Consideration of the Possibility That the Slow Step in Protein Denaturation Reactions Is Due to Cis–Trans Isomerism of Proline Residues†

John F. Brandts,* Herbert R. Halvorson,‡ and Maureen Brennan

ABSTRACT: A model is proposed to account for the observation that the denaturation of small proteins *apparently* occurs in two kinetic phases. It is suggested that only one of these phases—the fast one—is actually an unfolding process. The slow phase is assumed to arise from the cis–trans isomerism of proline residues in the denaturated protein. From model compound data, it is shown that the expected rate for isomerism is in satisfactory agreement with the rates actually observed for protein folding. It is also shown that a simple model of protein unfolding based on the isomerism concept is very successful in accounting for many known experimental characteristics of the kinetics and thermodynamics of protein denaturation. Thus, the model is able to predict that two kinetic phases will be seen in the transition region while none are seen in the base-line regions, that both the fast and slow refolding phases lead to the native protein as the product, that the fast phase becomes the only observable phase for jumps ending far in the denatured base-line region, that most or all small proteins show a limiting low-temperature activation energy of ca. 20,000 cal, and that the relaxation time for the slow phase seen in cytochrome *c* denaturation is much shorter than for all other small proteins. By utilizing "double-jump" experiments, it is shown directly that the slow phase is not part of the unfolding process but that it corresponds to a transition among two or more denatured forms which have identical spectroscopic (286.5 nm) properties. Thus, the slow relaxation is "invisible" except in the transition region where it couples to the fast unfolding equilibrium. Finally, since the present model assumes that only one of the major kinetic phases seen in denaturation reactions is concerned with the denaturation process per se, it is in agreement with numerous thermodynamic studies which show consistency with the two-state model for unfolding.

The mechanism of protein folding and unfolding has long been a controversial topic. One of the current areas of interest concerns the degree of cooperativity exhibited by major conformational rearrangements such as those involved in thermal denaturation. Opinions vary depending upon whether one emphasizes the picture which seems obviously consistent with available thermodynamic evidence or whether one places stronger emphasis on maintaining apparent consistency with recent kinetic evidence. Thus, the two types of information tend to highlight different aspects of the unfolding and refolding processes.

Thermodynamic data have generally been quantitatively consistent with the idea that the unfolding of small globular proteins is suitably approximated by a two-state model if it is assumed that the states are macroscopic in nature so that small within-state variations are allowed (Lumry et al., 1966; Brandts, 1969; Tanford, 1970). This being the case, it has been shown for several small proteins that the more critical thermodynamic tests for two-state behavior are obeyed to a good approximation. Although much evidence along these lines exists, the major point can be made by referring only to the recent paper by Privalov and Khechinashvili (1974). That paper reports the results of a calorimetric study of the reversible thermal denaturation of five small proteins (ribonuclease, chymotrypsinogen, cytochrome *c*, lysozyme, and metmyoglobin). It was shown that the ratio of the calorimetric enthalpy change to the van't Hoff enthalpy change is nearly unity (average $\Delta H_{cal}/\Delta H_{VH}$ of ca. 1.04 ± 0.03) for each of these transitions at a number of different pH values and temperatures. The heat capacity ratio, $\Delta C_{P_{cal}}/\Delta C_{P_{VH}}$, which should be even more sensitive to non-two-state behavior than the enthalpy ratio (Brandts, 1969), is also unity (within errors of ca. 10–15%) for all five protein transitions. These data are certainly strong prima facie evidence against the idea that the thermal denaturation of small proteins occurs with the formation of *high* concentrations of macroscopic states other than the principal native and denatured states.

On the other hand, recent kinetic data seem to support a very different picture of the unfolding process for many of the same proteins mentioned above. Of the thermal denaturation reactions which have been examined carefully, those of ribonuclease (Tsong et al., 1972), chymotrypsinogen (Tsong and Baldwin, 1972), cytochrome *c* (Tsong, 1973), and metmyoglobin (Summers and McPhie, 1972) exhibit complex kinetics in aqueous solution. A slow and a fast phase are seen in both the unfolding and refolding directions; the apparent relaxation times are separated by a factor of nearly 100. In the case of ribonuclease, for example, the percent of the total amplitude which occurs in the fast phase varies from nearly 0 to 100%, depending on conditions, so that sizable concentrations of "intermediates" are suggested.

In guanidine hydrochloride solutions, kinetic complexities similar to those described above are also found for the denaturation of cytochrome *c* (Ikai et al., 1973) and β-lactoglobulin (Ikai, 1971). It appears that lysozyme may be different from the rest of these proteins since Tanford et al. (1973) found that only one phase (slow) was present in the transition region for this protein in aqueous guanidine hydrochloride; however, the kinetics do become more complex

† From the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received April 17, 1975.* Supported by Grant No. GM-11071 from the National Institutes of Health.

‡ Present address: Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Michigan 48202.

in the base-line regions. Also, Segawa et al. (1973) reported that the unfolding of lysozyme in 4.5 *M* LiBr occurs with only a single relaxation time.

Kinetic studies, then, appear to detect complexities in the folding and unfolding of small proteins which go unnoticed even in very careful thermodynamic experiments. This likely reflects more than just a *general* lack of sensitivity of thermodynamic methods to the presence of structural intermediates. Rather, we feel that the apparent discrepancy which seems to exist between experimental rate data and equilibrium data does in fact lead to specific postulates about the nature of the additional states which are seen kinetically. The purpose of this paper is to show that a model for protein unfolding which assigns the slow kinetic phase to cis–trans isomerism of proline residues is consistent with all available data. Furthermore, it is a unique feature of this particular model that *it will lead to the occurrence of intermediates which will be easily detected in kinetic experiments but which will be virtually impossible to detect in thermodynamic experiments.* The model requires no ad hoc postulates about protein structure, since proline isomerism in model compounds is a well-documented phenomenon. Data are presented to show that the relaxation time for isomerism is in the right time range to account for the slow phase of protein folding and unfolding. Finally, it is shown that the slow phase of ribonuclease unfolding represents a transition between different forms of the denatured protein which have virtually identical spectroscopic properties (286.5 nm). This shows that structural changes associated with the slow phase are extremely subtle, as would be expected for the proposed model.

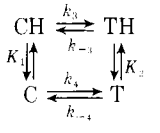
The Behavior of Proline-Containing Peptides

The ability of proline to isomerize has been demonstrated in numerous studies of polyproline. This polymer readily undergoes a transition from the all-cis form (polyproline I) to the all-trans form (polyproline II) which can be easily followed by proton or carbon nuclear magnetic resonance (NMR) since separate resonances are observed for the cis and trans isomers. The reaction follows zero-order kinetics throughout most of its course and exhibits a half-time of many hours at room temperature when the samples are of high molecular weight. It has been observed that the half-time becomes shorter as the degree of polymerization of the sample decreases. This observation, together with the zero-order kinetics, led Torchia and Bovey (1971) to propose that the isomerism is a concerted process which begins at one end of the molecule and proceeds, *one residue at a time,* to the other end. Consequently, the polyproline transition is really not a good one to study if one wants to learn about the characteristics of isolated prolines in the typical protein where nearest neighbors will probably be nonproline residues.

A limited amount of information exists on more suitable oligopeptides and other proline-containing compounds. Thomas and Williams (1972) reported the results of ^{13}C NMR studies which suggested that aqueous solutions of three simple proline dipeptides (Gly-Pro, Ala-Pro, and Val-Pro) contain nearly 40% of the cis configuration at neutral pH. This agrees well with the data of Evans and Rabenstein (1974), who found 35% cis for both Gly-Pro and Ala-Pro. They also found, as reported earlier by Wüthrich and Grathwohl (1974), that titration of the carboxy terminus leads to a marked decrease in the amount of the cis isomer to about 10–15%. Keim et al. (1974) found that the pentapeptide Gly-Gly-Pro-Gly-Gly has about 10–15% cis form and this is independent of the state of ionization of the terminal groups. Torchia (1972) examined the copolymers poly(Gly-Gly-Pro-Gly) and poly(Pro-Gly), both of which exist as random coils in aqueous solution. These two polymers show 20% cis isomer near room temperature.

