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Relationship between the Native-State Hydrogen Exchange and the Folding Pathways of Barnase

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ABSTRACT: The previous native-state hydrogen exchange experiment with barnase failed to detect any partially unfolded intermediate state which was contrary to the experimental results from kinetic deuterium hydrogen exchange pulse labeling and protein engineering studies. This has been taken to suggest that the native-state hydrogen exchange method cannot be used alone as an analytical tool to study the folding pathways of proteins. Here, we revisited the pulse labeling experiment with barnase and detected no stable folding intermediate. This finding allows a reconciliation of the native-state HX data and the folding pathway of barnase. Along with alternative theoretical interpretations for a curved chevron plot of protein folding, these data suggest that further investigation of the nature of the intermediate of barnase is needed.

How proteins fold still remains an unsolved problem in biochemistry. To understand the mechanism of protein folding, pathways or energy landscapes of protein folding should be studied in detail. Because of the potential power of equilibrium amide hydrogen exchange (HX) in identifying highly energetic species of proteins under native conditions, considerable efforts have been made to establish the relationship between equilibrium HX and folding pathways of proteins (*1*, *2*). A native-state HX method was recently proposed to relate the pattern of the HX free energies, $\Delta G_{HX} = -RT \ln(k_{ex}/k_{int})$, of amide protons under low concentrations of denaturant to partially unfolded forms of proteins (*3*−*6*), where k_{ex} is the measured exchange rate constant and k_{int} is the intrinsic exchange rate constant in unstructured polypeptides (*7*). It has also been suggested that the residues with slowly exchanging amide protons fold early on the kinetic folding pathways (*8*). However, native-state HX experiments with barnase failed to detect the partially unfolded intermediate that was observed in the previous D−H pulse labeling experiment and the protein engineering studies (*9*−*11*). It was, therefore, concluded that the native-state HX method cannot be used alone as an analytical tool to study protein folding pathways (*12*). Further, it was emphasized that equilibrium HX experiments cannot provide information about the order of a folding reaction in theory (*9*, *12*). There was an open exchange of opinions among Englander, Woodward, Fersht, and their co-workers on these issues (*13*−*15*). Despite these discussions, no consensus on the validity of the native-state HX method alone in studying the folding pathways of proteins was reached.

Although early folding events in a kinetic folding reaction may not be simply predicted by the proposal of Woodward (as stated) (*8*), a relationship between equilibrium HX data and stable folding intermediates does exist for the amide protons that have ΔG_{HX} values equal to or larger than the global unfolding free energy ΔG_{NU} under the EX2 condition (*3*). Simply stated, these amide protons must be protected

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from direct exchange with solvent protons in any stable intermediate populated in the kinetic folding experiment. This statement is self-evident by definition since if they were not protected in the stable intermediate state, the ΔG_{HX} values measured for these amide protons would be equal to the unfolding free energy of the intermediate state ΔG_{NI} under EX2 conditions (*3*). Because the intermediate is more stable than the unfolded state, ΔG_{NI} must be smaller than ΔG_{NU}. Therefore, one would expect the ΔG_{HX} ($=\Delta G_{NI}$) values of these amide protons to also be smaller than ΔG_{NU}. Any HX process due to local structural fluctuations will lead to an even smaller ΔG_{HX}. For barnase, 13 such amide protons have been found at pH 6.3 and 25 °C (*9*, *16*, *17*). Therefore, they can serve as diagnostic probes for understanding the relationship between equilibrium HX and the folding pathway of barnase.

