments (14) were performed with a Biologic QFM-4 (Grenoble, France; dead time ~1 ms). Folding of denatured barnase (~10 mg/ml) in 320 M deuterated GdmCl solution (pH 6.3, 10 mM Mes/D2O) was initiated by a 20-fold dilution with a buffer solution (pH 6.3, 50 mM Mes/H2O) at 25°C. Reactions were allowed to proceed for several minutes before pH was adjusted to 5.0 with 0.5 M HOAc. Solutions (50 ml) were concentrated, and buffer was exchanged with a 10% (vol/vol) D2O and 10 mM NaOAc solution with Amicon cells. The same experiment was performed at pH 7.23 (0.2 M K2HPO4/0.1 M Na2SO4). The 1H–15N heteronuclear sequential quantum correlation NMR spectra were collected on a Bruker AMX 500 spectrometer (Billerica, MA) and processed with Felix (version 97, Biosym Technologies, San Diego). Peak intensities were used to measure the proton occupancies with amide protons Q104, R110, and W94 (4NH) averaged as internal references. Calculations of exchange rate constants, \( k_{\text{ex}} \), and protection factors (\( P_i = k_{\text{int}}/k_{\text{ex}} \)) used Eq. 5 with 8 s\(^{-1}\) for \( k_i \) and intrinsic exchange rate constant, \( k_{\text{int}} \), based on peptide models (15).

Kinetic and Equilibrium CD and Fluorescence. Stopped-flow CD experiments were performed with the Biologic SFM4. Up to 30 kinetic traces with a 1.0-cm path-length observation cell were averaged for each data point. Kinetic runs were initiated by mixing denatured barnase (pH 1.6 or 9.4 M urea) with folding

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Abbreviations: GdmCl, guanidinium chloride; HX, hydrogen exchange.

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buffer (pH 6.3, 50 mM Mes) with a final protein concentration of 14.6 μM (extinction coefficient 25,900 M⁻¹-cm⁻¹ at 280 nm). Stopped-flow fluorescence was performed similarly at ≈3 μM protein concentration.

Three-State Chevron Curve Fitting. Chevron curves were fitted according to Eqs. 1–3 assuming U and I are in rapid equilibration, following Raschke and Marqusee (16).

\[ k_{II} \quad k_{IN} \]
\[ U \rightleftharpoons I \rightleftharpoons N \]
\[ k_{IU} \quad k_{NI} \]

and

\[ k_{obs}(I) = \frac{1}{[1 + K_{IU}H_2O^+e^{m_{IN}(Den)}]}k_{IN}H_2O^+e^{m_{IN}(Den)} + k_{II}H_2O^+e^{m_{IN}(Den)} \]

Intermediate Melting Curve Fitting. The unfolding equilibrium constant of the intermediate in water; \( K_{IU} \) and \( K_{II} \) are the parameters of the baselines for the intermediate (I) and unfolded (U) states, respectively; and \( m_{IN} \) is the coefficient for the denaturant dependence of the ln \( K_{IU} \). All curve fittings were carried out with SIGMAPLOT (SPSS, Chicago).

Results

Principles for Measurement of the Stability of the Early Folding State from HX Protection Factors. The stability of the early folding state of barnase can be determined based on the protection factors of the slowly exchanging amide protons, because 13 amide protons of barnase exchange with solvent protons only through global unfolding (9, 18). Persht and coworkers have shown: (i) the averaged \( \Delta G_{f} \) values for these protons are in excellent agreement with the \( \Delta G_{N} \) values measured by differential scanning calorimetry (18), where \( \Delta G_{f} \) is defined as \( -RT\ln(k_{ex}/k_{im}) \); (ii) the changes of the global unfolding free energy (\( \Delta \Delta G_{N} \)) are the same as \( \Delta \Delta G_{f} \) between wild-type barnase and the mutants for the 13 amide protons (18); and (iii) the HX rates of these amide protons are accelerated at very low concentrations of GdmCl (9). These results require that no exchange occurs for the 13 amide protons through any structural fluctuations in N or any intermediate state, other than the fully unfolded state (9, 18). Thus, the \( P_{f} \) measured after the intermediate is formed for these protons should reflect the stability (I to U) of the proposed intermediate state with the relationship between \( \Delta G_{f} = -RT\ln[1/(P_{f} - 1)] \) (19). For example, if the proposed folding intermediate of barnase has a stability of ≈5.0 kcal/mol as suggested previously (1), all of the 13 amide protons would be protected by a factor of >100 in the intermediate.