Although these data are incomplete, they do suggest that an isolated proline in a random chain might possess something on the order of 10–30% cis character, although this will probably depend to some extent on steric effects from neighboring side chains. Also, the fraction of cis isomer may possibly depend on nearby ionizable groups. It is expected, however, that this estimate will be relatively independent of the temperature. The enthalpy difference between cis and trans configurations of peptide bonds is very small. Madison and Schellman (1970) found that the isomer ratio for *N*-acetyl-L-proline-*N*,*N*-diisopropylamide was independent of temperature over the range 30–90°C in water, although a slight shift toward the trans isomer was noted at high temperature when the solvent was dioxane. Torchia (1972) found a small increase in enthalpy (ca. 1 kcal) upon conversion to the cis form for poly(Gly-Pro), while others have reported no detectable temperature effect for other proline derivatives (Maia et al., 1971).

Except for studies of polyproline, the kinetics of cis–trans isomerism have been relatively neglected. In fact, there are no studies in the literature which report rates for isomerism of proline peptides in water. In view of the absence of suitable kinetic data on proline derivatives in water, we have examined three dipeptides (Gly-Pro, Ala-Pro, and Val-Pro) in the pH region of carboxyl titration. Since it is known that the amount of cis isomer varies from neutral to acidic solution for these dipeptides (Evans and Rabenstein, 1974; Wüthrich and Grathwohl, 1974), it necessarily follows that the pK of the cis and the trans forms differ. Therefore, the isomerism reaction can be studied by following the time dependence of proton release after a pH jump. The following scheme pertains:



where K_1 and K_2 are proton dissociation constants for the cis and trans forms, respectively, and where k_3, k_{-3}, k_4, and k_{-4} are rate constants for the isomerism of the protonated and unprotonated forms. If the protonation reactions are assumed to be very fast, then there will only be one slow relaxation time for this system (Garel and Labouesse, 1971), i.e.:

$$\tau^{-1} = \frac{k_3[H^+] + k_4K_1}{[H^+] + K_1} + \frac{k_{-3}[H^+] + k_{-4}K_2}{[H^+] + K_2} \qquad (1)$$

The experimental relaxation times for the three dipeptides at 22.5°C are shown in Figure 1, plotted against the final pH (see Experimental Section). As might have been anticipated, the isomerism reaction becomes slower as the bulkiness of the side chain of the N-terminal residue increases in the series Gly-Pro, Ala-Pro, and Val-Pro. According to eq 1, the limiting value of τ^{-1} at high pH will be $k_4 + k_{-4}$, and it is seen that this parameter for Gly-Pro (0.004 sec^{-1}) is about 60% larger than that for Val-Pro (0.0025 sec^{-1}). Unfortunately, the amplitudes of these relaxations become unmanageably small as the pH is reduced below 3, so that it is not possible to obtain a precise value

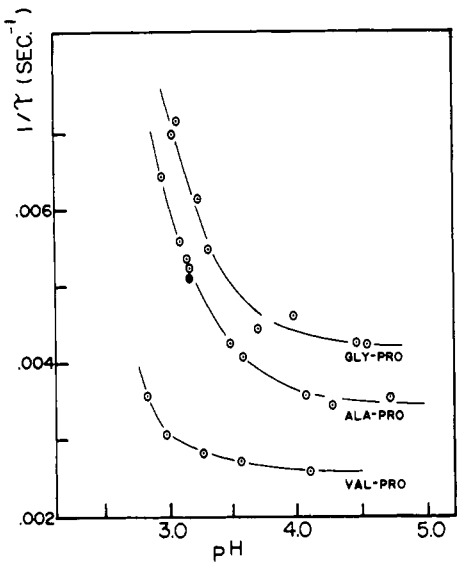


FIGURE 1: Reciprocal relaxation times for several proline-containing dipeptides, as a function of pH at 22.5°C. The single filled circle was determined spectrophotometrically, while all others were the result of direct pH measurements.

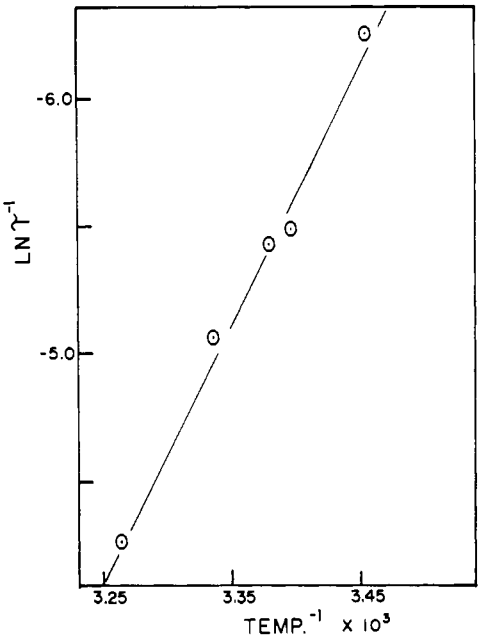


FIGURE 2: Arrhenius plot of the relaxation time for the isomerism of L-alanyl-L-proline. The final pH of the sample was 3.5 in all instances.

for the low pH limit, $k_3 + k_{-3}$. However, by utilizing the thermodynamic data in the literature, we can estimate k_3 and k_{-3} for Ala-Pro. From ^{13}C NMR, Evans and Rabenstein (1974) have estimated 11% cis (i.e., $K_3 = 8.1$) for the cationic form of Ala-Pro and 35% cis ($K_4 = 1.86$) for the zwitterionic form. The pK of the trans form was found to be 3.22, which means the pK for the cis form is 2.58 since the ratio K_3K_2/K_1K_4 must be unity. These thermodynamic data reduce the degrees of freedom in eq 1 from six to two, so that the remaining constants can be obtained by fitting to the data in Figure 1. Utilizing this approach, the resulting values of k_3 and k_{-3} are about 0.012 and 0.0015 sec^{-1}, respectively. These estimates suggest that the relaxation time for the isomerism of the cationic form of Ala-Pro is only about 25% as large as that for the zwitterionic form.

The relaxation time for Ala-Pro was also measured at several temperatures over the range 16–33°C. An Arrhenius plot of the data is shown in Figure 2, and this leads to an activation energy of 19,800 cal. These data were all taken at pH 3.5 where τ^{-1} is almost equal to $k_4 + k_{-4}$. Since the activation energy for the forward and reverse steps should be nearly identical, this will be very close to the true activation energy for isomerism of the zwitterionic form. This estimate is in good agreement with E_a values for polyproline (Torchia and Bovey, 1971) and other small proline and proline-like molecules (Maia et al., 1971; Portnova et al., 1970), where values range from 16,000 to 23,000 cal.

The relaxation times for Ala-Pro were found to be only slightly sensitive to the presence of electrolytes at pH 3.9 and 22.5°C. The addition of 0.5 M NaCl increased τ^{-1} by only about 10% while 0.3 M Na_2SO_4 caused a 25% increase. More striking than the change in rate was the loss in amplitude as Na_2SO_4 was added. At concentrations much higher than 0.3 M, the relaxation amplitude was too small to be studied.

Control experiments showed that a terminal proline was necessary in order that a pH relaxation be observed. Thus, no relaxation could be seen for Gly-Ala or for Gly-Pro-Ala under identical conditions with those for which the proline dipeptides showed large relaxations. On the other hand, the relaxations for the latter could be followed either by monitoring pH directly with a pH meter, as was done for nearly all of the above data, or by adding a small amount of an appropriate pH indicator (Methyl Orange) and following the relaxation spectrophotometrically. The two methods yielded identical relaxation times (see Figure 1).

