Equilibrium and Kinetic Analyses of Unfolding and Refolding for the Conserved Proline Mutants of Tryptophan Synthase α Subunit†

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ABSTRACT: To elucidate the role of conserved proline residues of the tryptophan synthase α subunit from *Escherichia coli* in stability and folding, equilibrium and kinetic studies of the unfolding−refolding induced by guanidine hydrochloride for six mutant α subunits (Pro → Ala) were carried out by peptidyl circular dichroism and aromatic fluorescence measurements at pH 7 and 25 °C. These results were analyzed assuming the presence of one intermediate (I) state in the denaturation process. (1) For all mutant and wild-type proteins, the Gibbs energy change ($\Delta G_{ni(H_2O)}$) in water between the native (N) and I states coincided with the difference ($\Delta G^{\ddagger}_{u(H_2O)} - \Delta G^{\ddagger}_{r(H_2O)}$) between the activation Gibbs energy changes in water for the unfolding ($\Delta G^{\ddagger}_{u(H_2O)}$) and refolding ($\Delta G^{\ddagger}_{r(H_2O)}$) reactions. This means that the early folding intermediate of the α subunit corresponds to the equilibrium intermediate. $\Delta G_{ni(H_2O)}$ values of all mutant proteins decreased compared with that of the wild-type protein. Gibbs energy change ($\Delta G_{id(H_2O)}$) in water between I and the denatured (D) states was not substantially affected by the substitutions. $\Delta G^{\ddagger}_{u(H_2O)}$ and $\Delta G^{\ddagger}_{r(H_2O)}$ decreased and increased, respectively, for all mutant proteins. (2) Six conserved prolines played roles in stability and folding of the α subunit in a different manner: prolines 28 and 96 by stabilizing the N state and prolines 28, 96, 132, and 207 by destabilizing the I state. The contributions of prolines 57 and 62 to the stability were marginal. (3) Cis proline 28 was not the origin of the slow phase in the refolding kinetics assumed to arise from the cis−trans isomerization reaction of proline.

A unique three-dimensional structure of a protein is essential for its biological function. The three-dimensional structure is determined by the information described in the amino acid sequence. Decoding the information is one of the main problems in modern life science research (Gros & Tocchini-Valentini, 1994). For this purpose, it is essentially important to find the contributions of individual amino acids to the stability and folding of a protein.

Among amino acids constituting a protein, a proline residue has unique aspects in the stability and folding of a protein [for reviews, see Kim and Baldwin, (1982, 1990), Nall, (1994), and Schmid (1992)]. The occurrence of the cis isomer of imido bonds (X-Pro) is greater than those of non-proline residues (Stewart et al., 1990; MacArthur & Thornton, 1991). The cis−trans isomerization of proline peptide bonds is involved in protein folding (Garel & Baldwin, 1973; Brandts et al., 1975; Kim & Baldwin, 1982, 1990; Nall, 1994; Schmid, 1992). Proline residues are therefore recognized to be of special significance in stability and protein folding. The replacements of proline residues often lead to large changes in stability and folding (Kelley & Richards, 1987; Matthews et al., 1987; Wood et al., 1988; Hurle et al., 1991; Schultz & Baldwin, 1992; Schultz et al., 1992; Mayr et al., 1993; Tweedy et al., 1993). However, in some cases, decreases in stability by replacements of prolines are small (Alber et al., 1988; Chen et al., 1992; Green et al., 1992; Herning et al., 1991). On the contrary, there is a proline residue which increases the stability by substitution (Green et al., 1992). These results indicate that the role of a proline residue in stability and folding of a protein changes depending on the location of each proline. However, the details remain to be elucidated.

In a series of studies on the stability and folding of the α subunit and its mutants of tryptophan synthase from *Escherichia coli* (Yutani et al., 1977, 1979, 1980, 1982, 1984, 1987), we have focused on highly conserved proline residues among the α subunits of bacteria. The α subunit is constituted of 268 residues without a disulfide bond and has 19 proline residues. A previous paper has revealed, based on calorimetry, that the conserved proline residues with low thermal factors play crucial roles in the thermal stability of the α subunit (Yutani et al., 1991). Some of the conserved proline residues have also played an important role in the stimulation of enzymatic activities of the tryptophan synthase (Ogasahara et al., 1992).

