[8] Amide Proton Exchange as a Probe of Protein Folding Pathways[1]

By PETER S. KIM

Introduction

The practical problems in determining a pathway of protein folding are quite formidable: a pathway has not been determined for any protein in structural terms. The methods that one would like to use (X-ray crystallography, NMR) to study structural aspects of protein folding are intrinsically slow, so that equilibrium folding intermediates are desired. In general, however, equilibrium intermediates are not significantly populated for single domain proteins, most likely due to marginal stability of intermediates inside the unfolding transition zone.

Kinetic studies of folding have the advantage that they can be carried out in conditions where the folded state is strongly favored and intermediates are more likely to be stable.[2] Although well-populated intermediates have been detected in kinetic folding experiments,[2a] the intermediates are transient and steps in folding are often fast (msec–min). Strategies for dealing with these problems include slowing down folding (e.g., at subzero temperatures in cryosolvents[3]) or adapting spectroscopic methods so that rapid measurements can be made (e.g., stopped-flow CD studies[4]). A third approach is summarized here: the protection from labeling that is provided by structure is measured at different stages during folding. The location and extent of labeling are determined after folding has gone to completion. Thus, analysis of the partially labeled protein is used to infer the structural state of the protein when it was labeled.

The choice of a labeling reagent is crucial. It is important that folding is not affected by the labeling reaction or conditions. Steps in folding are often fast, so the labeling reaction must be fast, and the ability to quench labeling is highly desirable. Since the labels will be located after folding has gone to completion, it is important that the native structure of the protein is not perturbed by labeling. An ideal labeling reaction, with regard to the above requirements, is amide proton exchange.

[1] Dedicated to the memory of Michael P. Graf.
[2] R. L. Baldwin, *Annu. Rev. Biochem.* **44,** 453 (1975).
[2a] Reviewed by P. S. Kim and R. L. Baldwin, *Annu. Rev. Biochem.* **51,** 459 (1982).
[3] A. L. Fink, this volume [10]
[4] A. M. Labhardt, this volume [7].

Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Methods for studying protein folding using amide proton exchange are described here. The feasibility of using amide proton exchange to study folding has been demonstrated using the radioactive isotope, ^{3}H. Recent work has begun to take advantage of the fact that the extent of labeling for individual amide protons can be determined using high resolution ^{1}H NMR and deuterium labeling. A strategy to characterize structural intermediates in folding using ^{1}H NMR and D_2O as a labeling reagent has been proposed.[5] It seems likely that these methods will provide a detailed and relatively high resolution picture of the folding process for small monomeric proteins.

Amide Proton Exchange

The probes for folding used here are the amide protons (peptide NH) of the polypeptide backbone. These nitrogen-bound protons exchange with solvent protons[6]; the exchange reaction is acid and base catalyzed, with a minimum exchange rate occurring near pH 2–3 (pH_{min}). Amide protons can thus be labeled with deuterium or tritium, using isotopic water (D_2O or $^{3}H_2O$).

The hydrogen-bond (H-bond) donors in both α-helices and β-sheets are the amide protons. When amide protons are H-bonded, their exchange rates drop dramatically, since the H-bond must break before exchange can occur; exchange involves the addition or removal of a proton by standard proton transfer mechanisms.[7,8] Solvent exclusion also retards exchange, since some solvent must be accessible to the peptide NH group in order for exchange to occur. H-bonding and solvent exclusion are thought to be chiefly responsible for the enormous reduction of exchange rates (up to 10^{10} times slower than the corresponding rate in a freely exposed amide) for some protons in native proteins. Amide proton exchange provides a very sensitive probe of structure formation.

The pH dependence of amide proton exchange rates offers a large dynamic range. In model compounds, exchange rates increase 10-fold for each pH unit increase in the base-catalyzed pH region (and vice versa in the acid-catalyzed region) of exchange. The half-time for exchange can be varied from greater than 1 hr (pH 3, 0°) to less than 1 msec (pH 10, 0°) by changing pH.[6] This makes it feasible to label a transient folding intermedi-

[5] K. Kuwajima, P. S. Kim, and R. L. Baldwin, *Biopolymers* **22,** 59 (1983).

[6] Reviewed by S. W. Englander, N. W. Downer, and H. Teitelbaum, *Annu. Rev. Biochem.* **41,** 903 (1972).

[7] M. Eigen, W. Kruse, G. Maass and L. DeMaeyer, *in* "Progress in Reaction Kinetics" (G. Porter, ed.), Vol. 2, p. 286. Pergamon, Oxford, 1964.

[8] M. Eigen, *Angew. Chem Int. Ed.* **3,** 1 (1964).

ate. For many small proteins it is also possible to quench exchange, if the protein can refold at low pH.

The replacement of ^1H with ^2H or ^3H is a minor perturbation as compared to most chemical labeling procedures. Where it has been checked, the structures of proteins are the same before and after the amide protons are replaced by deuterium atoms, as judged by X-ray crystallography.[9-11] Nevertheless, isotope effects and solvent perturbations (i.e., H_2O vs D_2O) must be considered in any amide proton exchange experiment (see Isotope and Solvent Effects).

Since deuterium atoms are not observed in an ^1H NMR spectrum, it is possible to use ^1H NMR to quantitate the extent of labeling at individual sites in a protein. Recent developments of ^1H NMR techniques to study proteins (in particular, the application of two-dimensional NMR techniques[12] by Wüthrich and co-workers[13,14] should make it feasible to "track" many individual amide protons in the folding processes of small, globular proteins.[5]

Exchange from the Native State

The mechanism of amide proton exchange from proteins, in conditions where the native conformation is stable, is the subject of much investigation and discussion.[15-20] For now, we simply note that studies of amide proton exchange in numerous proteins have shown that there is often a group of protons with substantially reduced rates of exchange (slowly exchanging protons). For example, approximately 50 amide protons in RNase A are resistant to exchange-out for at least 6 hr at pH 6, 10°. These protons have exchange rates that are reduced by at least a factor of $\sim 10^4$, since the average half-time for exchange in a freely exposed amide proton is a few seconds at pH 6, 10°.

