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Protein Folding Kinetics by Combined Use of Rapid Mixing Techniques and NMR Observation of Individual Amide Protons

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ABSTRACT **A method to be used for experimental studies of protein folding introduced by Schmid and Baldwin (*J. Mol. Biol.* 135: 199–215, 1979), which is based on the competition between amide hydrogen exchange and protein refolding, was extended by using rapid mixing techniques and ^{1}H NMR to provide site-resolved kinetic information on the early phases of protein structure acquisition. In this method, a protonated solution of the unfolded protein is rapidly mixed with a deuterated buffer solution at conditions assuring protein refolding in the mixture. This simultaneously initiates the exchange of unprotected amide protons with solvent deuterium and the refolding of protein segments which can protect amide groups from further exchange. After variable reaction times the amide proton exchange is quenched while folding to the native form continues to completion. By using ^{1}H NMR, the extent of exchange at individual amide sites is then measured in the refolded protein. Competition experiments at variable reaction times or variable pH indicate the time at which each amide group is protected in the refolding process. This technique was applied to the basic pancreatic trypsin inhibitor, for which sequence-specific assignments of the amide proton NMR lines had previously been obtained. For eight individual amide protons located in the β-sheet and the C-terminal α-helix of this protein, apparent refolding rates in the range from 15 s^{-1} to 60 s^{-1} were observed. These rates are on the time scale of the fast folding phase observed with optical probes.**

Key words: hydrogen exchange, BPTI, folding pathway, protein dynamics

INTRODUCTION

Considerable effort has been directed at determining the folding pathways of globular proteins. Although the existence of folding intermediates has been demonstrated in several cases (reviewed by Kim and Baldwin[1]), specific structural information is still lacking, especially with respect to the early events of structure condensation. A particularly promising approach introduced by Baldwin and co-workers[2–4] and applied to characterize the slow folding phase of RNase A relies on investigations of the competition between amide proton exchange from the unfolded protein and protein refolding, using tritium labeling of amide groups. The present paper describes an extension of this competition method, which can also be applied to proteins where a fast phase dominates refolding.

After inducing refolding of the protein by rapid mixing into a deuterated solvent medium, amide proton exchange is quenched after a predetermined time, and the extent of exchange for individual amide protons is determined by ^{1}H NMR experiments with the refolded protein. Sequence-specific assignments in the native protein can thus be directly applied for the interpretation of these experiments. The procedure is illustrated with studies on the refolding of BPTI. Rapid mixing is required since refolding of BPTI is dominated by a process in the 10-ms time range.[5]

METHODS

The mixing procedure used for the competition experiments is schematically illustrated in Figure 1. An H_2O solution of the unfolded protein (syringe S1) is rapidly mixed in the mixing chamber (M) with a larger volume of D_2O buffer (S2). Refolding and hydrogen-deuterium exchange are allowed to proceed during the time τ before the exchange reaction is quenched by rapid cooling (Q).

The kinetic analysis of the experiment is straightforward if we assume that the formation of folded structure affecting the exchange of a particular amide proton occurs irreversibly in a single step (in the discussion we will deal with more general kinetic situations). Equation (1) describes the competition between refolding and exchange for an amide proton that is protected against exchange upon refolding.

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Abbreviations: BPTI, basic pancreatic trypsin inhibitor; RCAM-BPTI, 14–38 reduced and carboxamidomethylated BPTI; RNase A, bovine pancreatic ribonuclease A; NMR, nuclear magnetic resonance; CD, circular dichroism.