The Protection Factors of the Amide Protons in the Early Folding State of Barnase Measured by Competition HX. To explore the stability of the proposed intermediate, we directly measured HX rate constants of amide protons in the early folding state of barnase by using the competition HX method (14). In this experiment, unfolded barnase in D2O and denaturant was diluted with H2O solution to initiate folding and HX reactions. Folding was allowed to proceed for several minutes to completion. In the native state, many amide protons are protected from exchange for weeks, which allows the fractions of the \( {\ ^{1}\!\!H} \ [P_{occ}(H)] \) exchanged during the HX-folding process to be measured using NMR from folded barnase. Because the stability of barnase decreases significantly, and the chevron plots became less curved at pHs above 7.5 (20), we performed competition experiments at two different pHs: 6.3 and 7.23. These experiments used the following relationship among \( k_{ex} \), \( k_{II} \), and \( P_{occ}(H) \) for barnase when the competition proceeded until native proteins were formed completely (14):

\[ P_{occ}(H) = 0.8 k_{ex}/(k_{ex} + k_{II}) + 0.2 \]

The factors 0.8 and 0.2 represent the fraction of the \( trans \) (fast folding) and \( cis \) (slow folding) proline isomers in the unfolded state, as determined by Matouschek et al. (1). If one measures \( P_{occ}(H) \) and \( k_{ex} \), then \( k_{II} \) can be calculated (Eq. 5), and \( P_{f} \) can be obtained (\( P_{f} = k_{im}/k_{ex} \)). At pH 6.3 and 25°C, 3 (K19, S50, and S91) of the 13 amide protons have large \( k_{im} \) (13, 10, and 9 s⁻¹, respectively), which is comparable to the \( k_{ex} \) (8 s⁻¹) of barnase at 0.16 M GdmCl. Therefore, the \( k_{ex} \) of these protons can be measured accurately in the competition experiment. The \( P_{f} \) values for the three amide protons K19, S50, and S91 were 1.7, 1.5, and 1.1, respectively. These numbers are typical of amide protons in unfolded polypeptide chains (15).

The same competition experiment was performed at a slightly higher pH (7.29) in the presence of 0.1 M Na2SO4. The stabilizing salt ensures that the proposed intermediate, if it exists,
should be more stable. This further stabilization is illustrated by
the right shift of the folding limb of the chevron plot (Fig. 1). At
pH 7.23, the HX protection of many additional amide protons
can be measured directly. All of the amide protons that could be
identified and resolved in the two-dimensional $^1$H--$^{15}$N correla-
tion spectrum (21) have $P_f$ values between 0.5 and 2.5, except for
one close to 4.5 (Fig. 2). Similar results were obtained in the
presence of 0.1 M Na$_2$SO$_4$ at 0.0 and 0.36 M urea and at higher
concentrations of Na$_2$SO$_4$ (up to 0.4 M, unpublished results).
Importantly, the 13 amide protons are not protected more than
others. The average $P_f$ for the slowly exchanging amide protons
is about 2. This value leads to a $K_{IU}$ of 1.0 and a $\Delta G_{IU}$ value of
$\approx -0.0$ kcal/mol.

