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Equilibrium amide hydrogen exchange and protein folding kinetics

Yawen Bai
Laboratory of Biochemistry, National Cancer Institute, NIH, Building 37, Rm 4A-01, Bethesda, MD 20892-4255, U.S.A. E-mail: Yawen@helix.nih.gov

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Abstract

The classical Linderstrøm-Lang hydrogen exchange (HX) model is extended to describe the relationship between the HX behaviors (EX1 and EX2) and protein folding kinetics for the amide protons that can only exchange by global unfolding in a three-state system including native (N), intermediate (I), and unfolded (U) states. For these slowly exchanging amide protons, it is shown that the existence of an intermediate (I) has no effect on the HX behavior in an off-pathway three-state system (I↔U↔N). On the other hand, in an on-pathway three-state system (U↔I↔N), the existence of a stable folding intermediate has profound effect on the HX behavior. It is shown that fast refolding from the unfolded state to the stable intermediate state alone does not guarantee EX2 behavior. The rate of refolding from the intermediate state to the native state also plays a crucial role in determining whether EX1 or EX2 behavior should occur. *This is mainly due to the fact that only amide protons in the native state are observed in the hydrogen exchange experiment*. These new concepts suggest that caution needs to be taken if one tries to derive the kinetic events of protein folding from equilibrium hydrogen exchange experiments.

Introduction

Hydrogen exchange (HX) from protein amides studied with two-dimensional NMR and mass spectrometry in a kinetic mode is a very powerful tool to elucidate folding pathways of proteins (Roder et al., 1988; Udgaonkar and Baldwin, 1988; Englander et al., 1992; Miranker et al., 1993). HX under native and equilibrium conditions provides an alternative way to study the structures and stability of the unfolded (U) and the intermediate state (I) (Kim and Woodward, 1993; Bai et al., 1994, 1995; Englander et al., 1996; Hosszu et al., 1997; Clarke and Itzhaki, 1998). The key to using this technique has been to design an experiment so that some of the properties of protein molecules can be correlated with the exchange rates in a simple way. To date, only a few HX experiments under native and equilibrium conditions have been performed to derive kinetic events of protein folding in the system that involves folding intermediate states (Zahn et al., 1996a,b; Dalby et al., 1998). Considering the keen interest in understanding the energy landscape of protein folding and the important role of the HX technique (Dill and Chan, 1997), it is expected that more work will be done in this area in the near future. However, several basic questions need to be addressed before the equilibrium HX can be used as an analytical tool for studying kinetics of protein folding: (a) How do folding intermediates affect hydrogen exchange rates? (b) What is the relationship between HX behaviors (such as EX1 and EX2; see definition below) and the kinetics of protein folding? (c) Do on- and off-pathway intermediates have the same effect on HX behavior? (d) Can equilibrium HX experiments in principle define the order of a protein folding reaction? In this paper, these questions are investigated from a theoretical point of view.

The HX of protein amide protons in D_2O is commonly described by the scheme proposed by Linderstrøm-Lang (Berger and Linderstrøm-Lang, 1955; Hvidt and Nielsen, 1966):

$$\mathrm{NH(C)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \mathrm{NH(O)} \xrightarrow{k_{int}} \mathrm{ND} \qquad (1)$$

where C represents the closed conformation as opposed to the opened conformation (O) where chemical exchange can actually occur. The k_{op} and k_{cl} are the opening and closing rate constants. k_{int} is the intrinsic exchange rate constant in the open state. As a first order approximation, the experimentally measured exchange rate constant k_{ex} can be written as $k_{ex} = k_{op}k_{int}/(k_{cl} + k_{op} + k_{int})$ (Hvidt and Nielsen, 1966). There are two extreme HX behaviors when the protein is stable ($k_{cl} \gg k_{op}$): (a) $k_{int} \gg k_{cl}$ and $k_{ex} = k_{op}$. This is defined as the EX1 mechanism. (b) $k_{int} \ll k_{cl}$ and $k_{ex} = (k_{op}/(k_{op} + k_{cl}))k_{int}$. This is defined as the EX2 mechanism. The most common method to obtain a transition from EX2 behavior to EX1 behavior is to increase the pH since k_{int} is base-catalyzed. The change of the slope in the plot of log k_{ex} versus pH from 1 to 0 is an indication of transition from EX2 to EX1 exchange behavior when protein stability is not affected by pH (Arrington and Robertson, 1997).