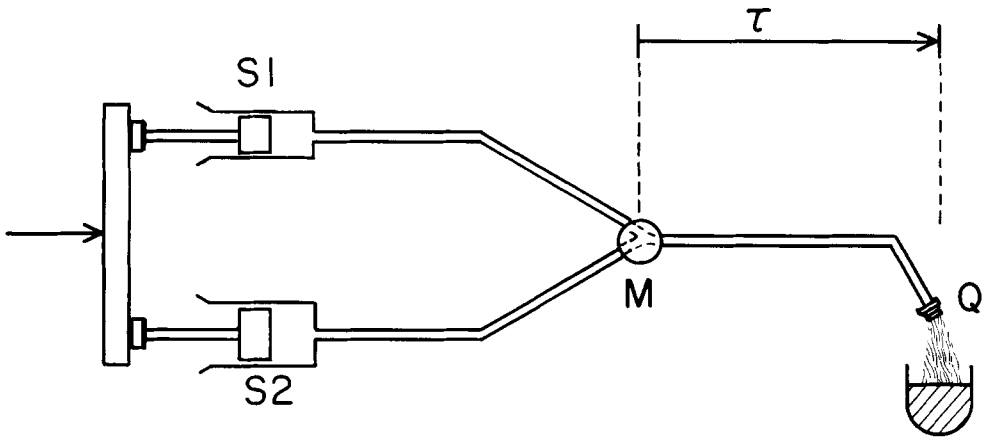

Fig. 1. Schematic illustration of the mixing apparatus. A mechanical actuator advances the syringe plungers by 13.9 mm in 2 revolution of a flywheel (maximum speed 700 rpm). The solutions contained in syringes S1 and S2 (8 and 12 mm diameter) are mixed in the four-jet mixing chamber M. At the quencher Q, consisting of a nozzle with five 0.1-mm holes, the solution is sprayed into cold liquid (ice water or isopentane at dry ice temperature). Syringes, mixing chamber, and tubing up to Q are thermostated at 70°C (± 0.5°). At a given flow rate, dV/dt, the volume V, between M and Q determines the competition time, $\tau = V/(dV/dt)$, during which exchange and refolding are allowed to proceed at 70°C.

$$\begin{array}{ccc} U^H & \xrightarrow{k_f} & F^H \\ k_c \downarrow & & \\ U^D & \xrightarrow{k_f} & F^D \end{array} \qquad (1)$$

Initially, all amide groups are protonated in the unfolded form, U^H. After mixing with deuterated buffer solution, the proton can either exchange at the intrinsic rate k_c, producing U^D, or it can be trapped within the structure formed during refolding (F^H). With the initial condition at $\tau=0$ (Fig. 1) as $U^H(0)=1$, $U^D(0)=F^H(0)=F^D(0)=0$, we obtain the following functions of the competition time τ.

$$U^H(\tau) = \exp\,[-(k_f+k_c)\tau] \qquad (2)$$

$$F^H(\tau) = \frac{k_f}{k_f+k_c} \cdot \{1 - \exp[-(k_f+k_c)\tau]\} \qquad (3)$$

When exchange is stopped by rapid cooling, the U → F transition goes to completion. Since the protons observed in this study have exchange times on the order of months in the native protein at room temperature and neutral or slightly acidic pH,[6] the experimentally observable quantity is the total remaining proton population ($U^H + F^H$) at the time of quenching. The measured proton occupancy, P, as a function of the competition time τ is then given by

$$\begin{aligned} P(\tau) &= U^H(\tau)+F^H(\tau) \qquad (4)\\ &= P_\infty+(1-P_\infty)\exp[-(k_f+k_c)\tau] \end{aligned}$$

$$P_\infty = k_f/(k_f+k_c) \qquad (5)$$

P_∞ is the final proton occupancy after a long competition time ($\tau >> 1/(k_f+k_c)$). The background due to the fraction f_H of H_2O present in the reaction mixture is taken into account by normalizing the measured NH intensity, I_m, as follows:

$$P(\tau) = [(I_m(\tau)/I_0) - f_H]/(1 - f_H) \qquad (6)$$

where I_0 is the resonance intensity of the fully protonated amide group.

Equations (4) and (5) suggest two different methods for measuring folding rates with observation of individual amide protons:

1. Measurement of $P(\tau)$ at constant pH and temperature. According to Eq. (4) the proton occupancy decays exponentially from unity to a final level $P_\infty = k_f/(k_f+k_c)$, with a rate constant k_f+k_c. Under conditions where k_f and k_c are similar in magnitude, both rates can be determined from an exponential fit of the normalized NH resonance intensity as a function of the competition time.

2. Measurement of the pH dependence of P_∞. In the base-catalysed regime the intrinsic exchange rate, k_c, increases by a factor of 10 per pH unit. Variation of pH can therefore be used to adjust k_c until it equals the folding rate, k_f. According to Eqs. (5) and (6), we then have that $P_\infty = 0.5$ (provided that the folded protein is stable under these conditions). At sufficiently low pH, where $k_c << k_f$, the proton will be fully trapped, so that $P_\infty = 1$; at high pH, where $k_c >> k_f$, the amide proton will be completely exchanged before folding is complete, and $P_\infty = 0$. Since a reliable calibration for the intrinsic amide proton exchange rates at variable pH and temperature is available from model peptide studies[7,8] and from measurements in thermally unfolded BPTI,[9] folding rates can be obtained from the pH location of the sigmoidal transition in plots of P_∞ vs. pH, as described by Eq. (5).

