Urea-Induced Unfolding of the α Subunit of Tryptophan Synthase: One-Dimensional Proton NMR Evidence for Residual Structure Near Histidine-92 at High Denaturant Concentration†

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ABSTRACT: The urea-induced unfolding reaction of the α subunit of tryptophan synthase was monitored by examining the chemical shifts and peak areas of the C^{ϵ} protons of the four histidine residues with 1D NMR spectroscopy. In a native base-line region defined by tyrosine absorbance and far-UV circular dichroism spectroscopy, histidine-146 appears to undergo a rapid, local unfolding reaction at increasing denaturant concentrations. As the native form is converted to a previously detected stable intermediate between 2 and 3 M urea [Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20,* 784], histidines-92 and -146 in the amino folding unit (residues 1–188) and histidines-195 and -244 in the carboxy folding unit (residues 189–268) all experience a change in their environments which is slow on the NMR time scale. The subsequent conversion of this intermediate to a newly detected, stable, partially folded form populated at 5 M urea appears to have no effect on any of the histidines at 25 °C when an intermolecular association process involving His-244 is taken into account. Strikingly, a slow exchange process involving only His-92 is observed to begin at 5 M urea where the unfolding transitions monitored by absorbance or far-UV circular dichroism spectroscopy are essentially complete. This residual tertiary structure unfolds in a cooperative fashion as the urea concentration is increased to 8 M.

A hallmark of naturally occurring proteins is the highly cooperative nature of the folding reaction. The great majority of proteins exist in either a native conformation or a manifold of rapidly interconverting unfolded conformers, depending upon the solvent and other environmental conditions. Partially folded states are, in general, not stable in aqueous solutions near neutral pH and ambient temperature. Consistent with these observations are the results of studies on proteolytic fragments of proteins which show that many of these partial sequences display little or no propensity for higher order structure (Epand & Scheraga, 1968; Wetlaufer, 1973; Tanaka & Scheraga, 1977; Högberg-Raibaud & Goldberg, 1977a,b; Matheson & Scheraga, 1978; Miles et al., 1987; Wright et al., 1988; Staley & Kim, 1990; Dyson & Wright, 1991). Thus, if folding intermediates are considered to consist of independently folding subdomains corresponding to such segments (Kim & Baldwin, 1990), it is not surprising that intermediates are present in very low levels at equilibrium.

An intriguing counterexample to the simple two-state unfolding behavior shown by most proteins is the α subunit of tryptophan synthase. This α/β-barrel protein is known to have a stable intermediate which can comprise up to 70% of the population at moderate guanidine hydrochloride or urea concentrations (Yutani et al., 1979; Matthews & Crisanti, 1981). Studies on the two proteolytic fragments produced by limited tryptic digestion have led to the proposal that the region containing the first six strands and five helices of the α_8/β_8 set (residues 1–188, designated the amino folding unit) is folded in a native-like fashion in the intermediate (Miles et al., 1982). The carboxy folding unit (residues 189–268) contains the final two strands and three helices and, on the basis of optical (Miles et al., 1982) and hydrogen exchange (Beasty & Matthews, 1985) experiments, has been proposed to be fully unfolded. More recent mutagenic studies of sets of single and double mutants in the β strands at the interface between these folding units, i.e., in strands 1, 6, 7, and 8, show that nonrandom structure is present in the carboxy folding unit, at least near position 209 in strand 7 and possibly near position 232 in strand 8 (Tsuji et al., 1993).

Because a stable intermediate provides an unusual opportunity to examine the evolution of the native conformation from the unfolded manifold, it is useful to examine this species from as many perspectives as possible. Although 2D nuclear magnetic resonance (NMR)[1] studies on a protein of this size, 28 600 g mol^{-1}, remain as a major challenge, a 1D proton NMR study of the C^{ϵ} protons of the four histidine residues is readily done. The amino folding unit contains two histidine residues, His-92 and His-146, which appear to be involved in salt bridges with anionic side chains in the native conformation (Hyde et al., 1988). The two histidines in the carboxy folding unit, His-195 and His-244, are exposed to solvent and are not apparently involved in intraprotein interactions. Thus, each folding unit has a pair of probes with which the native/intermediate and intermediate/unfolded transitions can be monitored.

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[1] Abbreviations: DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; $NaDodSO_4$, sodium dodecyl sulfate; His, histidine; QKHPT, acetyl-glutamine-lysine-histidine-proline-threonine-amide; LRHNV, acetyl-leucine-arginine-histidine-asparagine-valine-amide; LNHLV, acetyl-leucine-asparagine-histidine-leucine-valine-amide; EQHIN, acetyl-glutamic acid-glutamine-histidine-isoleucine-asparagine-amide; UV, ultraviolet; CD, circular dichroism; NMR, nuclear magnetic resonance; AB, absorbance.

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Yutani and his colleagues (Iwahashi et al., 1983; Yutani et al., 1987), in previous proton NMR studies, assigned one of the C^{ϵ} resonances to His-92 and made a tentative assignment of another to His-146. The peak area of His-92, unlike that of the other three histidines, was reported to be unaffected by the native/intermediate transition induced by urea. This result is suspect because the chemical shift of His-92 is nearly coincident with a resonance in the subsequent intermediate form; changes in peak area could easily be obscured. Furthermore, no changes in either the chemical shift or the peak area for any of the histidines for the intermediate/unfolded transition were detected by Iwahashi et al. (1983) at 5 °C and 20 mM phosphate, pH 7.8. The absence of effects on either chemical shifts or areas for the intermediate/unfolded transition is somewhat surprising in light of previous studies which indicate the existence of secondary and tertiary structure in the amino folding unit in the intermediate (Yutani et al., 1980; Matthews & Crisanti, 1981; Miles et al., 1982).

To test the possibility that increasing the working temperature might allow a distinction between His-92 in the native form and resonances corresponding to the unfolded conformation, the urea-induced unfolding reaction has been reexamined by NMR spectroscopy at 25 °C. The buffer concentration was increased to 58 mM phosphate because it was found empirically that the peak area of His-146 in the native conformation depends on the phosphate concentration (C. Froebe, G. Saab-Rincón, and C. R. Matthews, unpublished results). Above 50 mM phosphate, the area of the C^{ϵ} peak for His-146 is comparable to the areas of the other histidine protons.

When the results of the 25 °C and additional 5 °C NMR experiments were examined, all four histidines were found to be sensitive to the slow conformational change linking the native and intermediate forms. None of the histidines appear to be sensitive to the intermediate/unfolded transition. Significantly, organized structure involving His-92 persists at 5 M urea, after the disruption of secondary structure and the exposure of tyrosine residues to solvent. This residual structure unfolds in a cooperative fashion when the urea concentration is increased from 5 to 8 M.

MATERIALS AND METHODS

Materials

Chemicals and Reagents. Ultrapure urea was purchased from Schwarz/Mann and used without further purification; ^2H_2O, 99.8% and "100%" pure, was purchased from MSD Isotopes; ^2HCl, 99.0% pure, was purchased from MSD Isotopes; and NaO^2H, 99.0% pure, was purchased from ICN Biomedical. The oligonucleotide used to construct the H92Q mutant α subunit was purchased from the Penn State Biotechnology Institute. All other chemicals were reagent grade.

Peptides. Four pentapeptides containing the flanking sequence around each histidine residue in the protein were obtained from the Protein Structure Facility of the University of Iowa. The sequences are as follows: acetyl-LRHNV-amide, acetyl-EQHIN-amide, acetyl-LNHLV-amide, and acetyl-QKHPT-amide. The purity of these peptides was determined by high-pressure liquid chromatography analysis and exceeded 92%.