The conclusion that a stable (~3 kcal/mol) folding intermediate is populated in the sub-millisecond time scale on the folding pathway of barnase was based on the curved chevron plot and D−H pulse labeling experiment of Matouscheck et al. (*10*) and Bycroft et al. (*11*). In that pulse labeling experiment, it was found that several amide protons (Y13, A32, E73, and Y97) were completely protected within 6 ms. However, many amide protons were protected with time constants in 10−100 ms in a multiphastic way, indicating multiple intermediates were formed on the time scale that is longer than 10 ms. In particular, two of the amide protons (Ile25 and Ser50) with a ΔG_{HX} equal to ΔG_{NU} were not protected in any kinetic intermediate state (*10*, *16*). These complicated and controversial results have created a puzzling picture in the literature on how barnase folds and on the relationship between the native-state HX and the folding pathway of barnase (*18*). To resolve these discrepancies, we have revisited the pulse labeling experiment with barnase and found no stable intermediate state.

MATERIALS AND METHODS

Materials. Barnase was expressed in XL1-blue *Escherichia coli* cells using plasmid pMT 1002 (a gift from B. Hartley, National Institutes of Health) in M9 media for both normal and ^{15}N-labeled proteins. The purification procedure followed that of Serrano and Fersht (*19*). It was further purified on a reverse phase HPLC column and lyophilized. Ultrapure ganidinium chloride (GdmCl) and urea were from Sigma and Gibco BRL, respectively. Their concentrations were determined from refractive indexes according to the method of Pace (*20*). D_2O (99.8%) was from Cambridge Isotope Laboratory (Cambridge, MA). Deuterated GdmCl (D-GdmCl) and urea (D-urea) were prepared by evaporating D_2O in 6 M GdmCl or urea repeatedly (three times) on a rotary evaporator.

D−H Exchange Pulse Labeling. The D−H exchange pulse labeling experiments were performed using a Bio-Logic QFM-4 instrument with an estimated dead time of ~1 ms (*21*, *22*). Experiments were performed at 25 °C. Barnase was initially dissolved in 3.77 M deuterated GdmCl solution (pD 6.3, D_2O, and 10 mM Mes). The initial protein concentration was ~10 mg/mL. Refolding was initiated by a 10-fold dilution with buffer solution (pH 6.3, H_2O, 10 mM Mes, and 0.5 M NaCl). The refolding solution was further mixed with $^1/_4$ volume of the pulse buffer [1.0 M Tris (pH 8.6)] for 50 ms. The labeling pulse was terminated by mixing an equal volume of quench buffer [0.3 M citrate (pH 5.0)]. The quenched solution (~50 mL) was concentrated using an Amicon cell with membrane YM3 and adjusted to pH 4.7 with buffer solution [10% D_2O, 90% H_2O, and 10 mM NaAc (pH 4.7)]. A dead time refolding (~1 ms) and long pulse labeling (1 s) were also performed with initially unfolded barnase in 6.5 M D-urea (D_2O, pD 6.3) and in 0.33 M urea during the pulse period. The reference sample for the quantitative measurement of proton occupancy was prepared by dissolving barnase in 4.0 M GdmCl to allow the amide protons to exchange completely in the buffer solution described above. Further buffer exchange was used to refold the protein and remove GdmCl. The ^{1}H−^{15}N HSQC NMR spectra were collected on a Bruker DMX 500 MHz spectrometer (*23*). The data were processed using Felix (version 97) (Biosym, San Diego, CA).

Stopped-Flow and Steady-State Fluorescence. Stopped-flow fluorescence experiments were performed with a Biologic SFM4 machine (Grenoble, France). The fluorescence excitation wavelength was 280 nm, and emission was collected through a 320 nm cutoff filter. Kinetic folding experiments were initiated by diluting a solution of barnase in a high concentration of denaturant to various final concentrations of denaturant. Barnase folded in two measurable kinetic phases with 80% of the molecules in the fast phase and 20% in the slow phase due to the presence of a cis proline isomer in the unfolded state as first reported and studied by Matouschek et al. (*11*). All subsequent discussions will be related to the fast measurable kinetic phase. The kinetic refolding rate constant at 0.0 M denaturant was measured by refolding the acid-denatured barnase at pH 1.5. The melting experiments with denaturant were performed using a SLM 8100 fluorimeter (Spectronic, New York, NY). The samples at various denaturant concentrations were left overnight for equilibration at 4 °C. Excitation was at 280 nm. The emission spectra were scanned from 300 to 360 nm. The intensity of the spectra at 315 nm at different denaturant concentrations was used to plot the melting curves.