Intrinsic exchange rates, k_c, for the present study with BPTI were calculated with the equation

$$k_c(T,pH) = \tfrac{1}{2} \cdot k_c(T_o,pH_{min}) \cdot e^{-E_a(\frac{1}{T} - \frac{1}{T_o})/R} \cdot [10^{(pH - pH_{min})} + 10^{(pH_{min} - pH)}] \quad (7)$$

using the values for pH_{min} and k_c (T_o, pH_{min}) measured for the same protons in thermally unfolded RCAM-BPTI at $T_o = 86°C$.[9] An activation energy E_a = 17 kcal mol^{-1} was used for the base-catalyzed exchange reaction.[7] In addition, a correction was applied to account for the effect of solvent additives on NH exchange.[10,11] In 13% n-propanol, we find the intrinsic NH exchange to be slowed by a factor 1.3 (measured with poly-DL-alanine at pH 4.0, 25°C).

EXPERIMENTAL

BPTI (Trasylol) was a gift from Bayer AG, Leverkusen Germany. A home-built rapid mixing apparatus, kindly provided by Dr. H. Dutler, was modified for the present experiment (Fig. 1). An H_2O solution of the fully denatured protein at 70°C in 40% (v/v) n-propanol at pH 2.0 was mixed with 2 volumes of 0.2 M phosphate buffer in D_2O at a range of pD values between about 4.5 and 7.5. This mixture produces refolding conditions at pH values above 4.0 in a 1:2 H_2O/D_2O mixture containing 13% n-propanol. The initial protein concentration in the H_2O solution was 3 mM. pH was measured at 70°C in aliquots of the refolding mixture and is reported without correction for isotope effects or the presence of n-propanol. Syringes, mixer, and reaction tubing were thermostated at 70±0.5°C. For quenching, the mixture was injected through a nozzle into isopentane at −70°C or ice water. The nozzle was placed a few millimeters above the liquid. The reaction time was determined from the calibrated flow rate and the precisely measured reaction volume, i.e., the volume between mixer and quencher. The shortest reaction time achieved was 30 ms. Longer times were set by reduction of the flow rate and/or increased length of the reaction tubing (thermostated at 70°C).

The quenched protein samples were stored at dry ice temperature pending further processing. When isopentane was used for quenching, the frozen solution particles were collected by evaporating the organic solvent and immediately thereafter lyophilized. In experiments that involved injection into ice water, samples were concentrated by ultrafiltration at 4°C and lyophilized. Prior to the NMR measurements the lyophilized protein was redissolved in D_2O and adjusted to pD 4.0 by addition of DCl.

At 360 MHz on a Bruker HX 360 spectrometer ^1H NMR spectra were recorded to measure the extent of H-D exchange for resolved, individually assigned NH resonances.[12,13] Resonance intensities (I_m in Eq. 6) were determined by comparison with simulated spectra. The accuracy thus achieved was about ±5%. The intensity of a resolved line corresponding to a nonlabile proton was used for I_o. Since the determination of the proton occupancy, P, involves two intensity measurements (Eq. 6), its error is estimated to be about ±10%. (f_H was determined more accurately).

The exchange kinetics of poly-DL-alanine (Miles-Yeda Ltd., Revohot) in D_2O with and without addition of 13% (v/v) n-propanol (25°C, pH 4.0) was studied by the spectrophotometric method of Englander et al.[14] by using a Cary 118 spectrophotometer.

Circular dichroism experiments were performed on a Jasco 500 C spectopolarimeter by using thermostated quartz cuvettes with 1-mm pathlength.

RESULTS

In a search for suitable experimental conditions we performed CD measurements at 375 nm to monitor the thermal unfolding transition of BPTI. The high stability of BPTI requires addition of denaturants for complete unfolding at experimentally accessible temperatures. At pH 2.2 in the presence of 40% n-propanol, thermal unfolding was found to be fully reversible and had the characteristics of a two-state transition with a melting temperature of 60.5°C, an enthalpy change of 61 kcal mol^{-1}, and a heat capacity change of about 1 kcal mol^{-1} K^{-1}. The temperature chosen for the competition experiments, 70°C, lies at the top end of the transition (95% unfolded). In a 13% n-propanol solution at pH 4.5, the melting temperature was found to be above 90°C. Thus, our refolding conditions (70°C, 13% n-propanol, pH > 4.0) correspond to an equilibrium situation with at least 98% refolded protein. NMR spectra recorded after refolding were indistinguishable from those measured with a fresh solution.