These studies show that proline isomerism is a slow process near room temperature, but not nearly so slow as the polyproline I → II transition. The relaxation time for zwitterionic Ala-Pro is about 300 sec while that for the cationic form is *roughly* 75 sec at 22.5°C. An increase in the bulkiness of the side chain on the N-terminal side of proline in the dipeptides causes a significant increase in the relaxation time. Although it seems likely that the cationic form of these dipeptides would more closely mimic the behavior of a single proline in a long polypeptide chain, these model compound data can only be considered as crude approximations in any case. Even the cationic form may give misleading information at a quantitative level for at least two reasons. First, the presence of a residue on the carboxy-terminal side of proline might introduce additional steric interference in the isomerism. Second, it has been suggested that dipeptides such as these may be able to form cyclic hydrogen-bonded structures which involve the carboxylic acid proton (Evans and Rabenstein, 1974). These protons will not be present for single prolines in protein chains.

Other published kinetic data on model compounds pertain to nonaqueous solvents, usually deuteriochloroform. These include *N*-benzyloxycarbonyl-L-proline *tert*-butyl ester ($\tau_{25} = 0.5$ sec, $E_a = 16{,}000$ cal), *N*-benzyloxycarbonyl-*O*-*tert*-butyl-L-seryl-L-proline *tert*-butyl ester ($\tau_{25} = 13$ sec, $E_a = 21{,}000$ cal), and the methyl ester of *N*-acetyl-D-alanyl-L-methylalanine ($\tau_{25} = 1.2$ sec, $E_a = 21{,}000$ cal), where in all cases estimates were made from proton NMR line shapes (Maia et al., 1971; Portnova et al., 1970). Even though the precise relaxation times at any reference point depend quite strongly on the nature of the molecule and probably on the nature of the solvent as well, the activation energies seem to be fairly characteristic of the isomerism

Table I: Relaxation Times (25°C) and Limiting Low-Temperature Activation Energies for the Slow Phase of Refolding of Small Proteins.

Protein	τ(slow) (25°) (sec)	Limiting E_a (low T) (cal)	Bulkiness Parameter	Reference
Chymotrypsin (pH 2.7)	45	16,000	2.0	Pohl, 1970
Chymotrypsinogen (pH 2.2)	55	20,000	2.0	Pohl, 1970
Trypsin (pH 2.4)	45	17,000	2.2	Pohl, 1970
Trypsinogen (pH 2.2)	40	18,000	2.2	Pohl, 1970
RNase (pH 7.0)	52	18,000	2.8	Tsong et al., 1972
Cytochrome *c* (pH 4.0)	8	18,000	1.1	Tsong, 1973
Lysozyme (pH 6.3, 4.5 *M* LiBr)	17 (32°C)	N.A.	2.3	Segawa et al., 1973

process and only weakly dependent upon differences between molecules.

A Model for Folding and Unfolding of Small Proteins

Formulation of the Model. A large amount of experimental data exists that contributes to the formulation of a viable model for the folding and unfolding of small proteins. Although some of these observations have already been mentioned in the introductory statement, a more complete list is given here.

(1) Thermodynamic studies are usually consistent with or very nearly consistent with the existence of an equilibrium between only two macroscopic states (Privalov and Khechinashvili, 1974; Jackson and Brandts, 1970; Brandts, 1964, 1969; Brandts and Hunt, 1967; Lumry et al., 1966; Tanford, 1970; however, see also Tsong et al., 1970).

(2) Kinetic studies reveal at least biphasic behavior in both the folding and unfolding directions (Tsong et al., 1972; Tsong and Baldwin, 1972; Tsong, 1973; Summers and McPhie, 1972). Approximate relaxation times for the slow phase at 25°C are listed for several proteins in Table I. The fast phases are approximately 10–100 times faster.

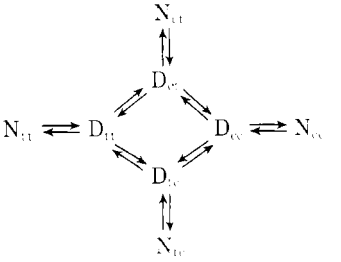
(3) Although the amplitudes for the fast and slow phases always have the same sign, the fraction of the total amplitude change which occurs in the fast phase depends strongly on conditions. At least in the cases of ribonuclease (Tsong et al., 1972) and cytochrome *c* (Tsong, 1973), the amplitude of the fast phase in unfolding becomes 100% of the total amplitude for jumps ending far into the denatured base-line region. For jumps ending on the native side of the transition point, the fraction of fast phase appears to reach a limiting non-zero value which is different for different proteins. Estimates of this limiting fraction of fast phase are given in Table II for several proteins.

(4) Neither the fast nor the slow phase is seen following "base-line jumps", i.e., jumps from one point in the denatured base-line region to another point in the denatured base-line region. The same is true for the native state base-line region.

(5) At least for ribonuclease, the fully active native enzyme is the product of both the fast and slow refolding processes (Garel and Baldwin, 1973).

From the data available, many of the above characteristics appear to be common to the denaturation of more than one protein. This suggests the possibility of a common mechanism which may not depend upon the precise details of three-dimensional structure. An unfolding model which is consistent with all of the above considerations and others to be discussed later is based upon the assumption that the slow phase in protein folding and unfolding is due directly to cis-trans isomerism about proline peptide bonds in the unfolded state. Therefore, in this model the slow phase constitutes a trivial structural event since it is not part of the unfolding process per se; however, it does lead to shifts in the unfolding equilibrium by mass action effects.

Considering a hypothetical protein with only two prolines, the general reaction scheme can be written as:



Here, the two subscripts are for the two different prolines; t signifies a trans configuration while c indicates cis. Because of the close packing which exists in most native proteins, the above assumes that proline isomerism must occur in the unfolded D state. All *conformational steps* N ⇄ D are considered to be fast while all *configurational steps* t ⇄ c are assumed to be slow. This model will then lead to a fast and slow phase in unfolding reactions *in all cases* except when each and every configurational probability is independent of conformation, i.e.:

$$[N_{tt}]/[N]_{tot} = [D_{tt}]/[D]_{tot}$$

$$[N_{ct}]/[N]_{tot} = [D_{ct}]/[D]_{tot}$$

$$\text{etc.} \qquad (3)$$

This situation will normally not be even approximately approached, since each proline in native proteins is thought to exist exclusively in one configuration or the other while in the denatured state the prolines will exist alternately in the cis and trans configurations while spending 70–90% of their time in the latter. In other words, all but one of the terms on the left-hand side of eq 3 may be essentially zero while none on the right will be zero.

A search through the protein crystallographic literature suggests that the trans configuration is adopted by most prolines in native proteins. There have been only nine *cis*-prolines reported thus far. These include Pro-93 and -114 in ribonuclease S (Wyckoff et al., 1970), Pro-168 in subtilisin (Alden et al., 1971), Pro-51 in thermolysin (Matthews et al., 1974), Pro-29 and -200 in carbonic anhydrase (K. K. Kannon, personal communication), Pro-EF3, -74 in erythrocruorin (Huber et al., 1971), and Pro-8 and -95 in the Bence-Jones protein Rei (Huber and Steigemann, 1974). In all of these cases, the cis residue occurs at the third position of a reverse open turn (Huber and Steigemann, 1974). However, for the vast majority of native proteins, no *cis*-prolines were incorporated into the crystallographic model. Overall, for all proteins where high-resolution data exist, only 5–10% of the total prolines have been suggested to be cis. This low estimate could be misleading, however, since Huber and Steigemann point out that "in

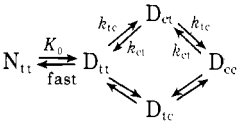
Table II: Limiting Fraction of Fast Phase for the Denaturation of Several Small Proteins.[a]

Protein	No. of Prolines	No. of *cis*-Prolines	Limiting Value α_2 Obsd	Limiting Value α_2 Calcd	Reference
RNase A	4	1	0.20	0.10	Garel and Baldwin, 1973
	4	2		0.025	
Cytochrome *c*	4	0	~0.50	0.41	Tsong, 1973
Chymotrypsinogen	9	0	~0.15	0.13	Tsong and Baldwin, 1972
Lysozyme	2	0	~0	0.64	Segawa et al., 1973

[a] In the case of each of these denaturation reactions, a very fast phase (microseconds) has been observed under certain conditions in addition to a fast phase (milliseconds) and a slow phase (seconds). The above estimates taken from experimental data have disregarded the very fast phase, so that α_2 represents the ratio of the amplitude of the fast phase to the sum of the amplitudes of the fast and slow phases. Limiting values of α_2 ($K_{den} << 1$) were estimated from unfolding data for chymotrypsinogen and lysozyme whereas both unfolding and refolding data on RNase and cytochrome *c* lead to nearly the same limiting value of α_2.

view of the uncertainty of protein models obtained from Fourier maps calculated with isomorphous phases, a reexamination of segments around proline residues in other protein structures might be advisable."