Proton Occupancy. The amide proton of K27 that is unprotected in the structure of native barnase was used as an internal reference, and peak intensities were used for the quantitative measurement of proton occupancy.

Chevron Curve Fitting. Chevron curves are fitted according to eq 1−3 assuming U and I are in rapid equilibrium (*24*):

$$\mathrm{U} \underset{k_{IU}}{\overset{k_{UI}}{\rightleftharpoons}} \mathrm{I} \underset{k_{NI}}{\overset{k_{IN}}{\rightleftharpoons}} \mathrm{N} \tag{1}$$

$$k_{obs} = [1/(1 + K_{IU})]k_{IN} + k_{NI} \tag{2}$$

$$k_{obs}([\mathrm{Den}]) = [1/(1 + K_{IU}{}^{H_2O} e^{m_{IU}[\mathrm{Den}]})]k_{IN}{}^{H_2O} e^{m^{\ddagger}_{IN}[\mathrm{Den}]} + k_{NI}{}^{H_2O} e^{m^{\ddagger}_{NI}[\mathrm{Den}]} \tag{3}$$

where $K_{IU}{}^{H_2O}$ is the unfolding equilibrium constant of the intermediate in water, $k_{IN}{}^{H_2O}$ and $k_{NI}{}^{H_2O}$ are the values of the corresponding microscopic rate constants in the absence of denaturant, and $m^{\ddagger}_{IN}$ and $m^{\ddagger}_{NI}$ are the coefficients for the denaturant dependence of the microscopic rate constants.

Global Melting Curve Fitting. The global unfolding equilibrium constant K_{NU} was obtained by assuming linear

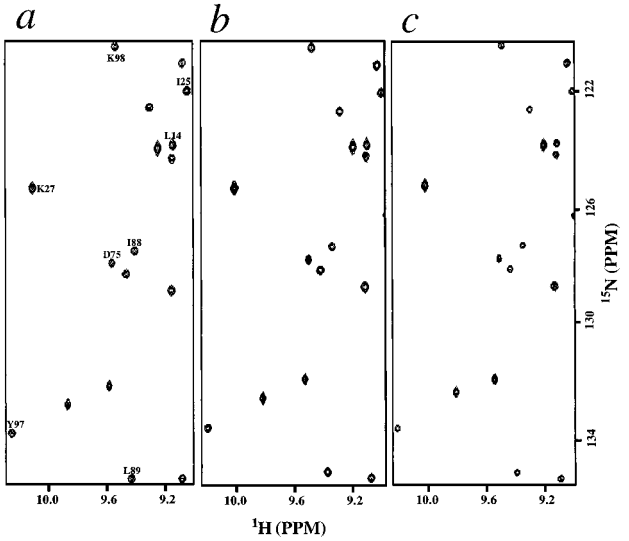

FIGURE 1: Portion of the $^{1}H-^{15}N$ HSQC spectra of barnase. (a) Reference spectrum. K27 is the internal reference. Other labels are for the amide protons that have ΔG_{HX} values equal to ΔG_{NU} and are expected to be protected on the sub-millisecond time scale according to the most recent conclusion about the folding pathway of barnase (*28*, *40*): (b) 10 ms refolding and (c) 60 ms refolding. The slight shift of some peaks in the spectra may be due to the slight change in pH.

dependence of the logarithm of the unfolding equilibrium constant as a function of denaturant and fitting the general equation with parameters for baselines (*20*, *25*). The complete equation used for the fitting is

$$I([\mathrm{Den}]) = [\alpha_N[\mathrm{Den}] + \beta_N + (K_{NU}{}^{H_2O}e^{m_{NU}[\mathrm{Den}]}) \times (\alpha_U[\mathrm{Den}] + \beta_U)]/(1 + K_{NU}{}^{H_2O}e^{m_{NU}[\mathrm{Den}]}) \quad (4)$$

where I is the intensity of the signal of fluorescence and $K_{NU}{}^{H_2O}$ is the global unfolding equilibrium constant in water. α_N, β_N, α_U, and β_U are the parameters of the baselines for the native and unfolded states, respectively. m_{NU} is the coefficient for the denaturant dependence of the logarithm of the K_{NU}.