Methods

Protein Isolation and Purification. The wild-type α subunit of tryptophan synthase (EC 4.2.1.20) was isolated from *Escherichia coli* strain W3110 (ΔtonB-trp)BA17 *his-* containing plasmid pBN55 (a gift from Brian Nichols at the University of Illinois, Chicago). The H92Q[2] α mutant was isolated from *Escherichia coli* strain CB149 transformed with plasmid pXH; the construction of this plasmid is described below. The purification procedure for the wild-type protein has been described previously (Beasty et al., 1986). The H92Q mutant protein was purified from inclusion bodies following the procedure developed by Tsuji et al. (1993). Protein purity was demonstrated by a single band on $NaDodSO_4$–polyacrylamide gel electrophoretograms. The proteins were stored in 70% saturated ammonium sulfate, 10 mM potassium phosphate, 1.0 mM K_2EDTA, and 1.0 mM DTE, pH 7.8 at 4 °C. Under these conditions, no change in the catalytic activity was observed for a period of several months.

The activity of the α subunit was measured by its ability to enhance the activity of the β_2 subunit of tryptophan synthase in the condensation of indole and serine to form tryptophan (Kirschner et al., 1975). Samples of α subunit used in the present studies had specific activities ranging from 5200 to 5400 units mg^{-1} for wild type and 2300 units mg^{-1} for the H92Q mutant. The maximum specific activity of the α subunit in this assay is 5500 units mg^{-1}. Protein concentration was determined by using a molar extinction coefficient of 12 600 M^{-1} cm^{-1} at 278 nm (Matthews et al., 1983) for both wild-type and H92Q mutant proteins.

Urea Unfolding Studies by NMR. The ammonium sulfate-precipitated protein was pelleted by centrifugation and dissolved in a buffer containing 1 mM potassium phosphate, pH 8.0, 0.1 mM K_2EDTA, and 1 mM 2-mercaptoethanol. The protein was dialyzed against this buffer and then lyophilized. To remove exchangeable amide protons that absorb in the same region of the NMR spectrum as the histidine C^{ϵ} protons, the lyophilized protein and buffer components were dissolved in a 1 mM 2-mercaptoethanol solution in ^2H_2O and incubated at 55 °C for 10 min. The protein concentration was held below 4 mg mL^{-1} to avoid aggregation during this heating step. The sample was then cooled in a water bath at room temperature and lyophilized again.

A stock solution of 2.0 mM DTE/56 mM potassium phosphate in ^2H_2O was prepared. A second stock solution of 9.0 M urea was also volumetrically prepared by adding to the urea the necessary amounts of potassium phosphate and DTE to get final concentrations of 56 mM and 2.0 mM, respectively, and then completing the volume with "100"% ^2H_2O. The p^{2}Hs of both solutions were adjusted to 7.80 either with 10 N NaO^2H or with 10 N ^2HCl. No correction for the isotopic effect on the pH measurement was made. Appropriate volumes of these solutions were mixed to yield the desired concentration of urea in each sample. The lyophilized protein then was dissolved in a volume of this solvent equal to half of the original volume of the protein solution. The samples, which initially contained 2 mM phosphate, thus became 58 mM phosphate buffer, 0.2 mM EDTA, and 2.0 mM DTE. The protein concentration was approximately 0.8 mM. The pH of each sample was finally adjusted to 7.80 with diluted ^2HCl or NaO^2H. In cases where the sample developed some turbidity after preparation, the aggregated material was removed by centrifugation at 15600*g*, and filtration through 0.2-μm Acrodiscs.

[2] The mutant protein is designated using the single-letter amino acid code with the first letter corresponding to the wild-type residue, the number indicating the position of the residue, and the second letter corresponding to the mutation. H92Q indicates the replacement of histidine-92 with glutamine.

The effect of this preparation procedure on the viability of the protein was examined by testing both the activity and the absorbance change upon unfolding. The activity and the amplitude of the unfolding equilibrium curve at 287 nm were approximately 95% of the values obtained with an aliquot of the same sample before treatment (data not shown). Therefore, the protein is not adversely affected to a significant degree by the lyophilization and exchange procedures.

The NMR spectra were obtained at 500 MHz on a Bruker AM 500 spectrometer in the Fourier transform mode. The spectral width was 8064 Hz, and the acquisition time was 1.02 s. Values for the spin–lattice relaxation times of the C^{ϵ} protons of the native α subunit were obtained by the "null method" (Farrar & Becker, 1971) and found to be approximately 1.5 s (data not published). A total recycle time of 6.02 s was employed to ensure that magnetization was fully recovered between pulses. The solvent peak was suppressed by saturation with the decoupler. The decoupling power was maintained throughout the relaxation delay and removed prior to data acquisition. Spectra are the average of 300 transients. Chemical shifts were measured relative to internal dioxane. These values were then corrected for the effect of urea by comparison to dioxane in an external capillary and finally reported relative to DSS by adding 3.75 ppm. The urea-induced unfolding study was carried out both at 5 °C and at 25 °C.

The number of histidine C^{ϵ} protons associated with each resonance in the spectrum was determined from measurements of the peak areas. The areas were obtained by cutting and weighing the peaks as well as by fitting the peaks to Lorentzians or the sum of Lorentzians using the program Glinfit. Both methods gave comparable results. The number of protons was then determined by multiplying the ratio of each individual peak area to the total area of all peaks found between 7.6 and 8.5 ppm (relative to DSS) by 4. The near-integer values observed for well-resolved resonances in various regions of the urea titration suggest that this is a valid method. The signal from the residual amide protons associated with urea made it impossible to use the aromatic region as an internal reference for normalizing the peak areas.

Urea Unfolding Studies by Absorbance and Circular Dichroism Spectroscopy. The difference absorbance unfolding equilibrium curve at 287 nm and the CD equilibrium curve at 222 nm were obtained as described elsewhere (Hurle et al., 1987). These data were fit to a three-state model in which the absorbance and CD properties of the intermediate were allowed to vary independently. The concentration of protein used in these experiments was 0.3–0.6 mg mL^{-1} in a buffer containing 58 mM potassium phosphate, 2 mM DTE, and 0.2 mM K_2EDTA.

Measurement of pK_a Values. The preparation of the samples for the measurement of pK_a values was similar to the above procedure for unfolding. The pH was adjusted by adding an appropriate volume of 10 N ^2HCl or 10 N NaO^2H. A nonlinear least-squares program was used to fit the dependence of the chemical shift on the pH to the Henderson–Hasselbalch equation (Westmoreland et al., 1975). The Hill coefficient was assumed to have a value of 1.0 in these fits; fits in which the Hill coefficient was allowed to vary independently consistently showed that it fell between 0.9 and 1.0 under a variety of solution conditions.