Although the X-ray crystallographic structure of the α subunit monomer from *E. coli* has not yet been determined, the structure of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* (Hyde et al., 1988) can be referred to, because the sequence homology between the α subunits from *E. coli* and *S. typhimurium* is very high. Figure 1 displays the locations of the six conserved proline residues in the tryptophan synthase $\alpha_2\beta_2$ complex from *S. typhimu-*

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[1] In the original coordinates IWSY, the proline in the α subunit at position 28 was not the cis form. It is corrected in the refined coordinates which should be submitted to the PDB as IWSY (personal communication). Proline 28 is in the cis form in the coordinates in the PDB files 1TTQ and 1TTP for the $\alpha_2\beta_2$ complexes with the K87T mutant of the β subunit of tryptophan synthase from *S. typhimurium* (Rhee et al., 1996).

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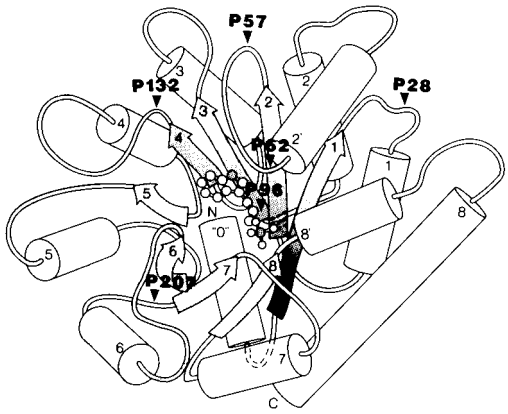

FIGURE 1: Positions of the six conserved proline residues in the α subunit of tryptophan synthase $\alpha_2\beta_2$ complex from *S. typhimurium* (Hyde et al., 1988). Arrowheads represent the prolines at positions 28, 57, 62, 96, 132, and 207.

rium. Prolines 57 and 62 are located in a flexible loop. Proline 132 is also in a loop, which is at the contact surface between α and β subunits. Proline 28 is in the turn of a loop which connects β sheet 1 and helix 1. The imido bond of proline 28 is the only one in a cis configuration[1] in the native state among the 19 proline residues. Proline 96 is in β strand 3. Proline 207 is in the loop following helix 6.

It has been proposed that an intermediate state in the denaturant denaturation process of the α subunit consists of an intact N-terminal domain (Residues 1−188) and an unfolded C-terminal domain (residues 189−268) (Yutani et al., 1982; Miles et al., 1982). However, recently, we have revised the model of the intermediate state to be in the molten globule state (Ogasahara et al., 1993). This intermediate has a substantial peptidyl CD spectrum without a specific ordered tertiary structure as judged by aromatic NMR and near-UV CD spectra and is in a thermodynamically denatured state based on the calorimetric study (Ogasahara et al., 1993). Furthermore, it has been reported that the undetectable early folding intermediate in the refolding kinetics of the wild-type α subunit is equivalent to the equilibrium intermediate (Ogasahara & Yutani, 1994). Most reports on stability and folding of the α subunit and its mutant proteins have been interpreted on the basis of the "old model" (Yutani et al., 1984, 1991; Matthews et al., 1983; Beasty et al., 1986; Tweedy et al., 1990). However, recent papers from Matthews' group have reported that the C-terminal folding unit adopts an organized structure in the intermediate (Tuji et al., 1993; Chen & Matthews, 1994). They have also reported that the intermediate contains approximately 40% of the secondary and tertiary structures found in the native state and that the environments of all four histidines in the N- and C-terminal domains in the intermediate state are altered relative to those in the native state from NMR study (Saab-Rincon et al., 1993). On the other hand, Choi and Hardman (1996) have suggested that the intermediate of the α subunit is heterogeneous, consisting of two species of a stable partially unfolded form of the N-terminal domain attached to either a completely folded or completely unfolded form of the C-terminal domain by using tryptophan fluorescence as a probe for the tryptophan-containing α subunit.

In this paper, to elucidate the role of the conserved proline residues in the folding of the α subunit, we performed equilibrium and kinetic studies using guanidine hydrochloride as a denaturant for the conservative proline mutant proteins of the α subunit from *E. coli*, in which one of the prolines at positions 28, 57, 62, 96, 132, or 207 (Figure 1) was replaced by alanine. The equilibrium and kinetic results were described by the denaturation Gibbs energy change and the activation Gibbs energy change, respectively. Combining both energy changes, we will discuss the role of the prolines in stability and folding in terms of the energy levels of the states in the folding pathway of the α subunit.