The slowly exchanging protons are particularly well suited as probes for the folding process. (1) They are sensitive probes of structure formation; the exchange rates of these protons decrease by factors of 10^4 to 10^{10}

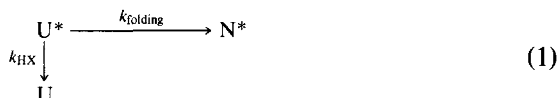
[9] A. A. Kossiakoff, *Nature (London)* **296,** 713 (1982).
[10] A. Wlodawer and L. Sjölin, *Biochemistry* **22,** 2720 (1983).
[11] B. P. Schoenborn, *Cold Spring Harbor Symp. Quant. Biol.* **36,** 569 (1971).
[12] W. P. Aue, J. Karhan, and R. R. Ernst, *J. Chem. Phys.* **64,** 4226 (1976).
[13] K. Wüthrich, G. Wider, G. Wagner, and W. Braun, *J. Mol. Biol.* **155,** 311 (1982).
[14] K. Wüthrich, *Biopolymers* **22,** 131 (1983).
[15] F. M. Richards, *Carlsberg Res. Commun.* **44,** 47 (1979).
[16] G. Wagner and K. Wüthrich, *J. Mol. Biol.* **160,** 343 (1982).
[17] C. Woodward, I. Simon, and E. Tüchsen, *Mol. Cell. Biochem.* **48,** 135 (1982).
[18] G. Wagner, *Q. Rev. Biophys.* **16,** 1 (1983).
[19] R. B. Gregory, L. Crabo, A. J. Percy, and A. Rosenberg, *Biochemistry* **22,** 910 (1983).
[20] S. W. Englander and N. R. Kallenbach, *Q. Rev. Biophys.* **16,** 521 (1983).

as the protein folds. (2) The slowly exchanging protons often represent a substantial fraction of the backbone amide protons in a protein (~50/119 in RNase A and ~30/53 in BPTI). (3) Since they are resistant to exchange in the native state, analysis of these protons can be carried out using methods that are intrinsically slow (e.g., NMR). (4) Most of the highly protected amide protons are H-bonded in secondary structure (α-helices, β-sheets) of the native protein in known cases (trypsin,[9] BPTI,[16,18] RNase A[10]). Therefore, exchange measurements of these protons in folding intermediates is likely to provide a readily interpretable picture, in terms of secondary structure units.

Overview of the Experimental Strategies

The Competition Method

Schmid and Baldwin[21] were the first to study a kinetic folding process with amide proton exchange. The experimental design was to set up a competition between refolding and exchange-out of the amide protons. Amide protons of the slow-folding species of RNase A were labeled with ^3H. The labeled, unfolded protein (in GuHCl and at the pH_{min}) was then rapidly diluted with nonradioactive buffer. This initiates the competition: the protein refolds since [GuHCl] is low, and exchange-out from the amide protons occurs at a rate that depends on the pH of the solution. In the absence of folding intermediates, the competition experiment can be represented as:



$$\begin{array}{ccc} U^* & \xrightarrow{k_{\text{folding}}} & N^* \\ {\scriptstyle k_{\text{HX}}}\downarrow & & \\ U & & \end{array} \tag{1}$$

where the asterisk indicates retention of label and k_{folding} and k_{HX} refer to the rate constants for folding and amide proton exchange, respectively. Label trapped in N* remains, since only the stable amide protons are studied.

In Eq. (1), a single reactant (U*) directly produces two products (U and N*). Thus, the relative concentrations of the two products gives the relative rate constants. Since those molecules that lose label (U* $\rightarrow$ U) will refold to give nonradioactive protein (N), we have

$$N^*/N = k_{\text{folding}}/k_{\text{HX}} \tag{2}$$

This equation assumes that there are no structural intermediates between U and N so that trapping of amide protons follows the same kinet-

[21] F. X. Schmid and R. L. Baldwin, *J. Mol. Biol.* **135,** 199 (1979).

ics as other probes of folding (e.g., tyrosine absorbance changes). The results obtained by Schmid and Baldwin[21] showed that substantially more protons were trapped than predicted by Eq. (2). For example, at pH 6, 10°, fewer than 3 protons are predicted to be trapped[21a] in the competition experiment using Eq. (2). The experimental results show that 20 protons are trapped under these conditions. A plot of the number of protons remaining ($^3H_{rem}$) vs pH showed that the experimental points were shifted to higher pH values by ~1.3 pH units from the curve predicted with Eq. (2). These results indicate that the effective rate of amide proton trapping during refolding was ~20-fold faster than $k_{folding}$ determined by tyrosine absorbance.[22] In other words, folding under these conditions is not an "all or none" process, but involves the rapid formation of at least one folding intermediate which protects protons from exchange.

The competition method is well suited for studying early folding intermediates. The drawbacks of the competition method are that it cannot be easily adapted to the study of later intermediates in folding, and it requires knowledge of exchange rates for different classes of amide protons in the unfolded protein (the empirical rules of Molday *et al.*[23] give reasonable estimates of these rates).

The Pulse-Labeling Method

A complementary method, pulse-labeling, has also been used to study the folding of RNase A.[24] Whereas the competition method measures trapping of protons by folding intermediates, the pulse-labeling method measures exclusion of label from amide groups by formation of folded structure.

The experimental design is as follows. Refolding is allowed to occur for a variable period of time before a labeling "pulse" is applied. Those protons whose exchange rates are retarded by structure will be protected from labeling. At the end of the pulse, exchange is quenched by lowering the pH, so that further exchange is slow compared to refolding. The extent of labeling is assayed after folding has gone to completion.

[21a] The term "trapped protons" is used throughout this chapter, and refers to the retention of label (i.e., during the competition) by amide protons that are slow to exchange in the native state. When reference is made to the number of trapped protons, it should be noted that if tritium methods are used, this number could correspond to partial labeling of many amides.

[22] This result is consistent with several mechanisms. The effective rate of amide proton trapping depends on both the rates of formation of the intermediates and the stability of the intermediates (see Ref. 21).

[23] R. S. Molday, S. W. Englander, and R. G. Kallen, *Biochemistry* **11**, 150 (1972).

[24] P. S. Kim and R. L. Baldwin, *Biochemistry* **19**, 6124 (1980).

When folding of the slow-folding species of RNase A is studied, the kinetics of protection from amide proton exchange measured with pulse-labeling are faster than the kinetics of tyrosine absorbance change.[24] This comparison (kinetic ratio test[25]) demonstrates that structure is formed, which protects amide protons from exchange, before burial of the tyrosine residues. The pulse-labeling results showed that ~20 protons were protected from label within 4 sec after folding was initiated,[24] confirming the existence of an early folding intermediate previously detected with the competition method.[21] This confirmation does not rely on knowing the exchange rates for amide proton exchange in the unfolded protein, since pulse-labeling conditions were chosen so that any freely exposed amide proton would be completely labeled by the pulse.