**Noncooperative Melting of the Early Folding State by Urea.** The
stability of the early folding state of barnase was also explored
directly by using CD and fluorescence. For barnase, the strongest
CD signal in the far UV region at wavelength of 230 nm provides
the information of secondary structures (2). The fluorescence
signal, excited at 280 nm and collected above 310 nm, reflects the
environment of the tryptophan residues in barnase. We mea-
sured the amplitude of these signals for the early folding state
from the kinetic traces for barnase folding at 10 ms as a function
of urea concentration by using a stopped-flow apparatus. By 10
ms, all of the burst phase intermediate is formed, and almost
none has gone forward to $N (k_t \approx 13 s^{-1})$. The urea dependence
of the amplitude therefore reflects how the proposed interme-
diate of barnase unfolds by denaturant at pH 6.3 and 25°C. Two
examples of the kinetic traces are shown in Fig. 3 A and B. The

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**Fig. 2.** HX protection factors in the early folding state. The filled bars are the
$P_f$ values of the 13 amide protons. The asterisks indicate the averaged $P_f$
measured at both pH 6.3 and 7.23. Others in open bars are at pH 7.23.

**Fig. 3.** Noncooperative unfolding of the postulated intermediate. Kinetic traces generated by the stopped-flow CD at 0.94 M urea (A) and fluorescence in water
(B) at pH 6.3 and 25°C. The values at 10 ms of folding as indicated by the arrows at different urea concentrations were plotted in C (solid circles) and D (solid
circles). The global unfolding of barnase is shown with the dotted line. The curved solid line is the fitting curve with a two-state model with parameters for both
pretransition and posttransition baselines (see Materials and Methods). The dashed line represents the posttransition baseline. Mdeg, millidegree.
values measured (shown in Fig. 3 C and D) did not show a sigmoid type of cooperative transition, although small deviations from linearity are observable. These results are very similar to the behavior of nonfolding cytochrome c fragments (4) and the unfolded, disulfide-broken state of ribonuclease A (5), suggesting that the early folding state of barnase may not be a discrete folding intermediate. The change in CD and fluorescence signals within submilliseconds may simply represent the conformational readjustment of the unfolded state under different solvent conditions. When the CD values were fitted to a two-state cooperative unfolding model with floating parameters for both pretransition and posttransition baselines (see Eq. 4), we obtained a value of 2.6 for $K_{1U} = [U]/[I]$, indicating that the putative intermediate state is even less stable than the unfolded state by 0.6 kcal/mol. This value is expected from an incomplete transition curve (Fig. 3C).

**Direct Observation of Nonlinear Behavior of log $k_o$ vs. GdmCl Concentrations.** If the postulated intermediate is not as stable as proposed previously or does not exist, one expects that $\Delta G_{\text{NU}}$, measured by equilibrium melting, will be equal to $-RT \ln (k_o/k_i)$. This expectation is consistent with measured rates (Fig. 1) only if log $k_o$ is downward curved at lower denaturant concentrations. A reanalysis of the previous native-state HX results by Clarke and Fersht (9) indicated that this nonlinear behavior is indeed the case (Fig. 4).

Because the 13 slowly exchanging amide protons can exchange only through global unfolding, their exchange process (in D$_2$O) can be written as in reaction scheme (6) with the intermediate state and the unfolded state included in the effective unfolded state and the unfolded state included in the effective unfolding model. When the CD values were fitted to a two-state cooperative model with floating parameters for both pretransition and posttransition baselines (see Eq. 4), we obtained a value of 2.6 for $K_{1U} = [U]/[I]$, indicating that the putative intermediate state is even less stable than the unfolded state by 0.6 kcal/mol. This value is expected from an incomplete transition curve (Fig. 3C).