Site-Directed Mutagenesis. The H92Q mutant was constructed using site-directed mutagenesis. The *Eco*RI/*Sal*I fragment from the vector pXH, a derivative of pTZ18 containing the gene for the α subunit of tryptophan synthase,

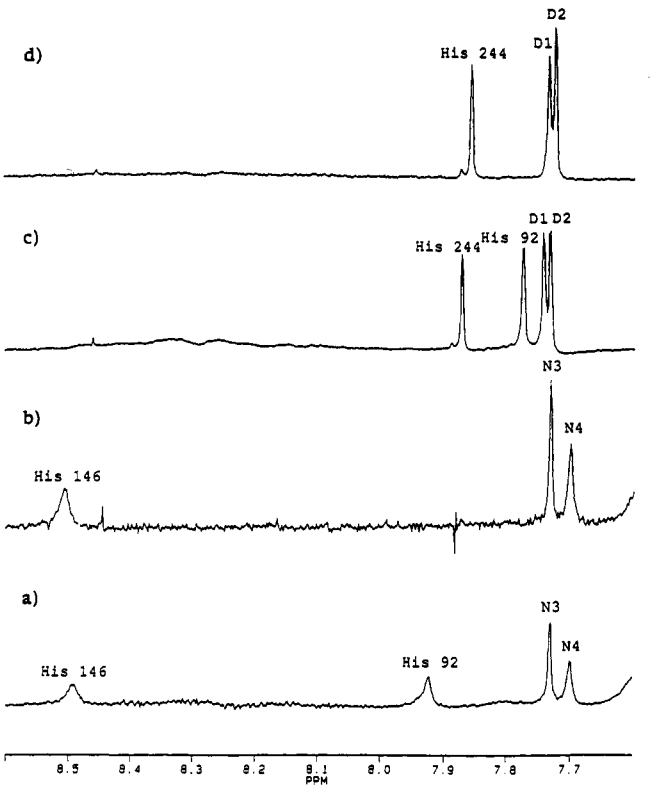

FIGURE 1: 500-MHz FT-NMR spectra of (a) the wild-type α subunit and (b) the H92Q mutant at 0.0 M urea, pH 7.60. Spectra of (c) the wild-type α subunit and (d) the H92Q mutant at 9.0 M urea, pH 7.80. Spectra were obtained at 25 °C in 58 mM potassium phosphate buffer, 0.2 mM K_2EDTA, and 2.0 mM DTE. Protein concentration was ≈0.8 mM.

was cloned into the M13mp19 vector. The uracil-phage method of Kunkel (Kunkel et al., 1987) was used for mutagenesis. A 24 base pair oligonucleotide coding for the desired CAC to CAG codon change was employed. Mutants were selected by direct sequencing of single-stranded DNA isolated from individual plaques using the Sequenase (U.S. Biochemicals) DNA sequencing kit. The entire gene of the mutant α subunit was sequenced to confirm that no other base changes had occurred. The *Eco*RI/*Sal*I fragment of the mutant double-stranded DNA was then cloned into the pXH vector for expression of the protein.

RESULTS

The proton NMR spectrum of the α subunit of tryptophan synthase in the region from 7.5 to 8.5 ppm (relative to DSS) is dominated by the four histidine C^{ϵ} protons (Figure 1, spectrum a). At pH 7.8 and 25 °C, His-92 and His-146 resonate at about 7.9 and 8.45 ppm, respectively, while the two resonances from the carboxy folding unit, His-195 and His-244, appear near 7.7 ppm. The assignment of His-92 was made previously by amino acid replacement (Yutani et al., 1987) and was reconfirmed in the present study (see below). The assignment for His-146 was taken from Yutani et al. (1987), who inferred that the broad line width in the NMR spectrum reflected the involvement of this residue in a salt bridge in the native conformation (Hyde et al., 1988). The carboxy folding unit histidines are not yet assigned in the native conformation and are designated as peaks N3 and N4 in Figure 1a.

Because His-92 displays unusual behavior at high urea concentrations (see below), it was of interest to reconfirm its assignment in the native conformation and to obtain the

Table I: pK_a Values for the Histidine Residues in the α Subunit of Tryptophan Synthase at Different Urea Concentrations[a]

	pK_a at [urea] (M) of				
His	0.0	1.0	4.0	6.5	8.0
His-92	6.42 (0.05)	6.47 (0.01)			
His-146	7.74 (0.05)	7.83 (0.02)			
N3	5.83 (0.03)	5.93 (0.01)			
N4	6.05 (0.01)	6.05 (0.02)			
His-244			7.04 (0.01)	7.18 (0.01)	7.22 (0.01)
D1			6.71 (0.01)	6.78 (0.01)	6.83 (0.01)
D2			6.56 (0.01)	6.68 (0.01)	6.75 (0.01)
His-92				6.79 (0.01)	6.85 (0.02)

[a] The pK_a values were determined at 25 °C. Buffer conditions were 58 mM potassium phosphate, 0.2 mM K_2EDTA, and 2.0 mM DTE. Errors represent standard deviations of the fits and are shown in parentheses.

assignment in 9.0 M urea. Comparison of the NMR spectra of the wild type and the H92Q mutant α subunit at 0.0 M urea (Figure 1a,b) shows that the peak at 7.9 ppm in the wild-type protein is absent in the mutant. The close similarity of the remaining peaks in the two spectra and the retention of substantial enzymatic activity in the mutant (2300 units mg^{-1}, ~50% of the activity of the wild-type protein) reconfirm the original assignment of the peak at 7.9 ppm to His-92 (Iwahashi et al., 1983). Comparison of the NMR spectra of the wild type and H92Q mutant α subunit at 9.0 M urea (Figure 1c,d) shows that the resonance near 7.8 ppm in the wild-type protein is missing in the mutant. The absence of an effect of this mutation on the chemical shifts of the remaining three resonances in 9.0 M urea (when small changes due to slight pH differences in the samples are taken into account) confirms the assignment of the peak at 7.8 ppm to His-92.

Effect of Urea on Histidine pK_a Values. To interpret properly the chemical shift behavior of the C^ϵ protons as the α subunit is unfolded by urea at pH 7.8, it is necessary to account for the effect of urea on the pK_a value of each histidine. The pK_a values of the four histidines in the native conformation at 0.0 and 1.0 M urea and those for the histidines in the intermediate and unfolded forms at 4.0, 6.5, and 8.0 M urea are listed in Table I. In the absence of denaturant, the pK_as of His-92, His-146, N3, and N4 are 6.42, 7.74, 5.83, and 6.05, respectively. The differences presumably reflect the local environments, e.g., the salt bridges for His-92 and His-146 observed in the X-ray structure and the high solvent exposure for His-195 and His-244 (Hyde et al., 1988). In 8.0 M urea where the protein is fully unfolded, peaks D1 and D2 and His-92 have very similar pK_a values near 6.8. Curiously, His-244, whose assignment is described below, has a significantly higher value, 7.22.

The pK_a values in the native form increase linearly as the urea concentration increases (Table 1), with the exception of the pK_a of the N4 proton which remains constant between 0.0 and 1.0 M urea. The increases range from 0.05 to 0.10 pK_a unit $(\text{molar urea})^{-1}$. These changes in the pK_a values result in downfield changes in the chemical shifts which must be eliminated before drawing any conclusions on fast exchange processes related to protein folding. This correction requires the computation of the predicted chemical shift for each peak using the Henderson–Hasselbalch equation (Westmoreland et al., 1975) and the observed pK_a at any given urea concentration. The correction for the native peaks (between 0 and 2.7 M urea) was then calculated by subtracting the chemical shift of the same peak in the absence of denaturant from this computed value. The correction for the chemical shifts of the resonances at and above 3.0 M urea was similarly computed; however, in this region, the reference chemical shifts were taken to be those at 3.0 M urea (where the intermediate and unfolded peaks first begin to appear). These correction factors were then subtracted from the observed chemical shifts to yield chemical shift values which are independent of the effects of urea on the histidine pK_a values.