It may not always be possible to quench exchange at the pH_{min} (e.g., if the protein is not stable at low pH). McPhie[26] has used amide proton exchange without a quench to study the folding of pepsinogen (pepsinogen activates itself to pepsin at low pH). Here the "pulse" was 15 min (long enough to permit complete refolding of the protein), and labeling was stopped by gel filtration. It may also be possible to use a partial quench (e.g., decreasing the pH as much as possible, followed by gel filtration). Optimal quench conditions are readily evaluated (see Experimental Details).

The advantages of the pulse-labeling method are that it can be used to study intermediates at any time in folding and does not require accurate knowledge of exchange rates in the unfolded protein.

Medium Resolution Studies Using ^3H and HPLC

Amide proton exchange measurements have been classified as low, medium, or high resolution.[27] High-resolution techniques are those that monitor individual amide protons (e.g., ^1H NMR, neutron diffraction). Low-resolution methods monitor exchange from the entire protein, usually with ^3H as a label. Medium-resolution methods[28,29] use HPLC to separate labeled fragments of a protein, which can be obtained with proteases.

The folding of RNase S has been studied with medium-resolution

[25] A. M. Labhardt and R. L. Baldwin, *J. Mol. Biol.* **135,** 231 (1979).
[26] P. McPhie, *Biochemistry* **21,** 5509 (1982).
[27] N. M. Allewell, *J. Biochem. Biophys. Methods* **7,** 345 (1983).
[28] J. J. Rosa and F. M. Richards, *J. Mol. Biol.* **133,** 399 (1979).
[29] S. W. Englander, D. B. Calhoun, J. J. Englander, N. R. Kallenbach, R. K. H. Liem, E. L. Malin, C. Mandel, and J. R. Rogero, *Biophys. J.* **32,** 577 (1980).

methods.[30] RNase S[31] is an enzymatically active derivative of RNase A cleaved by subtilisn at the peptide bond between residues 20 and 21. The cleavage products can be separated and are referred to as S-peptide (residues 1–20) and S-protein (residues 21–124). S-peptide and S-protein recombine to form the noncovalent complex, RNase S.

In the medium-resolution study of RNase S folding by Brems and Baldwin,[30] it was not necessary to use a protease to obtain fragments. After pulse-labeling, S-peptide and S-protein were separated rapidly by HPLC, under conditions where exchange is slow (pH 2.7, 0°). Previous NMR studies[32] had shown that the stable amide protons in the S-peptide moiety of RNase S correspond to residues 7–14, and therefore ^3H in the HPLC peak of S-peptide corresponds predominantly to H-bond donors of the 3–13 α-helix (i.e., amide protons of residues 7–13). It was thus possible to investigate the rate at which the 3–13 α-helix is stabilized in the folding of RNase S. The results (refolding conditions: pH 6, 10°) show that whereas S-protein (residues 21–124) protects amide protons from exchange early in the kinetic folding process, the S-peptide α-helix is not protected from exchange until a late stage in folding.

If proteases are used to produce fragments of labeled proteins, then an acid protease such as pepsin is desirable since digestion can be done near the pH_{min} of exchange. Medium-resolution techniques (proteolysis and HPLC separation of fragments; introduced by Rosa and Richards[28]) are reviewed in detail by Rogero *et al.*[33]

Matthews and co-workers[34] have used medium-resolution ^3H methods to characterize domains in kinetic folding intermediates of the α-subunit of tryptophan synthase. Limited trypsin digestion (pH 5.5, 0°) cleaves the α-subunit into three fragments. Two of these fragments result from the NH_2-terminal domain, and the third fragment is the COOH-terminal domain of the α-subunit. All three fragments are structurally stable enough to permit quantitative recovery of ^3H label following HPLC separation (125 protons are stable to exchange-out for 6 hr at pH 5.5, 0°; all of these protons can be recovered following trypsin digestion and HPLC separation[34]). This demonstrates the feasibility of using medium-resolution techniques in proteins that are not stable at acid pH (e.g., the α-subunit), provided that stable fragments can be obtained. The results of Beasty and Matthews[34] show that the NH_2-terminal domain is significantly more resistant to exchange than the COOH-terminal domain in early folding inter-

[30] D. N. Brems and R. L. Baldwin, *J. Mol. Biol.* **180,** 1141 (1984).

[31] F. M. Richards and P. J. Vithayathil, *J. Biol. Chem.* **234,** 1459 (1959).

[32] K. Kuwajima and R. L. Baldwin, *J. Mol. Biol.* **169,** 281 (1983).

[33] J. R. Rogero, J. J. Englander, and S. W. Englander, this volume [22].

[34] A. M. Beasty and C. R. Matthews, *Biochemistry* **24,** 3547 (1985).

mediates of the intact α-subunit. This supports the model for α-subunit folding proposed earlier,[35] based on hydrodynamic and spectroscopic properties of the intermediates.

Medium-resolution methods promise to be useful tools for analysis of folding intermediates labeled with ^3H_2O. These methods are particularly useful when studying large multidomain proteins, as demonstrated by the characterization of domain folding in the α-subunit of tryptophan synthase. The pulse-labeling studies of RNase S folding demonstrate the feasibility of monitoring individual structural units during folding, even without NMR techniques.

NMR Studies

In order to use amide proton exchange to characterize structures of folding intermediates by NMR, it is necessary to change from H_2O to D_2O (or vice versa) during folding, and then allow folding to go to completion. The ^1H NMR spectrum of the protein is used to analyze the location and extent of amide proton labeling at individual sites; deuterium is not detected in a ^1H NMR spectrum. Note that the NMR spectrum is taken after folding is complete—resonance assignments for folding intermediates are not needed.

Analysis of the ^1H NMR spectrum requires that amide proton resonances are resolved (i.e., not overlap other resonances) and assigned to individual protons in the protein. A solution to both of these requirements is provided by the elegant two-dimensional (2D) NMR studies of BPTI by Wagner, Wüthrich and co-workers.[13,14,16,18,36] These 2D-NMR techniques have been used to obtain resonance assignments in a systematic manner for almost all protons in BPTI.[36] Then, by measuring the amide proton–C^α proton cross-peak intensities in 2D-homonuclear correlated ^1H NMR spectra (COSY), it was possible to make quantitative exchange measurements for 38 of the 53 backbone amide protons in BPTI.[16] Overlapping resonances in a one-dimensional NMR spectrum are usually resolved in crosspeaks of the two-dimensional spectrum, so that the extent of labeling for many more amide protons can be determined. Although 2D-NMR methods have been used primarily to study small proteins like BPTI (58 residues), work has begun in several laboratories to apply these techniques to larger proteins.