Since no firm evidence exists to the contrary, it will be assumed for the moment that there is only one "correct" set of proline configurations for the native protein (possible exceptions to this will be discussed later). If the dominant form is the all-trans form, then the scheme of eq 2 simplifies to:



where K_0 is the equilibrium constant for the conformational change and where k_{tc} and k_{ct} are the forward and reverse rate constants for the trans to cis isomerism.

Thermodynamic Properties of the Model. The thermodynamic characteristics of systems which adhere to the scheme illustrated by eq 4 are very simple. Evidence cited earlier showed that proline isomerism has zero or very small enthalpy change associated with it. We would therefore expect that *all denatured forms have the same enthalpy.* Also, in terms of the usual spectroscopic variables used to follow transition curves (i.e., aromatic absorption, hemegroup absorption, fluorescence, circular dichroism, etc.), all of the denatured forms should be nearly equivalent assuming that proline isomerism leads to no additional conformational changes in the denatured protein. Within a good level of approximation then, *all denatured forms will have the same spectroscopic observables.* Thus, the usual thermodynamic tests for two-state behavior (enthalpy ratios, multiple variable tests, transition curve shapes, etc.) will fail to detect the complexities present in eq 4 since all denatured forms would be indistinguishable in terms of both direct (calorimetric) and indirect (spectroscopic) methods. *These transitions would therefore be judged as two-state thermodynamic transitions* if no complexities other than proline isomerism exist.

Nuclear magnetic resonance is one spectroscopic technique which must be considered more carefully than the others since it is influenced by kinetics as well as by thermodynamics. Complexities in denaturation reactions are frequently seen by this technique. The study of the ribonuclease thermal denaturation (Westmoreland and Matthews, 1973) is a typical example. Separate C-2 histidine resonances were observed for the native and denatured molecules, suggesting a slow equilibrium. On the other hand, the resonances for several of the histidines in the native state began to shift in the direction of the denatured peak as the temperature was increased near the transition temperature. This suggests (Pople et al., 1959) a moderately fast process with resonances in the region of *partial collapse,* i.e.

$$2\pi\tau\Delta\nu \simeq 10 \tag{5}$$

Since the shift was seen only for native histidine resonances as opposed to denatured resonances, this behavior was suggested to arise from a partial unfolding (fast) of the native molecule prior to the major denaturation reaction (slow). However, an equally plausible interpretation can be made in terms of the model illustrated by eq 4. Native and denatured histidine C-2 resonances are separated by about 50 Hz (Westmoreland and Matthews, 1973) and the relaxation time for the fast phase of ribonuclease denaturation is known (Tsong et al., 1972) to be about 50 msec under the conditions where shifts in the NMR signals were observed; this satisfies the criteria of partial collapse and consequently shifts would be expected as the transition temperature is approached. Also, these shifts should be much more prominent for the native resonances than for the denatured resonances. The reason for this can be readily seen by referring to eq 4. According to eq 4, 100% of the native protein will be in fast equilibrium with denatured protein. However, *only one form* of the denatured protein (the form with the "correct" native proline configurations) will be in fast equilibrium with the native form. For ribonuclease, as will be seen later, this amounts to something of the order of 20% of the denatured protein. With 80% of the denatured peak remaining stationary and only 20% shifting, the effect could easily have been overlooked for the denatured resonances even though easily detected for native resonances. At any rate, complexities in NMR behavior need not necessarily arise from partially unfolded intermediates since the presence of such complexities is consistent with mechanisms of the type we are proposing which involve no such intermediates.

Kinetic Properties of the Model. For the simplest case of a protein with only one proline, i.e.:

$$N_t \underset{}{\overset{K_0}{\rightleftharpoons}} D_t \underset{k_{ct}}{\overset{k_{tc}}{\rightleftharpoons}} D_c$$

the changes in the concentration of N_t will occur in two phases with the following reciprocal relaxation times:

$$\tau_{fast}^{-1} = k_0^f + k_0^b$$

$$\tau_{slow}^{-1} = \left(\frac{K_0}{1 + K_0}\right)k_{tc} + k_{ct} \tag{7}$$

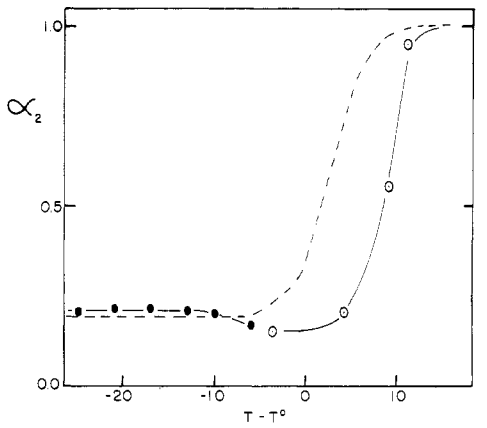


FIGURE 3: The fraction of fast phase for the ribonuclease A denaturation. The points designated with open circles are for unfolding at pH 3.9 (Tsong et al., 1972) and the filled circles for refolding at pH 5.8 (Garel and Baldwin, 1973). The dashed curve is a calculated curve, as explained in the text.

where k_0^f and k_0^b are the rate constants for the conformational step, which is assumed to be much faster than the isomerism step. The case discussed earlier (eq 4), where two prolines are involved, will have a homogeneous fast phase but a slow phase with contributions from two different relaxation times (Castellan, 1963), given by the two roots of the equation:

$$\tau_{slow}^{-1} = k_{tc}\left(\frac{3}{2} - \frac{1}{1+K_0}\right) + \frac{3}{2}k_{ct} \pm \left(k_{tc}^2\left[\frac{1}{4} + \frac{1}{(1+K_0)^2} - \frac{1}{1+K_0}\right] + k_{tc}k_{ct}\left[\frac{1}{2} + \frac{1}{1+K_0}\right] + \frac{k_{ct}^2}{4}\right)^{1/2} \quad (8)$$

This assumes that all prolines are identical in terms of the values of k_{tc} and k_{ct} so we are actually considering average velocities. Even so, the expressions for the slow relaxation times are somewhat complicated by the fact that they depend on constants associated with both the conformational and configurational steps. In the two limits as K_0 goes to either zero or infinity, the expressions become simpler since then they depend only on the rate constants for isomerism. The latter limiting case is not a practical one since the amplitude of the slow phase goes to zero as K_0 becomes large. Consequently, we will focus on refolding experiments where conditions are jumped far into the native base-line region where K_0 is very small. The two limiting relaxation times will then be:

$$\tau_{slow}^{-1} = \begin{cases} 2k_{tc} + k_{ct} \\ k_{tc} + 2k_{ct} \end{cases} \quad (9)$$

when $K_0 \ll 1$. The second relaxation will be of considerably lower amplitude since it depends upon having appreciable concentrations of the low-probability species D_{cc}.