MATERIALS AND METHODS

α Subunits and Chemicals. Mutant α subunits from *E. coli* (P28A, P57A, P62A, P96A, P132A, and P207A), in which each of the conserved prolines residues at position 28, 57, 62, 96, 132, or 207 was substituted by alanine, were constructed as described (Yutani et al., 1991), using the Amersham oligonucleotide-directed mutagenesis system. The wild-type and mutant α subunits were purified as described previously (Yutani et al., 1987). The purified wild-type and mutant α subunits gave a single peak on FPLC (MonoQ, Pharmacia). Guanidine hydrochloride (GuHCl) (specially prepared reagent grade) from Nacalai Tesque (Kyoto, Japan) was used without further purification. Other chemicals were of reagent grade.

Protein concentrations of the wild-type and mutant α subunits were estimated from the absorbance at 278.5 nm, using $E^{1\%} = 4.4$ with a cell having a 10-mm light path length (Adachi et al., 1974; Ogasahara et al., 1980).

Equilibrium Studies. GuHCl-induced denaturations of the proline mutants were monitored by CD at 222 nm as described (Yutani et al., 1979). CD measurements were carried out with a Jasco J-500 recording spectropolarimeter.

Kinetic Experiments of Unfolding and Refolding. The reactions of unfolding and refolding were monitored by CD at 222 nm and the fluorescence intensity above 300 nm with excitation at 280 nm. CD measurements were carried out with a Jasco J-720 recording spectropolarimeter by a manual mixing method. CD stopped-flow experiments were carried out with a Jasco J-720 recording spectropolarimeter equipped with a stopped-flow controller Model SFC-5 attached to a mixing device (Ohtsuka Electronics) with a 1:10 volume ratio of two solutions. Fluorescence stopped-flow experiments were carried out with a Photal RA-401 stopped-flow spectrophotometer equipped with a mixing device with a 1:10 volume ratio of two solutions (Ohtsuka Electronics). The systems for the CD and fluorescence measurements were as described previously (Ogasahara & Yutani, 1994).

The kinetic data were analyzed by software for curve-fitting (Sugaware et al., 1991) by a nonlinear least-squares method using

$$A(t) - A(\infty) = \sum A_i e^{-k_i t} \qquad (1)$$

where $A(t)$ is the value of CD or fluorescence intensity at a given time t, $A(\infty)$ is the value when no further change is observed, k_i is the apparent first-order rate constant of the ith kinetic phase, and A_i is the amplitude of the ith phase.

All of the equilibrium and kinetic experiments were performed in 20 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1 mM dithioerythritol at pH 7 and 25 °C. The concentrations of the α subunits were 0.04−0.05 mg/mL for CD measurements using a cell having a 10-mm light path length, and 0.1−0.15 mg/mL for fluorescence measurements

using a cell with 2-mm light path length. For calculation of the mean residue ellipticity for the α subunits, the mean residue weight was taken as 107.5.

RESULTS

Equilibrium Studies. GuHCl-induced denaturations of the proline mutant α subunits were monitored by CD at 222 nm at pH 7 and 25 °C. The negative CD values at 222 nm of the mutant proteins except for P207A are similar to that of the wild-type protein, but that of P207A is significantly lower than the value of the wild-type one (Yutani et al., 1991). The near-UV CD spectra of all of the mutant proteins are similar to that of the wild-type protein (Yutani et al., 1991).

Figure 2 shows two typical transition curves observed from the changes in CD values at 222 nm (Figure 2A) and the normalized denaturation curves of six mutant proteins with that of the wild-type protein (Figure 2B). The transition curves below 1.5 M GuHCl for P28A, P132A, and P207A clearly shifted to lower concentrations of GuHCl, and that of P96A largely shifted to the lower concentrations over the whole range compared with that of the wild-type protein (Figure 2B). The curves for all of the mutant proteins were biphasic as well as those for the wild-type and other mutant α subunits monitored by peptidyl CD and aromatic fluorescence (Yutani et al., 1979, 1980, 1984, 1987). To estimate the Gibbs energy change of denaturation in the absence of GuHCl, the denaturation curves for the mutant proteins were analyzed by the three-state denaturation model assuming the presence of one intermediate in the denaturation process as described (Yutani et al., 1979). The Gibbs energy changes in water obtained are represented in Table 1. The total Gibbs energy changes ($\Delta G_{nd(H_2O)}$) of all proline mutants were lower than that of the wild-type protein, indicating the destabilization by the substitutions.