One-dimensional NMR techniques are capable of characterizing trapped amide protons, if the number of protons studied in a given spec-

[35] C. R. Matthews, M. M. Crisanti, J. T. Manz, and G. L. Gepner, *Biochemistry* **22,** 1445 (1983).

[36] G. Wagner and K. Wüthrich, *J. Mol. Biol.* **155,** 347 (1982).

trum is limited to a few amides (~10 at a time). This is demonstrated by Kuwajima and Baldwin's ^{1}H NMR studies of the eight amide protons in residues 7–14 of RNase S.[32,37] Samples were prepared in which only these eight amide protons were in the ^{1}H form; the remaining amide protons of RNase S were deuterated. By using differential exchange, it is sometimes possible to divide amide protons of a protein into classes that are small enough to be resolved using one-dimensional NMR.[38] Neutron diffraction studies of trypsin[9] and RNase A[10] give the locations of stably protected amide protons and present the possibility of assigning amide protons by complementary ^{1}H NMR and neutron diffraction techniques.

Roder's[39,39a] study of BPTI is the first direct demonstration of the feasibility of using NMR and amide proton exchange to study kinetic folding processes. The competition method was used, and trapping of ^{1}H label in eight individual amide groups was analyzed with one-dimensional NMR. A rapid mixing apparatus was used to study the fast-folding (U_F) species of BPTI, which is known to be the major unfolded species.[40] BPTI was unfolded in H_2O (40% v/v *n*-propanol, pH 2, 70°) and the refolding/exchange-out competition was initiated by mixing with D_2O buffer (final conditions: 70°, 13% v/v *n*-propanol, pH 4 to 7.5). Samples prepared in this manner were lyophilized and redissolved in D_2O. ^{1}H NMR was used to determine amide proton resonance intensities, with nonexchangeable protons as an internal concentration standard.

The results of this competition experiment[39,39a] were analyzed using intrinsic exchange rates obtained for the same amide protons in thermally unfolded RCAM-BPTI.[39,41] The apparent rates of amide proton trapping during refolding [i.e., corresponding to $k_{folding}$ in Eq. (1)] were similar for seven of the eight amide protons studied (k = 30–70 sec^{-1}); these seven amides are in the β-sheet of BPTI. One of the amide protons studied (Met-52) which is in the C-terminal helix of BPTI, showed a reduced rate of trapping during refolding (k = 15 sec^{-1}).

Definitive conclusions about the nature of intermediates in the folding of BPTI cannot yet be made, since only one amide proton in the α-helix was studied and since the kinetics of folding have not been measured under these conditions by other probes (e.g., tyrosine absorbance). Nevertheless, these experiments[39,39a] represent an important first step in the

[37] K. Kuwajima and R. L. Baldwin, *J. Mol. Biol.* **169,** 299 (1983).
[38] A. Bierzynski, N. R. Kallenbach, P. S. Kim, and R. L. Baldwin, unpublished results with RNase A.
[39] H. Roder, Ph.D. thesis No. 6932, ETH Zürich (1981).
[39a] H. Roder and K. Wüthrich, personal communication.
[40] M. Jullien and R. L. Baldwin, *J. Mol. Biol.* **145,** 265 (1981).
[41] H. Roder, G. Wagner, and K. Wüthrich, *Biochemistry* **24,** 7407 (1985).

application of NMR and amide proton exchange to the study of folding pathways.

Determining the Degree of Protection for Structural Units in Intermediates

The methods described here can be used to determine the degree of protection from exchange for different amide protons in a folding intermediate (cf. Ref. 37). We define the degree of protection from exchange for a given amide proton, $[\theta]_P$, as the ratio of the intrinsic exchange rate, k_{int} (i.e., in the absence of structure) to the observed exchange rate for the amide, k_{obs}:

$$[\theta]_P = k_{int}/k_{obs} \tag{3}$$

In the simplest model of exchange from an intermediate, where exchange occurs only after unfolding of the intermediate, we have

$$\mathrm{I} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{U} \xrightarrow{k_{int}} \mathrm{U}^* \tag{4}$$

where k_{12} and k_{21} refer to the rates of unfolding and refolding, respectively, of the intermediate. If $k_{21} \gg k_{int}$, then the observed rate of exchange is given as[42]

$$k_{obs} = K_{12}k_{int} \tag{5}$$

where $K_{12} = k_{12}/k_{21}$. Thus, $[\theta]_P = 1/K_{12}$, and the degree of protection is equal to the stability constant of the intermediate ($=1/K_{12}$), in this simple model for exchange.

If direct exchange from I is not negligible, however, or if exchange can occur from an intermediate between I and U in Eq. (4), then Eq. (5) is not valid. Since the mechanism for exchange from folding intermediates is unknown, and likely to be different for different intermediates, we cannot directly relate $[\theta]_P$ to the stability constant of the intermediate.

Nevertheless, $[\theta]_P$ changes dramatically during folding (for the stable amide protons, $[\theta]_P = 1$ in U, and $[\theta]_P = 10^4$ to 10^{10} in N). Determination of $[\theta]_P$ for different amide protons at successive stages in folding will give information about the apparent stabilities of structural units in intermediates.

[42] This case is analogous to the EX2 mechanism of amide proton exchange from proteins (see Ref. 43). In the limit, $k_{int} \gg k_{21}$ (analogous to the EX1 mechanism), biphasic exchange kinetics will be observed for individual amide protons, with rate constants k_{int} and k_{12}. The relative amplitudes of the two phases will depend on the [U] : [I] ratio.

[43] Reviewed by A. Hvidt and S. O. Nielsen, *Adv. Protein Chem.* **21,** 287 (1966).

The pulse-labeling method provides one way of obtaining the degree of protection, $[\theta]_P$. By varying the duration of the pulse (or by changing the pH of pulse labeling), it is possible to change the sensitivity of the pulse-labeling assay. For example, in the experimental conditions used to study RNase A[24] (pH 7.5, 10°) the average half-time for exchange in unfolded RNase A is ~0.1 sec. Therefore, a 10 sec pulse will label amide protons whose exchange rates are retarded by a factor of ~100 or less (i.e., $[\theta]_P < 100$). In principle, it is possible to titrate the stability of an intermediate, and determine $[\theta]_P$ for different structural units in the intermediate, by changing the sensitivity of the pulse-labeling assay.