All curve fittings were carried out using SigmaPlot. It should be pointed out that the conclusion will not be affected if the intermediate is off-pathway (I ⇔ U ⇔ N) (*24*).

RESULTS

D−H Pulse Labeling by NMR. Figure 1 shows a portion of the $^{1}H-^{15}N$ HSQC reference spectrum of barnase and the spectra of the samples from D−H pulse labeling experiments after refolding for 10 and 60 ms. Figure 2 shows the proton occupancy derived from Figure 1 for the amide protons that exchange slowly at pH 4.7 and 25 °C, and their resonance assignments can be made unambiguously. From Figure 2, it is clear that these amide protons were labeled during the pulse period in a homogeneous way by about 90 and 70% for the 10 and 60 ms refolding, respectively. This result appears to be possible only if the amide protons in the native protein generated during the refolding at pH 6.3 were protected, indicating that there is no stable partially unfolded intermediates populated on the sub-millisecond time scale. Therefore, it is inconsistent with the result observed in the previous pulse labeling experiment in which the amide

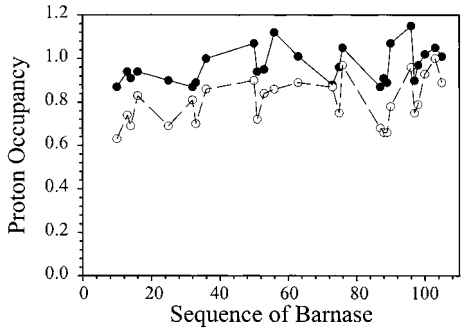

FIGURE 2: Proton occupancies with refolding times of 10 (●) and 60 ms (○).

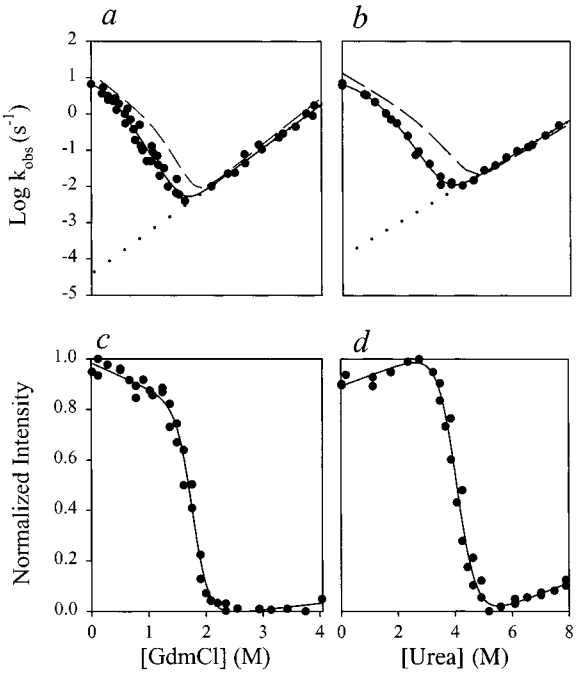

FIGURE 3: Kinetic chevron and equilibrium melting curves of barnase at pH 8.6 using GdmCl and urea monitored by fluorescence change. (a) Chevron curve in GdmCl. The solid curve is the three-state fitting curve. The dashed line is the curve at pH 6.3. The dotted line represents the linear extrapolation of log k_{NI}. (b) Similar to panel a except in urea. (c) GdmCl melting of baranse. The solid line is the fitting curve. (d) Similar to panel c except in urea.

protons of Y13, A32, E73, and Y97 were fully protected within 6 ms (*10*).