Measurements of $P(\tau)$ (method 1) were performed at pH 6.7 (70°C, 13% propanol, 0.13 M phosphate buffer). The incubation time τ of the mixture of 70°C was varied between 30 and 400 ms by adjusting the flow rate (Fig. 1). The exchange was quenched by injection of the solution into isopentane at −70°C (see Fig. 1). These procedures were repeated for eight values of τ with a common stock of fresh protein solution. NMR samples were prepared as described in the experimental section.

The fraction of amide protons retained in the refolded protein was measured in the ^1H NMR spectrum for six resolved lines and one pair of overlapped NH resonances. The proton occupancy, $P(\tau)$, for the NH of Phe 22, normalized according to Eq 6, is plotted in Figure 2 as a function of the competition time. Similar results were obtained for the other amide protons studied. The calculated curve in Figure 2 represents a least-squares fit of Eq. (4) with the parameters $k_f + k_c = 40\ s^{-1}$ and $P_\infty = 0.17$. With Eq. (5) we obtain the individual rate constants $k_f = 7\ s^{-1}$ and $k_c = 33\ s^{-1}$. For the exponential fit it was necessary to shift the zero time by 30 ms (Fig. 2), which probably reflects the mixing dead time. This adds considerably to the uncertainty of the analysis. Only

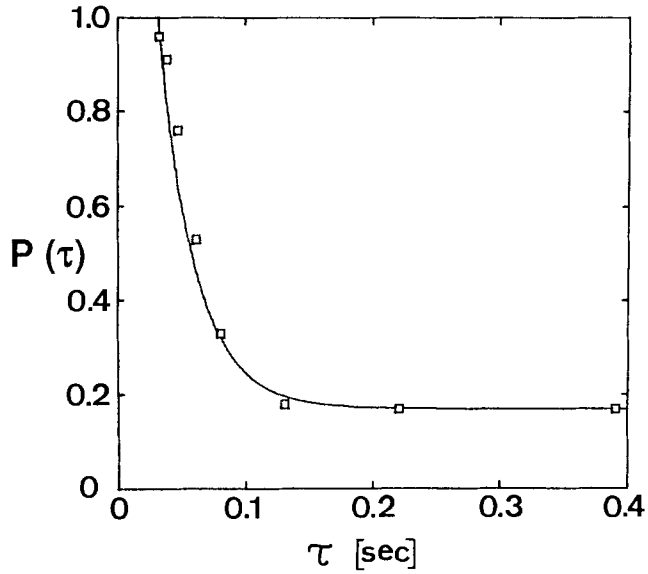

Fig. 2. Plot of the proton occupancy, $P(\tau)$, calculated with Eq. (6), vs. the competition time, τ, for the Phe 22 NH in BPTI at 70°C and pH 6.7 (0.13 M phosphate, 13% n-propanol). The data resulted from NMR analysis of eight different protein samples subjected to the mixing procedures outlined in the Methods section (method 1). The theoretical curve is a fit of Eq. (4) with $k_f + k_c = 40\ s^{-1}$ and $P_\infty = 0.17$. In this fitting procedure the zero point of the time axis was shifted by 30 ms (see text).

qualitative conclusions can be drawn from these results. Quantitiative results could be obtained from method 2, which is technically less demanding.

The pH dependence of P_∞ (method 2) was measured in the same refolding medium at a constant competition time of 0.4 s. Experiments were performed by using different D_2O phosphate buffer solutions producing pH values between 4.0 and 7.5 during the competition period. At this longer competition time, injection into ice water was used for quenching. The intensity of the resolved amide proton resonances in the ^{1}H-NMR spectrum was measured and Eq. (6) was used to calculate the residual protonation for eight individual amide groups in the protein. Results are displayed in Figure 3. In each case the trapped proton intensity decreases with increasing pH in a sigmoidal manner, with a midpoint near pH 6.0. The curves in Figure 3 were calculated with Eq. (5) and fitted to the data by variation of k_f. The values for k_c (pH) were calculated with Eq. (7). In independent experiments using method 1 it was found that $P(0.4\ s) = P_\infty$ at all pH values used for this study (Fig. 2).