Two-state system

For most amide protons that exchange relatively fast in a stable protein, their HX rates are determined by a local structure fluctuation process and their k_{op} and k_{cl} are simply formal kinetic parameters without clear meaning in terms of real physical events. But for some slowly exchanging amide protons, it has been shown that they can exchange with solvent protons only in the fully unfolded state (Englander et al., 1996). Therefore, the exchange process of these protons can be written as:

$$\mathrm{N} \underset{k_f}{\overset{k_u}{\rightleftharpoons}} \mathrm{U} \xrightarrow{k_{int}} \text{exchanged} \qquad (2)$$

where k_u and k_f are the unfolding and refolding rate constants. The k_{int} is close to the predicted peptide exchange rate constant k_{rc} in the unfolded state (Bai et al., 1993). If $k_f \gg k_{rc}$, the measured exchange rate constants for these amide protons become $k_{ex} = k_{rc}k_u/(k_u + k_f)$. If $k_f \ll k_{rc}$, $k_{ex} = k_u$. The two HX behaviors in a two-state system, therefore, can be adequately described by the two classical exchange mechanisms: EX2 and EX1.

Three-state system

For a three-state system, where the native state is the most stable state, three new factors have to be considered before an exchange mechanism can be determined for those amide protons that exchange only by global unfolding. (a) Two states, I and N, could both protect these amide protons from exchange with solvent protons. (b) The intermediate could be on-pathway (U↔I↔N) or off-pathway (I↔U↔N). (c) *Only amide proton signals from the native state are monitored in the equilibrium HX experiments for stable proteins because unfolded and intermediate states are much less populated and do not show up in the NMR spectra* (see illustrations in Figures 1 and 2). It should be noted that the intermediates may transiently populate in the kinetic folding experiment and can be studied directly using the D/H pulse labeling method, although they are not populated under equilibrium conditions (Roder et al., 1988; Udgaonkar and Baldwin, 1988). In this paper, we discuss the cases that hydrogen exchange rates are measured under equilibrium conditions.

The quantitative relationships between the exchange rates of amide protons that exchange only by global unfolding and the folding rate constants in a three-state system are derived here. Although analytical solutions of the above relationships can be derived for both on-pathway and off-pathway folding reactions (unpublished result), they are relatively complex. Therefore, certain approximations are made to derive simple equations that are valid for stable proteins. The results, based on these simple equations for the examples presented in the paper, are in excellent agreement with those from both the full analytical solutions, and from numerical simulations made without any approximations. The strategy is to convert a three-state problem into a two-state problem and to determine the effective closing rate constant and the effective intrinsic exchange rate constant.

For an off-pathway folding reaction, I↔U↔N, the exchange process can be written as

$$\mathrm{N} \underset{k_{UN}}{\overset{k_{NU}}{\rightleftharpoons}} \overset{\text{exchanged}}{\underset{\uparrow k_{rc}}{\mathrm{U}}} \underset{k_{IU}}{\overset{k_{UI}}{\rightleftharpoons}} \mathrm{I} \qquad (3)$$

where k_{NU}, k_{UN}, k_{UI}, and k_{IU} are the microscopic rate constants. Since only amide proton signals in the native state are monitored in the HX experiments of stable proteins, we have to treat [U⟺I] as an effective unfolded state, U^{eff}, rather than U alone. Then we

can rewrite the reaction as:

$$N \underset{k_{cl}^{eff}}{\overset{k_{op}^{eff}}{\rightleftharpoons}} U^{eff} \overset{k_{rc}^{eff}}{\Longrightarrow} \text{exchanged} \quad (4)$$

where k_{rc}^{eff} is the effective exchange rate constant and k_{cl}^{eff} is the effective rate constant for the formation of the N state in the following reactions, respectively:

$$I \underset{k_{UI}}{\overset{k_{IU}}{\rightleftharpoons}} U \overset{k_{rc}}{\Longrightarrow} \text{exchanged}$$

and

$$I \underset{k_{UI}}{\overset{k_{IU}}{\rightleftharpoons}} U \underset{k_{NU}}{\overset{k_{UN}}{\rightleftharpoons}} N$$

As a first order approximation (Hvidt and Nielsen, 1966),

$$k_{rc}^{eff} = k_{rc}k_{IU}/(k_{IU} + k_{UI} + k_{rc}) \quad (5)$$

$$k_{cl}^{eff} = k_{UN}k_{IU}/(k_{IU} + k_{UI} + k_{UN}) \quad (6)$$

since k_{NU} is very small under native conditions.