Stability of the Intermediate State at pH 8.6. A condition when using the pulse labeling approach to characterize protein folding intermediates populated at low pH is that the same intermediate remains stable at high pH during the period of the pulse. The result described above indicates that this may not be the case for barnase. If one assumes that the curved chevron plot of barnase is due to the population of the folding intermediate, the stability of the folding intermediate at pH 8.6 can be estimated from the chevron plot and the equilibrium melting curves by denaturant in the following two ways (Figure 3). (i) K_{IU} can be obtained directly from fitting the chevron plots in panels a and b of Figure 3 to a three-state model (U ⇔ I ⇔ N), assuming that U and I are in rapid equilibrium and the logarithms of the kinetic folding and unfolding rate constants are linear functions of denaturant concentration (*24*). (ii) K_{IU} can also

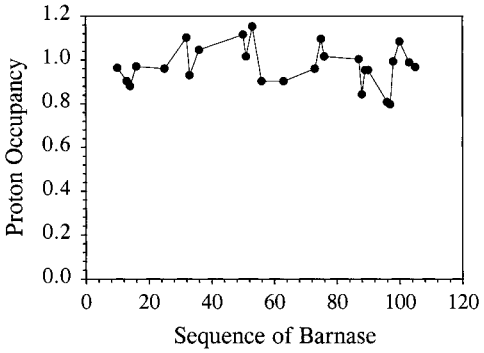

FIGURE 4: Proton occupancy measured during the dead time pulse labeling experiment.

Table 1: Kinetic and Thermodynamic Parameters of the Putative Intermediate of Barnase at pH 8.6

method	k_{NI} ($\times 10^{-5}$) (s^{-1})	k_{IN} (s^{-1})	K_{NI} ($\times 10^{-6}$)	K_{NU} ($\times 10^{-6}$)	K_{IU}[a]
chevron curve fitting					
in GdmCl	3.8	8.4			0.3 ± 0.2
in urea	10.0	7.4			0.08 ± 0.04
linear extrapolation					
in GdmCl	3.8	8.4	4.5	3.8	0.8 ± 0.5
in urea	10.0	7.4	13.5	4.9	0.4 ± 0.3

[a] The error is the estimated standard error from curve fitting.

be calculated from the ratio of the global unfolding equilibrium constant K_{NU} over the unfolding equilibrium constant between the native state and the intermediate state (K_{NI}) indirectly. K_{NU} can be determined directly from the equilibrium melting curves (panels c and d of Figure 3) using linear extrapolation methods, and K_{NI} can be calculated from the ratio of kinetic rate constants k_{NI}/k_{IN} (*11*, *20*). The thermodynamic and kinetic parameters measured via the methods described above are listed in Table 1.

It should be noted that the linear extrapolation method is not valid in the case of barnase (*26*, *27*). When nonlinear effects are considered, the value of K_{IU} becomes larger (*11*, *28*). Therefore, the above values underestimate the true K_{IU}.

Even with the low limit of the values of K_{IU} in Table 1, it can be shown that none of the amide protons studied in the previous pulse labeling experiments, in theory, can be protected by more than 50% under the condition used unless EX1 exchange has occurred (*10*). This is unlikely if the intermediate is formed on the sub-millisecond time scale. Indeed, we observe no significant protection for any amide protons in the sub-millisecond refolding using the dead time pulse labeling method under the previously described pulse labeling conditions (Figure 4) (*29*).

DISCUSSION

Native-State Hydrogen Exchange and Folding Pathways of Barnase

With the current pulse labeling result, the relationship between the native-state hydrogen exchange data and the kinetic folding pathway can now be reconciled in two ways.