Unfolding and Refolding Kinetics. Unfolding and refolding reactions of the mutant proteins were initiated by GuHCl concentration jumps from 0 and 3.0 M, respectively, to various concentratinos of GuHCl at pH 7 and 25 °C and monitored by the changes in CD at 222 nm and the fluorescence intensity above 300 nm excited at 280 nm. The kinetic progress curves were analyzed using eq 1. Figure 3 shows the typical kinetic progress curves of P28A measured together with the theoretical fitting curves. In both cases of unfolding and refolding, the kinetic curves for all of the mutant proteins examined in this report could be fitted to a single exponential or biexponentials with high quality, the same as reported for the wild-type protein [see the residuals in Figures 1C,D and 2 in Ogasahara and Yutani (1994)].

The unfolding progressions were fitted to a single exponential in eq 1 for P28A, P96A, and P207A, and to biexponentials in concentrations above 2 M GuHCl for P57A, P62A, and P132A as well as that of the wild-type protein as reported (Ogasahara & Yutani, 1994). For the biexponential unfolding, the amplitude of the fast phase was larger than that of the slow phase in all cases, indicating that the fast phase predominanted in the unfolding reaction. The observed total amplitudes were almost 100% for all the mutant proteins.

For the refolding reaction, most of the CD values at 222 nm rapidly recovered within the dead time (20 ms) of the stopped-flow apparatus. After this burst phase, the negative CD value increased toward the equilibrium value in a given concentration of GuHCl (curve a in Figure 3). The unfolding−refolding reactions of the proline mutant α subunits were completely reversible. In all the mutants, the time-dependent changes in CD values were well fitted to a biexponential function in the native region for each mutant protein below 0.4−0.7 M GuHCl and to a single exponential in the transition zone of denaturation.