The apparent folding rates resulting from this procedure are reported in Table I. The quality of the fits in Figure 3 indicates statistical errors of about 20%. Additional errors are introduced by the calibration of k_c[9]. The extrapolation of k_c and solvent effects may cause systematic errors affecting the absolute magnitude of the rates, but not the relative rates among different amide protons.

The competition data recorded with method 1 (Fig. 2) provide some important experimental controls. First, we note that the proton occupancy lies close to 1 for the points at the shortest competition times used. In these samples that were exposed to the D_2O refolding medium at 70°C only for a short time, the total amide proton intensity was recovered after the exchange was quenched by rapid cooling. This demonstrates that quenching was efficient and that no further exchange took place during the subsequent steps of sample handling and the NMR measurement.

The evaluation of the data obtained by measurement of the pH dependence of P_∞ (Fig. 3) relies on the condition that the long-time limit is reached before the end of the competition period used, i.e., the condition $\tau >> 1/(k_f + k_c)$ must be fulfilled. For the present experiments with BPTI the time course of $P(\tau)$ in Figure 2 shows that at pH 6.7 the proton level remains constant for competition times longer than ca. 0.15 s. The value of $\tau = 0.4$ s chosen for method 2 therefore ensures stationary conditions at pH 6.7, and the magnitude of the k_f values reported in Table I (15 s^{-1} or larger for all NH studied) shows that this is true at all other pH values of this study. In similar ways, suitable experimental conditions would have to be established for work with other proteins.

As a final control, we have to verify that the structure formed during competition experiments with BPTI at 70°C is able to protect the interior amide protons against exchange. In previous exchange studies of BPTI at elevated temperature[15] the amide protons studied here were found to exchange at rates between 10^{-3} and $2 \cdot 10^{-2}\ s^{-1}$ at 70°C and pH 8.0. Exchange from molecules refolded into nativelike conformations is thus expected to be negligible on the time scale of the mixing experiments, which were