(i) If the Curved Chevron Plot of Barnase Is Due to the Population of the Intermediate State, the Amide Protons Observed in the Native-State HX Experiment May All Be Protected in the Intermediate State of Barnase. For such an intermediate, even if it is stable, one does not expect the

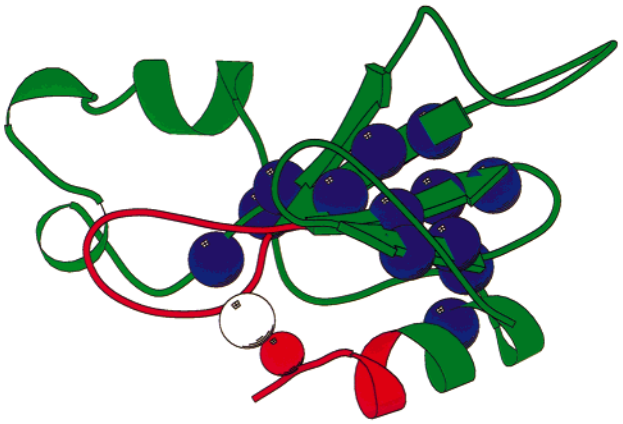

FIGURE 5: Molscript presentation of the structure of barnase (*41*, *42*). The blue balls represent the 13 slowly exchanging amide protons whose ΔG_{HX} values are equal to or larger than ΔG_{NU} at pD 6.8 (*9*). The white and the red balls represent the amide nitrogen of N77 and oxygen of N5, respectively. They form a hydrogen bond and connect the red loop (D75−I88) and the end of the N-terminal helix. The red region of the helix is from residue V3 to V10.

ΔG_{HX} values of the amide protons monitored in the native-state HX experiment to exhibit different patterns. This possibility is best illustrated from the wide distribution of the amide protons with ΔG_{HX} values equal to or larger than the ΔG_{NU} in the structure of baranse (Figure 5). Since all of these protons have to be protected in the stable intermediate state, the secondary structures are expected to fold in the intermediate state. Therefore, it is conceivable that other amide protons in the secondary structure are also protected, although they can still exchange with the protons of water through local structural fluctuations.

It is possible that some amide protons in the loop regions and at the ends of secondary structures may be exposed to solvent in the intermediate state. For example, the N-terminal helix may have frayed in the intermediate state. The amide proton of V10 has a relatively low ΔG_{HX} (6.4 kcal/mol) but a large *m* value (1 kcal/mol) (the slope in the plot of ΔG_{HX} vs denaturant concentration), indicating that some partial structural unfolding may have occurred (*9*). In the structure of the native barnase, a hydrogen bond between the backbone amide oxygen of N5 and the backbone amide proton of N77 connects the N-terminal helix and the loop that includes residues from D75 to I84. Interestingly, all of the residues in this loop have low Φ values (<0.3) in both the intermediate and the transition state characterized by protein engineering studies (*11*). Therefore, the HX behavior of amide proton V10 could be understood if this loop is unfolded together with the residues at the end of the helix in the intermediate state (Figure 5). Unfortunately, the exchange rates of these amide protons were not measured in the native-state HX experiment (*9*). Therefore, a definite conclusion could not be reached.

It should be pointed out, though, that equilibrium HX results may not be compared directly with those from protein engineering studies since protein engineering studies do not provide information about the formation of hydrogen bonds at the mutated site (or more accurately about the protection of the amide protons) except when the Φ value is 1.0. For example, I25 has a Φ value of -0.2 in both the intermediate and transition states, implying that the residue is unfolded

in the intermediate state (*30*). However it has a ΔG_{HX} equal to ΔG_{NU}, indicating that its amide proton must be strongly protected in the intermediate state if the intermediate is ~3 kcal/mol more stable than the unfolded state (*11*, *28*).

In general, the formation of a hydrogen bond alone in the intermediate state is not expected to cause much change of free energy since the same hydrogen bonding partners can also form hydrogen bonds with water in the unfolded state. Therefore, even if the Φ value is close to 0, one still cannot conclude that the hydrogen bond is not formed.