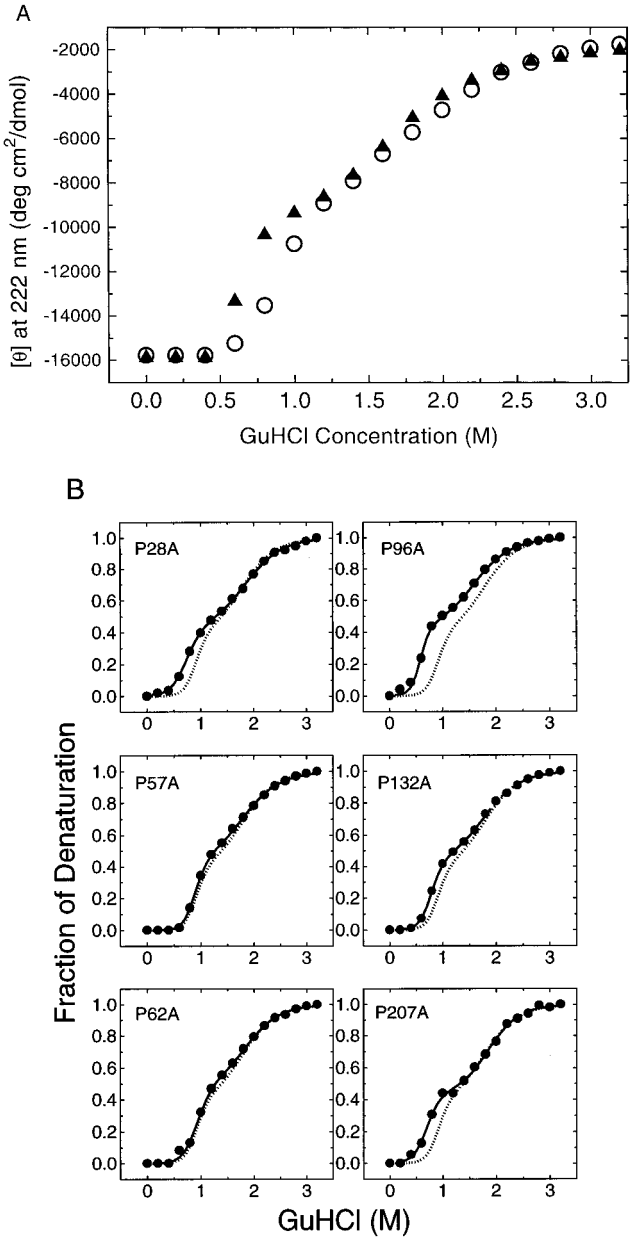

FIGURE 2: GuHCl-induced denaturation curves of the proline mutant α subunits at pH 7 and 25 °C. (A) Typical nonstandardized transition curves monitored by measurements of CD at 222 nm. (○) P57A; (▲) P28A. (B) Normalized denaturation curves monitored by CD at 222 nm. The fraction of denaturation (fd) was calculated from the equation $fd = (y_n - y)/(y_n - y_d)$, where y, y_n, and y_d are the CD values at 222 nm at a given GuHCl concentration, in the absence and in the presence of 3.2 M GuHCl, respectively. The closed circles are the experimental data for the mutant proteins in which each of the conserved prolines has been substituted with alanine. The solid lines represent the simulation curves analyzed assuming the three-state transition using the experimental points [eq 9 of Yutani et al. (1979)]. The dotted line is the simulation curve analyzing the data points for the wild type protein.

Amplitudes of Detected and Undetected Phases in Refolding Kinetics. Table 2 presents the amplitude for each phase

Table 1: Gibbs Energy Changes in Water of GuHCl-Induced Denaturation of the Proline Mutant and Wild-Type α Subunits of Tryptophan Synthase at pH 7 and 25 °C[a]

	$\Delta G_{nd(H_2O)}$ (kJ/mol)	$\Delta G_{ni(H_2O)}$ (kJ/mol)	$\Delta G_{id(H_2O)}$ (kJ/mol)	$\Delta\Delta G_{nd(H_2O)}$ (kJ/mol)	$\Delta\Delta G_{ni(H_2O)}$ (kJ/mol)	$\Delta\Delta G_{id(H_2O)}$ (kJ/mol)
wild type	34.3 ± 0.1	17.7 ± 0.8	16.7 ± 0.6			
P28A	26.3 ± 0.1	9.5 ± 0.5	16.9 ± 0.3	−8.0	−8.2	0.2
P57A	32.3 ± 0.1	16.7 ± 0.2	15.7 ± 0.1	−2.0	−1.0	−1.0
P62A	33.0 ± 0.3	14.8 ± 0.7	18.2 ± 0.4	−1.3	−2.9	1.5
P96A	25.4± 0.6	11.0 ± 0.2	14.4 ± 0.4	−8.9	−6.7	−2.3
P132A	29.9 ± 0.2	13.9 ± 0.3	16.1 ± 0.3	−4.4	−3.8	−0.6
P207A	29.4[b]	10.2[b]	19.3[b]	−4.9	−7.5	2.6

[a] $\Delta G_{nd(H_2O)}$, $\Delta G_{ni(H_2O)}$, and $\Delta G_{id(H_2O)}$ represent the denaturation Gibbs energy changes in water between the states of the native, intermediate, and GuHCl-induced denatured states. $\Delta\Delta G_{nd(H_2O)}$, $\Delta\Delta G_{ni(H_2O)}$, and $\Delta\Delta G_{id(H_2O)}$ show the differences in the Gibbs energy changes of the proline mutant and wild-type α subunits. [b] The values for P207A are taken from Yutani et al. (1991).