For folding with an on-pathway intermediate, such as U↔I↔N, the exchange process can be written as

$$N \underset{k_{IN}}{\overset{k_{NI}}{\rightleftharpoons}} I \underset{k_{UI}}{\overset{k_{IU}}{\rightleftharpoons}} U \overset{k_{rc}}{\Longrightarrow} \text{exchanged} \quad (7)$$

Again we need to treat [I⟺U] together as $U^{eff}$and rewrite the reaction as in Equation 4. The effective exchange rate constant k_{rc}^{eff} has the same terms as in the off-pathway case shown in Equation 5. But k_{cl}^{eff} is the effective rate constant for formation of the N state in the on-pathway reaction:

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

Because k_{NI} is small under native conditions, we have

$$k_{cl}^{eff} = k_{IN}k_{UI}/(k_{UI} + k_{IU} + k_{IN}) \quad (8)$$

The effective opening rate constant is simply given by the rate determining step for unfolding of N, i.e. $k_{op}^{eff} = k_{NI}$ for the on-pathway reaction and $k_{op}^{eff} = k_{NU}$ for the off-pathway reaction.

The observed exchange rate constant of an amide proton in stable proteins can be written as:

$$k_{ex}^{obs} = k_{op}^{eff}k_{rc}^{eff}/(k_{cl}^{eff} + k_{rc}^{eff}) \quad (9)$$

since k_{op}^{eff} is normally very small for stable proteins under native conditions.

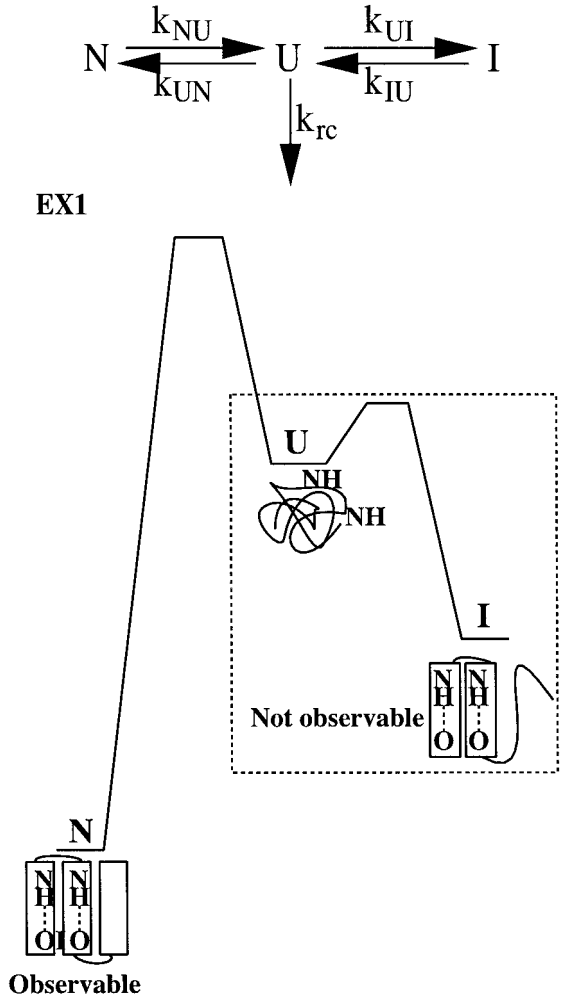

Figure 1. Illustration of the relationship between folding kinetics and EX1 behavior for an off-pathway three-state system. The intermediate is stable and $k_{UI} \gg k_{rc}$. Although the amide protons that can only exchange in the unfolded state may be protected rapidly by conversion from the unfolded to the stable intermediate state, such protection is not observable because only amide protons in the native state are monitored in the equilibrium HX experiments. Therefore, EX1 behavior will still occur when $k_{UN} \ll k_{rc.}$ The stability of the intermediate has nothing to do with the EX1 and EX2 behavior.

For both on- and off-pathway reactions, if $k_{cl}^{eff} \ll k_{rc}^{eff}$, EX1 behavior occurs. If $k_{cl}^{eff} \gg k_{rc}^{eff}$, EX2 behavior occurs.

Three typical examples are discussed below to illustrate how the kinetics of folding/unfolding affects HX behavior in a three-state system in which the native state is dominantly populated in the HX experiments.