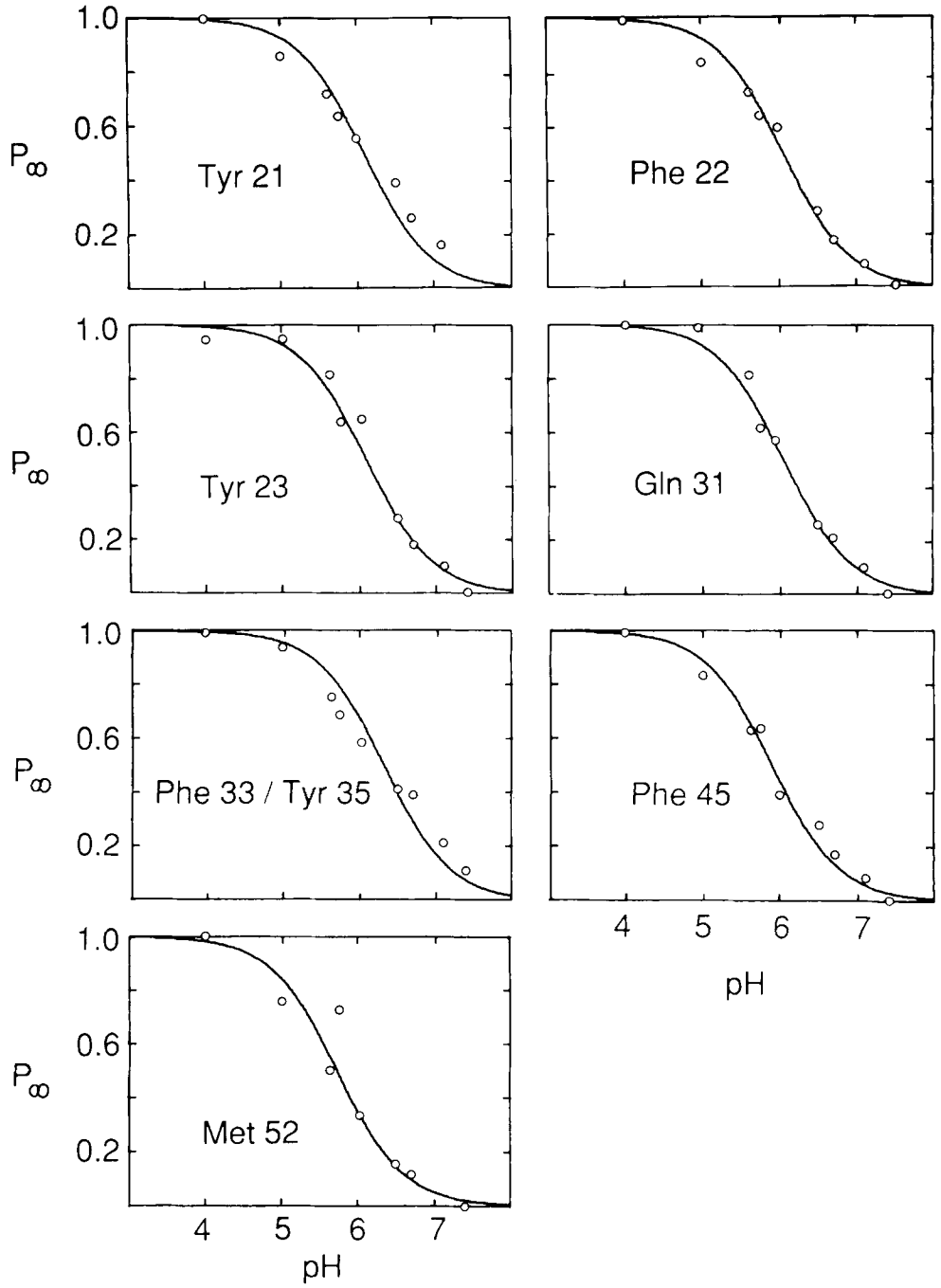

Fig. 3. Plot of the limiting proton occupancy, P_∞, vs. pH for individual amide groups in BPTI at 70°C (method 2). The theoretical curves are fits of Eq. (5) with adjustment of k_f (k_c was computed using Eq. (7)). Apparent folding rates, k_f, resulting from these fits are reported in Table I.

conducted at pH values below 7.3. This is further demonstrated by Figure 2, which shows that after the initial exponential phase, $P(\tau)$ does not change further at times $\tau \geqslant 0.15$ s. In contrast, if exchange from refolded BPTI were appreciable, we would have observed a second phase with the proton occupancy decaying toward the solvent proton background level ($P=0$).

DISCUSSION

The potential of experimental techniques using amide protons as probes of structure formation in protein folding studies has been recognized for some time.[2-4,16-18] Most such studies so far used tritium labeling and manual mixing and were therefore limited to slow folding steps. The present experiments

TABLE I. Apparent Folding Rates for Individual Amide Protons in BPTI From the pH Dependence of the Competition Between Refolding and NH Exchange*

	Tyr 21	Phe 22	Tyr 23	Gln 31	Phe 33/ Tyr 35†	Phe 45	Met 52
$pH_{½}$‡	6.1	6.0	6.1	6.0	6.3	6.0	5.6
k_f [s^{-1}]**	30	25	30	60	50	50	15

*Refolding conditions were 70°C, 2:1 D_2O:H_2O, 13% (v/v) n-propanol, 0.15 M phosphate.
†The NH resonances of Phe 33 and Tyr 35 were overlapped.
‡pH where 50% of the amide protons in this position is trapped in the refolded protein (Fig. 3).
**Apparent folding rates, k_f, determined by fitting Eq. (5) to the data in Figure 3 (method 2). The average standard deviation of the fit is 20%. Intrinsic NH exchange rates, k_c, were obtained by extrapolation of the data from Roder et al.[9], using Eq. (7) with E_a = 17 kcal mol^{-1}.

combine rapid mixing techniques with NMR observation of individual amide protons at known sequence positions.[12,13] This enables extension of folding studies to faster processes and leads to the structural resolution of folding rates for individual amide proton sites.

The approach described here relies on measurements of the competition between two rate processes, i.e., between the exchange of solvent deuterons for amide protons in unfolded parts of the protein, and the formation of folded structure which protects the amide protons against solvent exchange. This competition method makes it possible to label short-lived folding intermediates in a nonperturbing way, so that the intrinsically slow NMR technique can be used subsequently to obtain a high degree of structural resolution. The detailed interpretation of competition data depends on the structural features responsible for slowing hydrogen exchange, among which intramolecular hydrogen bonding appears to be the most important factor.[19] As a general observation, in previously studied proteins one notes that the most slowly exchanging amide protons are involved in the hydrogen bonds of regular secondary structures.[20,21–23] The time resolution of the competition method is determined by the intrinsic amide proton exchange rate, which can be varied over a wide range by the choice of pH and temperature, and by the speed of mixing, which can be controlled by the choice of the experimental setup (Fig. 1).