For small single domain proteins, determination of $[\theta]_P$ for structural units (e.g., α-helices, β-sheets) in folding intermediates is best done using NMR techniques to observe individual amide protons. This eliminates the problems associated with ^3H methods, involving heterogeneity of intrinsic exchange rates. $[\theta]_P$ for the S-peptide α-helix in the equilibrium unfolding transition of RNase S has been determined by ^1H NMR measurements of individual amide proton exchange rates,[37] using RNase S that was specifically labeled as described earlier. When measurements are made within the unfolding transition zone, $[\theta]_P$ is the same for all seven amide protons in the S-peptide helix, within a factor of two.[37]

These results demonstrate the feasibility of using NMR and amide proton exchange to determine apparent stabilities ($[\theta]_P$) of structural units in equilibrium unfolding intermediates. The extension to kinetic intermediates is straightforward, but includes the usual difficulties encountered with a short-lived species. With kinetic intermediates, the labeling time must be short compared to individual steps in refolding (e.g., a short pulse should be used).

As a first step to obtain $[\theta]_P$ in kinetic intermediates, the sensitivity of the labeling assay could be changed (e.g., by changing the pH or duration of the pulse), and the extent of labeling for individual amide protons determined by ^1H NMR. A series of measurements with different sensitivities would give the observed exchange rate (k_{obs}) for an amide proton, at a particular point in folding.[44] $[\theta]_P$ could then be determined using Eq. (3).

Characterization of Folding Intermediates

As mentioned previously, the major factors responsible for retardation of amide proton exchange rates in folded proteins are believed to be H-bonding and solvent exclusion. Proposed mechanisms for exchange include penetration of solvent to the site of exchange,[15,17] and local unfold-

[44] The observed rate of exchange may be complicated by the presence of multiple populated intermediates.

ing of structural units[20] in the otherwise folded protein. These and other mechanisms are used to explain how exchange occurs under conditions where the native conformation is very stable. Under conditions where the protein is marginally stable (e.g., high temperatures) there is more general agreement—most workers believe that some form of unfolding (local or total) is responsible for exchange.

The rate of amide proton exchange from *unfolded* proteins can be predicted quite well using data obtained with solvent-exposed model compounds, as demonstrated by Molday *et al.*[23] Small amounts of residual structure have been detected in thermally unfolded proteins as compared to their denaturant-unfolded counterparts.[45] Amide proton exchange from thermally unfolded RNase S, however, is independent of urea concentration (0–5 *M* urea), after correction for the effects of urea on intrinsic exchange rates.[46,47] The polypeptide chain of unfolded RNase in aqueous solutions is accessible to solvent, at least with regard to amide proton exchange.

The retardation of exchange rates observed in kinetic folding experiments with RNase A and RNase S are large (>100-fold), demonstrating that populated folding intermediates exist. It seems likely that the major determinant retarding exchange in folding intermediates is H-bonding, and that methods described here will lead to determination of the H-bonded structures in intermediates. Exclusion of solvent may also contribute to inhibition of amide proton exchange in folding intermediates, since there is a large decrease in solvent accessible surface area when an α-helix or β-sheet is formed.[48] In any case, these methods will show what parts of the molecule are involved in structure that retards exchange.

For RNase A, results obtained with both the competition[21] and pulse-labeling[24] methods demonstrate that exchange rates for many amide protons are retarded by structure formed early in the folding process, before tyrosine absorbance changes are complete. The aromatic absorbance changes observed during folding occur when solvent is excluded from the environment of aromatic rings.[49] These data suggest that a substantial part of the secondary structure framework of RNase A is formed before tertiary structure in folding. Most results obtained with other methods also support a framework model of folding for single domain proteins.[2a] Amide

[45] Reviewed by C. Tanford, *Adv. Protein Chem.* **23**, 121 (1968).

[46] D. J. Loftus, G. O. Gbenle, P. S. Kim, and R. L. Baldwin, *Biochemistry* **25**, 1428 (1986).

[47] These effects were evaluated in the model compounds poly(DL-alanine) and *N*-acetyl-lysine methyl ester.

[48] Reviewed by F. M. Richards, *Annu. Rev. Biophys. Bioeng.* **6**, 151 (1977).

[49] S. Yanari and F. A. Bovey, *J. Biol. Chem.* **235**, 2818 (1960).

proton exchange methods should permit characterization of these early H-bonded frameworks.

Concluding Remarks

The methods described here permit labeling of transiently populated kinetic intermediates in a relatively nonperturbing manner. The large dynamic range of amide proton exchange rates makes it feasible to study fast steps in folding. Although most studies of folding by amide proton exchange have centered on slow-folding species, some preliminary results have been obtained with the fast-folding species (U_F) of BPTI.

A major feature of using amide proton exchange to study folding is that the locations and amount of label can ultimately be obtained with high resolution and precision, using ^1H NMR techniques after folding has gone to completion. A fairly detailed picture of protein folding pathways should emerge from these studies, since individually assigned amide protons in different parts of the protein can be studied.

It may be possible to use amide proton exchange methods to evaluate the stability of structural units in intermediates, in addition to the rate at which they form during folding. Eventually, one would like to quantitate the degree of protection from exchange ($[\theta]_P$) for individual amide protons, at successive stages in folding.

Low and medium resolution information can be obtained without NMR, using ^3H_2O as a labeling reagent. This offers a probe of secondary structure formation in most proteins. When combined with methods based on proteolysis and HPLC, information about the folding of structural units and domains can be obtained even in proteins that are too large to be studied by traditional NMR methods. ^3H methods are particularly important in determining suitable conditions for high resolution studies.

Experimental Details

^3H Methods: General Aspects

As with all studies involving amide proton exchange, it is important that the pH and temperature of solutions are carefully controlled. An error of 0.1 pH unit can result in a 25% change in intrinsic exchange rate, and the activation energy for exchange in model compounds in ~20 kcal/mol.[6] Buffers and temperature baths should be used, and the pH of solutions should be checked in trial runs containing protein (^3H_2O can be eliminated in these trials). Since charge effects on amide proton exchange

can be substantial,[50,51] a moderate ionic strength (>0.1 *M*) should be maintained. The required concentration of ^3H_2O depends on the number of protons that are labeled and on the amount of protein that is assayed. For RNase A, we have found that an ^3H_2O concentration of ~20 mCi/ml, and a sample size of ~1 mg (~0.1 μmol) works well. Since ^3H_2O is volatile, all manipulations should be done in a fume hood or enclosed glove box.