Table 1. Summary of the examples illustrated in the text

On-pathway or off-pathway	K_{IU}	k_{UN} (off-pathway) k_{IN} (on-pathway)	$k_{rc}^{eff}/k_{cl}^{eff}$	HX behavior
Off-pathway	0.01	0.1	9.2	EX1
On-pathway	0.01	0.001	9.1	EX1
On-pathway	0.01	0.1	0.1	EX2
On-pathway	10	0.1	99.2	EX1

k_{UN} and k_{IN} have the unit of k_{rc}. In all cases, $k_{UI} = 10k_{rc}$.

Example 1. Let us consider an off-pathway folding reaction with $k_{UI} = 10k_{rc}$, $k_{IU} = 0.1k_{rc}$, and $k_{UN} = 0.1k_{rc}$, i.e. the folding from U to I is relatively fast but it is relatively slow from U to N, as illustrated in Figure 1. The intermediate is more stable than the unfolded state ($K_{IU} = k_{IU}/k_{UI} = 0.01$). The values of k_{rc}^{eff} and k_{cl}^{eff} are calculated to be $(1/111)k_{rc}$ and $(1/1020)k_{rc}$, respectively, using the above equations for the off-pathway reaction. Since $k_{rc}^{eff} \gg k_{cl}^{eff}$, EX1 behavior will occur even though $k_{UI} \gg k_{rc}$. This example demonstrates that, although the unfolded state may fold rapidly to the more stable intermediate state and protect these amide protons from exchange in the intermediate state, it does not display EX2 behavior because the amide protons in the intermediate are not monitored in the HX experiment. In fact, the HX behavior of an off-pathway three-state system is determined by the two-state kinetics between U and N when folding from U to I is fast. This can be easily demonstrated using Equations 5 and 6 to calculate k_{rc}^{eff} and k_{cl}^{eff} for the off-pathway reaction. Under the conditions $k_{UI} \gg k_{rc}$ and $k_{UI} \gg k_{UN}$, $k_{rc}^{eff} = k_{rc}k_{IU}/(k_{UI} + k_{IU})$ and $k_{cl}^{eff} = k_{UN}k_{IU}/(k_{UI} + k_{IU})$. Therefore,

$$k_{rc}^{eff}/k_{cl}^{eff} = k_{rc}/k_{UN} \qquad (10)$$

In other words, when EX1 behavior occurs, it means the refolding rate constant from U to N must be smaller than k_{rc}. Therefore, it is not possible to determine whether the amide proton exchanges through EX1 or EX2 behavior by simply comparing k_{UI} with k_{rc} even if the intermediate is more stable than the unfolded state.

Example 2. Consider an on-pathway folding reaction with $k_{UI} = 10k_{rc}$, $k_{IU} = 0.1k_{rc}$ and $k_{IN} = 0.001k_{rc}$, i.e. folding from U to I is fast and the intermediate is more stable than the unfolded state as illustrated in Figure 2a. The calculated k_{rc}^{eff} and k_{cl}^{eff} using the equations for an on-pathway reaction are $(1/111)k_{rc}$ and $(1/1010)k_{rc}$, respectively. Since $k_{rc}^{eff} \gg k_{cl}^{eff}$, EX1 behavior should occur. However, if $k_{IN} = 0.1k_{rc}$, the calculated k_{cl}^{eff} will be $(10/101)k_{rc}$ (see Figure 2b) Since $k_{rc}^{eff} \ll k_{cl}^{eff}$, EX2 behavior will occur. Therefore, fast refolding from U to a stable I alone is again not sufficient to guarantee EX2 behavior. The refolding rate constant from I to N also plays an important role in determining the HX behavior.

Example 3. Consider an on-pathway reaction with $k_{UI} = 10k_{rc}$, $k_{IU} = 100k_{rc}$, and $k_{IN} = 0.1k_{rc}$, i.e. folding from U to I is fast and the intermediate is less stable than the unfolded state (Figure 2c). Using the equations for an on-pathway reaction, k_{rc}^{eff} and k_{cl}^{eff} are calculated to be $(100/111)k_{rc}$ and $(10/1101)k_{rc}$, respectively. EX1 exchange behavior should occur. Since k_{IN} is normally small and k_{rc} can be very large at high pH, it is likely that EX1 should occur if the intermediate is destabilized. In fact, when $k_{UI} \gg k_{rc}$ and $k_{UI} \gg k_{IN}$, for an on-pathway reaction, one can use Equations 5 and 8 to calculate $k_{rc}^{eff}/k_{cl}^{eff}$:

$$k_{rc}^{eff}/k_{cl}^{eff} = k_{rc}k_{IU}/k_{IN}k_{UI} = k_{rc}K_{IU}/k_{IN} \qquad (11)$$