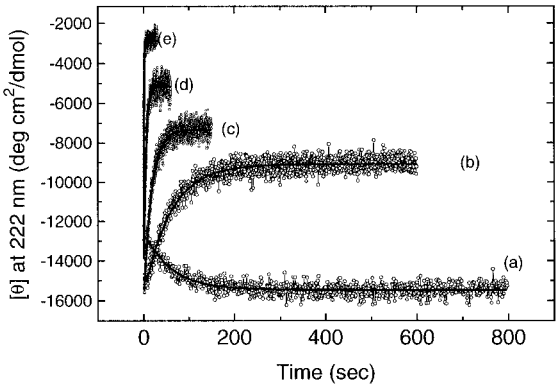

FIGURE 3: Typical kinetic progress curves of unfolding and refolding for P28A monitored by CD at 222 nm at pH 7 and 25 °C. The unfolding and refolding reactions were initiated by concentration jumps of GuHCl from 0 and 3 M, respectively, by manual (traces a and b) and stopped-flow (traces c−e) methods. (a) GuHCl jump is 3 to 0.27 M; (b) 0 to 1.27 M (c) 0 to 1.72 M; (d) 0 to 2.23 M; (e) 0 to 2.86 M. Small open circles are data points. The continuous lines represent the theoretical curves for single- or biexponential fittings according to eq 1. The best-fit values of parameters are as follows: (a) $k_1 = 6.48 \times 10^{-3}$ s^{-1}, $A_1 = 0.75 \times 10^{-1}$, $k_2 = 1.54 \times 10^{-2}$ s^{-1}, $A_2 = 1.71 \times 10^{-1}$; (b) $k_1 = 1.68 \times 10^{-2}$ s^{-1}, $A_1 = 9.41 \times 10^{-1}$; (c) $k_1 = 5.75 \times 10^{-2}$ s^{-1}, $A_1 = 1.00$; (d) $k_1 = 2.05 \times 10^{-1}$ s^{-1}, $A_1 = 9.76 \times 10^{-1}$; (e) $k_1 = 1.42$ s^{-1}, $A_1 = 1.31$.

Table 2: Amplitude of Each Phase in Refolding Kinetics of the Proline Mutant and Wild-Type α Subunits of Tryptophan Synthase at a Final GuHCl Concentration of 0.27 M at pH 7 and 25 °C[a]

	A_s (%)	A_f (%)	A_i (%)
wild type	5.7[b]	30.0[b]	62.5[b]
P28A	7.5	17.1	75.4
P57A	7.1	31.5	61.4
P62A	5.5	28.6	65.9
P96A	11.3	21.9	66.8
P132A	6.4	28.6	65.0
P207A	7.4	25.6	67.0

[a] A_s and A_f are the amplitudes of the slow and fast phases, respectively, in the refolding kinetics. A_i is that of the undetected phase, in which the early folding intermediate is formed. The amplitudes were obtained from the refolding kinetics monitored by CD at 222 nm. [b] The values for the wild-type α subunit are taken from Ogasahara and Yutani (1994).

of the refolding kinetics in the native region in all mutant proteins. The amplitudes for the undetected burst phase accounted for 61−75% at a final GuHCl concentration of 0.27 M. This suggests the presence of the intermediate state formed at an early stage of the refolding reactions for the proline mutants as well as the wild-type protein (Ogasahara & Yutani, 1994).

In the wild-type α subunit, the CD values at 222 nm recovered at an undetectable early stage in the refolding reaction have been reported to be almost independent on the GuHCl concentration below 0.9 M and are −9500 deg cm^2/dmol on the average [Figure 4 in Ogasahara and Yutani (1994)]. The value corresponds to that of the equilibrium intermediate (Ogasahara et al., 1993). CD values at 222 nm of the early folding intermediates of the proline mutant proteins were also independent of the GuHCl concentration and were nearly equal to the wild-type values (data not shown), suggesting that the early folding intermediates of all mutants have structures similar to that of the wild-type protein.

The amplitudes of the fast phase out of the two phases observed in the refolding kinetics were greater than the slow-phase amplitudes for all proteins, indicating that the fast phase is predominant in the refolding reaction. Replacement of cis proline at position 28 (P28A) did not abolish the slow phase (Table 2). This suggests that the slow phase is not due to cis-trans isomerization of cis proline at position 28.

Dependences of Apparent Rate Constants of Unfolding and Refolding Kinetics on GuHCl Concentration. Figure 4 shows the GuHCl concentration dependence of the logarithm of the apparent rate constant (k_{app}) for each phase observed in the unfolding and refolding kinetics. For all the mutant proteins, the log k_{app} values of the major fast phases in the unfolding and refolding kinetics increased and decreased, respectively, with increasing concentrations of GuHCl, characterized by a V-shape with a minimum near the midpoint of the first step transition of the equilibrium denaturation (Figure 2) of each mutant. The unfolding rates of P28A, P96A, and P207A were higher than that of the wild-type protein. These mutant proteins were less stable than the others (Table 1). In P57A, P62A, and P132A, the log k_{app} values for the major fast and minor slow phases of the unfolding kinetics were almost the same or slightly higher relative to the wild-type ones. For the refolding kinetics, the log k_{app} values of the minor slow phases for the mutant proteins were independent of the concentration of GuHCl and almost equal to the wild-type one.