Table II: pK_a Values for the Histidine Residues in Pentapeptides from the α Subunit in 8.0 M Urea[a]

peptide	pK_a
QKHPT (His-92)	6.81 (0.02)
LRHNV (His-146)	6.80 (0.02)
LNHLV (His-195)	6.88 (0.01)
EQHIN (His-244)	7.25 (0.02)

[a] The pK_a values were determined at 25 °C. Buffer conditions were 58 mM potassium phosphate, 0.2 mM K_2EDTA, and 2.0 mM DTE. Errors represent standard deviations of the fits and are shown in parentheses.

Assignment of His-244 in the Unfolded α Subunit. The distinctively higher value for the pK_a of one of the histidines in the α subunit at 8.0 M urea (see above) prompted an investigation of its assignment. Reasoning that the higher pK_a might reflect local sequence effects, four blocked pentapeptides corresponding to the sequences surrounding each histidine were prepared by solid-state synthesis. The pK_a values of the histidines in these peptides dissolved in 8.0 M urea were determined by NMR and compared to those for the α subunit in 8.0 M urea. As shown in Tables I and II, the pK_a of the histidine in the EQHIN peptide is identical with that for the histidine with the highest pK_a in the unfolded α subunit and is quite different from any of the others. The good agreement between the pK_a values supports the assignment of this resonance to His-244 in 8.0 M urea; however, this assignment is considered tentative until it is directly confirmed by site-directed mutagenesis.

Equilibrium Unfolding Experiments by NMR at 25 °C. The equilibrium unfolding transition of the α subunit at 25 °C can be divided into four different zones according to the observed changes in chemical shifts and/or peak areas. Zone 1 (0–2.0 M urea) highlights the behavior of the native form; zone 2 (2.0–3.0 M urea), the transition from the native to a previously observed (Matthews & Cristanti, 1981) stable intermediate form; zone 3 (3.0–5.0 M urea), the subsequent transition to a second, newly discovered, partially folded form; and zone 4 (5.0–9.0 M urea), the complete unfolding of the α subunit. Figure 2 shows representative spectra from each of these zones. As the urea concentration is increased, the native peaks diminish in area and are ultimately replaced by four peaks of nearly equal areas at 9.0 M urea. Chemical shift changes with increasing urea concentration are also apparent.

Zone 1 (0–2.0 M urea): As noted previously (Iwahashi et al., 1983), the two amino folding unit His C^ϵ protons, His-92 and His-146, have substantially broader line widths than their carboxy folding unit counterparts, His-195 and His-244. All

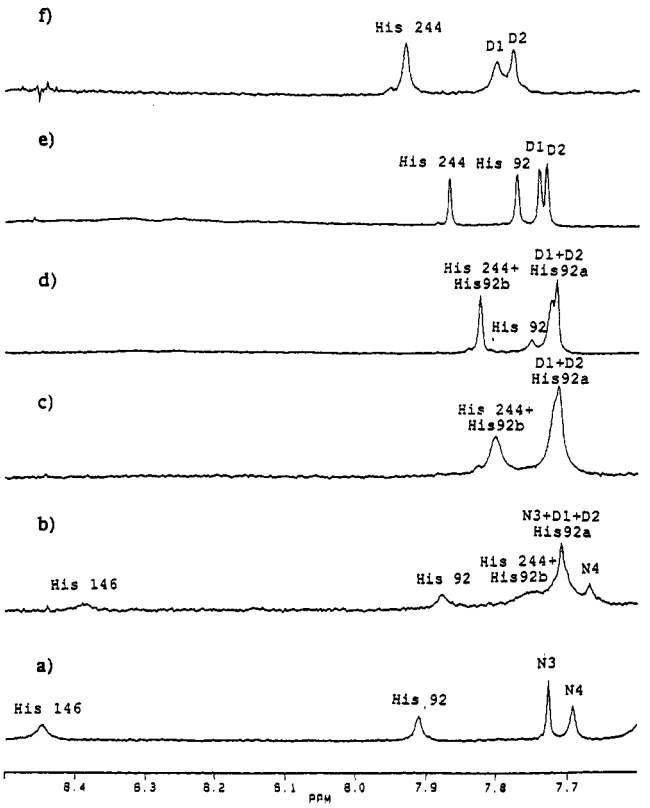

FIGURE 2: 500-MHz FT-NMR spectra of the α subunit at 25 °C: (a) zone 1, 0.0 M urea; (b) zone 2, 2.7 M urea; (c) zone 3, 4.0 M urea; (d) zone 4, 5.5 M urea; (e) zone 4, 9.0 M urea; (f) at 5 °C in zone 3, 3.0 M urea. Spectra were obtained in 58 mM potassium phosphate, pH 7.80, 0.2 mM K_2EDTA, and 2.0 mM DTE. Protein concentration was ≈0.8 mM.

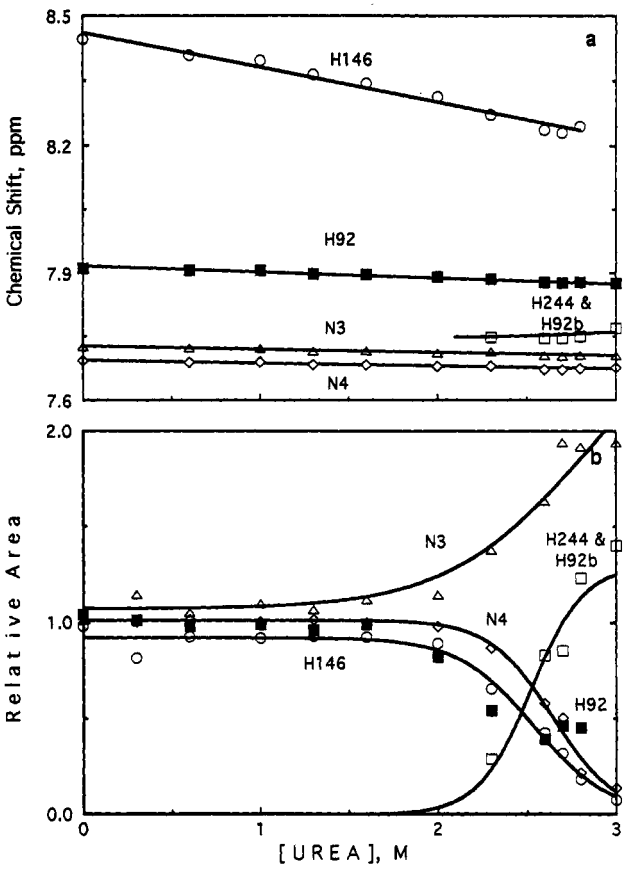

FIGURE 3: (a) Chemical shifts and (b) relative areas for the histidine C^{ϵ} protons of the α subunit between 0 and 3.0 M urea, monitored at 25 °C and pH 7.80: His-146 (○), His-92 (■), N3 (△), N4 (◇), and denatured His-244 + His-92b (□). The solvent is described in the caption for Figure 1. The chemical shift values have been corrected for the effect of urea concentration on the pK_a values of the histidines, as described under Materials and Methods. The figure displays the portion of the titration curves from 0 to 3 M urea to illustrate the chemical shift and peak area behavior in the adjacent zone.

four resonances show a linear upfield shift with increasing urea concentration (Figure 3a). The changes in chemical shift for His-92, N3, and N4 are similar and relatively small, suggesting that they all reflect a common origin, e.g., urea binding. In contrast, His-146 undergoes a significant upfield shift which may reflect a rapid, local unfolding reaction (see Discussion). The peak areas in this zone are not affected by the increasing urea concentration (Figure 3b), demonstrating that no slow exchange processes are occurring.