(ii) If the Curved Chevron Plot Is Partly or Completely Due to Movement of the Transition- or Unfolded-State Ensemble, Then the Intermediate of Barnase May Only Be Marginally Stable or Does Not Exist. Without the support of the previous pulse labeling result (*10*), the experimental evidence for the existence of the stable intermediate (~3 kcal/mol) can only be inferred indirectly by the curved chevron plot (*11*, *28*). However, it was pointed out recently that a curved chevron plot could also exist due to movement of the transition- or unfolded-state ensemble (*31*−*34*). One way to distinguish an intermediate from the movement of the transition- or unfolded-state ensemble is to determine the unfolding equilibrium constant K_{NI} for the equilibrium between the native and the putative intermediate state from k_{NI}/k_{IN} and compare it with the value of K_{NU} measured in thermodynamic equilibrium experiments (*11*). If K_{NU} is equal to K_{NI}, it can be concluded that the putative intermediate does not exist. This behavior was shown for the two-state protein U1A and some mutants of CI2 (*32*, *33*).

For barnase, it was shown that the "structure" of the transition state could be changed with single mutations (*35*). The values of k_{NI} in low concentrations of denaturant at 25 °C and pH 6.3 have been extrapolated indirectly from the values at higher temperatures using the Eyring equation (*28*). Although it was concluded that the intermediate is 2.8 kcal/mol more stable than the unfolded state, theoretically, however, one cannot simply apply the Eyring equation to protein folding and unfolding reactions without knowing the pre-exponential factor. The factor used by Dalby et al. can only be applied for simple chemical reaction in the gas phase. It is unlikely to be valid for protein folding reactions. Therefore, the stability of the putative intermediate of barnase still needs to be determined directly by other means to confirm or reject the existence of the intermediate state.

Native-State HX Method

It has been argued that native-state HX is not a good analytical method for studying protein folding pathways since it could not identify any partly unfolded forms of barnase and CI2, but the folding pathways of baranse and CI2 can be characterized fully by other methods (*15*). The available data do not appear to support this conclusion.

As discussed above, the partly formed intermediate of barnase characterized in the previous pulse labeling experiment must be an artifact, although there were claims that it was in excellent agreement with and supported the results from the protein engineering studies (*10*, *11*). It is also clear that a fractional or 0 value of Φ from protein engineering studies provides no reliable information about specific hydrogen bond formation. On the contrary, it was the result from the native-state HX experiment that has motivated us to question and revisit the previous pulse labeling experiment of barnase. Without the support of the previous pulse labeling result, the existence of the stable folding intermediate of barnase derived from the curved chevron plot alone is questionable since alternative interpretations exist.

For ribonuclease H, the partially unfolded intermediate was identified early by the native-state HX method and was further confirmed later by the kinetic pulse labeling experiment (*4*, *24*). In the case of CI2, the native-state HX detects no partly unfolded forms, which is consistent with the two-state folding behavior determined with other kinetic methods (*36*, *37*). Therefore, available data on the refolding of CI2, in our opinion, have further validated the native-state HX method instead of refuting it.

It is correctly emphasized by Clarke et al. that the native-state HX data alone cannot define the order of a folding reaction and other experiments are needed (*15*, *38*, *39*). However, it may be equally emphasized that the other available kinetic approaches cannot define the order of a folding reaction in a straightforward manner either. It may be potentially misleading to assume that protein engineering methods can define the order of a folding reaction since there is no prior reason Φ values derived from protein engineering studies can define the reaction order of barnase (*12*).

The results from our current reinvestigation of the pulse labeling experiment and the stability of the intermediate of barnase at high pH underscore the importance of combining all available approaches to cross-check them against each other and form a consistent and complete picture of how specific proteins fold. In particular, the studies presented here suggest that the nature of the folding intermediate of barnase needs to be investigated further.

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