For P28G, P57A, P96A, and P207A, the log k_{app} values measured by fluorescence intensity above 300 nm showed good agreement with those from the CD measurements for both the unfolding and the refolding kinetics (Figure 4). This indicates that the disruptions and formations of the secondary and tertiary structures of the mutant proteins simultaneously proceed like those of the wild-type protein (Ogasahara & Yutani, 1994).

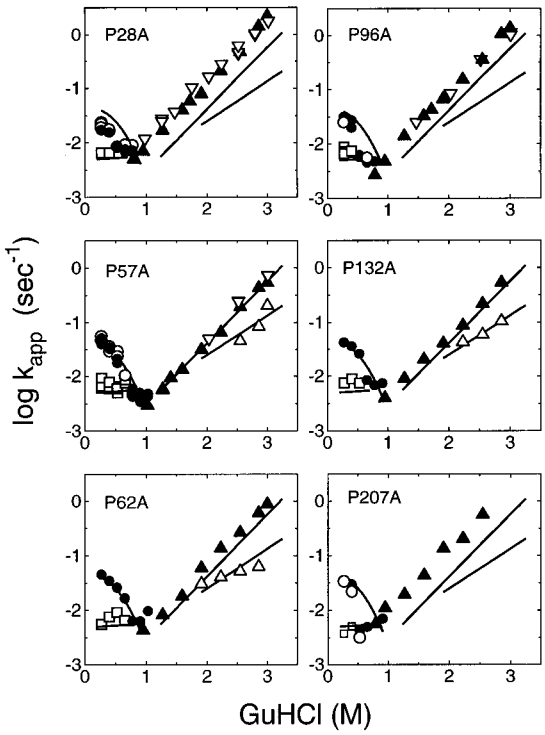

FIGURE 4: GuHCl concentration dependences of the logarithms of the apparent rate constants (k_{app}) for unfolding and refolding of the proline mutant α subunits at pH 7 and 25 °C, compared those of the wild-type α subunit. The log k_{app} values of the unfolding are as follows: (▲) fast phase monitored from CD at 222 nm; (△) slow phase from CD at 222 nm; (▽) fast phase from fluorescence intensity above 300 nm with excitation at 280 nm. For the log k_{app} of the refolding, (●) fast phase from CD at 222 nm; (□) slow phase from CD at 222 nm; (○) fast phase from the fluorescence intensity. Solid lines represent GuHCl concentration dependences of the log k_{app} of the fast and slow phases in the unfolding and refolding reactions monitored from CD at 222 nm for the wild-type α subunit from Ogasahara and Yutani (1994).

Activation Gibbs Energy Changes of Unfolding and Refolding Reactions. Changes in the kinetic rate constants induced by amino acid replacements could reflect changes in the energy level of the transition state existing in the unfolding−refolding process. The activation Gibbs energy change ($\Delta G^{\ddagger}_{(H_2O)}$) in water to the transition state can be calculated from the rate constants ($k_{(H_2O)}$) in water by Eyring's equation (Eyring, 1935):

$$\Delta G^{\ddagger}_{(H_2O)} = RT \ln(k_B T/hk_{(H_2O)}) \qquad (2)$$

where k_B and h are the Boltzmann and Planck constants, respectively. Because linear relationships were observed between the GuHCl concentration and log k_{app} values for the major fast phase of unfolding kinetics in all mutant proteins, we estimated the unfolding rate constants in water ($k_{u(H_2O)}$) by extrapolation to 0 M GuHCl concentration from the linear correlations. The refolding rate constants in water ($k_{r(H_2O)}$) were also estimated by extrapolation to 0 M GuHCl concentration from polynomal regression of the GuHCl concentration dependences of the log k_{app} values of the major fast phases for all mutant proteins. Table 3 presents the rate constants in water and the calculated activation Gibbs energy changes. The value of the activation Gibbs energy change in water for the unfolding ($\Delta G^{\ddagger}_{u(H_2O)}$) in Table 3 for the wild-type protein is equal to the reported value obtained using urea as a denaturant at 25 °C and pH 7.8 and the activation Gibbs energy change in water for the refolding ($\Delta G^{\ddagger}_{r(H_2O)}$) was slightly higher (Beasty et al., 1986; Tsuji et al., 1993).