Zone 2 (2.0–3.0 M urea): The chemical shifts of the four native peaks continue to exhibit linear upfield changes with increasing urea concentration (Figure 4a). A new resonance appears at 7.75 ppm and increases in area in a sigmoidal fashion to represent approximately 1.3 protons by 3.0 M urea (Figure 4b). This peak also moves downfield (Figure 4a) and eventually assumes the chemical shift observed for His-244 at higher urea concentrations. Therefore, one of the resonances associated with this peak can be assigned to His-244 in the folding intermediate. As will be discussed below, a fraction of the His-92 proton population coincidentally resonates at this chemical shift in zones 2 and 3. The peak areas of the resonances corresponding to His-92, His-146, and N4 all decrease sigmoidally with increasing urea concentration and approach zero at 3.0 M urea (Figure 4b). The total area at the chemical shift exhibited by N3 in the native form, 7.72 ppm, increases with increasing urea concentration in zone 2. This result implies that resonances from the folding intermediate overlap with that for N3 at 25 °C. At 4.0 M urea, this composite peak contains about 2.7 protons and includes, as will be shown below, His-146, His-195, and the remaining population of the His-92 proton. At 25 °C, unlike its behavior at 5 °C (Iwahashi et al., 1983), His-92 in the native form (zone 2) is well resolved from the resonances in the intermediate and clearly shows a change in the peak area for transition between the native and intermediate forms. As the urea concentration approaches 3.0 M, the line widths of all of the peaks increase (Figure 2, spectrum b). Because sample turbidity is also observed at this urea concentration, this effect may reflect the aggregation of the intermediate which is expected to be highly populated under these conditions (Matthews & Crisanti, 1981).

Zone 3 (3.0–5.0 M urea): The chemical shift of His-244 undergoes a substantial downfield shift between 2.0 and 5.0 M urea and attains a limiting value of 7.80 ppm (Figure 5a). The increase in the urea concentration results in the resolution of the peak at 7.72 ppm into a pair of peaks designated D1 and D2. Both show small, linear upfield changes in chemical shift as the urea concentration is further increased, similar in magnitude to those observed for three of the four native peaks (Figure 3a). The peak areas in this zone are essentially constant (Figure 5b); the small increase for the composite peak is probably within the error of the measurement. Therefore, no slow exchange processes occur in this zone. Attempts to decompose the composite peak into individual areas representing three protons did not give unique results and were abandoned.

A striking feature of the data in zone 3 is that the line widths of all of the resonances decrease significantly as the

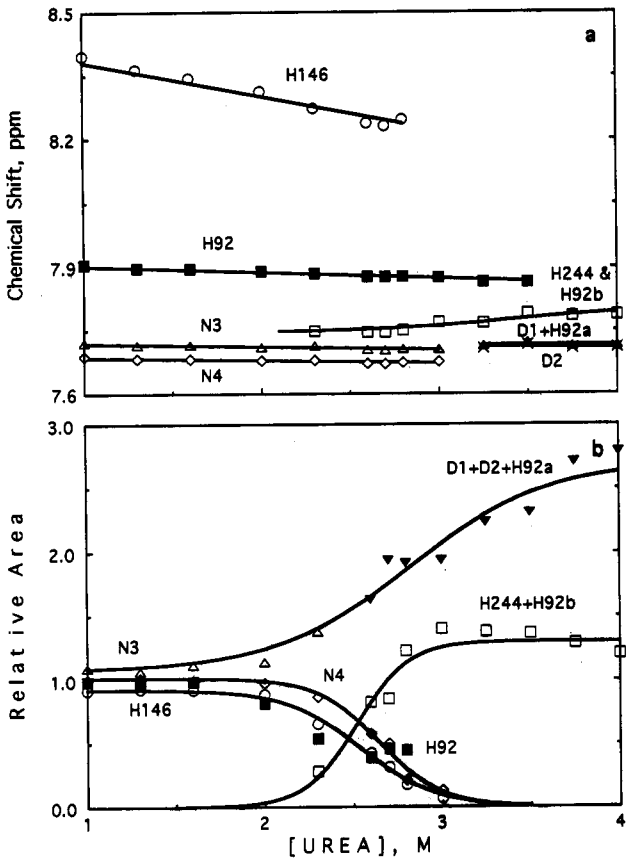

FIGURE 4: (a) Chemical shifts and (b) relative areas for the histidine C^{ϵ} protons of the α subunit between 1.0 and 4.0 M urea, monitored at 25 °C and pH 7.80: His-146 (○), His-92 (■), N3 (△), N4 (◇), denatured His-244 + His-92b (□), D1 + His 92a (▲), and D2 (×). The change in the symbols for the upper curve in panel b reflects the change in the protons assigned to this peak from N3 (△) below 2.3 M urea to D1 + D2 + His-92a (▼) above 2.3 M urea. The solvent is described in the caption for Figure 1. The chemical shift values have been corrected for the effect of urea concentration on the pK_a values of the histidines, as described in the text. The figure displays the portion of the titration curves from 1 to 4 M urea to illustrate the chemical shift and peak area behavior in the adjacent zones.

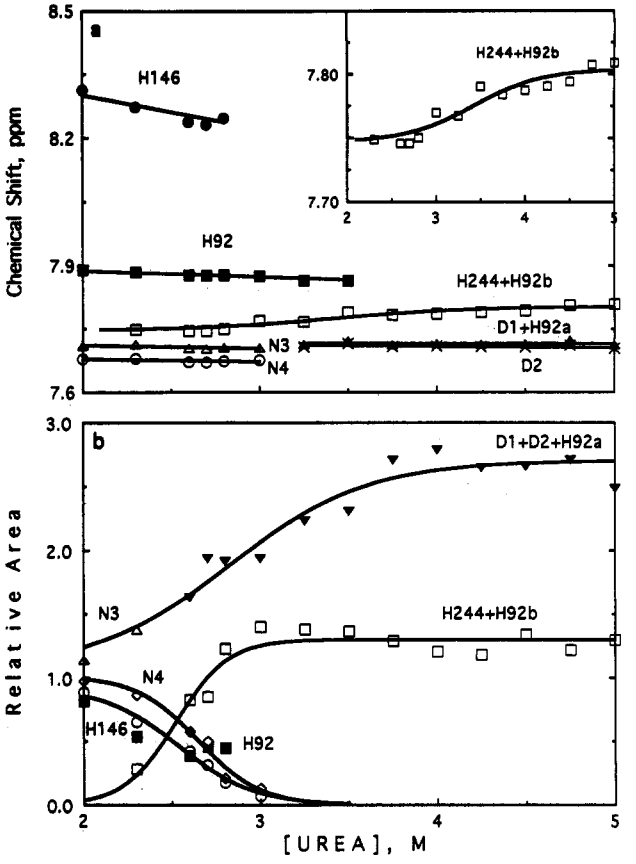

FIGURE 5: (a) Chemical shifts and (b) relative areas for the histidine C^{ϵ} protons of the α subunit between 2 and 5 M urea, monitored at 25 °C and pH 7.80. The inset in panel a highlights the change in chemical shift of His-244 + His-92b. Symbols are as defined in Figure 4. The solvent is described in the caption for Figure 1. The chemical shift values have been corrected for the effect of urea concentration on the pK_a values of the histidines, as described in the text. The figure displays the portion of the titration curves from 2 to 5 M urea to illustrate the chemical shift and peak area behavior in zone 2.

urea concentration is increased. For example, the line width of the well-resolved peak corresponding to His-244 decreases from about 12 Hz at 3.5 M urea to about 3.7 Hz at 5.0 M urea (data not shown). Because this behavior correlates with the decrease in turbidity of the samples at these same urea concentrations, it seems likely that an aggregated species is being disrupted. This interpretation is supported by the results of an unfolding study at 5 °C (see below).