Typically the protein is unfolded with GuHCl or urea, under conditions where previous equilibrium measurements (e.g., of a spectroscopic probe) have demonstrated that the protein is completely unfolded. Thermal unfolding can be used, but should be avoided where possible, since the rates of refolding and amide proton exchange are both temperature dependent. In general, the protein should be kept unfolded long enough to allow complete equilibration of fast- and slow-folding species.[52]

Refolding is initiated by adding a small aliquot of unfolded protein to refolding buffer, so that the final denaturant concentration is low (a 1 : 10 to 1 : 20 dilution is usually used). In general, folding intermediates are stabilized by low temperatures (0–10°), low denaturant concentrations, and stabilizing salts [e.g., $(NH_4)_2SO_4$].

Labeled protein is separated from excess ^3H_2O on a short Sephadex column,[6] equilibrated at low temperature and low pH (if possible). Coarse or medium grade Sephadex will result in a faster separation, and up to 5 lb/in.^{2} of nitrogen pressure can be applied to the top of the column to increase the flow rate. It is possible to separate RNase A from ^3H_2O in less than 5 min using this procedure.[21]

At the end of all manipulations (including an exchange-out procedure if only the stable amide protons are to be studied), the protein must again be separated from tritium that has exchanged-out. A second Sephadex column[6] or a convenient filter paper assay[53] (utilizing cation-exchange paper) can be used. The filter assay is very fast and works very well with RNase A; however, each protein should be checked since some proteins do not give consistent results with the filter assay.[54,55]

It is necessary to correct for differences in protein recovery. This can be done spectroscopically, using known molar extinction coefficients or by including trace amounts of protein labeled with another isotope. Radioactive protein can be made by chemical modification (e.g., we reduc-

[50] P. S. Kim and R. L. Baldwin, *Biochemistry* **21,** 1 (1982).
[51] J. B. Matthew and F. M. Richards, *J. Biol. Chem.* **258,** 3039 (1983).
[52] F. X. Schmid, this volume [4].
[53] A. A. Schreier, *Anal. Biochem.* **83,** 178 (1977).
[54] M. Lennick and N. M. Allewell, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6759 (1981).
[55] K. R. Shoemaker and R. L. Baldwin, unpublished results.

tively methylate[56] the lysines of RNase A with $H^{14}CHO$ and $NaCNBH_3$) or, if possible, by growth of microorganisms in the presence of radioactive amino acids.

The Competition Method[21]

This method uses labeled, unfolded protein that has been separated from excess ^3H_2O. For this reason, it is desirable that the protein can be unfolded with a denaturant at low pH (pH_{min}) and low temperature. It is also desirable that refolding rates are independent of pH in the pH range where the competition will take place (or at least that the pH dependence of refolding rates is known).

If the slow-folding species (U_S) are to be studied, it may be advantageous to use the selective labeling procedure of Schmid and Baldwin[21] which does not label the fast-folding species (otherwise $U_F^* \rightarrow N^*$ contributes to the protons trapped during folding). The procedure is as follows. (1) Unfold in the absence of ^3H_2O until the $U_F \rightleftharpoons U_S$ reaction has reached equilibrium. (2) Refold for a short time under conditions where $U_F \rightarrow N$ is fast but $U_S \rightarrow N$ is slow. (3) Add ^3H_2O under conditions where exchange into U_S is fast, but exchange into the stable protons of N (formed from U_F) is slow. (4) Adjust pH to the pH_{min} and add denaturant to keep the protein unfolded. (5) Rapidly separate the labeled protein from ^3H_2O on a Sephadex column at 0°, equilibrated with denaturant at the pH_{min}. (6) This procedure gives U_F and U_S^*, which is used immediately in the competition experiment. Selective labeling of U_S can be obtained in this manner only if the $U_S \rightleftharpoons U_F$ equilibrium is slow compared to the column separation time.

The alternative to selective labeling of U_S is to label both U_S and U_F, and then correct for contribution of the $U_F^* \rightarrow N^*$ reaction to the amount of label trapped. This approach is simpler than selective labeling, but is less precise when the concentration of U_F is significant. Unfolded protein ($U_F \rightleftharpoons U_S$ equilibrium) is labeled with ^3H_2O and then separated from excess label on a Sephadex column equilibrated (pH_{min}, 0°) with denaturant to keep the protein unfolded. The competition experiment is carried out with the labeled mixture (U_F^*, U_S^*). The contribution of the $U_F^* \rightarrow N^*$ reaction can be evaluated if refolding of U_F is fast compared to exchange in the pH range used, and if the relative concentration of U_F molecules in the equilibrium unfolded mixture is known. Schmid and Baldwin[21] also used completely labeled RNase A (U_F^*, U_S^*) in their competition experiments. After correction for label trapped by the $U_F^* \rightarrow N^*$ reaction, the amount of label trapped by U_S^* was the same, within experi-

[56] N. Jentoft and D. G. Dearborn, *J. Biol. Chem.* **254**, 4359 (1979).

mental error, as that obtained using selectively labeled slow-folding species (U_S*).

If the fast-folding reaction is to be studied, then the completely labeled protein (U_S*, U_F*) can usually be used (cf. Refs. 39, 39a), since the competition conditions required to study the $U_F \rightarrow N$ reaction will typically result in complete loss of label from U_S* species. If the concentration of U_F is low in the equilibrium-unfolded mixture, then a high concentration of U_F (and hence, U_F*) can be obtained using the "double-jump" technique that has been used extensively in kinetic studies of refolding.[52] Briefly, this involves using unfolding conditions (high denaturant concentrations and low temperature) where $N \rightarrow U_F$ is fast and $U_F \rightleftharpoons U_S$ is slow, to populate U_F kinetically. The folding of U_F can then be studied without interference from U_S.

Competition between exchange-out and refolding is initiated by diluting the unfolded, labeled protein with refolding/exchange-out buffer. Typically, refolding is allowed to continue to completion, although exchange-out can be quenched after a shorter time of competition. When refolding is complete, the partially labeled protein is exchanged-out[56a] so that only the stably protected protons remain. Exchange-out can be carried out at the pH of competition. Alternatively, one set of exchange-out conditions can be used for all samples. In either case, the number of protons that remain after the competition is compared to that obtained with labeled native protein (N*), exchanged-out in the same conditions.