Equation 9 is particularly important, since it shows that the limiting relaxation times ($K_0 \ll 1$) for the slow phase of protein refolding should be directly related to the rate constants for the elementary step of proline isomerism. As proteins with more prolines are considered, the above expressions will of course become more complicated, but it is still expected that every relaxation time will involve some additive combination of elementary rate constants for isomerism; this follows because we are assuming that each proline behaves identically and independently. Taking advantage of the additional fact that there is a zero or very small enthalpy change associated with isomerism, it then follows that:

$$\frac{\partial \ln \tau_{slow}^{-1}}{\partial 1/T} = \frac{E_a}{R} \qquad (\text{when } K_0 \ll 1) \quad (10)$$

where E_a is the activation energy for the cis-trans isomerism of a single proline. Since this will be true for each of the slow relaxation times, it should also be approximately true for the average relaxation time which comes from treating refolding data for the slow phase in terms of a linear Arrhenius plot. Thus, the model makes the specific prediction that *the limiting low-temperature activation energy for the slow phase of refolding should be the same for all proteins and equal to the activation energy for cis-trans isomerism of model compounds.*

Another kinetic property which is of interest (Tsong and Baldwin, 1972) is the fraction of the total amplitude change which occurs in the fast phase following a jump in conditions. This parameter, α_2, reflects the fast perturbation in the conformational equilibrium which occurs prior to any adjustments in the concentrations of the various denatured forms. For a protein containing n prolines, it can be easily shown that this will have the value:

$$\alpha_2 = \frac{(1 + AK_0)}{A(1 + K_0)} \quad (11)$$

The derivation of this equation is based on the assumption that there is only one "correct" set of proline configurations in the native state, although it need not necessarily be the all-trans form. If the native protein has $n - j$ *trans*-prolines and j *cis*-prolines, then A is given by:

$$A = (1 + K_{tc})^{n-j}(1 + 1/K_{tc})^j \quad (12)$$

It has been assumed that each proline has the same equilibrium constant for isomerism ($K_{tc} = k_{tc}/k_{ct}$) in the denatured form and that this equilibrium constant has the same value under initial and final conditions. The conformational equilibrium constant K_0 is the value for the final conditions. Equations 11 and 12 hold equally well for jumps in the unfolding or refolding direction. Another very important assumption contained in these two equations is that the observable used to measure α_2 is not inherently sensitive to isomerism, i.e., all denatured forms have the same value of the observable.

The behavior predicted by eq 11 is fairly obvious. With jumps which end far in the denatured base-line region, only the fast phase will be expected. For jumps ending closer to the transition point, the slow phase will appear. The fast phase will then become a smaller and smaller fraction of the total amplitude change until it reaches a limiting value of $1/A$ under final conditions such that K_0 is small. This same limiting value is predicted for both unfolding and refolding, so long as K_0 is small. It has a simple physical significance which is:

$$\text{limit}_{K_0 \to 0}\, \alpha_2 = 1/A = \text{(concn of denatured form which has native Pro configuration)/(concn of all denatured forms)} \quad (13)$$

Further Comparison of the Model with Conformational Reactions of Small Proteins. The most complete experimental characterization of the kinetics of protein folding has been carried out for RNase A in Baldwin's laboratory. The fraction of the fast phase for both unfolding and refolding is shown in Figure 3 as a function of the distance away

from the transition temperature. Shown also is a curve calculated from eq 11 using an A value of 5 and assuming that ΔH for unfolding is 80 kcal/mol. The calculated values reproduce the general shape of the curve for the unfolding reaction although the large change in the fraction of the fast phase is displaced toward lower temperatures relative to the experimental data.

From more fragmentary data, other proteins appear similar to ribonuclease in that the amount of fast phase reaches a limiting non-zero value at low temperature while it becomes the dominant phase in unfolding for jumps ending at high temperature. This appears to be true for chymotrypsinogen (Tsong and Baldwin, 1972), myoglobin (Summers and McPhie, 1972), and cytochrome *c* (Tsong, 1973) in addition to ribonuclease. Shown in Table II are estimates of the limiting low-temperature fraction of fast phase in all cases where sufficient data exist to make an estimate. It is seen that large differences exist between different proteins, as α_2 varies from 0% for lysozyme to 50% for cytochrome *c*.

The limiting values of α_2 predicted from eq 11 and 12 are also listed in Table II. Since no *cis*-prolines have been reported for the crystal structures of any of these proteins except RNase, we have assumed that each proline is 100% trans in the native state and 80% trans in the denatured state in order to arrive at an estimate of A in eq 12. Somewhat incomplete evidence exists for RNase. In the case of RNase S, Pro-93 and -114 appear to be cis while -42 and -117 are trans (Wyckoff et al., 1970). However, interpretation of the electron density map of RNase A is more difficult (G. Kartha, personal communication). Although Pro-114 is almost definitely in the cis state while -42 and -117 are trans, the configuration for Pro-93 is open to question. Thus, two estimates are included in Table II for the limiting value of α_2; one for the case where one proline is cis and the other for the case where two are cis. The first estimate is in closer agreement with the experimental estimate obtained under most conditions, which is about 0.20. However, Garel and Baldwin (1975) have found very recently that the fraction of fast phase varies in refolding experiments depending on the pH in the initial denaturing buffer. It changes from a value of 0.20 at acid pH to a value near zero with an apparent pK of ca. 4. They have interpreted this to mean that there is a pH-dependent change in the structure of the denatured protein to yield less of the fast-refolding form and that this may be linked to the titration of a carboxyl side chain.

With the exception of lysozyme, the calculated values of α_2 are in satisfactory accord with the experimental values. Thus, the low value of α_2 for RNase relative to cytochrome *c*, which also has four prolines, is rationalized in terms of the crystallographic models which show one or more cis-prolines for RNase and none for cytochrome *c* (Dickerson et al., 1971). The low value of α_2 for chymotrypsinogen relative to cytochrome *c*, both of which apparently have the all-trans native configuration, is also expected since chymotrypsinogen has twice as many prolines.

Although no estimate of α_2 has been made for the thermal denaturation of lysozyme in water, it has been reported that no fast phase is present in the transition region when the denaturation is carried out in 4.5 M LiBr (Segawa et al., 1973) or in high concentrations of guanidine hydrochloride (Tanford et al., 1973). Since there are only two prolines in lysozyme, we would expect that the fast phase would be very large in amplitude if both of these are in the trans configuration in the native molecule, which apparently is the case (Blake et al., 1967). Although we do not know why this discrepancy exists between the predicted and observed values of α_2, one suggestion can be made. In contrast to proline residues, the probability of any residue other than proline being in the cis configuration in an unfolded protein could be considerably less than 1% (Lakshminarayanan et al., 1967; Tonelli, 1971; LaPlanche and Rogers, 1964). Thus, if only one non-proline peptide unit were constrained to be in the cis configuration in lysozyme or any other *native* protein, the limiting value of α_2 would be virtually zero as is evident from eq 13. Such a cis residue has not been detected for lysozyme although apparently His-196 is cis in native carboxypeptidase (Libscomb et al., 1968). On the other hand, the difficulty in determining the configuration of proline residues in protein crystals was noted previously. The problems are perhaps worse with nonproline residues since electron density maps are routinely fit by assuming the all-trans configuration for nonproline residues. Normally, only in the case of an extremely poor fit would the possibility of a cis configuration be explored.

Strong support for the idea of proline involvement comes from examination of rates and activation energies for denaturation reactions under final conditions where the native protein is very stable, i.e., $K_{den} \ll 1$. Although the temperature dependence of τ_{slow} is complex in the transition region and the nature of the complexities varies from protein to protein, this is not true at temperatures substantially below the transition temperature. Thus, Arrhenius plots become linear for refolding reactions as the final conditions are changed such that K_{den} becomes progressively smaller. These are exactly the conditions where refolding kinetics should reflect the characteristics of the elementary process of proline isomerism, as illustrated previously in connection with eq 9 and 10. Relaxation times for refolding at 25°C and limiting low-temperature activation energies (obtained from data at or below 25°C) are shown in Table I for all proteins where suitable data exist in the literature. In the case of lysozyme, the data are fragmentary and not exactly comparable because of the higher temperature and the high concentration of LiBr.