Zone 4 (5.0–9.0 M urea): At 5.0 M urea, where the α subunit is apparently unfolded by the criteria of near-UV absorbance and far-UV CD spectroscopy (Matthews & Crisanti, 1981), a new peak appears at 7.75 ppm (Figure 6a). The chemical shifts of this peak and the remaining three peaks move linearly upfield in this zone by very small amounts, similar to the behavior of all but one of the native peaks in zone 1 (Figure 3a). The area of this new peak increases in a sigmoidal fashion to the equivalent of one proton above 7.5 M urea (Figure 6b). As described above, this peak was assigned to His-92 by comparison with the spectrum of the H92Q mutant at 9.0 M urea. Simultaneously, the areas of the two remaining peaks each decrease by approximately 0.5 proton to reach constant, near-integer values above 7.5 M urea. Note that because D1 and D2 are not completely resolved at high urea concentrations, the combined area is reported in Figure 6b.

The peak area changes in zone 4 imply that His-92 occupies two different environments at 5.0 M urea and that exchange between them is slow on the NMR time scale. His-92a is coincident with the peak representing D1 + D2 and His-92b with the peak representing His-244 (Figure 6). The two different conformers then unfold in a concerted fashion to produce a single environment for His-92 above 7.5 M urea. This final unfolding reaction is also slow on the NMR time scale.

Equilibrium Unfolding Experiments by NMR at 5 °C. As described above, Iwahashi et al. (1983) did not observe an effect of urea on the chemical shifts of any of the histidines in the range expected for the transition from the intermediate to the unfolded form (3.0–5.0 M urea). This result contrasts with the behavior of the His-244 proton presented in the present paper (Figure 5a). The observation that the chemical shift effect observed for His-244 at 25 °C is accompanied by increased turbidity and line widths prompted an NMR study of the urea-induced unfolding transition at 5 °C, the same temperature used in the earlier study.

At the lower temperature, the samples did not become turbid near 3 M urea, and the line widths of the peaks remained narrow throughout the transition (Figure 2, compare spectra c and f). Once again, only His-146 shows a significant, upfield change in chemical shift between 0.0 and 2.0 M urea at 5 °C (Figure 7). The resonances for His-146, N3, and N4 are well resolved from those in the stable intermediate and exhibit

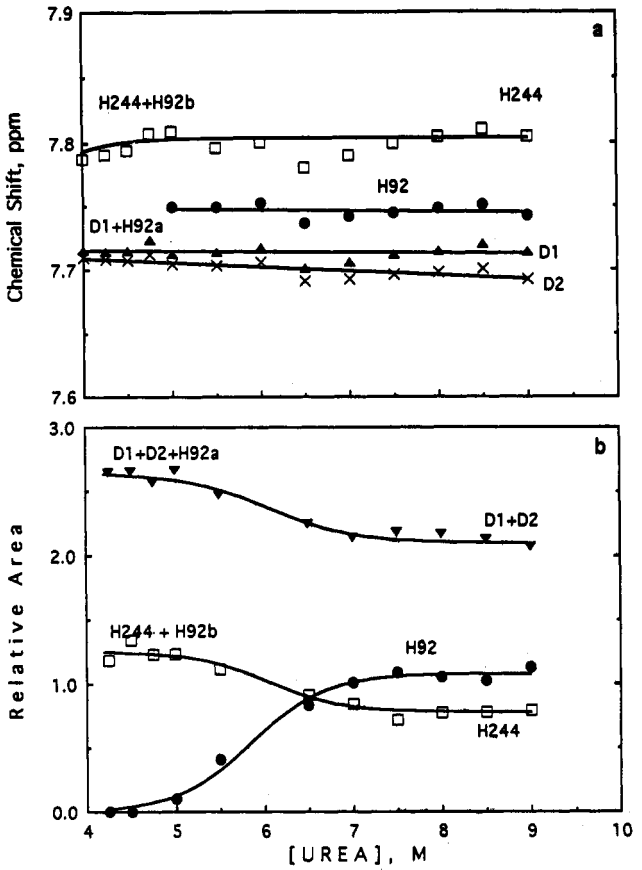

FIGURE 6: (a) Chemical shifts and (b) relative areas for the histidine C^{ϵ} protons of the α subunit between 4 and 9 M urea, monitored at 25 °C and pH 7.80. His-244 (□), His-92 (●), D1 (▲), and D2 (×). In panel b, the sum of the areas of D1, D2, and His-92a is represented by (▼). The solvent is described in the caption for Figure 1. The chemical shift values have been corrected for the effect of urea concentration on the pK_a values of the histidines, as described in the text. The figure displays the portion of the titration curves from 4 to 9 M urea to illustrate the chemical shift and peak area behavior in the adjacent zone.

peak area changes corresponding to the transition from the native to the intermediate forms (data not shown). As was observed in the previous study (Iwahashi et al., 1983), His-92 overlaps with one of the peaks in the intermedaite conformation. Therefore, it is not possible to measure the decrease in area of His-92 as was done at 25 °C (Figure 4b). Concerning the behavior of His-244 between 3.0 and 5.0 M urea, there is no evidence for the intermediate/unfolded transition at 5 °C in terms either of chemical shift (Figure 7) or of peak area changes (data not shown). The peak area change for His-92 between 5.0 and 7.0 M urea is still observed at 5 °C (data not shown).

Thermodynamic Analysis. The NMR data presented above can be combined with the results of absorbance and circular dichroism studies of the urea-induced unfolding of the α subunit to generate a thermodynamic model for this process. Previous optical studies (Matthews & Crisanti, 1981) have shown that the native form is converted to a stable intermediate between 2 and 3 M urea, which is then converted to the unfolded form between 3 and 5 M urea. Because the thermodynamic parameters are sensitive to the solvent, absorbance and far-UV CD spectroscopies were used to examine the unfolding of the α subunit in the same buffer as the NMR experiments (with the exception that ^2H_2O was replaced with H_2O for the optical studies).

The results of fitting these optical data (not shown) to a three-state model, N ↔ I ↔ U, are shown in Table III. The

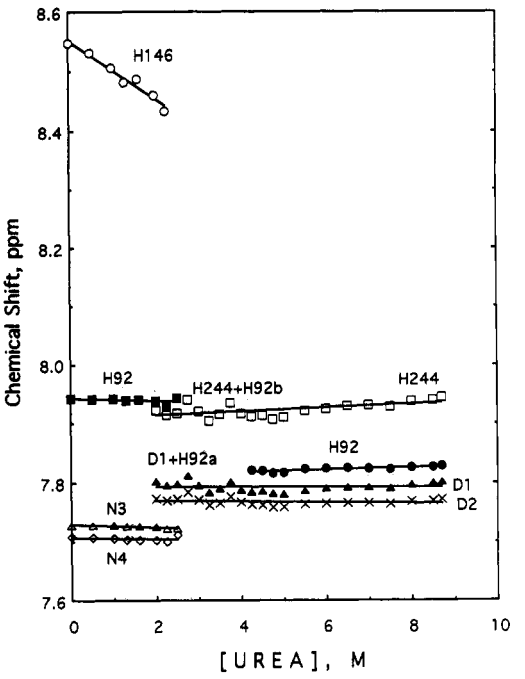

FIGURE 7: Chemical shift as a function of urea concentration for the histidine C^{ϵ} protons of the α subunit between 0.0 and 9.0 M urea monitored at 5 °C and pH 7.80: His-146 (○), His-92 (■), N3 (▲), and N4 (◊) in the native form and His-244 (□), D1 (▲), D2 (×), and His-92 (●) in the intermediate and unfolded forms of the α subunit. The solvent was 58 mM potassium phosphate, 0.2 mM K_2EDTA, and 2.0 mM DTE. Protein concentration was ≈0.8 mM.