It is necessary to obtain values for k_{HX} and $k_{folding}$ in order to predict trapping of label in the absence of populated intermediates [Eqs. (1) and (2)]. Reasonable estimates of k_{HX} can be obtained using the rules of Molday *et al.*[23] It is also possible to measure amide proton exchange directly from the unfolded protein. This can be done by measuring exchange from (1) thermally unfolded protein—significant errors may result from uncertainties in extrapolation to lower temperatures, (2) proteins unfolded due to chemical modification (e.g., reduction and blocking of S–S bonds), where errors (usually small) may result from residual structure, and (3) urea-unfolded proteins, where errors may result from uncertainties in the correction for urea effects on exchange rates (these effects have been calibrated in model compounds[46] and are small compared to temperature effects).

Values for $k_{folding}$ are obtained preferably with a probe for folding that monitors formation of tertiary structure. These include absorbance or fluorescence from chromophores (e.g., aromatic residues) in the protein,

[56a] This final exchange-out procedure should not be confused with exchange that occurs during the competition.

and probes of the active site (e.g., specific inhibitor binding, enzymatic activity). If there are multiple unfolded forms of the protein, then it is important to determine accurately the concentration as well as folding rates of each unfolded species. The pH dependence of relative amplitudes and rates of folding should be determined.

The amount of label trapped in the absence of folding intermediates is predicted using the "null equation."[21] The null equation is essentially Eq. (2), modified to include the following sources of complexity. (a) Exchange rates for amide protons in the unfolded protein are not all the same, so that k_{HX} in Eq. (2) is replaced by a summation over all amides, each with its own k_{HX}. (b) If there are multiple unfolded species, then $k_{folding}$ in Eq. (2) is also a summation, weighted according to the relative concentration of each unfolded species. (c) Only a fraction of the amide protons is stable to the exchange-out procedure used. This fraction is determined in control experiments with N*, as described previously. Unless there are data to suggest otherwise, one assumes that the stable protons are a representative sample of all amide protons, with regard to their exchange rates in the unfolded protein. When NMR methods are used to monitor trapping by individual amide protons, the only source of complexity comes from multiple unfolded species.

A good control is to repeat the competition experiment under conditions where intermediates are not populated during folding (i.e., to directly test the null equation). For this purpose, marginally native folding conditions have been used. The refolding/exchange-out competition is performed just outside the unfolding transition zone, where folding is slower but goes to completion. In these conditions, folding usually occurs without populated intermediates, which are destabilized near the transition zone. Moderate concentrations of denaturants can be included in refolding/exchange-out buffers to achieve marginally native folding conditions. Corrections for the effects of denaturants on k_{HX} can be made by using the data of Loftus *et al.*,[46] who calibrated GuHCl and urea effects on intrinsic exchange rates. When the competition experiment was carried out with RNase A in the presence of 2.5 *M* GuHCl (conditions where folding goes to completion), the amount of ^3H label retained coincided with that predicted by the null equation.[21]

The Pulse-Labeling Method[24]

From a practical point of view, the pulse-labeling method offers the advantage that unfolding can be done at any pH, and refolding can be studied at one pH, so that the pH dependence of refolding rates does not need to be investigated. The method does not require knowledge about differences in exchange rates of amide protons in the unfolded protein. Amplitudes and rate constants for individual kinetic phases in refolding

do not need to be characterized extensively. This is because intermediates that protect amide protons are demonstrated by a direct comparison of kinetic progress curves (kinetic ratio test[25]) rather than by calculation of expected results in the absence of intermediates. In general, the pulse-labeling method is more time consuming than the competition method, since the samples taken at each time point must be individually manipulated through all steps that follow.

Unfolded protein ($U_S \rightleftharpoons U_F$ equilibrium) is refolded by rapid dilution into buffer. It is preferable, but not necessary, to refold at the same pH as the labeling pH to be used. Since the $U_F \rightarrow N$ reaction typically occurs in a fraction of a second, it does not usually interfere with measurements of the $U_S \rightarrow N$ reaction.

The protein is allowed to refold for a variable period of time before the labeling pulse is applied. For a given set of labeling conditions, the time of refolding before addition of label is the only variable. If the pH of refolding is the same as the labeling pH, then the pulse can be initiated by adding ^3H_2O directly to the buffered refolding solution. If the labeling pH is different from refolding pH, then ^3H_2O should be added as a buffered solution. Errors in pH and/or duration of the pulse are the largest sources of scatter in the data.

Some guidelines for initial pulse-labeling studies are as follows. The temperature of labeling and refolding should be the same. When possible, the pulse should be short compared to folding (if necessary, a rapid-mixing device can be used). The pH of labeling is chosen based on the average exchange rate of an exposed amide proton. We want to label completely any amide proton that is not protected from exchange (i.e., $[\theta]_P = 1$), without labeling protected protons in the native protein (i.e., $[\theta]_P = 10^4$ to 10^{10}). A good starting point is to use a pulse that on average labels protons with $[\theta]_P < 50$. The sensitivity (S) of the pulse-labeling assay is defined here as the average value of $[\theta]_P$ for amide protons that are half-labeled by the pulse:

$$S = t/(t_{1/2})_{HX} \qquad (6)$$

where $(t_{1/2})_{HX}$ is the average half-time for exchange in the absence of structure at the pH and temperature used in the pulse, and t is the duration of the (short) pulse. Values of $(t_{1/2})_{HX}$ are readily available from model compound studies of amide proton exchange.[6,23] Since the kinetic ratio test[25] will be used, it is not necessary to know the distribution of intrinsic exchange rates [i.e., different $(t_{1/2})_{HX}$ values]. However, it should be recognized that S will be different for different amide protons in the protein due to individual differences in $(t_{1/2})_{HX}$. Since amide proton exchange rates are similar[6] within a factor of 10 (with a few exceptions), values of S

below 50 are not generally recommended. Two control experiments should be mentioned here. It is important to demonstrate that the pulse does not significantly label stable protons in the folded protein (N), and that the pulse completely labels unfolded protein (U). The latter can be checked by pulse-labeling U in the presence of denaturants, since the effects of GuHCl and urea on intrinsic exchange rates are small.

At the end of the labeling pulse, exchange is quenched, if possible by lowering the pH to the pH_{min} (for RNase A, pH_{min} 2.7). A weak acid (e.g., formic) or buffered solution can be used. If the protein is not stable at the pH_{min}, then a partial quench can be achieved by lowering the pH as much as possible. Decreasing the temperature may also help. The best pH to use for a quench will usually be one where the ratio of folding rate to intrinsic amide proton exchange rate is largest.

