Characterization of the Slow Steps in the Folding of the α Subunit of Tryptophan Synthase†

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ABSTRACT: The two slow phases detected by ultraviolet spectroscopy in the refolding of the urea-unfolded α subunit of tryptophan synthase have been analyzed for their dependence on final urea concentration, pH, and temperature. The decay time of the slower of the two phases is independent of final urea concentration and pH and has an activation energy of 17.6 ± 2.5 kcal mol^{-1}. This behavior is consistent with the explanation that proline isomerization is the rate-limiting process in the step corresponding to this phase. The decay time for the faster phase depends on both the final urea concentration and the pH; the variation with pH is well described by a single protonation event with a pK of 7.6 ± 0.3. The activation energy for this phase is 14.5 ± 2.6 kcal mol^{-1}. It appears that the step corresponding to this phase is not simply related to proline isomerization. The binding of a competitive inhibitor for the α subunit, indolepropanol phosphate, during refolding displays biphasic kinetics; the decay times for the two phases are identical with those observed for the folding of the protein in the absence of inhibitor. All of the results can be explained in terms of a folding model in which the unfolded protein exists in two kinetically different forms. These two species rapidly collapse to two intermediate forms, one of which proceeds directly to the native conformation by the pH-dependent step, with the intermediate decay time. The second intermediate must first execute a proline isomerization before proceeding to the native form.

Kinetic studies of the folding of globular proteins have provided insights into the mechanisms involved that cannot be obtained from equilibrium studies. The advantages of a kinetic approach are exemplified by the studies of Baldwin and his colleagues who have used a variety of techniques to monitor the behavior of ribonuclease A during folding (Garel & Baldwin, 1973; Blum et al., 1978; Nall et al., 1978; Hagerman et al., 1979; Schmid & Baldwin, 1979a–c). Their results show that transient intermediates appear during the folding process that are not detected in equilibrium experiments (Ginsberg & Carroll, 1965; Greene & Pace, 1974; Privalov & Khechinashvili, 1974). Another key finding of this work is that the unfolded form actually consists of two kinetic species, one of which refolds rapidly (10^{-2}-s time range) and one slowly (10^{1}-s time range) to the native conformation. Brandts et al. (1975) provided a plausible explanation for this observation by noting that the relative populations of the cis and trans isomers of the peptide bond at X–Pro linkages are comparable in model compounds and that the rate of isomerization is extremely slow at room temperature, with a decay time in the range 10^{1}–10^{2} s. These workers proposed that only that fraction of the unfolded protein that has all of the X–Pro peptide bonds in the same isomeric form as found in the native conformation can refold rapidly. The remainder of the population must execute at least one slow proline isomerization and thus refold slowly. The results of studies on the slow kinetic phases in RNase A[1] (Schmid & Baldwin, 1979a–c), parvalbumin (Lin & Brandts, 1978), and other proteins (Pohl, 1976; Hagerman, 1977) are generally consistent with the hypothesis that proline isomerization plays a role in protein folding. However, it appears that this role is more complex than originally postulated (Schmid & Baldwin, 1979a,c).

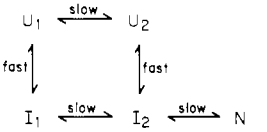
In an effort to obtain more information on the mechanism of folding of globular proteins, we have been studying the urea-induced unfolding of the α subunit of tryptophan synthase from *Escherichia coli*. The α subunit is a monomeric protein of molecular weight 28 700 which contains no prosthetic groups and no disulfide bonds. In a previous study (Matthews & Crisanti, 1981), the kinetics of the urea-induced unfolding and refolding of the α subunit were monitored by following the absorbance changes at 286 nm. The following observations were made.

(1) Unfolding jumps resulted in a single exponential decrease in absorbance. The total change in amplitude is identical with that expected from an equilibrium unfolding study.

(2) Refolding jumps from the unfolded base line into the transition region resulted in two phases, a slow phase that accounted for less than half the expected increase in absorbance and a fast phase that could not be directly observed in stopped-flow measurements and which accounted for the remainder of the absorbance change. The fast phase was estimated to have a half-time of less than 10 ms.

(3) Refolding jumps into the native base-line region resulted in three kinetic phases: a slow phase, an intermediate phase, and, as before, a fast phase, whose presence was postulated to account for the remainder of the expected absorbance change.

On the basis of these kinetic experiments and the results of a urea gradient gel electrophoresis study, the following model was postulated to describe the folding of the α subunit:



where U_1 and U_2 are unfolded forms with different kinetic properties, I_1 and I_2 are partially folded intermediates, and N is the native form.

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol; $NaDodSO_4$, sodium dodecyl sulfate; IPP, indolepropanol phosphate; RNase A, ribonuclease A.

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Several questions were left unanswered in the previous study: How do the slow and intermediate phases detected in jumps into the native base-line region relate to this model? Are either or both of the slow and intermediate steps related to proline isomerization? Can the relative populations of U_1 and U_2 be understood in terms of the extended two-state model of Brandts et al. (1975) for protein folding? The purpose of this paper is to provide answers for these questions and, thereby, a better understanding of how the α subunit of tryptophan synthase folds to its native conformation. Presumably the common factors derived from this study and others on protein folding will lead to a better understanding of how the amino acid sequence of a protein determines its native conformation.

Experimental Procedures

The α subunit of tryptophan synthase (EC 4.2.1.20) from *E. coli* was isolated from strain B8/F'B8 by the methods of Kirschner et al. (1975). The purity of the enzyme was determined by both $NaDodSO_4$ and native polyacrylamide gel electrophoresis; the electrophoretograms exhibited one symmetrical band indicative of only a single protein component. Urea gradient gel electrophoresis experiments reported previously (Matthews & Crisanti, 1981) also failed to detect any microheterogeneity. The activity of the α subunit was measured by its ability to enhance the activity of the β_2 subunit in the condensation of indole and serine to form tryptophan; the reported maximum specific activity of the α subunit in this assay is 5500 units/mg (Kirschner et al., 1975). The specific activity of the α subunit used in this study was 4700 ± 400 units/mg. The protein concentration was calculated by using a molar extinction coefficient of 12 600 M^{-1} cm^{-1} (Adachi et al., 1974).

Ultrapure urea was purchased from Schwarz/Mann and was used without further purification. Indolepropanol phosphate (IPP) was a generous gift of Dr. K. Kirschner and was provided as the bis(cyclohexylammonium) salt; the concentration of IPP was determined by using a value of 5350 M^{-1} cm^{-1} (Kirschner et al., 1975) for the molar extinction coefficient at 281 nm. All other chemicals were purchased from commerical sources and were reagent grade.

Studies of the kinetics of the absorbance change at 286 nm accompanying folding were performed as described previously on a Cary 118 CX spectrophotometer by using difference spectroscopy (Matthews & Crisanti, 1981). The α subunit in a buffered solution containing no urea was used as a reference. The unfolding (refolding) was initiated by using microliter syringes to add accurately measured volumes of protein (protein in urea) to premeasured volumes of urea (buffer). The solutions were manually mixed, and the absorbance at 286 nm was recorded as a function of time. The dead time for this procedure was 4 s. The inherent instrumental drift, <0.0005 absorbance units/h, was always less than 2% of the total observed change. Studies of the transient response of the fluorescence emission of IPP at 365 nm during folding were performed in a similar fashion except that IPP was present in the premeasured volume of buffer. Unless otherwise indicated, the final protein concentration was in the range 1.0–1.5 mg mL^{-1}.

Measurements of decay times as a function of pH were made by dialyzing stock solutions of the α subunit against buffer containing 10 mM potassium phosphate, 0.2 mM Na_2EDTA, and 1 mM 2-mercaptoethanol at various pH values. The urea and buffer solutions used to initiate unfolding and refolding jumps were titrated to the same pH value as the stock protein solution by using microliter amounts of concentrated HCl. Alternatively, kinetic jumps at several pH values were made by titrating the urea, buffer, and stock protein solutions to consecutively lower pH values with 0.01 M HCl; decay times obtained with solutions prepared by either method were identical within experimental error.

The rate constant, k, and its associated decay time, τ, where $\tau = k^{-1}$, for each kinetic phase were determined from semilogarithmic plots of the change in absorbance or fluorescence intensity as a function of time by an exponential stripping procedure (Hagerman & Baldwin, 1976). In this procedure, the long time behavior of the largest decay time was typically extrapolated back to zero time to provide a τ value for the slowest phase. This component was then subtracted from the semilogarithmic plot and the process repeated until all the observed amplitude was accounted for. A linear least-squares fitting program was used to fit each phase to a straight line.

UV difference measurements were made by using a Cary 118CX spectrophotometer equipped with thermojacketed cell holders; the temperature of the cell was maintained at the desired value to within ±0.1 °C by using a Laude Model T-2 circulating water bath. The sample temperature was measured with a copper–constantin thermocouple and a digital voltmeter at the end of a kinetic run. Fluorescence measurements were made on a Perkin-Elmer MPF 44A spectrofluorimeter equipped with a thermojacketed cell holder; transient responses were recorded by a Houston Instrument Model B5247-15 strip chart recorder. The excitation wavelength was 305 nm, and the fluorescent emission was detected at 365 nm in kinetic studies. Both the excitation and the emission slit widths were set at 6 nm. A 1.5-s time constant was used to improve the signal-to-noise ratio.

Results

The possibility that one or more of the kinetic phases observed in the folding of the α subunit are related to proline isomerization prompts several experiments involving the dependence of the decay times on environmental parameters such as urea concentration, pH, and temperature. The rate of proline isomerization is expected to be independent of urea concentration at pH near neutrality; the isomerization is acid catalyzed (Steinberg et al., 1960). The dependence of the rate constants for isomerization on temperature yields an activation energy of 20 ± 3 kcal mol^{-1} for a range of model compounds (Brandts et al., 1975; Cheng & Bovey, 1977).

Urea Dependence. In the previous study of the urea-induced unfolding of the α subunit (Matthews & Crisanti, 1981), it was found that at pH 7.8, 25 °C, the native conformation is stable up to approximately 2 M urea. Between 2 and 6 M urea, the changes in the UV difference spectrum shows that the α subunit undergoes a cooperative unfolding transition that exposes a number of buried tyrosine residues to the solvent. Above 6 M urea, the α subunit is completely unfolded as judged by the absence of any further cooperative changes in the absorbance at 286 nm. This information is summarized in the inset in Figure 1.

The dependence on the final concentration of urea of the decay times for the kinetic phases involved in unfolding and refolding jumps of the α subunit at pH 7.8, 25.0 °C, is shown in Figure 1. As discussed in the introduction, unfolding jumps result in a decrease in absorbance that follows a single exponential decay. The logarithm of the decay time decreases linearly as a function of urea concentration above 3 M urea. Refolding jumps ending in the transition region give rise to a fast phase that could not be directly detected and a slow phase whose decay time is shown in Figure 1. The decay times of the single phase in unfolding jumps and the slow phase in the refolding jumps that end at the same final urea concen-

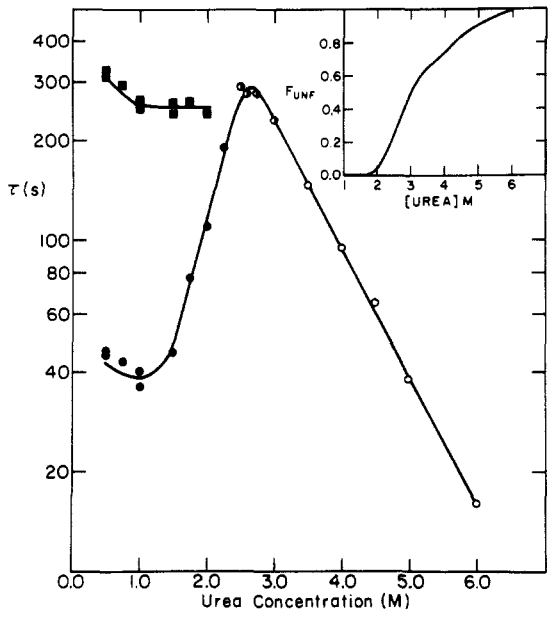

FIGURE 1: Semilogarithmic plots of the dependence of the observed decay times on final urea concentration as monitored by $\Delta\epsilon_{286}$ in 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol, 25 °C. The decay times of the slow (■) and intermediate (●) phases in refolding were measured in jumps starting at 6 M urea and ending at the indicated urea concentration. Unfolding jumps (○), which always resulted in a single exponential change, were initiated at 0 M urea and ended at the indicated final urea concentration. In the transition region, it was possible to measure the single decay time by both unfolding and refolding jumps (◑). The inset shows the fraction of the unfolded form, F_{UNF}, as a function of urea concentration for the equilibrium process, as determined previously by the change in extinction coefficient at 286 nm (Matthews & Crisanti, 1981).

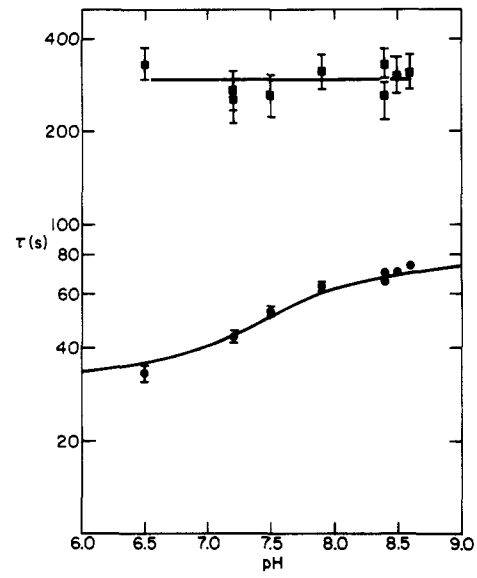

FIGURE 2: The pH dependence of the decay times of the slow (■) and intermediate (●) phases observed in refolding jumps from 6 to 1.5 M urea as monitored by UV spectroscopy in 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol, 21.7 °C. The solid line through the intermediate phase data (●) represents a fit of the data to the Henderson–Hasselbach equation with a single ionizable group whose pK is 7.6. The error bars indicate the estimated experimental error.

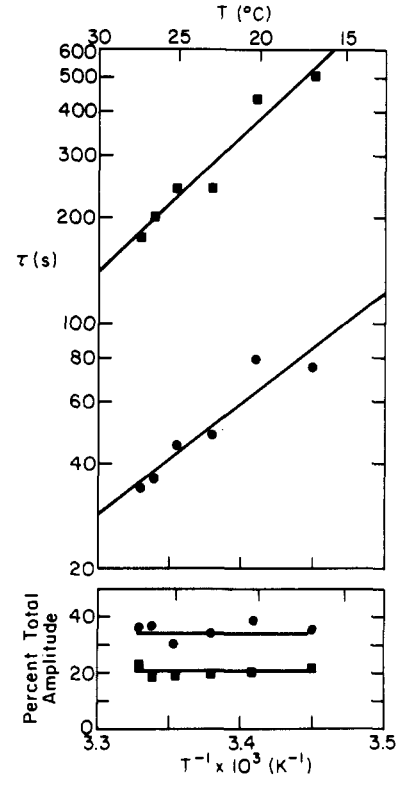

FIGURE 3: (Top) The temperature dependence of the decay times of the slow (■) and intermediate (●) phases observed in refolding jumps from 6 to 1.5 M urea as monitored by UV spectroscopy. The buffer solutions contained 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol. Solid lines indicate the linear least-squares fit of the decay times to the Arrhenius equation. (Bottom) The relative amplitudes of the slow (■) and intermediate (●) phases observed in refolding jumps from 6 to 1.5 M urea as monitored by UV spectroscopy. Not shown is the amplitude of an additional faster phase (Matthews & Crisanti, 1981).

tration are identical, demonstrating that the unfolding process is reversible.

For refolding jumps ending below the transition zone, a slow and an intermediate phase, in addition to the fast phase, were detected. The decay times for these two phases were obtained by an exponential stripping procedure (Hagerman & Baldwin, 1976). In Figure 1, it can be seen that the decay time for the slow phase in refolding is independent of the final urea concentration, within experimental error. In contrast, the decay time of the intermediate phase increases as the final urea concentration increases up to the point where the decay times of these two phases become too similar to be resolved.

pH Dependence. The pH dependences of the decay times for the slow and intermediate phases in refolding jumps into the native base-line region are shown in Figure 2. The pH of the solution was checked before and after each jump to ensure that it remained constant. The decay time of the slow phase has a pH-independent value of 300 s at 21.7 °C over the pH range from 6.5 to 8.6. In contrast, the decay time of the intermediate phase increases from 33 s at pH 6.5 to 72 s at pH 8.6. The behavior is well described by a single protonation–deprotonation event whose pK value is 7.6 ± 0.3. Two lines of evidence show that this pH dependence does not reflect a major change in the conformation of the native form. First, the value of K_m for the hydrolysis of indoleglycerol phosphate by α subunit is independent of pH from 6 to 9 (Hardman & Yanofsky, 1965). Second, the mean residue ellipticity of the α subunit at 222 nm is constant from pH 6.5 to 7.8 (M. M. Crisanti and C. R. Matthews, unpublished results). Thus, refolding is occurring to the same final conformation over this pH range.

Temperature Dependence. Further information on the nature of the slow and intermediate phases in refolding was obtained by determining their temperature dependence under strongly refolding conditions. Arrhenius plots of the rate constants for jumps ending at 1.5 M urea, pH 7.8, are shown in Figure 3. The activation energies of the slow and inter-

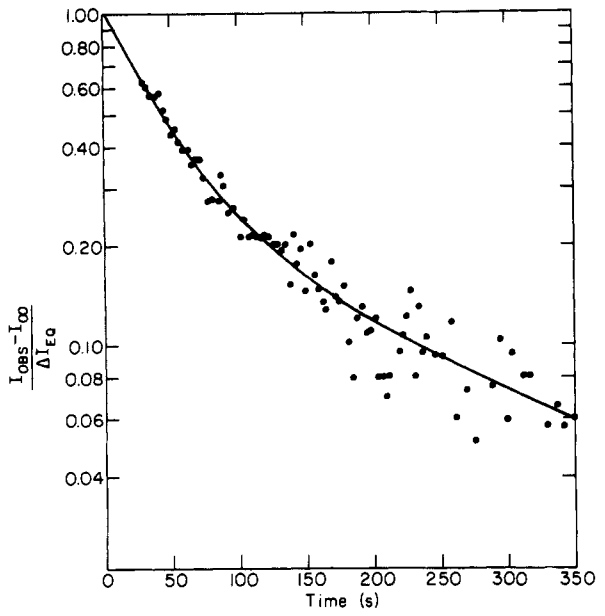

FIGURE 4: A semilogarithmic kinetic plot of the fluorescence intensity of IPP at 365 nm for a refolding jump from 6 to 1.5 M urea, pH 7.8 and 26 °C. Solid points represent fluorescent intensities taken from a continuous trace at approximately 3-s intervals. The solid line indicates the double-exponential fit to the data by using values for the relative amplitudes of the slow and intermediate phases of 0.25 and 0.75, respectively, and decay times of 235 and 43 s, respectively. The change in fluorescence intensity was normalized by using the total change expected from equilibrium measurements, ΔI_{EQ}. The IPP and protein concentrations were 40 and 70 μM, respectively.

mediate phases were calculated from the slopes and found to be 17.6 ± 2.5 and 14.5 ± 2.6 kcal mol^{-1}, respectively. Also shown in Figure 3 is the fact that the relative amplitudes of these two phases are independent of temperature over this range.

Inhibitor Binding. Considerable insight into the folding mechanism of RNase A has been obtained from studies of the kinetics of binding of an inhibitor, 2′-CMP, during refolding (Garel & Baldwin, 1973). The availability of a competitive inhibitor for the α subunit, indolepropanol phosphate (IPP) (Kirschner et al., 1975), makes possible similar studies on the α subunit. A simple means of monitoring the binding of IPP to the native conformation is provided by the fluorescent properties of the inhibitor (Heyn & Weischet, 1975). The fluorescence maximum of free IPP is ~370 nm. Upon binding to the α subunit, the maximum shifts to ~350 nm and decreases in intensity. Since the α subunit contains no tryptophan residues, the background fluorescence from the protein in this region is negligible. By following the decrease in fluorescence intensity at 365 nm, where the difference between the emission spectra of free and bound IPP is a maximum, the kinetic phases that lead to the binding of the inhibitor can be studied.

Before the results of such experiments can be interpreted, it is necessary to ascertain that the rate of binding of the IPP to the native conformation is rapid compared to the rates of the folding steps that lead to the native form. It was found in a separate experiment that the fluorescent changes accompanying the addition of IPP to a solution containing the α subunit in 10 mM potassium phosphate, pH 7.8, 26 °C, containing 1.5 M urea occur rapidly compared to the mixing time. The conditions of this experiment, 50 μM α subunit and 20 μM IPP, were such that 44% of the IPP should be bound at equilibrium. Thus, fluorescent changes observed after the mixing is complete will reflect the kinetics of folding of the protein. Further evidence supporting the conclusion that the rate-limiting steps in the binding of IPP during refolding jumps are due to protein folding will be presented later.

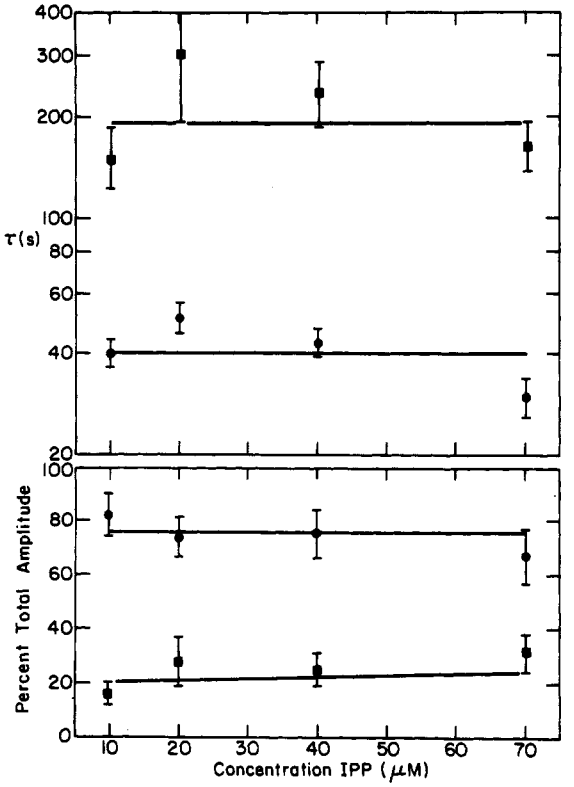

FIGURE 5: The dependence of the decay times and the amplitudes of the slow (■) and intermediate (●) phases observed in refolding jumps from 6 to 1.5 M urea on IPP concentration as monitored by fluorescence intensity at 365 nm. The buffer contained in 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol, 26 °C. The error bars indicate the estimated error. The protein concentration was held constant at 70 μM.

The α subunit, initially unfolded in 6 M urea, pH 7.8, 26 °C, was refolded by dilution of the urea to 1.5 M with a buffered solution containing IPP. A semilog plot of the time dependence of the fluorescent intensity at 365 nm from a typical experiment is shown in Figure 4. Two kinetic phases were detected: a slow phase, with a decay time of 235 s, that accounted for 25% of the total change in fluorescence, and a faster phase, with a decay time of 43 s, that accounted for the remainder of the change. These decay times agree, within experimental error, with the values obtained for the slow and intermediate phases observed in the refolding of the α subunit in the absence of inhibitor (Figure 3). The total amplitude of the fluorescent change agrees well with the value determined from the difference of the fluorescent intensity of the same concentration of IPP in a buffered solution containing 1.5 M urea and the intensity of IPP fluorescence in a solution containing native α subunit in 1.5 M urea. Thus, no fast phases that are complete in the dead time of mixing occur. The fluorescence spectra of IPP in 6 M urea in the presence and absence of α subunit were identical, indicating that the IPP does not bind to the unfolded protein. Since IPP absorbs strongly in the region from 250 to 300 nm, it was not possible to monitor the folding of the protein in the presence of the inhibitor.

A series of refolding jumps at constant protein concentration but varying concentrations of IPP were performed to determine the effect of varying the inhibitor concentration of the decay times and amplitudes of the slow and intermediate phases. As is shown in Figure 5, both of these parameters are independent of IPP concentration, within experimental error, over a range where the saturation of the α subunit varied from 8% to 43%. In principle, this experiment permits one to determine if the biphasic binding kinetics of the inhibitor reflect binding to two different conformations of the protein or binding only to the

native conformation which is formed by two kinetic processes (Garel & Baldwin, 1973). Unfortunately, the scatter in the data on the relative amplitudes (Figure 4) is large enough that it is not possible to choose between these alternatives if binding occurs to an intermediate form with a dissociation constant only 10-fold less than that of the native form. The absence of a dependence of the decay times on the IPP concentration supports the previous conclusion that the rate-limiting steps in inhibitor binding are involved with the folding of the α subunit.

Discussion

The slow and intermediate phases in the folding of the α subunit observed in the optical experiments can now be assigned to particular steps in the folding model proposed previously on the basis of the urea gradient gel electrophoresis experiments (Matthews & Crisanti, 1981). A key observation from the electrophoresis experiments was that the rate of the step linking the native to preceding forms in the folding pathway is pH dependent. At pH 7.2, 16 °C, the exchange was fast, on the electrophoresis time scale (Creighton, 1979), while at pH 8.6, the exchange decreased into the intermediate to slow range. From the gel electrophoresis results, it can be estimated that the half-time of the pH-dependent phase should increase from less than 1 min at pH 7.2 to greater than 1 min at pH 8.6. The decay times of the intermediate phase when corrected for the difference in temperatures between the gels, 16 °C, and those of the data in Figure 3 vary in a fashion consistent with these predictions. This correspondence supports the assignment of this phase to the step linking N to I_2 in the model. The decay time of the pH-independent slow phase falls into the intermediate to slow time range expected for this phase from the gel electrophoresis studies.

In the previous study (Matthews & Crisanti, 1981), it was found that for refolding jumps initiated at 2.5 M urea only the intermediate and slow phases were detected; no fast phase was observed. As the initial urea concentration was increased above 2.5 M, a fast phase appeared whose magnitude increased up to 6 M. From this behavior, it was presumed that the slow and intermediate phases reflect the folding of intermediates, designated I_1 and I_2 in the model, one or both of which are present initially at 2.5 M urea; the fast phase reflects folding of the unfolded protein to these intermediates. Since the intermediate phase has been assigned to the step linking I_2 and N, the slow phase can be assigned to the step linking I_1 and I_2. The placement of these steps and the observation of biphasic refolding kinetics starting at 2.5 M urea combine to show that both intermediates must be present initially at 2.5 M urea. If only I_1 were stable, a monophasic slow kinetic phase would have been detected. If only I_2 were stable, only a monophasic, intermediate phase would have been seen. Thus, both intermediates are stable, in a thermodynamic sense.

The placement of the step with an intermediate rate between N and subsequent forms in the pathway rules out the following simple linear model for the folding of the α subunit:

$$U \overset{\text{fast}}{\leftrightarrow} I_1 \overset{\text{slow}}{\leftrightarrow} I_2 \overset{\text{intermediate}}{\leftrightarrow} N$$

On the assumption that each species has a different extinction coefficient at 286 nm, refolding jumps would show biphasic kinetics, fast and slow, and unfolding jumps would show biphasic kinetics, intermediate and slow. Similar extinction coefficients for any of the species would only decrease the number of observed phases. The triphasic fast, intermediate, and slow kinetics observed in refolding and monophasic kinetics observed in unfolding (Matthews & Crisanti, 1981) are inconsistent with this model. The linear model also does not provide an explanation for the appearance of two intermediates each in fast exchange with the unfolded forms that were observed in urea gradient gel electrophoresis studies at low temperature (Matthews & Crisanti, 1981).

For completeness, it should be noted that the folding model for the α subunit described in the introduction, like the linear model, predicts that unfolding jumps into the transition zone where the intermediates I_1 and I_2 are populated should result in biphasic intermediate and slow kinetics. The observation that the absorbance change is characterized by only a single exponential is most likely due to the fact that the decay times for the two phases become equivalent in the region near 2.5 M urea. Although the decay times are sufficiently different below this urea concentration to be resolved, the amplitude of the absorbance change rapidly diminishes to zero (Figure 1).

The evidence on the independence of the decay time for the slow phase in folding on final urea concentration and on pH and an activation energy of 17.6 kcal mol^{-1} is consistent with the assignment of the step linking I_1 and I_2 to proline isomerization. Although an activation energy of 14.5 ± 2.6 kcal mol^{-1} for the intermediate phase does not rule out the possibility that proline isomerization is involved, the dependence of the decay time for the intermediate phase on pH and final urea concentration suggests that simple proline isomerization is not the explanation for this phase. The observation that the pH dependence of the decay time of the intermediate phase is well described by a single protonation event with a pK of 7.6 suggests that a key histidine residue is involved in the rate-limiting step between I_2 and N. Another possible candidate is the amino group of the methionine residue at the amino terminus of the protein; the protonation of the amino terminus usually has a pK value near 8 (Ellenbogen, 1952).

According to the proposed folding model, the collapse of U_1 and U_2 to I_1 and I_2 is rapid compared to exchange between U_1 and U_2 and between I_1 and I_2. Therefore, the populational distribution between I_1 and I_2 at zero time also reflects the initial distribution between U_1 and U_2 at high urea concentrations. As is shown in Figure 3, the absence of a temperature dependence in the relative amplitudes for the intermediate and slow phases for folding implies that the enthalpy difference between U_1 and U_2 must be small, certainly less than 6 kcal mol^{-1}. A difference of this magnitude suggests that a major, cooperative conformational change is not involved; such differences are typically in the range 100–200 kcal mol^{-1} (Privalov, 1979). However, the enthalpy difference is consistent with an isomerization process linking U_1 and U_2. As has been suggested previously (Brandts et al., 1975), proline isomerization is a possibility; the enthalpy difference is 0–1 kcal mol^{-1} between the cis and trans isomers (Steinberg et al., 1960; Cheng & Bovey, 1977).

The selection of a folding model in which two intermediates are each rapidly formed from unfolded species suggests that the absence of such fast phases in the binding of IPP during folding can be most easily explained by assuming that IPP binds only to the native form. Binding to either intermediate would have resulted in the observation of a fluorescent change on the millisecond time range.

The kinetic scheme proposed for the folding of the α subunit, in which U_1 and U_2 collapse to the intermediates I_1 and I_2 within the dead time of mixing but are themselves separated by a step which must be several orders of magnitude slower, makes it possible to calculate the initial distribution of the two unfolded forms. Under strongly refolding conditions, the kinetic model becomes formally equivalent to an irreversible

linear three-state mechanism in which the two intermediates present after mixing are converted to the native form:

$$I_1 \xrightarrow{k_1} I_2 \xrightarrow{k_2} N$$

The populations of I_1 and I_2 present after the collapse of the unfolded forms can be calculated from the integrated form of the appropriate rate equation (Frost & Pearson, 1953), the data on the relative amplitudes of the two phases observed in IPP binding (Figure 5), and the rate constants for the two phases in which IPP binds at pH 7.8, 25 °C, 4.8×10^{-3} and 2.4×10^{-2} s^{-1} for the slow and intermediate phases, respectively. In the limit that $k_1 \ll k_2$, the relative concentrations would be equal to the relative amplitudes of these two phases. Since k_2 is only ~5 times larger than k_1, a small correction is required. The initial relative concentrations of I_1 and I_2 are 0.21 and 0.79, respectively, using 0.25 and 0.75 as the relative amplitudes of the slow and intermediate phases in IPP binding (Figure 5). Since U_1 and U_2 rapidly collapse to these intermediates, the initial relative concentrations of U_1 and U_2 must also be 0.21 and 0.79, respectively.

Approximate values for the extinction coefficients at 286 nm of the two intermediates, relative to the native conformation, can be determined from data presented in this paper and the previous study (Matthews & Crisanti, 1981). The total change in extinction coefficient at 286 nm for a jump from 6 to 1.5 M urea is 3900 M^{-1} cm^{-1}; the fractions of that change that are due to the intermediate and slow phases are 34% and 20%, respectively. From the IPP binding experiments, the relative populations of the intermediates I_2 and I_1 are 0.79 and 0.21, respectively. A simple calculation reveals that $\epsilon_{I_2} - \epsilon_N = -1660$ M^{-1} cm^{-1} and that $\epsilon_{I_1} - \epsilon_N = -3900$ M^{-1} cm^{-1}. When Donovan's value of -700 M^{-1} cm^{-1} for the exposure of a buried tyrosine to solvent (Donovan, 1973) is used, a net of 2.4 Tyr residues are exposed to solvent in the conversion of N to I_2. A further 3.2 net Tyr residues are exposed in the conversion of I_2 to I_1. The extinction coefficient of I_1 is the same as the extinction coefficients of U_1 and U_2, which are presumably fully unfolded. Given that two to three Tyr are exposed to solvent in the native conformation (Higgins et al., 1979), it appears that four to five Tyr are exposed in I_2 and all seven Tyr in I_1.

Although these values can only be regarded as approximate, it is clear that I_1 and I_2 have different secondary and/or tertiary structures. They do not differ simply in the state of isomerization of one or a few proline residues. Support for this conclusion is provided by the observation from urea gradient gel electrophoresis that the two intermediates migrate with substantially different rates. One intermediate migrates at nearly the same rate as N at low urea concentration; the other migrates more slowly. Since more rapid migration presumably correlates with a more compact structure in which more Tyr residues should be excluded from solvent, I_2 is likely to be the more rapidly moving intermediate and I_1 the more slowly moving form. The rate of migration of I_1 is clearly faster than that of the totally unfolded forms, indicating that it is not a random coil. Whatever the nature of the compact structure in I_1, the UV data indicate that most, if not all, of the Tyr residues are still exposed to solvent.

The distribution of unfolded forms and the proposed nature of the slow and intermediate phases in folding suggest that the state of isomerization of one or a few key proline residues is important in the folding of only a fraction of the α subunit; a majority of the unfolded form proceeds to the native conformation without being rate limited by proline isomerization. This conclusion contradicts the prediction of the extended two-state model of Brandts et al. (1975) for the α subunit. With 19 proline residues, over 98% of the unfolded α subunit should have executed a slow step governed by proline isomerization. Thus, the simple extended two-state model for protein folding does not accurately describe the folding of the α subunit.

If the distribution of cis–trans isomers at X–Pro linkages in the unfolded protein is similar to that observed in model compounds (Torchia, 1972; Dorman & Bovey, 1973; Grathwohl & Wüthrich, 1976), then it appears that the α subunit can adopt a native-like conformation, capable of binding inhibitor, in which many proline residues are in the incorrect isomeric form. Cook et al. (1979) have found that RNase A is able to fold to a quasi-native conformation with at least one proline in an incorrect isomeric form; isomerization occurs *after* folding.

The picture of folding for the α subunit that emerges from this work and the previous study (Matthews & Crisanti, 1981) is one in which there is an initial rapid collapse of the unfolded protein into two more compact forms. That these forms differ from the unfolded conformation and from each other in their degrees of compactness is indicated both by the differing degrees of exclusion of solvent from the tyrosine residues and from the differing mobilities through polyacrylamide gels. A possible explanation for this collapse is the rapid formation of α helices (Hammes & Roberts, 1969; Cummings & Eyring, 1975; Zana, 1975). The formation of hydrophobic clusters is another, but not necessarily exclusive, possibility (Richards et al., 1971; Chothia, 1976). The relaxation time for the formation of a helical intermediate from the guanidine hydrochloride unfolded form of α-lactalbumin has been reported to be less than 1 ms (Kuwajima, 1977).

The products of this initial collapse of the unfolded α subunit are stable, in a thermodynamic sense. The stability of the intermediates may be a reflection of the moderately large size of the α subunit (28 700 daltons) and the possibility of relatively large structural domains in the protein. Higgins et al. (1978) have found that a limited tryptic digestion of the α subunit yields two fragments, 1–188 and 189–268, each of which possesses secondary structure when isolated. The relationship of I_1 and I_2 to the structure in these complementing fragments must await the results of future studies.

The conversion of the intermediate I_2 to the native form appears to be rate limited by a pH-dependent step with a relatively high activation energy. The conclusion that the slow step in folding occurs late in the pathway, close to the native form, is similar to that reached by Creighton in analyzing results from the re-formation of disulfide bonds in both reduced trypsin inhibitor (Creighton, 1977a) and reduced ribonuclease A (Creighton, 1977b) and also the results of a urea gradient gel electrophoresis study of several proteins (Creighton, 1980). Labhardt & Baldwin (1979), on the basis of a study of the refolding of RNase S, have proposed that the rate-limiting step in folding occurs not in the initial stages where nucleation occurs but rather at a later stage and involves the association of intermediates. An analysis of the available data on the rates of formation of α-helical structures and the rate of collision of these species by diffusion by Baldwin (1980) showed that both of these rates are considerably faster than the rates observed for protein folding. Baldwin (1980) postulated that the docking of rapidly formed substructures may well be the rate-limiting steps in folding.

Further studies on the folding of the α subunit are required to identify the molecular events involved in the rate-limiting step. The existence of over two dozen missense mutants for the α subunit may provide a means of accomplishing this goal.

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