Table III: Thermodynamic Parameters for the Urea-Induced Unfolding of the α Subunit of Tryptophan Synthase at 25 °C, pH 7.8[a]

technique	$\Delta G^{app}_{NI}(H_2O)$	A_{NI}	$C_{m_{NI}}$	$\Delta G^{app}_{IU}(H_2O)$	A_{IU}	$C_{m_{IU}}$	Z
AB	6.8	−2.8	2.5	5.1	−1.3	3.9	0.65
	(0.4)	(0.2)	(0.4)	(0.3)	(0.1)	(0.5)	(0.03)
CD	6.2	−2.5	2.5	4.6	−1.2	4.0	0.58
	(0.5)	(0.2)	(0.4)	(0.5)	(0.1)	(0.8)	(0.06)

technique	$\Delta G^{app}_{NI_1}(H_2O)$	A_{NI_1}	$C_{m_{NI_1}}$	$\Delta G^{app}_{I_2U}(H_2O)$	A_{I_2U}	$C_{m_{I_2U}}$
NMR						
His-146	7.2	−2.8	2.5			
	(0.8)	(0.3)	(0.6)			
N4	9.0	−3.4	2.6			
	(0.9)	(0.3)	(0.5)			
His-92				8.2	−1.4	5.9
				(0.9)	(0.2)	(1.3)
His-244+				8.7	−1.4	6.1
His-92b				(2.2)	(0.4)	(3.0)
D1+D2+				7.6	−1.3	6.1
His-92a				(1.4)	(0.2)	(2.2)

[a] Units are as follows: $\Delta G^{app}_{XY}(H_2O)$, apparent free energy of unfolding in the absence of denaturant, kilocalories per mole; A denotes the urea concentration dependence of the free energy of unfolding, kilocalories per mole of protein per molar urea concentration; C_m denotes the transition midpoint, molar urea; Z represents the remaining fractional spectral properties of the intermediate(s), dimensionless, $Z = (\epsilon_I - \epsilon_N)/(\epsilon_U - \epsilon_N)$; errors represent standard deviations of the fit and are shown in parentheses.

thermodynamic parameters derived from these two methods agree within experimental error, supporting the choice of the three-state model. The average stability of the native form relative to the intermediate in the absence of denaturant is 6.5 ± 0.5 kcal mol^{-1}, and the midpoint of this transition is 2.5 M urea. The average stability of the intermediate relative to the unfolded form in the absence of denaturant is 4.8 ± 0.5 kcal mol^{-1}, and the midpoint of this transition is 4.0 M urea. The optical properties of the intermediate, reflected in the Z values for these fits, indicate that 58 ± 6% of the secondary structure

is disrupted and 65 ± 3% of the seven tyrosines are exposed to solvent.

These results can be compared to the transitions observed in the NMR experiment to emphasize similarities and highlight differences. The decreases in area detected for His-146 and N4 in zone 2 were fit independently to a two-state model describing the transition from the native to an intermediate form. Such a fit for the His-92 peak was not attempted because its larger line width precluded accurate measurements above 2.7 M urea. The thermodynamic parameters derived from His-146 and N4 are summarized in Table III. The apparent free energy differences between the native and intermediate form, I_1, in the absence of denaturant, approximately 7 kcal mol^{-1}, are in good agreement with those for the N ↔ I transition detected in optical experiments when the errors are considered. Note that the better resolution of His-146 makes these data more reliable than the data derived from N4. The midpoints and slopes obtained from all three methods are in excellent agreement: C_m is near 2.5 M urea, and the slope is about −2.8 kcal mol^{-1} (molar urea)$^{-1}$. Thus, it is clear that all three methods are sensitive to the same conformational transition in the α subunit.

Although the downfield chemical shift observed for one of the peaks between 3.0 and 5.0 M urea at 25 °C (Figure 5a) shows that a fast exchange process occurs in the range of the I ↔ U transition seen by AB and CD spectroscopies, the absence of this effect at 5 °C suggests that it reflects an association reaction (see Discussion). If this interpretation is correct, then the NMR data provide no information on this transition.

The slow exchange process seen in zone 4 for His-92 implies that there is a conformational change beginning at 5 M urea which is not apparent in the absorbance or far-UV CD spectra (Matthews & Crisanti, 1981). This additional equilibrium must link a second intermediate form, I_2, with the unfolded α subunit. When the increase in area for His-92 and the decreases for the peaks representing His-244 + His-92b and D1 + D2 + His-92a were independently fit to a two-state model, an average stability for I_2 of 8.2 ± 1.5 kcal mol^{-1} and a midpoint of 6.0 M urea for the I_2 ↔ U transition were obtained (Table 3).

DISCUSSION

The results of the present NMR investigation demonstrate that the histidine side chains experience a variety of changes in their local environments, some of which correspond to the conformational changes detected by optical spectroscopy and others which do not.

Native Base-Line Region (Zone 1). In the region from 0.0 to 2.0 M urea, where no changes in secondary structure or exposure of tyrosine residues to solvent are evident, His-146 experiences a greater change in chemical shift than the remaining histidine residues in the α subunit. This upfield shift may reflect the disruption of the hydrogen-bonded salt bridges with Asp-112 and Glu-119 seen in the X-ray crystal structure (Hyde et al., 1988). These salt bridges probably account for the high pK_a value of His-146 observed in the native conformation of the α subunit (pK_a = 7.74, Table I) by stabilizing the protonated form of the imidazole. Assuming that the alternative conformation places His-146 in a more unfolded-like environment, the relaxation time for this fast exchange process must be at least less than 12.6 ms (based upon a chemical shift difference of 13 Hz for this peak between 0 and 2.8 M urea).[3] Another potential explanation is that the larger perturbation on the chemical shift reflects binding of urea to a particularly favorable site near or at His-146. Additional studies are required to select between these alternative possibilities.

Native/Intermediate 1 Transition (Zone 2). Between 2.0 and 3.0 M urea, all four histidine C$^\epsilon$ protons experience a change in their local environments as the native form is converted to the stable intermediate detected in prior equilibrium unfolding studies (Matthews & Crisanti, 1981). This conclusion is based upon the observation of coincident peak area changes both at 5 °C and at 25 °C. The very good agreement between the predicted apparent stabilities, midpoints, and slopes of the denaturation curves obtained by techniques which are sensitive to tyrosine exposure to solvent, secondary structure, and the local environments of two histidines (one in each of the folding units) demonstrates the high cooperativity of the folding reaction between the native and intermediate forms. Because these NMR experiments also reveal that presence of another partially folded form at higher urea concentration (see below), the intermediate which is highly populated at 3 M urea is designated as I_1.