The kinetic interpretation of competition data is straight forward if the simple two-state situation described by Eq. (1) applies; i.e., a particular amide proton is protected against exchange in a single step without transient formation of intermediate states that would be sufficiently long-lived to observably retard exchange. In this case the apparent folding rate, k_f, obtained in the competition experiment (method 1 or 2) can be attributed to the rate of local structure formation near the observed amide proton. It should be stressed that a two-state situation for individual amide protons does not imply that the overall folding process has to be a two-state reaction. Even if individual segments of secondary structure are formed in a concerted way, different parts of the protein may fold at different times. The competition method can thus in principle resolve the sequence of folding events throughout the structure.

In general, however, more complicated kinetic situations than Eq. (1) have to be considered. While it is usually possible to ensure essentially irreversible formation of the final folded state by choosing strongly native refolding conditions, unstable folding intermediates are likely to be encountered in the early phases of refolding. Such a situation is described by the following generalized scheme (cf.[2]):

$$\begin{array}{ccccc} U^H & \underset{k_U}{\overset{k_I}{\rightleftharpoons}} & I^H & \overset{k_N}{\rightarrow} & N^H \\ k_c\downarrow & & & & \\ U^D & \underset{k_U}{\overset{k_I}{\rightleftharpoons}} & I^D & \overset{k_N}{\rightarrow} & N^D \end{array} \qquad (8)$$

where k_N is the rate-limiting step ($k_N << k_I$). The intermediate state, I, affects the competition behavior of a particular amide proton only if the structure formed in I is able to protect that proton against exchange. If this is the case, then three limiting situations can be distinguished:

1. If I is stable on the timescale of the exchange reaction and formed at a rate k_I comparable to k_c ($k_c \sim k_I >> k_U, k_N$), the model reduces to the scheme of Eq. (1) with k_I substituted for k_f in Eqs. (4) and (5); i.e., formation of I is now the kinetic step competing with exchange.

2. If I is only marginally stable ($k_I \sim k_U$) and $k_I >> k_c$, the folding rate k_f in Eqs. (4) and (5) can be interpreted as an effective rate of protection, given by

$$k_f = k_N \cdot (1 + k_I/k_U) \qquad (9)$$

i.e., the rate k_f determined on the basis of the simple scheme of Eq. (1) would overestimate the rate constant k_N. (Protons are lost via the backreaction to the unfolded form).

3. If I is unstable ($k_U >> k_I$), it is not detected by the competition experiment. Eq. (9) reduces to $k_f = k_N$ and we have again the simple situation of Eq. (1).

Different limiting cases in folding processes with nonnegligible transient accumulation of structured intermediate states cannot a priori be distinguished by the competition method, so that in general a unique interpretation in terms of elementary rates in Eq. (8) is not possible.

The competition experiment can, however, still provide structural characterization of complex folding pathways by comparing the apparent folding rates measured in different parts of the protein. Obtaining structural resolution relies on the following. First, the amide proton resonances in the folded protein must be assigned to specific residues in the amino acid sequence.[13] Second, the spatial protein structure in solution must be known, either from direct determination by NMR[24–26] or from experiments (primarily NMR) enabling detailed comparison with the crystal structure. Resolution in the spatial protein structure is then achieved, since each data point measured with the competition technique can be attributed to a specified location. This spatial resolution can be obtained also in the absence of knowledge on the individual elementary rates in the scheme of Eq. (8). This is in the following illustrated with the data on BPTI in Figures 2 and 3 and Table I.

An important initial observation for BPTI is that the close agreement between the data in Figure 3 and the curve shape predicted by the simple kinetic model of Eq. (1) demonstrates that the assumption of pH-independent folding rates is reasonable. This was to be expected, since BPTI has no titrating groups in the range between about pH 4.5 and 7.5.[27]

The groups observed in this initial competition study with BPTI include seven amide protons located in the core of the β-sheet and one amide proton in the C-terminal α-helix.[28] The variation in apparent folding rates for these sites is relatively small (Table I), indicating that the observed folding transition is quite cooperative. On a finer level we can distinguish three groups of protons on the basis of the different apparent folding rates. The smallest rate is observed for Met 52 located in the C-terminal α-helix; its pH transition is displaced by ca. 0.5 pH units to lower pH relative to the other amide protons (Fig. 3, Table I). The amide protons of Tyr 21, Phe 22, and Tyr 23 on the central strand of the β-sheet form a group with intermediate rates. The amide protons on the two peripheral strands, i.e., Gln 31, Phe 33, Tyr 35, and Phe 45, are protected at somewhat higher rates. While the lower rate of protection for Met 52 can be rationalized by the fact that this proton is in a different structural element, and also in terms of the more peripheral location of the C-terminal helix, inspection of the native BPTI structure reveals no obvious reason for the distinction among the different β-sheet protons.