All of these proteins show similar values for the slow relaxation time under these limiting conditions, suggesting a common mechanism. Cytochrome *c* is somewhat atypical and the possible reason for this will be discussed presently. Not only are the relaxation times similar from protein to protein, but they are also in order of magnitude agreement with what might be expected for proline isomerism since it was previously shown that the relaxation time for the protonated form of Ala-Pro is about 75 sec at 22.5°C. Perhaps more significant is the fact that *the limiting low-temperature activation energies for all of these denaturations are nearly identical and also in excellent accord with the predicted value of about 20,000 cal which would be expected if the slow phase were rate limited by proline isomerism.*

It is interesting that cytochrome *c* shows a slow phase which is about six times faster than the slow phase for other proteins where comparable data are available. Although this might at first glance appear to suggest a different mechanism of unfolding, this now appears not necessarily to be the case since a reasonable explanation exists in terms of the present considerations. Inspection of space-filling models suggests that isomerism will be sterically influenced by the bulkiness of side chains which are immediately adjacent to prolines in the polypeptide chain. This is also evident in the experimental data of Figure 1 where isomerism is seen

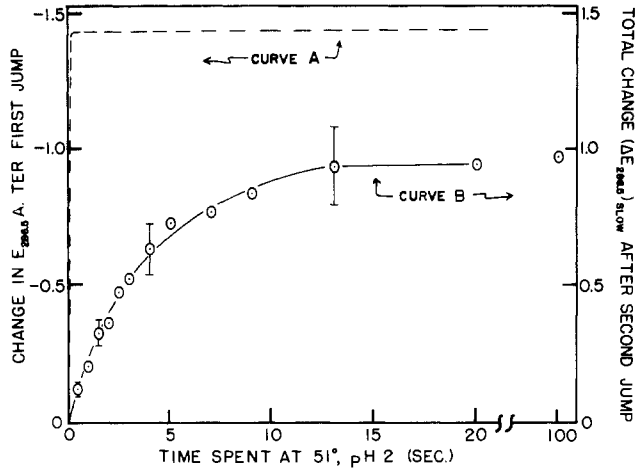


FIGURE 4: Kinetic results on ribonuclease. Curve A shows the change in the extinction coefficient (286.5 nm) for ribonuclease after the pH jump 7 → 2 at 51°C. No slow phase is seen. Curve B shows the *total amplitude* of the slow phase following the second jump of a double jump sequence. The first jump is the same as above, from pH 7 to 2 at 51°C. The second jump is (pH 2, 51°C) → (pH 4.5, ~0°C). In this double jump experiment, the time scale on the abscissa corresponds to the time lapse between the first and second jumps.

to become significantly slower in moving down the series Gly-Pro, Ala-Pro, and Val-Pro. To try to reflect this nearest neighbor effect, an arbitrary bulkiness parameter B has been devised. This parameter is calculated by assigning index numbers of zero to glycyl side chains, 1 to all straight-chain aliphatic side chains, 2 to aliphatic side chains where branching occurs at the γ-carbon, 3 to Cys and to aliphatic side chains where branching occurs at the β-carbon, and 4 to the very bulky aromatic side chains. The magnitude of B is then determined by finding the average value of this index number for all side chains which occur adjacent to prolines in the primary sequence of each protein. These values are shown in Table I. Cytochrome *c* stands out among all the proteins listed, since the average bulkiness (1.1) of its proline nearest neighbors is considerably less than for any of the other proteins (2.0–2.8). This might account for the unusually short relaxation time found for this protein.

Proof that the Slow Phase of Ribonuclease Unfolding Causes No Changes in Absorbance. Definitive proof that the slow phase of protein folding and unfolding is linked to proline isomerism probably must come from NMR experiments. For model compounds, the two isomers can always be seen as separated peaks in ^{13}C spectra and are sometimes resolved in ^{1}H spectra. However, in proteins the identification of the isomeric resonances of individual proline residues is not an easy job so that direct proof or disproof of the model by this route will be slow.

However, other spectroscopic evidence has been obtained which provides considerable support for the model proposed here. One of the difficulties which has been largely responsible for the lack of progress in understanding the slow phase of unfolding is the fact that it couples to the fast reaction. Consequently, any structural changes which occur in the fast reaction will also be manifest in the slow phase by mass action effects. In order to learn more about the nature of the structural changes in the slow phase, it must first be uncoupled from the fast reaction. There is one simple way of doing this, if the present model is correct. When jumps are made from native state conditions to *strongly* denaturing ($K_{den} \gg 1$) conditions, essentially all of the conformational changes will occur in the fast phase. According to the simple scheme of eq 4, this represents a shift of 100% of the protein from the N_{tt} state to the D_{tt} state. The slow phase can then be expected to occur until the equilibrium distribution of the various proline configurations has been achieved. However, during this slow phase, there will be no further change in the concentration of N_{tt} since that equilibrium has already been shifted completely to the D_{tt} state during the fast phase. Consequently, the slow phase will occur uncoupled to the equilibrium for the fast reaction.

Experiments have been carried out in order to see if this uncoupling can be achieved in the case of ribonuclease A unfolding. In curve A of Figure 4, it is seen that upon changing conditions from strongly native (pH 7, 51°C) to strongly denaturing (pH 2, 51°C) essentially all of the change in $E_{286.5}$ is complete within 1 sec; i.e., the entire optical change occurs in the fast phase. The fact that no slow phase is seen in $E_{286.5}$ readings means one of two things: (1) no slow phase occurs; (2) a slow phase occurs following the fast phase, but it is not seen because all of the states involved in the slow phase have identical optical properties. It has sometimes been implicitly assumed that the inability to observe a slow phase means that it does not occur. However, the proline isomerism model predicts that a slow phase always occurs but that it will not be seen in the absence of coupling to the fast phase since the optical properties of denatured forms with different cis–trans configurations will be virtually identical.

In order to decide which of these two alternatives is correct, "double jump" experiments have been carried out. The first jump is identical with the one carried out for curve A, i.e., from native to strongly denaturing conditions. The sample is then allowed to remain at these latter conditions for a measured time of x seconds. Following this time lapse under denaturing conditions, the second jump is carried out to a final state (pH 4.5, 0°C) favoring the native form. The resultant transition from 100% denatured state to 100% native state is then monitored on the Cary 14 at 286.5 nm. Because of the rather large "dead time" (see Experimental Section) in these experiments, only the slow phase is seen following the second jump. The *total amplitude* of the slow refolding phase for various values of x has been estimated and these values are shown in curve B of Figure 4. The comparison of curves A and B clearly shows that alternative 2 above is the correct one. Even though no further change occurs in $E_{286.5}$ after the first second (curve A), it is apparent that a slow reaction is nevertheless taking place (curve B). After spending only 1 sec at the high temperature, all of the denatured protein is in a form which is capable of fast refolding subsequent to the second jump. With increasing time spent at 51°C, pH 2, part of the denatured protein is converted into a different denatured form which can only refold slowly to the native state, as seen from the increase in the amplitude of the slow refolding phase in curve B. This transition, i.e.:

$$\begin{matrix}\text{(fast refolding)} \\ \text{denatured state}\end{matrix} \rightleftarrows \begin{matrix}\text{(slow refolding)} \\ \text{denatured state}\end{matrix}$$

is an "invisible" process, i.e., it occurs with no detectable change in $E_{286.5}$ as seen in curve A. The first-order relaxation time for the transition is about 4.7 sec according to the data of curve B. This relaxation time is precisely in the time range expected for the slow phase of protein denaturation, as judged by comparison with results from experiments car-

ried out under conditions where coupling with the fast reaction permits the slow phase to be observed directly (an interpolated value of about 6 sec can be estimated from the data of Tsong et al. (1972) for the observable slow phase of unfolding at 51°C following the jump pH 7 → pH 3.9). Therefore, it seems likely that the two processes are, in fact, the same process and that the changes in absorbance normally seen in the slow phase of denaturation are entirely due to coupling with the fast phase.

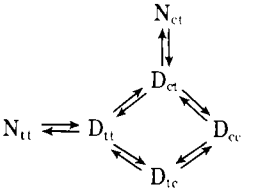
These experiments then show conclusively that *the slow phase of the ribonuclease unfolding reactions corresponds to the interconversion of different denatured forms, all of which are spectroscopically equivalent (286.5 nm)*. Even though spectroscopically equivalent, the different denatured forms can be recognized since only part of them are capable of fast refolding. The experimental results of these double jump experiments are precisely what would be predicted from the proline isomerism model (eq 4). Other models, which attempt to explain the existence of two kinetic phases by postulating the occurrence of a state or states whose structure is "intermediate" between the native and denatured forms, do not seem nearly so consistent with these findings since one is then forced into an additional ad hoc explanation of why states with significantly different conformations might have identical optical properties.

Recent experiments of Garel and Baldwin (1975) have also led to the conclusion that a fast and slow refolding species may coexist in the denatured base-line region. Also, earlier results from the same authors (Garel and Baldwin, 1973) had shown that the molar value of ΔA_{286} during refolding is the same in the fast and slow refolding reactions, an observation which is consistent with the idea that the denatured forms which participate in the slow and fast refolding processes have identical spectroscopic properties.

Heterogeneity in the Prolines Involved in the Slow Process. Considerations thus far have been based on the assumption that all proline residues in a protein are kinetically and thermodynamically equivalent in the denatured state. However, the data of Figure 1 show that rates of isomerism may be strongly influenced by at least the nearest neighbors of each proline residue. The results from studies of Wüthrich and Grathwohl (1974) suggest that large effects might even be exerted by residues more distant than nearest neighbors. They have found that the oligopeptide L-Phe-L-His-L-Thr-L-Phe-L-Pro contains 15% *cis*-proline while the related oligopeptide L-Thr-L-Phe-L-Pro contains about 60% *cis*-proline in deuterated Me_2SO! All of these considerations suggest the possibility that multiple relaxation times will be involved in the decay curve for the slow phase because of differences in neighboring residues for each proline. Also, when many prolines are present, multiple relaxation times would be expected even for equivalent prolines as seen in eq 8 and 9. Thus, the slow phase should not be strictly first order if it is controlled by isomerism. Although some apparent curvature in first-order plots can be detected in some of the data in the literature, it is questionable whether it is experimentally significant. Even if substantial heterogeneity exists, it may be difficult to detect in relaxation studies. For example, for a hypothetical protein where the slow phase is due to four relaxation times having relative magnitudes of 1, 2, 2, and 3, the curvature in the first-order plot would be difficult to detect unless absorbance readings were accurate to about 1% of the total change in absorbance. In view of the fact that relaxation data are not usually extremely accurate (particularly the infinite time base line), careful analysis is necessary to demonstrate the presence or absence of heterogeneity.

The Possibility of Multiple Native Forms. An additional complication may arise if more than one set of proline configurations can exist in the native protein. Since a cis configuration is intrinsically only about 500–1000 cal less stable than the trans, this represents a real possibility. The difference in intrinsic stability may not be the crucial consideration, however, since a change in the configuration of a proline residue in a native protein could have serious effects on the folding of the entire polypeptide chain. For example, of the nine *cis*-prolines which have been reported to occur in native crystalline proteins, all occur in the third position of a reverse open turn (Huber and Steigemann, 1974). It may be that these serve a very important function in the overall folding of a protein chain; thus, the folded protein might have no stability with these critical prolines in the trans state. Still, there are numerous other ways to accomplish reverse bends which do not require a peptide bond to be in the cis state (Crawford et al., 1973). Also, there are many prolines which do not occur at bend positions in the chain and the sense of configuration may not be critical in all cases. Therefore, the possibility of multiple native forms can certainly not be ruled out by present evidence.

Considering a hypothetical protein with two prolines, one of which can exist in the cis or trans form in the native protein, we have:



This scheme will give rise to two fast relaxations, although perhaps with similar relaxation times. The case where the native form having a *cis*-proline (N_{ct}) is somewhat more stable than that having a trans (N_{tt}) is interesting. In refolding following a jump from denaturing to native conditions, for example, most of the protein will initially go into the less stable N_{tt} state (because of the higher concentration of D_{tt} relative to D_{ct}). To arrive at the more stable N_{ct} state, it must *unfold again,* isomerize, and refold. Consequently, if jumps are made to low temperatures where the native form is very stable and where isomerism is slow, it might be possible to "trap" some of the native protein in the secondary state for long periods of time. Once trapped in such a metastable state under strongly native conditions, the recovery to the thermodynamically stable state will require long periods of time if isomerism must occur in the unfolded form.

The Possible Existence of a Slow Phase Associated with Isomerism of Other Residues besides Proline. For nonproline residues, no reliable estimate can be made as to the fraction of cis form at equilibrium in denatured proteins. The concentration is low enough so that no evidence of the cis form is present in NMR spectra of oligopeptides or monosubstituted amides, which probably means that it is 1% or less. However, because of the large number of nonproline residues relative to proline residues in most proteins, even very low equilibrium concentrations of the cis form could produce slow relaxations of significant amplitude. For example, consider a protein that has 100 nonproline residues in the cooperative unit which is involved in unfolding. For simplicity, it will be assumed that all of these are 100% in

the trans configuration in the native protein. Even if the probability of the trans configuration is as high as 99.90% for each residue in the denatured form, then this will still lead to a slow phase accounting for nearly 10% of the total amplitude in the low-temperature limit (calculated from eq 12 and 13, using $n = 100$, $j = 0$, and $K_{tc} = 0.001$). Therefore, it is not possible to state with certainty that the magnitudes of the slow phases listed in Table II are *solely* reflective of proline isomerism.

On the other hand, it is entirely possible that the isomerism of nonproline residues occurs with a significantly different relaxation time than does the isomerism of proline residues. On steric grounds alone, it is reasonable to expect that it might be faster. If this were the case, then the isomerism of proline and nonproline residues should be seen as separate kinetic phases.

Conclusion

The available evidence argues rather convincingly that the slow phase of protein unfolding arises directly from proline isomerism in the denatured state, i.e., the slow phase corresponds to an intra-state, rather than an inter-state, relaxation. This was demonstrated most clearly by the double-jump experiments where it was shown that the slow phase is not part of the unfolding process per se but is a base-line phenomenon. This has been overlooked in the past because the usual spectroscopic observables cannot see the slow relaxation except in the transition region where it couples to the unfolding reaction. If this picture is correct in principle, then it follows that *the existence of multiple kinetic phases in unfolding reactions is not in itself sufficient justification for the assumption that structural "intermediates" or partially unfolded molecules exist in the transition region.* It must, in addition, be shown that such relaxations do not also occur following base-line jumps. The *inability to observe* a relaxation in the base-line region by conventional methods of monitoring denaturation reactions cannot by itself be taken as proof that the relaxation is not occurring in the base-line region. Methods capable of detecting relaxations between structures with very similar physical and optical properties, such as the double-jump method, must also be employed.

Experimental Section

Isomerism Rates for Model Compounds. The oligopeptides (glycyl-L-proline, L-alanyl-L-proline, L-valyl-L-proline, glycyl-L-alanine, and glycyl-L-prolyl-L-alanine) were purchased from Sigma Chemical Co. and used without further purification. For the kinetic studies, aqueous solutions of the peptides were prepared at a concentration of about 0.1% and titrated to a pH of 1.8 with a concentrated HCl solution. A 10-ml aliquot of this solution was placed into a thermostated titration cell and allowed to equilibrate for about 20 min. At zero time, a predetermined amount of KOH solution (1 *M*) was added quickly from a mechanical microburet. The slow pH transient was then followed with a Radiometer pH Meter 26, equipped with a combination glass–calomel electrode and stripchart recorder. The amplitude associated with the slow transient varied depending upon the final pH and the particular peptide being studied, but was of the order of 0.1 pH unit for those peptides where cis–trans relaxations occurred. The slow relaxation was always in the direction opposite to the direction of the pH jump. The relaxation time observed was dependent only on the final pH. That is, the same relaxation times would be observed in the two-jump sequence pH 1.8 → pH 3.0 → pH 4.0 as was observed for the two single jumps, pH 1.8 → pH 4.0 and pH 1.8 → pH 3.0. In no case was a significant relaxation observed for any peptide which lacked a C-terminal proline.

As a check upon the method, the relaxation time for Ala-Pro was determined spectrophotometrically in a single instance. In this case, the peptide solution was prepared and treated exactly as above except for the addition of 2.5×10^{-5} *M* Methyl Orange. Following the addition of KOH, the slow transient was followed spectrophotometrically at 530 nm on the 0.1 slidewire of the Cary 14.

Kinetic Studies of Ribonuclease Unfolding. The ribonuclease A was purchased from Sigma Chemical Co. and chromatographed on CM-Sephadex immediately prior to use. Conventional relaxation studies were carried out on the Durrum stopped-flow spectrophotometer. Two separate thermostating baths were used: one to thermostat the two syringes and another to thermostat the mixing chamber and observation block. In control experiments involving water in both syringes, the temperatures of the baths were individually adjusted so that the thermal mixing artifact was minimal.

The double-jump experiments were carried out using two special hand-operated syringes, each equipped with its own thermostating jacket and its own thermostating bath. Initially, 0.250 ml of a ribonuclease solution (~4% RNase, pH 7, unbuffered) was pipetted into a quartz spectrophotometer cell (3.175 cm pathlength) and placed in a bath thermostated at 51°C. From a syringe which was also thermostated at 51°C, 0.250 ml of HCl (pH 1.60) was quickly added to the protein solution, resulting in a pH drop to 2.0. A prescribed amount of time was then allowed to elapse before the second jump was carried out. This was accomplished by adding 7.5 ml of a solution (0.01 *N* sodium acetate buffer, pH 4.5) from the second syringe, which was thermostated at 0°C. The final temperature of the spectrophotometer cell and its contents was about 8°C, and the final pH was 4.50. The cell was then quickly placed in a thermostated receptacle (0°C) in the Cary 14 and the slow refolding phase followed on the sensitive slidewire at a wavelength of 286.5 nm. In determining the total amplitude of the slow refolding phase, the recorded transient was extrapolated back to that time at which the second jump was initiated. This "dead time" amounted to about 25 sec and resulted in amplitude corrections of about 30%. This resulted in rather large uncertainties in the absolute value of $\Delta A_{286.5}$, as indicated in Figure 4, although the relative errors would be considerably smaller as is apparent from the scatter of points.

Note Added in Proof

Since this manuscript was written, a recent study by Hagerman and Baldwin (1975) has come to our attention. Using improved methods of analysis, these authors show that kinetic data on RNase A are more consistent with the existence of three major kinetic phases rather than the two which had been suggested earlier. Their analysis supports the minimal mechanism:

$$U_1 \rightleftharpoons U_2 \rightleftharpoons U_3 \rightleftharpoons N$$

where U_1, U_2, and U_3 are three forms of the heat-denatured protein that apparently have nearly identical optical properties. These three forms exist in an equilibrium ratio of 0.78 to 0.20 to 0.02, respectively, at acid pH. Although the dis-

cussion in the present paper has assumed the existence of only two major kinetic phases, it is nevertheless consistent with the improved analysis of Hagerman and Baldwin. Most of our arguments have been based on data obtained by refolding to strongly native conditions. Under these circumstances, the third kinetic phase represents only about 2% of the total amplitude so that the old picture is essentially in agreement with the newer one. In terms of the new analysis, we still feel that it is the slowest step ($U_1 \rightleftharpoons U_2$ in the above mechanism) which most likely involves proline isomerism while the fastest step ($U_3 \rightleftharpoons N$) is the actual unfolding process. The interesting question which is posed by these new results has to do with the molecular events which give rise to the step of intermediate velocity ($U_2 \rightleftharpoons U_3$). Since this reaction also involves little or no change in the optical properties of aromatic residues, it seems at least possible that it could represent a second configurational relaxation. For example, it was noted above that the isomerism of nonproline residues could lead to a separate phase of configurational relaxation whose complications would be superimposed on those due to proline isomerism as well as those due to protein unfolding. However, a multitude of other possibilities also exist and additional information is necessary before any conclusions can be reached.

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References

Alden, R. A., Birktoft, J. J., Kraut, J., Robertus, J. D., and Wright, C. S. (1971), *Biochem. Biophys. Res. Commun. 45,* 337.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. R. Soc. London, Ser. B 167,* 365.

Brandts, J. F. (1964), *J. Am. Chem. Soc. 86,* 4302.

Brandts, J. F. (1969), *Biol. Macromol. 2,* 213.

Brandts, J. F., and Hunt, L. (1967), *J. Am. Chem. Soc. 89,* 4826.

Castellan, G. W. (1963), *Ber. Bunsenges. Phys. Chem. 67,* 898.

Crawford, J. L., Libscomb, W. N., and Schellman, C. G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 538.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971), *J. Biol. Chem. 246,* 151.

Evans, C. A., and Rabenstein, D. L. (1974), *J. Am. Chem. Soc. 96,* 7312.

Garel, J., and Baldwin, R. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3347.

Garel, J., and Baldwin, R. L. (1975), *J. Mol. Biol. 94,* 611.

Garel, J., and Labouesse, B. (1971), *Biochimie 53,* 9.

Hagerman, and Baldwin, R. L. (1975), *Biochemistry* (in press).

Huber, R., Epp, O., Steigemann, W., and Formanek, H. (1971), *Eur. J. Biochem. 19,* 42.

Huber, R., and Steigemann, W. (1974), *FEBS Lett. 48,* 235.

Ikai, A. (1971), Ph.D. Thesis, Duke University.

Ikai, A., Fish, W., and Tanford, C. (1973), *J. Mol. Biol. 73,* 165.

Jackson, W. M., and Brandts, J. F. (1970), *Biochemistry 9,* 2294.

Keim, P., Vigna, R., Nigen, A., Morrow, J., and Gurd, F. (1974), *J. Biol. Chem. 249,* 4149.

Lakshminarayanan, A. V., Sasisekharan, V., and Ramachandron, G. N. (1967), *Conform. Biopolym., Pap. Int. Symp. 1,* 61.

LaPlanche, L. A., and Rogers, M. T. (1964), *J. Am. Chem. Soc. 86,* 337.

Libscomb, W. N., Hartsuck, J. A., Reeke, G. N., Jr., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Symp. Biol. 21,* 24.

Lumry, R., Biltonen, R., and Brandts, J. F. (1966), *Biopolymers 4,* 917.

Madison, V., and Schellman, J. (1970), *Biopolymers 9,* 511.

Maia, H. L., Orrell, K. G., and Rydon, H. N. (1971), *Chem. Commun.,* 1209.

Matthews, B. W., Weaver, L. H., and Kester, W. R. (1974), *J. Biol. Chem. 249,* 8030.

Pohl, F. (1970), Ph.D. Thesis, University of Konstanz, Konstanz, Germany.

Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, p. 222, New York, N.Y., McGraw-Hill.

Portnova, S. L., Bystrov, V. F., Balashova, T. A., Ivanov, V. T., and Ovchinnikov, Y. A. (1970), *Izv. Akad. Nauk SSSR, Ser. Khim.,* 825.

Privalov, P. L., and Khechinashvili (1974), *J. Mol. Biol. 86,* 665.

Segawa, S., Husimi, Y., and Wada, A. (1973), *Biopolymers 12,* 2521.

Summers, M. R., and McPhie, P. (1972), *Biochem. Biophys. Res. Commun. 47,* 831.

Tanford, C. (1970), *Adv. Protein Chem. 24,* 1.

Tanford, C., Aune, K., and Ikai, A. (1973), *J. Mol. Biol. 73,* 185.

Thomas, W. A., and Williams, M. K. (1972), *J. Chem. Soc., Chem. Commun.,* 994.

Tonelli, A. E. (1971), *J. Am. Chem. Soc. 93,* 7153.

Torchia, D. (1972), *Biochemistry 11,* 1462.

Torchia, D. A., and Bovey, F. A. (1971), *Macromolecules 4,* 246.

Tsong, T. Y. (1973), *Biochemistry 12,* 2209.

Tsong, T. Y., and Baldwin, R. L. (1972), *J. Mol. Biol. 69,* 145.

Tsong, T. Y., Baldwin, R. L., and Elson, E. L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1809.

Tsong, T. Y., Hearn, R. P., Wrathall, D. P., and Sturtevant, J. M. (1970), *Biochemistry 9,* 2666.

Westmoreland, D. G., and Matthews, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 914.

Wüthrich, K., and Grathwohl, C. (1974), *FEBS Lett. 43,* 337.

Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970), *J. Biol. Chem. 245,* 305.