The relaxation time for the slow exchange process between N and I_1 must be greater than 0.5 ms, based upon the largest shift difference between native and intermediate resonances (346 Hz). This lower limit on the relaxation time is consistent with previous kinetic studies (Crisanti & Matthews, 1981; Hurle et al., 1987) which demonstrated that the interconversion of N and I_1 is one of the rate-limiting steps in folding. The relaxation times observed in these studies exceeded 40 s over the same range in urea concentrations examined in the present NMR study.

Intermediate 1/Intermediate 2 Transition (Zone 3). When I_1 is unfolded at 25 °C, the peak representing His-244 + His-92b displays a change in chemical shift between 3.0 and 5.0 M urea. The absence of this behavior at 5 °C, where the protein is more soluble and the line widths of the C$^\epsilon$ protons are considerably narrower, suggests that I_1 undergoes an association reaction which is driven by the hydrophobic effect. Because peaks D1 and D2 do not display this behavior, it appears that this is not a random association between α subunit molecules. Brems and his colleagues (Brems et al., 1986, 1987, 1988; Brems, 1988) have previously observed a similar association/aggregation phenomenon with bovine growth hormone and assigned the effect to a specific helical segment. If the chemical shift behavior of His-244 + His-92b does reflect the disruption of an associated species, then none of the histidines in the α subunit are actually sensitive to the transition responsible for the disruption of secondary and tertiary structure detected by optical spectroscopy at both 25 °C (Matthews & Crisanti, 1981) and 5 °C (data not shown).

Unfolded Base-Line Region (Zone 4). When the urea concentration reaches 5 M, three of the four histidines in the α subunit exist in an unfolded-like environment. The lone exception is His-92. The sigmoidal change in the peak area for its C$^\epsilon$ proton as the denaturant concentration increases from 5.0 to 9.0 M urea shows that cooperative, residual structure exists near this side chain *after* the secondary structure is disrupted and the tyrosines are fully exposed to solvent. This behavior implies that a second, stable folding

[3] A chemical exchange process which is fast on the NMR time scale has a relaxation time, τ, such that $\tau \ll (2\pi\Delta\nu)^{-1}$, where $\Delta\nu$ is the difference in chemical shift in hertz between the two frequencies at which the individual nucleus absorbs in the two electromagnetic environments. For a slow exchange process, $\tau \gg (2\pi\Delta\nu)^{-1}$.

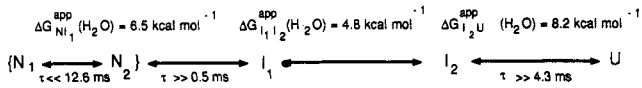

FIGURE 8: Diagram of the equilibrium folding model for the α subunit of tryptophan synthase. The apparent free energy difference for the $N \leftrightarrow I_1$ transition is the average of the values obtained by absorbance and CD measurements of the native to intermediate transition. The apparent free energy difference for the $I_1 \leftrightarrow I_2$ transition is the average of the intermediate to unfolded transition detected by absorbance and CD spectroscopies. The average apparent free energy difference for the I_2 to U transition is the average of the values obtained from the NMR data assuming a two state-transition.

intermediate is highly populated at 5 M urea; this species is designated as I_2. The relaxation time for the unfolding of I_2 must be larger than 4.3 ms, on the basis of the largest chemical shift difference between the peaks representing His-92 in I_2 and U, 36.5 Hz.

Equilibrium Folding Model. The integration of the NMR and optical results into an equilibrium folding model for the α subunit requires the introduction of two additional species: one native-like and the other a second, stable intermediate. The model is presented in Figure 8. N_1 represents the fully folded α subunit, and N_2 is a native-like species in which the salt bridges linking His-146 to Glu-112 and Asp-119 may be broken. I_1 is a stable intermediate containing approximately 40% of the secondary and tertiary structure found in N_1, and corresponds to the intermediate designated as I in the previous, three-state model (Matthews & Crisanti, 1981). The environments of all four histidines in I_1 are altered relative to the native conformers. I_2 is a newly discovered, stable intermediate which has most if not all of its secondary structure disrupted and its tyrosines exposed to solvent. However, I_2 does contain residual tertiary structure near His-92, which unfolds cooperatively as the urea concentration is increased above 5 M. U represents the fully unfolded form and is the predominant species above 7.5 M urea. The apparent free energy differences in the absence of denaturant and the limits on the relaxation times for each step are also shown in Figure 8. No estimate of the free energy difference between N_1 and N_2 is available. The apparent free energy difference between these two native conformers and I_1 can be calculated from the AB and CD data for the $N \leftrightarrow I$ transition (Table 3). Because I_2 has optical properties which closely resemble the unfolded α subunit and is the predominant species at 5 M urea, the $I \leftrightarrow U$ transition observed by absorbance and CD spectroscopies corresponds to the $I_1 \leftrightarrow I_2$ transition in this more complete model.

Although proteins dissolved in high concentrations of chemical denaturants are usually thought to be fully unfolded (Nozaki & Tanford, 1963), Wüthrich and his colleagues (Neri et al., 1992a,b) have recently reported residual structure in the 434 repressor dissolved in 7 M urea. In this case, 2D NMR techniques revealed a hydrophobic cluster involving a pair of valines, a tryptophan, and a leucine, which occur at positions 54, 56, 58, and 59, respectively. Inspection of the α subunit sequence near His-92 shows a downstream hydrophobic stretch which could well play a similar role for the α subunit.

The well-known propensity of the Xaa–Pro peptide bond to adopt both cis and trans isomers in significant proportions (Brandts et al., 1975; Schmid, 1993) suggests that Pro-93 could also be playing a role in the stabilization of the residual structure in I_2. The observation that the increase in the peak area of His-92 in fully unfolded α subunit is derived from two different peaks in roughly equal proportions (Figure 6b) is consistent with this proposal. However, a pair of cis/trans isomers cannot be the entire explanation for the residual structure in the α subunit because proline isomerization is not sensitive to the urea concentration (Schmid & Baldwin, 1979; Schmid et al., 1984; Nall, 1985). Preliminary results with the P93S mutant of the α subunit indicate that the slow unfolding reaction is still present at high urea concentration; however, His-92 only appears as a single peak at 5 M urea (Saab-Rincón and Matthews, unpublished results).

One is led to the conclusion that the conformation of I_2 is defined not by secondary structure or the burial of tyrosines in nonpolar pockets but by an association of nonpolar side chains which persists at high concentrations of urea. The absence of significant secondary structure rules out a molten globule structure (Ptitsyn, 1987; Kuwajima, 1989) for this intermediate. Although 5 M urea appears to be sufficient to disrupt backbone hydrogen bonding and solvate the tyrosine hydroxyl groups in the α subunit, it may not be capable of dissolving a hydrophobic cluster composed of contiguous, nonpolar side chains in the sequence. The magnitude of the stability of I_2, 8.2 kcal mol^{-1}, suggests that multiple elements containing such sequences may be involved in a higher order structure which defines this intermediate. Site-directed mutagenesis near His-92 and in other regions where strings of nonpolar residues appear, e.g., β strands, will test their role in stabilizing this residual structure in the α subunit of tryptophan synthase.

The magnitude of the apparent free energy difference between I_2 and U, 8.2 kcal mol^{-1}, is somewhat surprising considering that it is as large as the difference between the native and unfolded forms for many proteins (Alber, 1989). If the residual structures detected in the α subunit of tryptophan synthase and the 434 repressor (Neri et al., 1992a,b) reflect common rather than unusual properties of globular proteins, it is possible that the stabilities of many proteins have been underestimated. NMR spectroscopy with its ability to resolve individual side chains and, thereby, monitor their behavior may be capable of testing the generality of these two initial observations.

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