From this equation, one could expect the HX behavior of an on-pathway scheme to be related to the stability of the intermediate state. A comparison between Figures 2b and 2c also illustrates how an EX2 behavior could be switched to EX1 due to destabilization of the intermediate state and the second transition state of refolding. The effect of the stability of intermediate state, K_{IU}, on k_{rc}^{eff} is shown in Figure 3. A summary of these examples is shown in Table 1.

The parameters used in the above examples are in the realistic range. For example, if the experiment was done at pD 9.0 in D_2O at room temperature, k_{rc} is about 5 ms for the amide proton of alanine residues in an unfolded polypeptide (Bai et al., 1993). Early folding intermediates of many proteins form in the sub-millisecond range and late folding processes occur normally after more than 100 ms.

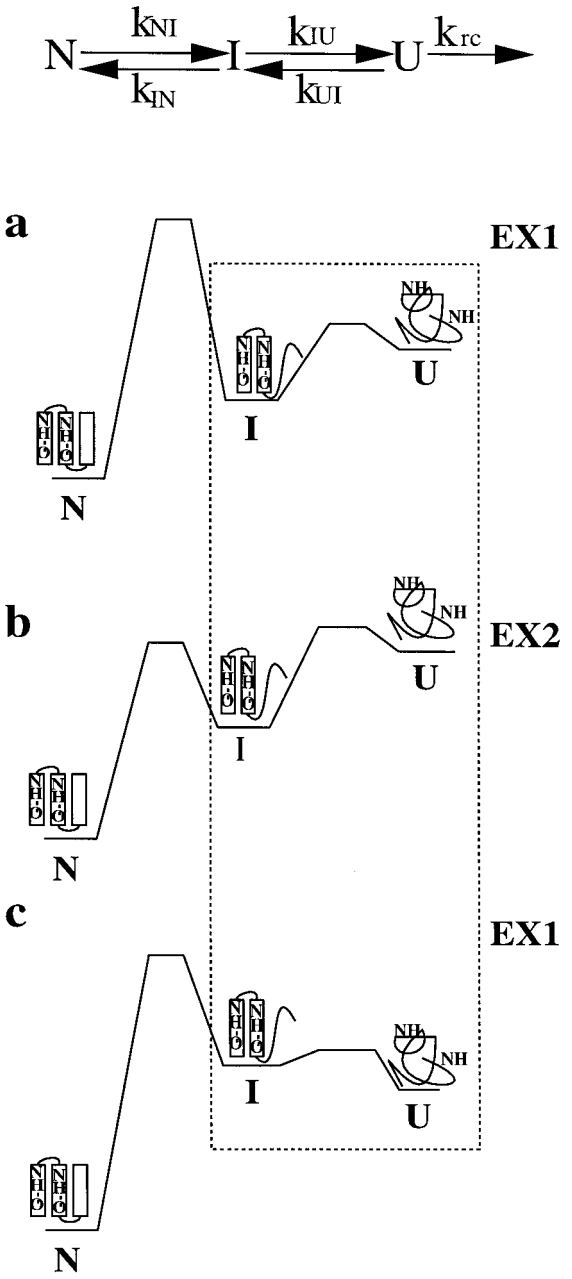

Figure 2. Illustration of exchange behavior and folding kinetics for an on-pathway three-state system. Again, the species in the dashed frame are not observable in the HX experiment. (a) EX1. The intermediate is stable and $k_{UI} \gg k_{rc}$. If the kinetic barrier between I and N is sufficiently high, EX1 exchange behavior occurs. In such a case, once a protein molecule unfolds to the intermediate state it has no chance to refold before the slowly exchanging amide protons are exchanged, although k_{rc}^{eff} is decreased due to the stability of the intermediate. (b) EX2. The intermediate is stable and $k_{UI} \gg k_{rc}$. If the kinetic barrier between I and N is not sufficiently high, the EX2 exchange behavior could occur. In such a case, when a protein molecule unfolds to the intermediate and the unfolded states, it still has the chance to fold back before the slowly exchanging amide protons are exchanged. (c) EX1. Destabilization of the stable intermediate and late transition states as shown in b could switch EX2 behavior to EX1 behavior.