**Table 1. $\Delta G_{\text{HX}}$ values for the slowly exchanging amide protons at 0.0 M GdmCl, 30°C, pH 6.8 (pD$_{\text{read}}$ + 0.4) in D$_2$O**

<table>
<thead>
<tr>
<th>Residue number</th>
<th>$k_{\text{ex}}$, s$^{-1}$</th>
<th>$k_{\text{int}}$, s$^{-1}$</th>
<th>$\Delta G_{\text{HX}}$, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>$2.83 \times 10^{-7}$</td>
<td>7.3</td>
<td>10.3</td>
</tr>
<tr>
<td>50</td>
<td>$4.27 \times 10^{-7}$</td>
<td>8.5</td>
<td>10.1</td>
</tr>
<tr>
<td>52</td>
<td>$1.63 \times 10^{-7}$</td>
<td>3.0</td>
<td>10.1</td>
</tr>
<tr>
<td>98</td>
<td>$1.77 \times 10^{-7}$</td>
<td>2.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>10.1</td>
</tr>
</tbody>
</table>

Here, $k_{\text{ex}}$ and $k_{\text{int}}$ are the effective exchange rate constant and the effective rate constant of generating the native state, respectively. The measured exchange rate constant $k_o$ can be written as $k_{\text{ex}} = k_o k_{\text{int}}/(k_o^{\text{eff}} + k_{\text{int}}^{\text{eff}})$. It follows that $k_{\text{ex}} = k_o$ if $k_{\text{int}}^{\text{eff}} >> k_o^{\text{eff}}$ (EX1 mechanism) and $k_{\text{ex}} = k_o/k_{\text{int}}^{\text{eff}} = K_u$ if $k_{\text{int}}^{\text{eff}} << k_o^{\text{eff}}$ (EX2 mechanism; see discussions in ref. 22). Simply stated, equilibrium HX experiments provide a means to measure the unfolding rate constant $k_u$ when HX occurs through EX1 mechanism and the global unfolding equilibrium constant $K_u$ when HX occurs through an EX2 mechanism.

In the native-state HX experiments on barnase at pH 6.8 (pD$_{\text{read}}$ + 0.4) and 30°C, it was demonstrated that the 13 slowly exchanging amide protons exchanged with solvent protons by an EX2 mechanism at 0.0 M GdmCl and by an EX1 mechanism at above 0.51 M GdmCl (9). These conclusions were based on the following findings. (i) The HX rate constants of these amide protons were sensitive to pH changes at 0.0 M GdmCl but became independent of pH at GdmCl concentrations higher than 0.51 M GdmCl. (ii) The HX rate constants were different for different amide protons at 0.0 M GdmCl, but they converged to the same value at above 0.51 M GdmCl (Fig. 4). Therefore, at 0.0 M GdmCl, the $\Delta G_{\text{HX}}$ for these amide protons should represent the global unfolding free energy $\Delta G_{\text{NU}}$. Above 0.51 M GdmCl, the measured $k_u$ should represent $k_o$.

Table 1 lists the $\Delta G_{\text{HX}}$ values for the four slowly exchanging amide protons measured at 0.0 M GdmCl. The averaged value is 10.1 kcal/mol. Fig. 4 shows that the log $k_o$ at 0.0 M GdmCl extrapolated from the HX rate constants measured under EX1 mechanism (above 0.7 M GdmCl) is $-5.92$. Because the directly measured folding rate constant $k_f$ is 14.7 s$^{-1}$, the $\Delta G_{\text{app}}$ [$-RT \ln (k_o/k_i)$] for barnase at 0.0 M GdmCl is calculated to be 9.8 kcal/mol. This number is only 0.3 kcal/mol smaller than the value 10.1 kcal/mol of the averaged $\Delta G_{\text{HX}}$ for the slowly exchanging amide protons (Table 1). If a linear extrapolation is used to obtain a log $k_o$ in D$_2$O from the unfolding values of the right limb of the chevron plot (Fig. 4) as done by Matouschek et al. (1), the value of log $k_o$ is $-4.3$. This value leads to a $\Delta G_{\text{app}}$ of 7.6 kcal/mol, which is 2.5 kcal/mol less than the global unfolding free energy 10.1 kcal/mol. The missing stability was postulated by Matouschek et al. (1) to represent the stability of the early intermediate. However, the...
directly measured nonlinear \( k_u \) shown in Fig. 4 explains the missing stability and leaves only 0.3 kcal/mol for the postulated intermediate.

Matouschek et al. (1) initially used an unstable mutant (L14A) to obtain \( k_u \) values down to 0.5 M urea at pH 3.0 to justify the linear extrapolation. A linear behavior was observed in the range between 0.5 M and 3.5 M urea for this mutant. However, the slope shown from this linear fitting was clearly larger than that obtained between 3.7 M and 4.7 M urea for this mutant. The value was obtained by fitting the CD signals (Fig. 3C) to a two-model state with floating parameters for both pretransition and posttransition baselines (Eq. 4). If \( k_D \) is fixed with a value of 0.25 (\( \Delta G_U = 0.8 \) kcal/mol), the fitting yields an \( m_{HU} \) value that is 60% of the \( m_{HU} \) value for global unfolding (20). This value is significantly larger than the 43% value obtained from the chevron curve fitting (see Fig. 1), indicating that the \( \Delta G_U \) is unlikely larger than 0.8 kcal/mol.

This value was obtained assuming an average protection factor of 2 (Fig. 2).

Simulation studies that use Eq. 5 show that a \( \pm 10\% \) error in \( P_{\text{nat}}(H) \) measurement may contribute an error less than a factor of 2 in \( P_h \), which may contribute an uncertainty in \( \Delta G_{IU} \) by \( \pm 0.4 \) kcal/mol.

The value was calculated from \( \Delta G_{U-HX}^\text{mix} - \Delta G_{U-HX}^\text{mix} \) (see text) based on the HX data of Clarke and Fersht (9). Possible uncertainties in \( k_u \) by a factor of 2 may contribute another error in free energy by 0.4 kcal/mol. Thus, the \( \Delta G_{U-HX} \) determined from both HX experiments are unlikely larger than 0.8 kcal/mol.

### Table 2. Stability of the proposed intermediate of wild-type barnase measured by different methods in kcal/mol at pH 6.3 and 25°C

<table>
<thead>
<tr>
<th>Method</th>
<th>Competition HX</th>
<th>Native-state HX</th>
<th>Indirect methods*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta G_{IU} )</td>
<td>-0.6</td>
<td>-0.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Values are 2.8 kcal/mol from Dalby et al. (24), 2.7 kcal/mol from the chevron plot fitting that used a three-model state with fixed intermediate and transition state (see Materials and Methods and Fig. 1), and 3.2 kcal/mol from Matouschek et al. (1).

The value was obtained by fitting the CD signals (Fig. 3C) to a two-model state with floating parameters for both pretransition and posttransition baselines (Eq. 4). If \( k_D \) is fixed with a value of 0.25 (\( \Delta G_U = 0.8 \) kcal/mol), the fitting yields an \( m_{HU} \) value that is 60% of the \( m_{HU} \) value for global unfolding (20). This value is significantly larger than the 43% value obtained from the chevron curve fitting (see Fig. 1), indicating that the \( \Delta G_U \) is unlikely larger than 0.8 kcal/mol.

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### Possible Interpretations for the Curved Chevron Plot of Barnase

The significant curvature in the folding limb of the chevron plot of barnase was concluded to be due to the population of a stable intermediate by Matouschek et al. (1). This interpretation, however, is questionable and contrary to the experimental results presented in this article. Two alternative models have been proposed to account for the curvature in chevron plots. (i) Movement of the transition state ensemble as a function of denaturant alone may be responsible for the curved chevron plot. This model was suggested by Otzen et al. (7) and was used to explain the curved chevron plot of U1A. The curved log \( k_u \) shown in Fig. 4 seems to be consistent with this model. Movement of the transition state ensemble toward the unfolded state as protein stability increases is also expected by the Hammond postulate and was observed from earlier mutation studies on barnase (25, 26). (ii) The early folding state of barnase may be an ensemble of differently collapsed unfolded states. A redistribution of this ensemble as a function of denaturant concentrations may be responsible for the curvature in the chevron plot of barnase. This model was used to explain the curved chevron plots of several proteins by Parker and Marqusee (27). A combination of i and ii is also possible.

### Native-State HX and Protein Engineering Approaches

Clarke et al. (28) questioned the usefulness of the native-state HX approach as an analytical tool to study folding pathways of proteins. They concluded that the native-state HX approach was not a reliable approach, because it was not able to identify the very stable intermediate of barnase that has been studied fully by the protein-engineering approach and the H/D pulse-labeling methods. Previously, we found that the H/D pulse-labeling experiment by Bycroft et al. (3) could not be reproduced, and our recent H/D pulse-labeling experiment did not detect any stable intermediate (8). The experimental results presented herein, including (i) the small HX protection factors, (ii) the noncooperative melting of the early folding state by urea, and (iii) the downward curved log \( k_u \), are all consistent

### Discussion

Taken together, (i) the directly measured small HX protection factors, (ii) the noncooperative unfolding behavior of the putative early folding state, and (iii) the downward shifted unfolding rate constants at very low GdmCl concentrations all argue that a stable folding intermediate with a stability of \( \approx 3 \) kcal/mol does not exist on the folding pathway of wild-type barnase. The proposed intermediate, if it exists, is unlikely to be more stable than the unfolded state by 0.8 kcal/mol (see footnotes in Table 2 for error estimation).

### Uncertainties in the Earlier Characterization of the Intermediate of Barnase

Matouschek et al. (1, 12) used the protein engineering approach to determine side-chain interactions in the proposed intermediate state of barnase. This approach measures the changes of the unfolding free energy of the intermediate (\( \Delta G_{IU} \)) and the global unfolding free energy (\( \Delta G_{NU} \)) between the wild-type protein and mutants. A \( \phi_1 \) value, defined as \( \Delta G_{IU}/\Delta G_{IU} \), was used to indicate the extent of the interactions between the residue that is mutated and other residues in the intermediate. If the \( \phi_1 \) value is 1.0, it suggests that the mutated residues interact with other residues in the intermediate state as strongly as in the native state. If the \( \phi_1 \) value is zero, then the interactions are as weak as in the unfolded state. A fractional \( \phi_1 \) value represents partial interactions. Because the stability of the intermediate state of wild-type barnase is less than 0.8 kcal/mol, the experimentally measured \( \Delta G_{IU} \) for any mutant should not exceed this value. Thus, it provides a criterion to test the accuracy of the earlier measurements. A large number (more than 40%) of \( \Delta G_{IU} \) values measured in the earlier studies by Matouschek et al. (12) were larger than 0.8 kcal/mol (see Table 2 in ref. 12). Some of them were even larger than 2 kcal/mol. These large values suggest that there are large uncertainties in the \( \Delta G_{IU} \) and \( \phi_1 \) values and in the structure of the intermediate characterized based on them. The large \( \Delta G_{IU} \) values obtained by Matouschek et al. (1, 12) seem to result from the assumption that the measured log \( k_u \) vs. denaturant concentration, which must be extrapolated to zero denaturant, has similar linear behavior for both wild-type and mutant proteins. That is, the computed \( \phi_1 \) values incorporate the same (apparently incorrect) assumption that leads to an estimated 3 kcal/mol in stability for the supposed intermediate.
with the native-state HX results, i.e., no stable intermediate seems to exist.

To conclude, the initial burst phase folding of barnase seems to reach the ensemble of forms with some characteristics that one may call the unfolded state under the conditions of the folding experiment (4, 5). Whether particular interactions or structures exist in this ensemble that help to predetermine the folding pathways remains to be seen (29). We show that any such interactions provide no net favorable free energy to the overall ensemble.

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