The effectiveness of the quench can be evaluated by measuring the amount of label that is incorporated into protein that has been refolded at the quench conditions, in the presence of ^3H_2O. Exchange-in rather than exchange-out is measured since the former has a lower background and is therefore more sensitive. Checking the quench in this manner gives the maximum effect of incomplete quenching on pulse-labeling, since this test starts with unfolded protein whereas intermediates (with some protected protons) are quenched in pulse-labeling experiments. When RNase A ($U_S \rightharpoonup U_F$) is refolded at pH 2.7, 3° (i.e., conditions corresponding to the quench), in the presence of ^3H_2O, approximately 3 protons are stably trapped.[24] The most likely sources of these protons are (1) nonspecific, low-level labeling of backbone amide protons and (2) specific labeling of some protons with significant exchange rates at pH 2.7, especially the side-chain primary amide protons of asparagine and glutamine residues.[57]

Exchange-in experiments during refolding can be used to define the optimum pH for quenching exchange relative to folding. This is particularly useful for proteins that are not stable at the pH_{min}.

The partially labeled protein is separated from excess ^3H_2O on a Sephadex column, equilibrated at the quench pH. The column is kept cold, and refolding continues during the separation. The separated, pulse-labeled protein solution is adjusted to exchange-out conditions that have been determined previously, so that only the stable amide protons re-

[57] The side-chain amide protons of Asn and Gln residues have minimum exchange rates near pH 4–5 (Ref. 23). At pH 2.7, 0°, exchange from these side-chain protons has a rate approximately 1000-fold faster than the average rate of exchange from backbone amide protons. In native proteins, exchange from side-chain amides can also be retarded by structure; for example, the side-chain amide of Asn-43 is one of the slowly exchanging protons in BPTI (Ref. 58).

[58] R. Richarz, P. Sehr, G. Wagner, and K. Wüthrich, *J. Mol. Biol.* **130,** 19 (1979).

main. The number of stable protons trapped in pulse labeling is determined as a function of refolding time before the pulse was applied.

The pulse-labeling results are analyzed with the kinetic ratio test[25] which states that, if normalized kinetic progress curves obtained with two probes are not superimposable (i.e., the kinetic ratio test is positive), then a populated folding intermediate is detected. The kinetic ratio test requires that (1) the reaction is monomolecular (e.g., folding of a polypeptide chain) and (2) different initial species (i.e., unfolded proteins) are indistinguishable by the probes used.

The kinetic ratio test can be extended to multimolecular processes only if the observed time-dependent changes arise from the participation of exactly one of the components (e.g., in the RNase S pulse-labeling study,[30] S-peptide contains no tyrosine, and the amounts of label in S-peptide and S-protein can be quantitated separately following HPLC separation). If there are multiple unfolded forms (e.g., different U_S species) which refold at different rates, the kinetic ratio test is still valid provided that different unfolded forms are indistinguishable by the probes used[24,25] (e.g., same number of exposed amide protons, same absorbance extinction coefficient, lack of enzymatic activity). For the derivation, and a more complete discussion of the kinetic ratio test, see Labhardt and Baldwin.[25]

The pulse-labeling data are normalized and compared to the normalized changes of another probe of folding. Since pulse-labeling is likely to be most sensitive to secondary structure formation, it is usually best to use a tertiary structure probe in the kinetic ratio test.

As a control, it is desirable to find conditions where the kinetic ratio test is negative. This is likely to occur in marginally native folding conditions. When the folding of RNase A (U_S) was studied in 2.5 *M* GuHCl (pH 7.5, 10°), the normalized kinetics of protection from exchange and of tyrosine absorbance changes during folding were superimposable.[24]

Isotope and Solvent Effects

The equilibrium isotope effect for amide proton exchange has been measured ($^3H/^1H = 1.21$; $^3H/^2H = 1.05$), and results should be corrected accordingly.[6] Kinetic isotope effects have also been measured and found to be small.[59,60] When changing solvent from H_2O to D_2O (or vice versa), it is probably best *not* to correct pH for the glass electrode effect—both exchange rates[60] and apparent ionization constants[61] in model compounds

[59] R. S. Molday and R. G. Kallen, *J. Am. Chem. Soc.* **94,** 6739 (1972).

[60] J. J. Englander, D. B. Calhoun, and S. W. Englander, *Anal. Biochem.* **92,** 517 (1979).

[61] A. Bundi and K. Wüthrich, *Biopolymers* **18,** 285 (1979).

are similar in H_2O and D_2O when pH meter readings without corrections are used.

It is possible that the stability of intermediates will be altered by changes in solvent (H_2O to D_2O) or deuteration of H-bond donors. ^3H_2O is used only in tracer amounts and is likely to be much less perturbing than D_2O. In general, D_2O has a stabilizing effect on native proteins as compared to H_2O.[43] The isotope-induced effects on stability are likely to be minor perturbations as compared to the large stability changes that occur in protein folding. Nevertheless, it will be important to check this directly in labeling experiments utilizing ^1H NMR. This can be done by using H_2O when D_2O is called for (and vice versa) in folding studies. If complementary experiments give the same results, this suggests that isotope-dependent effects do not significantly perturb intermediates in the folding pathways.

Acknowledgments

It is a pleasure to thank Dr. Robert L. Baldwin for his advice and discussions. I am also grateful to A. Bierzynski, D. N. Brems, J. Carey, K. H. Cook, N. R. Kallenbach, K. Kuwajima, D. Loftus, C. R. Matthews, C. Mitchinson, H. Roder, F. X. Schmid, and K. R. Shoemaker for discussions. This research was supported by grants from the National Institutes of Health (GM19988-22) and the National Science Foundation (PCM77-16834) to R. L. Baldwin, and by a Medical Scientist Training Program Grant from the National Institutes of Health (GM07365). Use of the Stanford Magnetic Resonance Facility (supported by NSF Grant GP23633 and NIH Grant RR00711) is acknowledged.

[9] Detection of Folding Intermediates Using Urea-Gradient Electrophoresis

By THOMAS E. CREIGHTON

The pathway of protein folding must be studied kinetically because the partially folded intermediates that define it are inherently unstable and not present in significant concentrations at equilibrium.[1] Unstable intermediates might accumulate transiently as kinetic intermediates in either unfolding or refolding, although there is no fundamental requirement that they do so. The only intermediates that will accumulate are those that both precede the rate-limiting step in the pathway and, under the final conditions, have free energies close to, or lower than, the initial form of the protein. These are severe restrictions, for the fully folded and unfolded states under most conditions differ only slightly in free energy.[1]

[1] P. L. Privalov, *Adv. Protein Chem.* **33,** 167 (1979).

Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved.