# Urea-Induced Unfolding of the α Subunit of Tryptophan Synthase: Evidence for a Multistate Process†

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ABSTRACT: The urea-induced unfolding of the α subunit of tryptophan synthase from *E. coli* was monitored by optical spectroscopy and by urea-gradient gel electrophoresis. Three independent lines of evidence support the conclusion that one or more stable intermediates are present in this process: (i) Satisfactory fits of the equilibrium unfolding transitions obtained from difference spectroscopy at 286 nm and circular dichroism spectroscopy at 222 nm require a model which involves a stable intermediate in addition to the native and unfolded forms. (ii) Kinetic studies of the change in the extinction coefficient at 286 nm show that while the unfolding is well described by a single exponential change the refolding kinetics are complex. The nature of the dependence of the refolding kinetics on the initial concentration of urea supports the conclusion that at least one stable intermediate exists. (iii) The patterns obtained from urea-gradient gel electrophoresis experiments on the α subunit show that at least one and possibly two stable intermediates are involved; the intermediates have markedly different degrees of compactness. A kinetic model for the folding of the α subunit, consistent with all of these results, can be formulated.

The mechanism by which proteins fold to their unique native conformations from an initially disorganized form is one of the fundamental problems in molecular biology that is yet unsolved. Although a variety of equilibrium and kinetic techniques have been applied to the study of the folding of a number of globular proteins (Tanford, 1968; Wetlaufer & Ristow, 1973; Baldwin, 1975; Anfinsen & Scheraga, 1975; Creighton, 1978a), as yet few details are available on the essential molecular events. The high degree of cooperativity observed in the equilibrium unfolding transitions for many proteins has frustrated the attempts to determine the folding pathway by characterizing the conformation of partially folded forms; only the native and unfolded forms are present in significant concentrations at equilibrium. Stable intermediates have been observed in the unfolding of carbonic anhydrase (Wong & Tanford, 1973), penicillinase (Robson & Pain, 1976a,b; Creighton & Pain, 1980), α-lactalbumin (Kuwajima et al., 1976; Nozaka et al., 1978), and, recently, the guanidine hydrochloride-induced unfolding of the α subunit of tryptophan synthase (Yutani et al., 1979), but little progress has been made in identifying the conformation of these partially folded forms.

In an effort to obtain information on mechanisms involved in protein folding at the molecular level, recent investigations in this laboratory have focused on the reversible unfolding of the α subunit of tryptophan synthase from *E. coli*. This protein is an ideal candidate for folding studies since it is a monomer in solution and contains no prosthetic groups; the folding process should involve only unimolecular phenomena. An especially important attribute of this system is the existence of over two dozen mutant strains of *E. coli* containing missense mutations in the gene for the α subunit. The site and identity of the amino acid replacements in the α subunit for these mutants have been determined by Yanofsky and his colleagues (Yanofsky, 1967; Yanofsky & Horn, 1972). Comparative studies of the unfolding behavior of the protein from the wild-type strain and various mutants may elucidate the importance of various individual amino acids in the folding process. It has been observed that the replacement of glycine at position 211 in the α subunit with either glutamic acid or arginine has a significant effect on the energetics of the thermal unfolding transition (Matthews et al., 1980).

In this paper, the results of equilibrium and kinetic studies of the urea-induced unfolding of the α subunit of tryptophan synthase from *E. coli* are reported. Analysis of the transition curves obtained from UV difference spectroscopy at 286 nm and circular dichroism (CD)[1] measurements at 222 nm shows that at least three conformations, the native, partially unfolded, and fully unfolded forms, appear at significant concentrations in the equilibrium unfolding process. Kinetic studies of the UV difference spectrum confirm that the process is more complex than a simple two-state transition and that at least one and possibly two stable, partially folded forms must be involved. The results of urea-gradient polyacrylamide gel experiments support this conclusion and provide a more detailed picture of the folding transition. A folding model is proposed that is consistent with all of these results.

## Materials and Methods

### *Materials*

*α Subunit of Tryptophan Synthase.* The α subunit of tryptophan synthase (EC 4.2.1.20) was isolated from *E. coli*, strain *trp* B8/F′B8, by the method of Kirschner et al. (1975). There is no possibility of heterogeneity in this protein arising from multiple, nonidentical operons. This strain contains a single intrinsic tryptophan operon and a second identical operon on an F′ episome. The episome has been introduced to increase the quantity of protein synthesized. The purity and homogeneity of the protein were ascertained by both native and $NaDodSO_4$–polyacrylamide gel electrophoresis, where only a single band was apparent in the electrophoretograms. The enzyme was stored as a crystalline suspension in 100 mM potassium phosphate, pH 7.8, 55% ammonium sulfate, 1 mM EDTA, and 1 mM DTE at 4 °C; the activity remained unchanged under these conditions for at least 2 months.

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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The activity of the α subunit was determined by measuring its ability to enhance the activity of the $\beta_2$ subunit in the condensation of indole and serine to form tryptophan (Kirschner et al., 1975); the maximum specific activity of the α subunit in this assay is 5500 units $mg^{-1}$. Samples of α subunit used in the folding studies had specific activities ranging from 4600 to 5300 units $mg^{-1}$. Protein concentration was determined from the optical spectrum by using a specific absorption $E_{278}^{1\%} = 4.4$ (Adachi et al., 1974); the molar extinction coefficient was calculated to be 12 600 $M^{-1}$ $cm^{-1}$ by using a molecular weight of 28 700 (Yanofsky et al., 1967; Li & Yanofsky, 1972).

*Chemicals.* Ultrapure urea was purchased from Schwarz/Mann and used without further purification. The concentration of urea was determined by measuring the density as described by Kawahara & Tanford (1966). The activities of urea solutions were calculated by using the data of Bower & Robinson (1963). All other chemicals were reagent grade.

## Methods

*Spectroscopy.* Ultraviolet difference spectroscopy measurements were made by using the tandem-cell technique described by Herskovits (1967) on a Cary 118CX spectrophotometer. The protein concentration was held constant throughout each experiment and was generally in the range 0.4–1.5 mg $mL^{-1}$.

Kinetic studies of the folding were performed on either a Cary 118CX spectrophotometer or a Durrum Model D-130 stopped-flow spectrophotometer, depending on the time range of interest. Kinetic measurements of transient responses in the time range of 10 s or longer were performed on the Cary 118CX spectrophotometer by using difference spectroscopy. The α subunit in a buffered solution containing no urea was used as a reference. The unfolding (refolding) was initiated by using a microliter syringe 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 A/h, was always less than 2% of the total observed change.

Kinetic measurements in the time range of 10 ms to 10 s were performed on a Durrum Model D-130 stopped-flow spectrophotometer equipped with variable-diameter drive syringes. Separate thermostating water baths were used to independently control the temperature of the drive syringes and the observation chamber. This arrangement minimizes thermal artifacts arising from mixing highly concentrated urea solutions with buffer. The temperatures of the drive syringes and observation chamber were adjusted until the response of a sample of tyrosine and phenylalanine amino acids appropriate to the α subunit was minimized. The changes in transmittance at 286 nm were recorded on a Textronix D15 storage oscilloscope. This trace was then photographed with Polaroid film (type 107) for subsequent analysis.

Circular dichroism measurements were performed on a Jasco Model J-40 spectropolarimeter. The CD spectra were recorded over the wavelength range 200–350 nm. Since no absorptions in the CD spectrum of the 8 M urea stock solution were observed in this range, no corrections had to be made for varying concentrations of urea. Mean residue ellipticity values were calculated according to the equation

$$[\theta]_\lambda = \frac{\theta_{obsd}\mathrm{MRW}}{10dc}$$

where $[\theta]_\lambda$ is the mean residue ellipticity at wavelength λ, $\theta_{obsd}$ is the observed ellipticity in degrees, MRW is the mean residue weight, $d$ is the cell path length in centimeters, and $c$ is the protein concentration in g $mL^{-1}$. The mean residue weight was calculated to be 107.2 g from the amino acid sequence of the α subunit. A 0.1-cm cell path length was used to keep the absorbance at 222 nm below 0.8 $A$ unit. The protein concentration was 0.3 mg $mL^{-1}$.

*Equilibrium Sedimentation.* Equilibrium sedimentation studies were performed on a Beckman Model E analytical ultracentrifuge by using a meniscus depletion method. Samples of α subunit were centrifuged on an AnFTi rotor at 30 000 rpm at 25 °C for approximately 72 h. The protein concentration was 1.5 mg $mL^{-1}$. The partial specific volume of the α subunit was calculated from the amino acid composition by the procedure of Cohn & Edsall (1943) and was found to be $\bar{v} = 0.748$ $cm^3$ $g^{-1}$.

*Urea-Gradient Gel Electrophoresis.* The urea-gradient gel electrophoresis experiments were performed by Dr. Thomas Creighton. The urea-gradient gels were prepared in the manner described previously (Creighton, 1979). The gels contained a horizontal, linear urea gradient from 0 to 8 M and a compensatory, inverse gradient of 15–11% acrylamide. The acrylamide gradient ensures uniform porosity across the gel. The electrophoresis buffers for pH 7.2 and 7.8 were 50 mM Bistris–acetate and 1 mM EDTA and for pH 8.6 Tris–borate (5.45 g of Tris, 1.55 g of boric acid, and 0.29 g of EDTA per liter). No dithiothreitol was incorporated into the gels, as it inhibits the polymerization of the acrylamide.

Samples of the native α subunit were prepared for electrophoresis by mixing 40 μL of α subunit dissolved in 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 0.2 mM DTE with 170 μL of $H_2O$, 20 μL of 10 mM dithiothreitol, ~2 μL of a saturated solution of bromophenol blue, and a small drop of glycerol to increase the density. Samples of the unfolded protein were prepared by substituting a saturated urea solution for the water, and omitting the glycerol. The protein concentration was about 1.1 mg $mL^{-1}$. Volumes of 20–80 μL were layered across the top of each gel. A water-jacketed electrophoresis cell was maintained at constant (±1 °C) temperature throughout electrophoresis by a refrigerated water bath. The gels were stained for visualization as described previously (Creighton, 1979).

## Results

*Equilibrium Spectroscopic Studies.* The urea-induced unfolding of the α subunit of tryptophan synthase in a buffered solution containing 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM mercaptoethanol, 25 °C, was monitored by UV difference spectroscopy in the region between 250 and 350 nm and by CD spectroscopy in the range from 200 to 350 nm. The changes in absorption at 286 nm were converted to changes in the molar extinction coefficient and plotted as a function of urea concentration (Figure 1A). The small, linear increase in $\Delta\epsilon_{286}$ that is observed from 0 to ~2 M urea is due to a solvent perturbation effect of urea on the electronic absorption of exposed tyrosines in the native conformation. Between urea concentrations of 2 and 6 M, the large decrease in $\Delta\epsilon_{286}$ shows that the α subunit undergoes a cooperative unfolding transition in this range. Above 6 M urea, where the unfolded conformation is stable, the linear increase in $\Delta\epsilon_{286}$ is again due to solvent perturbation of the exposed tyrosines in the unfolded conformation by increasing concentrations of urea. The reversibility of the urea-induced unfolding transition was ascertained spectroscopically (Figure 1A) and by the nearly quantitative (>90%) recovery of enzymatic activity following refolding by dilution.

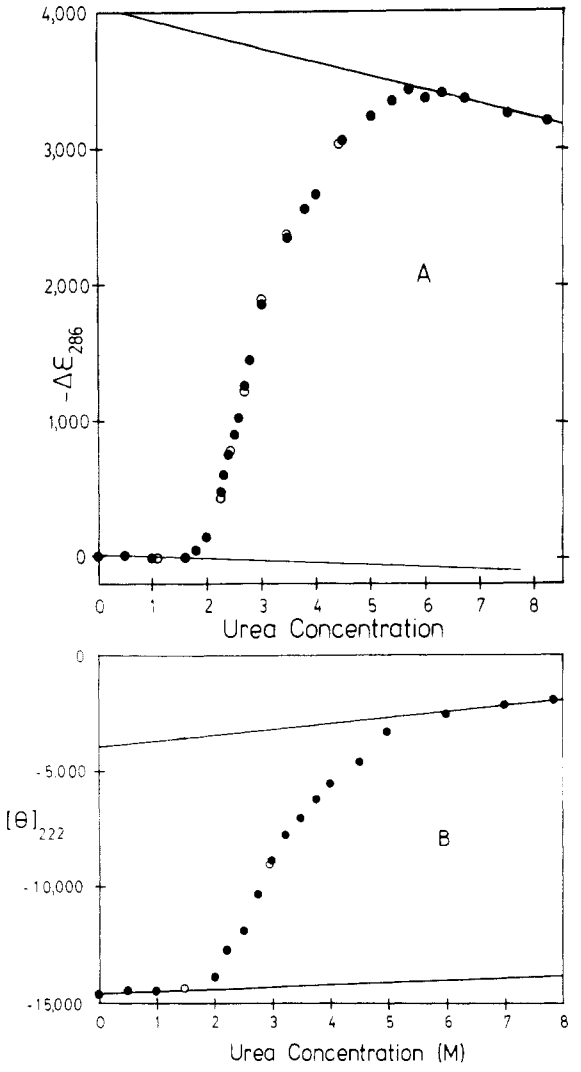



FIGURE 1: Dependence of (A) the difference extinction coefficient at 286 nm, $\Delta\epsilon_{286}$, and (B) the mean residue ellipticity at 222 nm, $[\theta]_{222}$, on urea concentration in unfolding (●) and refolding (○) experiments in 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol at 25 °C. The solid lines indicate the assumed dependence of coefficients of native and unfolded forms of the $\alpha$ subunit on urea concentration. The protein concentration ranged from 0.3 to 1.6 mg $mL^{-1}$.

The urea-induced unfolding at pH 7.8, 25 °C, was also monitored by CD spectroscopy. As is shown in Figure 1B, the mean residue ellipticity at 222 nm, $[\theta]_{222}$, which is sensitive to secondary structure, depends slightly on urea concentration up to approximately 2 M urea. Between 2 and 6 M urea, the increase in $[\theta]_{222}$ indicates that the $\alpha$ subunit undergoes a cooperative unfolding transition that results in the loss of secondary structure. The transition monitored by CD spectroscopy is fully reversible.

The unfolding transitions detected by these two spectroscopic methods can be compared by converting the data shown in Figure 1A,B to plots of the fractional change in either parameter as a function of urea concentration. The fractional change is defined by $F_{app} = (Y_{obsd} - Y_N)/(Y_U - Y_N)$ where $F_{app}$ is the apparent fractional change, $Y_{obsd}$ is the observed value of the parameter, and $Y_N$ and $Y_U$ are the values of the parameter for native and unfolded forms, respectively, at the given urea concentration. For the UV difference and CD results, values for $Y_N$ and $Y_U$ at urea concentrations in the transition region were obtained by linear extrapolation of the native and unfolded base lines into the transition region. The base lines are shown in Figure 1.

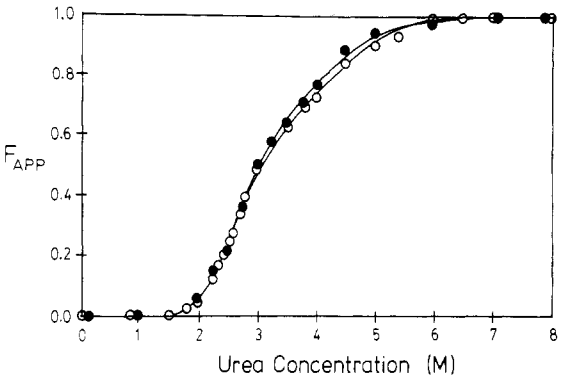



FIGURE 2: Dependence of the fractional change, $F_{app}$, in $\Delta\epsilon_{286}$ (●) and $[\theta]_{222}$ (○) on urea concentration at pH 7.8, 25 °C, as determined from the data in Figure 1.

The dependence of $F_{app}$ on urea concentration determined by UV and CD spectroscopy is shown in Figure 2. Up to 3 M urea, the transition curves are completely coincident. Although small differences between these curves are observed between 3 and 6 M urea, these differences are similar in magnitude to the errors introduced by experimental limitations and by the choice of base lines used to describe the dependence of $\Delta\epsilon_{286}$ or $[\theta]_{222}$ on urea concentration for the native and unfolded forms. As a result, the differences may not be significant. The apparent coincidence of the transition curves indicates that the conformational changes that expose tyrosines to solvent also disrupt the secondary structure in the $\alpha$ subunit.

In order to characterize these transitions and obtain quantitative thermodynamic parameters for the unfolding process, both the UV difference and CD data were computer fit to a two-state model involving only a native (N) and an unfolded (U) form:

$$N \underset{}{\overset{K}{\rightleftharpoons}} U$$

The dependence of the equilibrium constant on urea concentration was assumed to follow the linear denaturant binding model (Tanford, 1970):

$$K_{app} = K^{H_2O}(1 + ka)^{\Delta n} \quad (1)$$

where $K_{app}$ is the apparent equilibrium constant at a urea activity, $a$, $K^{H_2O}$ is the equilibrium constant in the absence of urea, and $\Delta n$ is the difference in the number of binding sites for urea between the native and unfolded forms of the protein. The value of $k$ was taken to be 0.1. The values for $\Delta G^{H_2O}$, calculated from $K^{H_2O}$ at 25 °C, and $\Delta n$ that give the best fit are shown in Table I. The calculated values for $F_{app}$ at any urea concentration are shown in Figure 3. The fit of either the UV difference or the CD data to a two-state model is rather poor, even when the extrapolations required for the unfolded base lines were considered.

The data could only be fit adequately by incorporating a third species in the unfolding model:

$$N \underset{}{\overset{K_1}{\rightleftharpoons}} I \underset{}{\overset{K_2}{\rightleftharpoons}} U$$

where I is a partially unfolded form and $K_1$ and $K_2$ are the appropriate equilibrium constants. A nonlinear least-squares program was used to fit the data to the following equation (Tanford, 1970):

$$K_{app} = \frac{K_1K_2 + ZK_1}{1 + (1 - Z)K_1} \quad (2)$$

where $K_{app}$ is the apparent equilibrium constant, and the

Table I: Parameters for Two- and Three-State Fits to Observed Transition Curves

| | pH | spectroscopy | $\Delta G_1^{H_2O}$ (kcal/mol) | $\Delta G_2^{H_2O}$ (kcal/mol) | $\Delta G_{total}^{H_2O}$ (kcal/mol) | $\Delta n_1$ | $\Delta n_2$ | $Z$ |
|---|---|---|---|---|---|---|---|---|
| three-state fits | 7.8 | UV | 6.4 | 5.3 | 11.7 | 50.6 | 28.0 | 0.54 |
| | | CD | 6.4 | 3.9 | 10.3 | 50.0 | 28.9 | 0.53 |
| | 7.2 | UV | 8.1 | 5.5 | 13.6 | 59.0 | 27.5 | 0.57 |
| | | CD | 7.6 | 4.4 | 12.0 | 55.8 | 31.1 | 0.54 |
| | 6.5 | UV | 7.3 | 5.3 | 12.6 | 51.0 | 26.5 | 0.57 |
| | | CD | 6.1 | 5.3 | 11.4 | 43.0 | 27.7 | 0.54 |
| two-state fits | 7.8 | UV | | | 3.5 | 23.2[a] | | |
| | | CD | | | 3.7 | 24.3[a] | | |

[a] Difference in the number of binding sites for urea on the unfolded and native conformations.

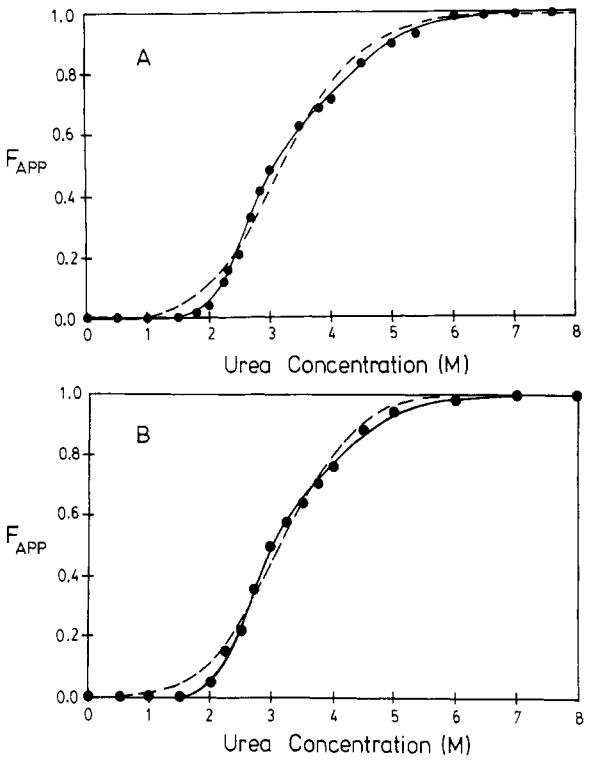



FIGURE 3: Comparison of the fit of the experimentally observed transition curve (●) from (A) UV difference spectroscopy and (B) CD spectroscopy to a two-state (---) and a three-state (—) model. The parameters for the fits to two- and three-state models are given in Table I.

equilibrium constants $K_1$ and $K_2$ each were assumed to depend on urea according to the linear denaturant binding model shown in eq 1. The parameter $Z$ is the fractional change in the optical parameters in the transition from N to I and is defined by $Z = (Y_I - Y_N)/(Y_U - Y_N)$ where $Y_I$ is the value of a given optical parameter for the intermediate species, I. The values for $\Delta G_1^{H_2O}$ and $\Delta G_2^{H_2O}$, calculated from $K_1^{H_2O}$ and $K_2^{H_2O}$ at 25 °C, $\Delta n_1$, $\Delta n_2$, and $Z$ are shown in Table I. The best fit of $F_{app}$ determined from these values to the experimental data is shown in Figure 3. Both data sets fit very well to the three-state model over the entire transition region. We conclude that the unfolding transition of the α subunit is better characterized by a three-state model that includes a stable, partially folded form.

The unfolding transitions were also monitored by UV difference and CD spectroscopy at pH 7.2 and 6.5 to determine if the failure of the simple two-state model to describe adequately the urea-induced unfolding at pH 7.8, 25 °C, was due to an artifact at this particular pH. In all cases, the three-state model provided a better fit to the data (results not shown).

The thermodynamic parameters for the three-state model used to generate the theoretical curves at various pH values

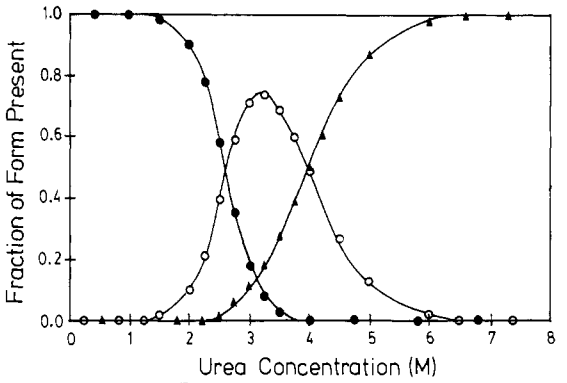



FIGURE 4: Fraction of native (●), intermediate (○), and unfolded (▲) forms of the α subunit as a function of urea concentration at pH 7.8, 25 °C.

are listed in Table I. At each pH, the values for $\Delta G_1^{H_2O}$, $\Delta G_2^{H_2O}$, $\Delta n_1$, $\Delta n_2$, and $Z$ obtained from the UV difference and CD data are in reasonable agreement, considering the possible sources of error and the approximations involved. The magnitude of $Z$, ~0.5–0.6, for both $\Delta\epsilon_{286}$ and $[\theta]_{222}$ indicates that approximately one-half of the total change in each of these parameters observed in complete unfolding occurs in the transition from the native to the intermediate form. Thus, the intermediate retains substantial residual structure.

From the parameters in Table I, the fractions of the native ($f_N$), intermediate ($f_I$), and unfolded ($f_U$) forms at various urea concentrations can be calculated. The appropriate equations are the following:

$$\exp[-\Delta G_1/(RT)] = K_1 = f_I/f_N$$

$$\exp[-\Delta G_2/(RT)] = K_2 = f_U/f_I$$

$$f_N + f_I + f_U = 1$$

The values for the unfolding transition at pH 7.8, 25 °C, are shown in Figure 4. According to this model, the intermediate is the predominant species in solution near 3 M urea. The unfolded form does not appear until 2.5 M and becomes the predominant species above ~4 M urea. The populational distribution of the native, intermediate, and unfolded forms was insensitive to the choice of the value of $k$ in eq 1; varying $k$ from 0.01 to 1.0 produced no significant changes in $f_N$, $f_I$, or $f_U$ as a function of urea concentration.

*Kinetic Studies.* Kinetic studies of the reversible unfolding of α subunit were performed to gain further insight into the folding mechanism. The results for an unfolding jump from 0 to 2.5 M urea and a refolding jump from 6 to 2.5 M urea at pH 7.8, 25 °C, are shown in Figure 5. In these plots, the difference between the absorbance at 286 nm at a given time, $A(t)$, and the absorbance when no further changes were observed, $A(\infty)$, was normalized by dividing by the total change in absorbance expected for each jump from equilibrium studies.

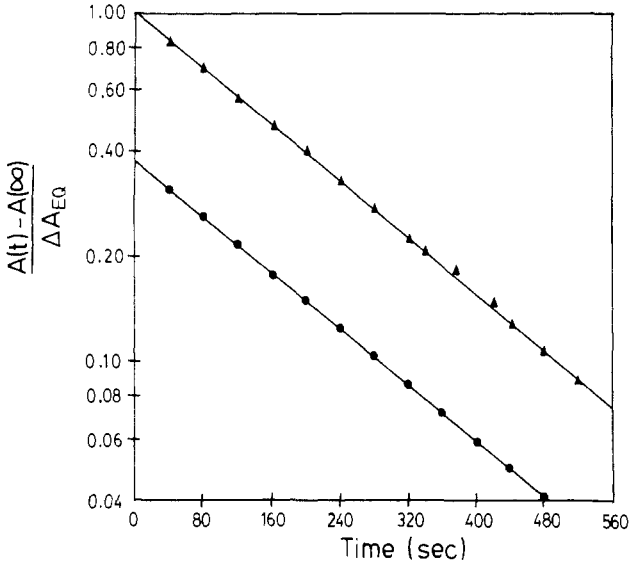



FIGURE 5: Semilogarithmic kinetic plots for unfolding (▲) and refolding (●) jumps from initial concentrations of 0.0 and 6.0 M urea, respectively, to a final concentration of 2.5 M urea, pH 7.8. Solid lines represent the linear least-squares fit to the data points.

Using this procedure, it is possible to determine if the observed kinetic phases account for all of the expected absorbance changes. The unfolding process is well described by a simple exponential decay whose half-time is 154 ± 10 s. Extrapolation to zero time shows that this kinetic phase accounts for all the expected change in absorbance at 286 nm. The same behavior was observed for other unfolding jumps which ended at a series of urea concentrations from 2.5 M, where the transition is 25% complete, to 6.0 M, where the protein is fully unfolded (data not shown). The half-times of these optical changes did not depend on the protein concentration, indicating that the unfolding process is a unimolecular phenomenon.

In contrast to the simple exponential behavior observed for unfolding, the refolding jump from 6 to 2.5 M urea exhibited a complex response (Figure 5). A slow phase with a half-time of 158 ± 10 s was observed that only accounts for 38% of the expected absorbance change. The remainder of the increase in absorbance accompanying refolding is attributed to a fast phase that could not be directly detected by either manual or stopped-flow mixing. The fast phase was obscured by optical effects that were attributed to light scattering. For both methods of mixing, a decrease in absorbance was observed not only at 286 nm but also at 320 and 350 nm, where the protein difference spectrum is zero. The subsequent slow increase in absorbance at 286 nm that reflects protein folding was not observed at the longer wavelengths. This decrease in absorbance may reflect the breakup of aggregates that are formed in mixing. Attempts to eliminate this problem by lowering the protein concentration by a factor of 5 were not successful. Refolding at lower protein concentrations did demonstrate, however, that the slow phase is related to folding and not aggregation, since its half-time was unaffected.

It should be noted that, at equilibrium, there was no evidence for aggregated protein. In the UV difference spectra, the base lines were independent of wavelength from 240 to 350 nm at all urea concentrations. The absence of aggregated species at the beginning of the refolding jumps was confirmed by measuring the molecular weight of the protein in 6 M urea by equilibrium sedimentation. The molecular weight, determined by equilibrium sedimentation, was 30000 ± 1000, in satisfactory agreement with that calculated from the amino acid sequence, 28700. From these results, it can be concluded

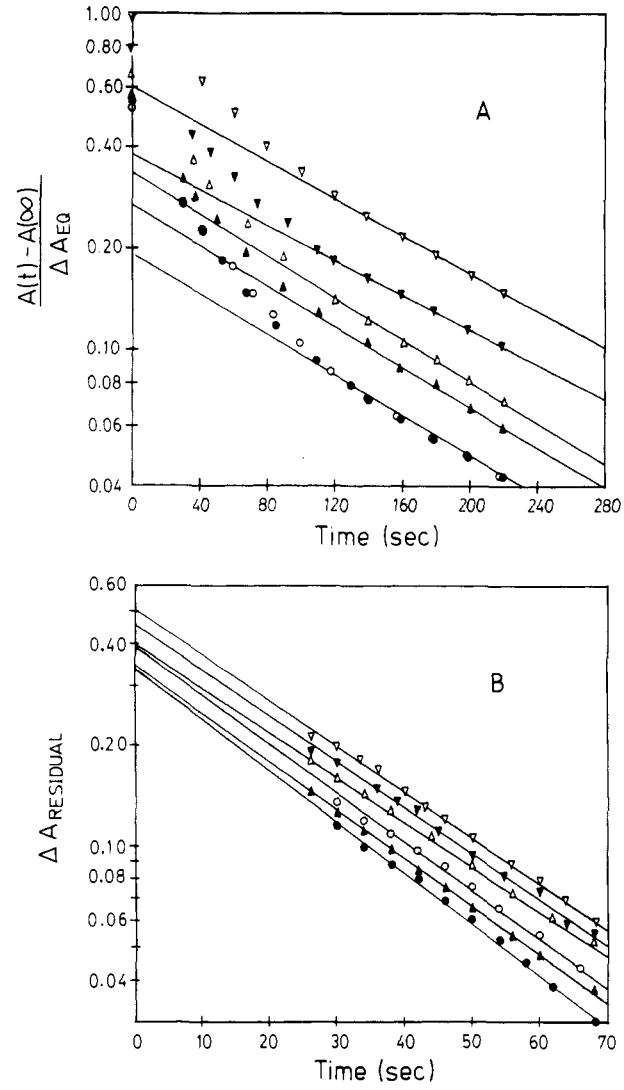



FIGURE 6: (A) Semilogarithmic kinetic plots for refolding jumps from initial concentrations of 6.0 (●), 5.0 (○), 4.0 (▲), 3.0 (△), 2.7 (▼), and 2.5 (▽) M urea to a final concentration of 1.5 M urea at pH 7.8, 25 °C. Symbols on the ordinate indicate the total amplitude accounted for by both slow and intermediate phases as a function of the initial urea concentration. Solid lines represent the linear least-squares fits to the data points for the slow phase. (B) Semilogarithmic kinetic plots for the intermediate phase obtained by exponential stripping for refolding jumps from initial concentrations to a final urea concentration of 1.5 M urea at pH 7.8, 25 °C. $\Delta A_{residual}$ is the difference between the actual absorbance at a given time and the absorbance due to the slow phase at that time.

that the aggregation obscuring the fast phase in folding is only a transient phenomenon.

An estimate of the upper limit of the half-time for the fast phase can be obtained from the observation that it must be complete by the time the light scattering effects have disappeared in the stopped-flow mixing experiment (~50 ms). If the fast-folding phase is considered to be complete in 5 half-times, then the half-time must be less than 10 ms. The persistence of the light scattering effects at much longer times (5–10 s) when the solutions were manually mixed is attributed to the slower and less efficient mixing process.

In order to probe further the nature of the refolding process, a series of refolding jumps from various initial conditions to the same final conditions were performed. Semilog plots of a series of such refolding jumps to 1.5 M urea, where the native form is stable at pH 7.8, 25 °C, are shown in Figure 6A. As before, the changes in absorbance have been normalized to the value determined from equilibrium experiments. The

Table II: Rate Constants and Relative Amplitudes of Observed Phases for Refolding Jumps to 1.5 M Urea, pH 7.8, 25 °C

| initial urea concn (M) | protein concn (mg mL$^{-1}$) | slow phase | | intermediate phase | | fast phase |
|---|---|---|---|---|---|---|
| | | $t_{1/2}$ (s) | relative amplitude (%) | $t_{1/2}$ (s) | relative amplitude (%) | relative amplitude (%) |
| 6.0 | 1.6 | 95 ± 15 | 22 ± 3 | 22 ± 2 | 32 ± 4 | 46 ± 7 |
| | 0.8 | 102 ± 15 | 19 ± 3 | 20 ± 2 | 36 ± 5 | 45 ± 8 |
| | 0.3 | 133 ± 32 | 18 ± 5 | 22 ± 2 | 37 ± 5 | 47 ± 10 |
| 5.0 | 0.8 | 110 ± 30 | 17 ± 3 | 22 ± 2 | 33 ± 4 | 50 ± 7 |
| 4.0 | 0.8 | 108 ± 10 | 25 ± 3 | 23 ± 2 | 29 ± 5 | 46 ± 8 |
| 3.0 | 1.6 | 116 ± 10 | 33 ± 8 | 23 ± 2 | 35 ± 5 | 32 ± 13 |
| | 0.8 | 133 ± 32 | 29 ± 7 | 22 ± 2 | 38 ± 5 | 33 ± 12 |
| | 0.3 | 133 ± 32 | 18 ± 8 | 22 ± 2 | 50 ± 8 | 32 ± 16 |
| 2.7 | 0.8 | 136 ± 25 | 33 ± 4 | 24 ± 2 | 43 ± 4 | 24 ± 8 |
| 2.5 | 1.6 | 114 ± 22 | 52 ± 4 | 23 ± 2 | 55 ± 4 | −7 ± 8 |
| | 0.8 | 131 ± 13 | 55 ± 4 | 22 ± 2 | 52 ± 4 | −7 ± 8 |
| | 0.3 | 136 ± 25 | 37 ± 9 | 22 ± 2 | 57 ± 4 | 6 ± 13 |
| | | av 120 ± 15 (SD) | | av 22 ± 1 (SD) | | |

response observed is even more complex than that for jumps ending at 2.5 M urea. For jumps initiated at concentrations of urea over 2.5 M, at least three kinetic phases are apparent, a slow phase with a half-time of 120 s, an intermediate phase with a half-time of 22 s, and a fast phase whose presence is again inferred from the observation that the two previous phases cannot account for all of the absorbance change calculated from the equilibrium data. The half-time and magnitude of the intermediate phase were obtained by an exponent "stripping" procedure whereby the amplitude of the slower phase is subtracted from the observed data and the logarithm of the resultant difference replotted as a function of time. The replots for each of the refolding jumps are shown in Figure 6B. The amplitudes and rate constants for the three phases are shown in Table II.

The relative amplitude of the fast phase increases from 0% at 2.5 M urea to 46% at 6 M urea. The near coincidence of the normalized kinetic curves for jumps beginning at 5 and 6 M urea shows that the effect of urea in altering the kinetic response is complete by 6 M. Comparison of these observations with the relative fraction of the unfolded form estimated from the equilibrium data (Figure 4) suggests that the fast phase is related to the folding of the completely unfolded protein to a partially folded intermediate. The unfolded form only appears above 2.5 M urea and is virtually the only species present at 6 M urea. Consistent with this hypothesis is the fact that the fraction of the total change in absorption accounted for by the fast phase for complete refolding jumps from 6 to 1.5 M urea (46%), identical with the value for the fraction of the change in $\Delta\epsilon_{286}$ associated with the transition from U to I for a three-state fit (Table I) (46%). Although the fast kinetic phase could be related to the collapse of the unfolded form into an incorrectly folded conformation, the increase in amplitude as the initial concentration of urea is increased makes this possibility less likely. In general, increasing concentrations of urea destabilize folded structures and favor the unfolded forms. Thus, it appears that one or more partially folded forms, as judged by $\Delta\epsilon_{286}$, exist in significant concentrations at intermediate urea concentrations.

As is shown in Table II, the half-times and relative amplitudes of the intermediate and slow phases do not depend on the protein concentration, within experimental error. Therefore, the absorbance changes detected in these phases reflect the unimolecular protein folding process and not the intermolecular aggregation. The appearance of an intermediate phase for jumps ending at 1.5 M urea, in addition to the slow and fast phases previously observed, means that at least four kinetic species must be included in a complete description of the folding of the α subunit.

*Urea-Gradient Gel Electrophoresis.* The urea-induced unfolding of the α subunit was probed further with the urea-gradient gel electrophoresis technique developed by Creighton (1979). The urea-gradient gels for the α subunit at a series of pH values are shown in Figure 7. At pH 7.2, 16 °C, a continuous band extends from 0 to 8 M urea, starting with either the native (N) or the unfolded (U) forms. The mobility decreases significantly between 3 and 4 M urea, reflecting the unfolding of the protein in this range. The observation that identical patterns involving a single continuous band are obtained when starting from either N or U indicates that the unfolding and refolding are rapid on the time scale of electrophoresis; the half-time must be less than 2 min at all urea concentrations. The patterns are similar to those predicted for a rapid two-state transition, except that there is a slight tendency for the mobility of the unfolded form to increase near the transition zone. At pH 7.2, the spur that appears at the upper portion of the transition zone in the gels, starting from either N or U, is probably due to a fraction of the unfolded molecules that becomes modified during electrophoresis so that they cannot refold.

The patterns observed at pH 8.6, 17 °C, differ from those at pH 7.2 in one important aspect: the band of stained protein on the native side of the transition region has become extremely diffuse. In fact, when the protein is initially in the native conformation, a discontinuity is observed in this region. The native band remains horizontal as unfolding occurs; however, the unfolded band displays a definite downward curvature in the transition zone when the protein is initially in either the native or the unfolded form. The absence of stained material in the early stages of the transition region indicates that the exchange between the native and subsequent forms must be slow at this pH. The patterns at pH 7.8, 15 °C, are intermediate to those at pH 7.2 and 8.6.

These gels do not resemble those predicted for a simple two-state model in either fast or slow exchange; other forms must be present. The curvature of the unfolded band reflects a rapid exchange between the fully unfolded form and a more compact form which is stable in the transition zone. If this compact form were only a transient species, this curvature would not have been observed on the gel when the protein was initially in the native form; both native and unfolded bands would have been horizontal. The urea gels at pH 8.6 support the conclusion that a stable, partially folded form exists in the urea-induced unfolding of the α subunit.

It should be pointed out that none of the patterns observed in Figure 7 are those predicted for a heterogeneous mixture

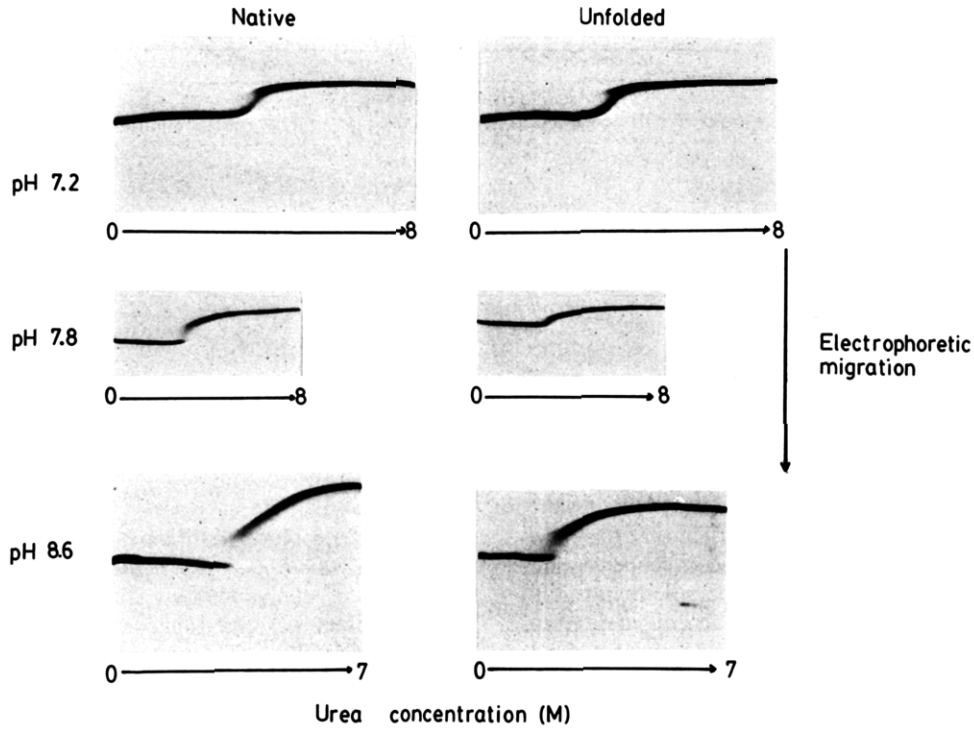



FIGURE 7: Urea-gradient gel electrophoresis of the $\alpha$ subunit of tryptophan synthase, starting in the native and unfolded forms at pH 7.2, 7.8, and 8.6. The time of electrophoresis was 3.7 h at pH 7.2, 16 °C, and 1.5 h at pH 7.8, 15 °C. At pH 8.6, the temperature was 17 °C, and the electrophoresis time was 4.0 h, and 15 °C and 1.5 h, for protein starting in the native and unfolded forms, respectively.

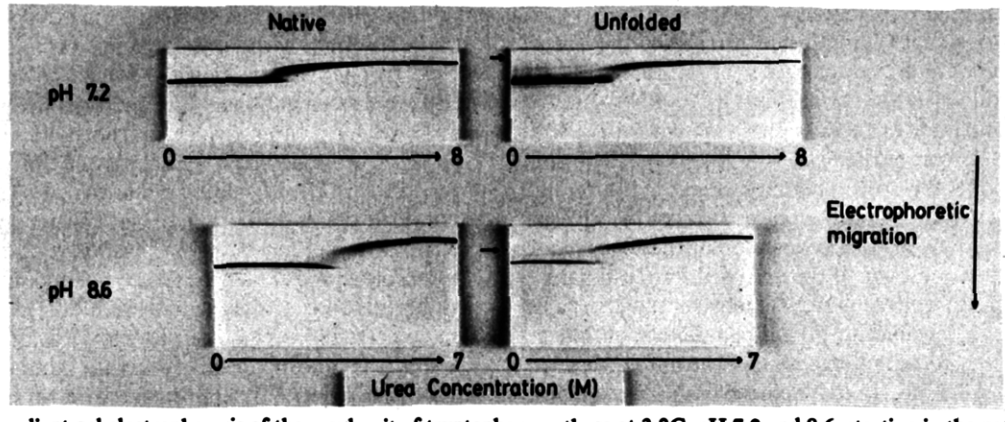



FIGURE 8: Urea-gradient gel electrophoresis of the $\alpha$ subunit of tryptophan synthase at 3 °C, pH 7.2 and 8.6, starting in the native and forms. The electrophoresis time was 1.0 h.

of proteins with differing stabilities to urea unfolding (Creighton, 1980). Therefore, this trivial explanation for the optical results described above can be ruled out.

Since the patterns obtained on the urea gels depend upon the rates of exchange between various conformations, it has proven useful to run the gels at lower temperatures in order to slow these rates down (Creighton, 1980). The patterns for samples of the $\alpha$ subunit run at 3 °C at both pH 7.2 and 8.6 are shown in Figure 8. The effect of lowering the temperature is quite dramatic at both pH values. At pH 7.2, starting with N, the pattern is close to that expected for a two-state transition which is slow in the unfolding region. The half-time must be greater than 6 min. The pattern is far more complex when starting with U at pH 7.2. Although a majority of the protein has refolded to the native conformation at low urea concentration, a substantial fraction of the unfolded protein (U) refolds to N only very slowly. The residual unfolded band divides into two bands at low urea concentration which have different mobilities, one quite close to that of the native form. The continuous nature of the band connecting U and the two compact, nonnative forms indicates that both are in rapid equilibrium with U. There is also a very faint band of protein, denoted by the arrow at 0 M urea, that retains the mobility of U at all urea concentrations. This band is clearly distinct from the major band of protein at 4–5 M urea, demonstrating that the majority of the unfolded protein becomes slightly more compact in the upper part of the transition region. There is probably a similar phenomenon occurring at 16 °C, but there is no U band with which to compare mobilities.

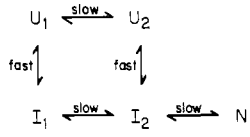
At pH 8.6, 3 °C, the pattern starting with the protein in the N form is similar to that observed at pH 7.2, 3 °C. In the pattern starting with U, the intensity of the more slowly migrating band of protein in fast exchange with U is substantially decreased relative to the intensity of the more rapidly migrating band. The position of this band is indicated with an arrow to aid in its visualization. Also, the band of protein with the mobility of the U form observed at pH 7.2 at all urea concentrations was not observed for refolding at pH 8.6. The absence of this band at pH 8.6 suggests that it was an artifact at pH 7.2, possibly attributable to a small fraction of the

material that was irreversibly denatured. The absence of bands with intermediate mobilities at higher temperatures for both pH 7.2 and 8.6 shows that the bands are not due to protein which has become irreversibly denatured.

## Discussion

The results of equilibrium and kinetic spectroscopic experiments and the urea-gradient gel electrophoresis experiments demonstrate that the urea-induced unfolding of the α subunit of tryptophan synthase from *E. coli* is a complex process. A simple two-state model can be ruled out by the poor fits of the equilibrium unfolding data, the observation of more than a single kinetic phase in refolding, and the patterns on the urea gels. The results also strongly suggest that the extended two-state model (Brandts et al., 1975), which predicts that the rate of protein folding is limited by the rate of isomerization of the peptide bond in X–Pro linkages, does not apply. According to this model, the folding of approximately 98% of the unfolded α subunit, with 19 prolines, should be rate limited by at least one proline isomerization. The half-time for refolding can be estimated from the computer simulations of Creighton (1978b) to be approximately 10 min at room temperature. The half-time would be expected to be at least an order of magnitude longer at 3 °C, since the activation energy for proline isomerization is ~20 kcal mol$^{-1}$. The urea-gradient gels at 3 °C when the protein is initially in the unfolded form show quite clearly that the amount of protein that remains unfolded at low urea concentrations is much less than would be expected if the extended two-state model described the folding of the α subunit. The data do not rule out the possibility that one or a few key prolines play a role in limiting the rate of folding of the α subunit.

From the urea gel results at low temperature, a kinetic model can be proposed to describe the folding of the α subunit:




In this model, N represents the native form, $U_1$ and $U_2$ represent a pair of unfolded forms with different kinetic properties, and $I_1$ and $I_2$ represent two compact forms with different, intermediate mobilities.

The relative rates assigned to each transition are those required to explain the urea gel results at 3 °C for both pH 7.2 and 8.6, where more details of the folding mechanism are evident. The $U_1 \rightleftharpoons I_1$ and $U_2 \rightleftharpoons I_2$ fast steps account for the appearance of two bands of intermediate mobility which appear in refolding and that are continuous with the band for the unfolded protein. The step linking $U_1$ and $U_2$ and that linking $I_1$ and $I_2$ must be slow to observe two separate bands. If either of these steps was fast, only a single band of intermediate mobility would be observed. A possible explanation for the structural differences between $U_1$ and $U_2$ is the state of isomerization of one or a few key proline residues. Previous studies on the refolding of ribonuclease A (Nall et al., 1978) and one of the isozymes of carp parvalbumin (Lin & Brandts, 1978) have demonstrated that the unfolded forms of these proteins also consist of more than one kinetic species. The differences were attributed to the state of isomerization of proline residues. The step linking N to subsequent conformations must be slow to explain the horizontal band for the native protein and the absence of material between this band and the other observed bands.

When the temperature is raised from 3 to 16 °C at pH 7.2, all the rates must become fast on the electrophoresis time scale (Creighton, 1980) so that the pattern on the gels resembles that for a two-state model in fast exchange. At pH 8.6, when the temperature is increased from 3 to 17 °C, the step connecting N with subsequent forms remains slow. The appearance of only a single band of intermediate mobility in fast exchange with U at 17 °C may be due either to the increase in the rates of all the other steps so that they become fast or to the destabilization of one of the partially folded forms at the higher temperature.

Correlation of the kinetic results from the optical experiments with those from the urea-gradient gels must be done on a qualitative basis since the gels only provide estimates of the limiting values of the half-times for the observed processes. With this caveat in mind, the kinetic model proposed from the urea gel results at low temperature is consistent with the optical results. The fast phase inferred from $\Delta\epsilon_{286}$, with a half-time of less than 10 ms, is sufficiently rapid that any conformational changes corresponding to this change in extinction coefficient should be in fast exchange on urea gel electrophoresis. The steps linking $U_1$ and $I_1$ and $U_2$ and $I_2$ are consistent with such a rate. In the unfolding transition region, the half-time of the slow phase observed in the optical experiments at pH 7.8, 25 °C, is 154 s. At lower temperatures, where the urea gels were run, the half-time should become sufficiently long that the conformational transition to which it corresponds should result in intermediate to slow exchange in the electrophoresis experiment. The slow step postulated on the basis of the urea gel results to link N to subsequent forms is consistent with this rate.

Equilibrium spectroscopic studies on the urea and guanidine hydrochloride-induced unfolding of the α subunit have been reported previously (Yutani et al., 1977, 1979). A three-state model was required to adequately fit CD and tyrosine fluorescence data for the guanidine hydrochloride-induced unfolding, indicating that the partially folded form(s) is (are) also stable in this process. Although the urea-induced unfolding was fit to a two-state model, inspection of the data shows that a stable intermediate was probably present in this case also.

The nature of the two intermediates detected in the present study is not clear, but an intriguing possibility is that they are related to subdomains in the native conformation of the α subunit. Higgins et al. (1979) have found that a limited tryptic digestion of the α subunit results in two fragments, which, when separated, each contain organized structures. The amino-terminal fragment consists of residues 1–188, and the carboxyl-terminal fragment contains the remaining residues, 189–268. If the folding proceeds by the initial collapse of either subdomain, the more rapidly migrating intermediate may be that fraction of the population with the larger subdomain formed and the more slowly migrating intermediate that with the smaller subdomain formed. Further kinetic studies are required to determine if such a hypothesis is correct. Carrey & Pain (1978) have suggested that the folding of penicillinase may proceed by the folding of subdomains. The existence of a series of missense mutants for the α subunit provides an approach for probing the structure of the partially folded forms detected in the urea-induced unfolding and the nature of the molecular events that link these intermediates to the native and unfolded forms.

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References

Adachi, O., Kohn, L. D., & Miles, E. W. (1974) *J. Biol. Chem. 249*, 7756.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453.
Bower, V. E., & Robinson, R. A. (1963) *J. Phys. Chem. 67*, 1524.
Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953.
Carrey, E. A., & Pain, R. H. (1978) *Biochim. Biophys. Acta 533*, 12.
Cohn, E. J., & Edsall, J. T. (1943) in *Proteins, Amino Acids, and Peptides* (Cohn, E. J., & Edsall, J. T., Eds.) p 370, Reinhold, New York.
Creighton, T. E. (1978a) *Prog. Biophys. Mol. Biol. 33*, 231.
Creighton, T. E. (1978b) *J. Mol. Biol. 125*, 401.
Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235.
Creighton, T. E. (1980) *J. Mol. Biol. 137*, 61.
Creighton, T. E., & Pain, R. H. (1980) *J. Mol. Biol. 137*, 431.
Herskovits, T. T. (1967) *Methods Enzymol. 11*, 748.
Higgins, W., Fairwell, T., & Miles, E. W. (1979) *Biochemistry 18*, 4827.
Kawahara, K., & Tanford, C. (1966) *J. Biol. Chem. 241*, 3228.
Kirschner, K., Wiskocil, R. L., Loehn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513.
Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359.
Li, S.-L., & Yanofsky, C. (1972) *J. Biol. Chem. 247*, 1031.
Lin, L.-N., & Brandts, J. F. (1978) *Biochemistry 17*, 4102.
Matthews, C. R., Crisanti, M. M., Gepner, G. L., Velicelebi, G., & Sturtevant, J. M. (1980) *Biochemistry 19*, 1290.
Nall, B. T., Garel, F.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317.
Nozaka, M., Kuwajima, K., Nitta, K., & Sugai, S. (1978) *Biochemistry 17*, 3753.
Robson, B., & Pain, R. H. (1976a) *Biochem. J. 155*, 325.
Robson, B., & Pain, R. H. (1976b) *Biochem. J. 155*, 331.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1.
Wetlaufer, D. B., & Ristow, S. S. (1973) *Annu. Rev. Biochem. 42*, 135.
Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518.
Yanofsky, C. (1967) *Harvey Lect. 61*, 145.
Yanofsky, C., & Horn, V. (1972) *J. Biol. Chem. 247*, 4494.
Yanofsky, C., Drapeau, G. R., Guest, J. R., & Carlton, B. C. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 296.
Yutani, K., Ogasahara, K., Sugino, Y., & Matsushiro, A. (1977) *Nature (London) 267*, 274.
Yutani, K., Ogasahara, K., Suzuki, M., & Sugino, Y. (1979) *J. Biochem. (Tokyo) 85*, 915.

# Tryptophanyl Fluorescence Heterogeneity of Apomyoglobins. Correlation with the Presence of Two Distinct Structural Domains†

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ABSTRACT: The individual fluorescence of the two tryptophan residues (Trp-7 and Trp-14) of mammalian apomyoglobins has been resolved by comparing the fluorescence properties of these proteins to those of bluefin tuna apomyoglobin, which contains only Trp-14. The two tryptophan residues have been found to have different emission maxima, i.e., 321 for Trp-14 and 333 for Trp-7. The fluorescence of Trp-14 depends on the protonation of a sterically related histidyl residue in the pH range between 8.3 and 5.6, where no conformational change was detected. This residue has been identified as His-119. At pH 8.3 the quantum yield of Trp-7 is lower than that of Trp-14. An increase of the fluorescence intensity of Trp-7 occurs when the heme binding site of apomyoglobin is destroyed by acid or a low concentration of guanidine hydrochloride. An independent unfolding of the N-terminal district of the apomyoglobin molecule occurs on increasing the guanidine concentration. The two distinct structural transitions have been discussed in terms of two domains of tertiary structure.

The luminescence properties of tryptophan residues are, in general, dependent upon their microenvironments. The widespread location of chromophores into the proteic matrix is responsible of the great diversity in emission behavior for the single residues. This fact leads into the consideration that the observed protein fluorescence arises from unequal contributions of individual residues to the total emission. The resulting luminescence heterogeneity is detectable by various fluorescence techniques (Kronman, 1976; Longworth, 1977), but the discrimination and the study of the fluorescence properties characterizing each residue always present difficulties.

Most of the myoglobins have two tryptophans which fill the invariant positions 7 and 14 (Dayhoff, 1972; Jones, B. N., et al., 1976; Lehman et al., 1977). Few attempts have been made to distinguish the fluorescence behavior of the two tryptophans.

Kirby & Steiner (1970) reported that the two tryptophan residues of sperm whale apomyoglobin differ, one of the two being more fluorescent than the other one, but they were not able to distinguish the individual contribution. An interesting

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