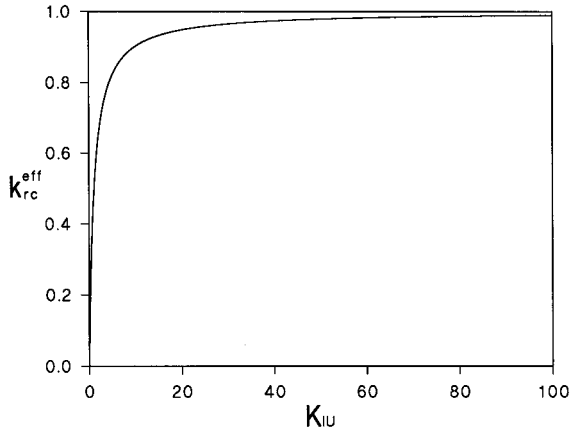

Figure 3. k_{rc}^{eff} as a function of K_{IU}. The parameter k_{UI} is chosen as $10k_{rc}$. $K_{IU} = k_{IU}/k_{UI}$. When the intermediate is stable ($K_{IU} \ll 1$), $k_{rc}^{eff} = K_{IU}k_{rc}$; therefore a stable intermediate decreases the effective intrinsic exchange rate constant. When the intermediate is unstable, k_{rc}^{eff} becomes close to k_{rc}. The unstable intermediate has little effect on HX behavior.

The model proposed here indicates that one cannot simply conclude that folding from U to I is slower than k_{rc} using the classical Linderstrøm-Lang model when EX1 behavior is observed in a system that involves an intermediate state (Zahn et al., 1996a,b). The fast refolding from an unfolded state to a stable intermediate alone does not guarantee an EX2 behavior (Clarke and Itzhaki, 1998; Dalby et al., 1998). Further, the on-pathway intermediate may, in principle, be distinguished from an off-pathway intermediate by studying the HX behavior of the amide protons that exchange only by global unfolding.

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References

Arrington, C.B. and Robertson, A.D. (1997) *Biochemistry*, **36**, 8686–8691.

Bai, Y., Milne, J.S., Mayne, L. and Englander, S.W. (1993) *Proteins*, **17**, 75–86.

Bai, Y., Milne, J.S., Mayne, L. and Englander, S.W. (1994) *Proteins*, **20**, 4–14.

Bai, Y., Sosnick, T.R., Mayne, L. and Englander, S.W. (1995) *Science*, **269**, 192–197.

Berger, A. and Linderstrøm-Lang, K. (1955) *Arch. Biochem. Biophys.*, **69**, 106–118.
Clarke, J. and Itzhaki, L.S. (1998) *Curr. Opin. Struct. Biol.*, **8**, 112–118.
Dalby, P.A., Clarke, J., Johnson, C.M. and Fersht, A.R. (1998) *J. Mol. Biol.*, **276**, 647–656.
Dill, K.A. and Chan, H.S. (1997) *Nat. Struct. Biol.*, **4**, 10–19.
Englander, S.W. and Mayne, L. (1992) *Annu. Rev. Biophys. Biomol. Struct.*, **21**, 243–265.
Englander, S.W., Sosnick, T.R., Englander, J.J. and Mayne, L. (1996) *Curr. Opin. Struct. Biol.*, **6**, 18–23.
Hosszu, L.L.P., Craven, C.J., Parker, M.J., Lorch, M., Spencer, J., Clarke, A.R. and Waltho, J.P. (1997) *Nat. Struct. Biol.*, **4**, 801–804.
Hvidt, A. and Nielsen, S.O. (1996) *Adv. Protein Chem.*, **21**, 287–386.
Kim, K. and Woodward, C. (1993) *Biochemistry*, **32**, 9609–9613.
Miranker, A., Robinson, C.V., Radford, S.E., Aplin, R.T. and Dobson, C.M. (1993) *Science*, **262**, 896–900.
Roder, H., Elöve, G. and Englander, S.W. (1988) *Nature*, **335**, 700–704.
Udgaonkar, J.B. and Baldwin, R.L. (1988) *Nature*, **335**, 694–699.
Zahn, R., Perrett, S. and Fersht, A.R. (1996a) *J. Mol. Biol.*, **261**, 43–61.
Zahn, R., Perrett, S., Stenberg, G. and Fersht, A.R. (1996b) *Science*, **271**, 642–645.