Jullien and Baldwin[5] studied the folding kinetics of reduced BPTI (RCAM-BPTI) by stopped-flow measurements monitored by tyrosine absorption. The structure of this derivative, which is obtained by selective cleavage of the 14–38 disulfide bond, has been shown to resemble closely that of the native protein.[29] In 1.9 M GuHCl at 25°C and pH 6.8, three distinct folding phases were observed, with the major fraction (75% of the amplitude) formed at a rate of 25 s^{-1} and two minor fractions with rates of 0.07 and 0.005 s^{-1}. At 60°C and pH 6.1, a fast phase with a rate of 70 s^{-1} and a single slow process at 0.3 s^{-1} were observed with about equal amplitude. Although different experimental conditions were chosen for our competition experiments, it is apparent that the proton trapping manifested in Figures 2 and 3 occurs on the time scale of the fastest optically detected process. Furthermore, our observation of complete proton trapping on the acidic side of the pH-dependent competition data (Fig. 3) indicates that structure capable of protecting the central β-sheet amide protons is formed rapidly not only in the fast refolding form, but also in the slowly refolding forms of BPTI[5]. (A significant fraction (> 10%) of the protein remaining fully unfolded over a period of seconds would lead to a lower protonation level than that observed at the low pH end of the curves in Fig. 3).

The narrow range of folding rates for the β-sheet protons suggests that an early intermediate is formed with nativelike secondary structure in the core of the molecule. The limited evidence from the single amide proton in the C-terminal helix suggests that this more peripheral secondary structure is folded in an independent step. Optical probes[5] apparently monitor much slower, minor structure rearrangements which set in after the main features of the folded structure have been formed. These probably also include local structural rearrangements associated with proline isomerization (cf.[1]).

Further information on local folding rates resulted from NH exchange studies of the core protons in the BPTI β-sheet under destabilizing conditions,[15] where the transition predicted by the structural opening model[30] between the exchange-limited (EX_2) and the opening-limited (EX_1) region of the exchange kinetics was observed. Structurally resolved folding rates estimated from the location of the transition on the pH scale ranged between $1.5 \cdot 10^3$ and $4 \cdot 10^3$ s^{-1} at 68°C, and between 200 and 600 s^{-1} at 55°C in the presence of 3 M GuHCl. Additional observations suggested that these rates reflect refolding from only partially unfolded conformations. While the absolute rates should therefore not be directly compared, it is inter-

esting that the pattern of relative rates for the different β-sheet protons (k_2^g in Table II in[15]) is comparable with the results of the competition experiments reported in Table I.

The present results demonstrate that early events in protein folding can to a certain extent be structurally and kinetically characterized by ^{1}H-NMR observation of the competition between hydrogen exchange and structure condensation. A number of improvements and extensions of the method can be considered. Two-dimensional (2D) correlated spectroscopy (COSY)[31,32] or 2D nuclear Overhauser enhancement spectroscopy (NOESY)[33] could be used to resolve a more complete set of amide protons throughout the protein structure (cf[20]). The mixing technique could be improved by increasing the D_2O:H_2O ratio during the competition period. This would reduce the H_2O background present in the mixture and permit more extensive dilution of the denaturant; the resulting more strongly native conditions favor the population of folding intermediates.[1] Complementary information might be gained by using the pulse labeling method developed by the Baldwin group.[3,4] This variation of the competition method would offer improved flexibility and the possibility to characterize intermediates at any stage of folding, though this is achieved at the cost of further reduced time resolution compared to the methods used in this study. Finally and probably most important in practice, the number of proteins for which sequence-specific resonance assignments are available is growing rapidly, and it can be forseen that we shall soon be able to work with a number of proteins for which the solution conformation has been determined by NMR. On this basis we expect the presently proposed competition techniques to become instrumental in future efforts to unravel the protein folding puzzle.

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