DISCUSSION

Energy Profile among the Native, Intermediate, Denaturant-Induced Denatured, and Transition States. The stability and folding features of a protein are determined by the energy levels of the states existing in the folding pathway, involving the native, denatured, intermediate, and major transition states. For understanding the role of the amino acid residue in the stability and folding of a protein, it is important to find the effect of substitution on the energy level of each state under physiological conditions. Although the absolute values of the energies of the states cannot be experimentally obtained, the change in the energies between any states can be estimated. For the α subunits, we estimated the Gibbs energy changes in water between states, assuming the three-state denaturation mechanism, and also the activation Gibbs energy changes in water, assuming that the major fast phases of the unfolding and refolding reflect the major transformation between the native (N) and GuHCl-induced denatured (D) states. According to the assumption that the early folding intermediate corresponds to the equilibrium intermediate (I) state (Ogasahara & Yutani, 1994), the major transition ($T^{\ddagger}$) state was postulated to lie between the N and I states, because the reaction of the D to I states was too fast to follow. The energy profile of the unfolding−refolding process of the α subunit can then be illustrated as shown in Figure 5. This profile indicates the following relations:

$$\Delta G_{ni(H_2O)} = \Delta G_{nd(H_2O)} - \Delta G_{id(H_2O)} \qquad (3)$$

$$\Delta G_{ni(H_2O)} = \Delta G^{\ddagger}_{u(H_2O)} - \Delta G^{\ddagger}_{r(H_2O)} \qquad (4)$$

$\Delta G_{ni(H_2O)}$ can be obtained in different ways: equilibrium (eq 3) and kinetic (eq 4) experiments. The values of $\Delta G_{ni(H_2O)}$ obtained from the equilibrium (eq 3) and kinetic (eq 4) methods for the mutant proteins coincided well with each other within experimental error as shown in Table 4, although a discrepancy of 1.9 kJ/mol for the wild-type protein was the maximum among the proteins. The agreement of the values of $\Delta G_{ni(H_2O)}$ obtained from eqs 3 and 4 indicates that the energy profile for the states under the unfolding−refoplding process of the α subunit illustrated in Figure 5 is reasonable. This also confirms the validity of the proposal that the early folding intermediate corresponds to the equilibrium intermediate (Ogasahara & Yutani, 1994) and supports the "revised model", in which the intermediate corresponds to the denatured state in water, that is, a molten globule state, because the equilibrium intermediate is in a denatured state from a thermodynamic point of view (Ogasahara et al., 1993). This means that the early folding intermediate is a molten globule state and corresponds to a denatured state that equilibrates with the native state under physiological conditions. The fact that the difference between the activation Gibbs energy changes [($\Delta G^{\ddagger}_{u(H_2O)} - \Delta G^{\ddagger}_{r(H_2O)} = -RT \ln(k_{u(H_2O)}/k_{r(H_2O)})$] of the unfolding and refolding reactions coincides with $\Delta G_{ni(H_2O)}$ (Table 4) for all proline mutants supports the validity that the major transition ($T^{\ddagger}$) state in the folding pathway of the α subunit lies between the N and I states and the I state is the initial state of folding of the α subunit under physiological conditions.

Table 3: Kinetic Parameters of Refolding and Unfolding Reactions in Water of the Proline Mutant and Wild-Type α Subunits of Tryptophan Synthase at pH 7 and 25 °C[a]

	$k_{r(H_2O)}$ ($\times 10^{-1}$ s^{-1})	$k_{u(H_2O)}$ ($\times 10^{-4}$ s^{-1})	$\Delta G^{\ddagger}_{r(H_2O)}$ (kJ/mol)	$\Delta G^{\ddagger}_{u(H_2O)}$ (kJ/mol)	$\Delta\Delta G^{\ddagger}_{r(H_2O)}$ (kJ/mol)	$\Delta\Delta G^{\ddagger}_{u(H_2O)}$ (kJ/mol)
wild type	1.24[b]	2.14[b]	78.2 ± 0.2	94.0 ± 0.6		
P28A	0.21	5.10	82.6 ± 0.5	91.8 ± 0.3	4.4	−2.2
P57A	1.07	1.77	78.6 ± 0.3	94.4 ± 0.3	0.4	0.4
P62A	0.94	3.17	78.9 ± 0.3	93.0 ± 0.4	0.7	−1.0
P96A	0.35	4.09	81.3 ± 1.1	92.4 ± 0.2	3.1	−1.6
P132A	0.73	3.56	79.5 ± 0.3	92.7 ± 0.2	1.3	−1.3
P207A	0.52	8.32	80.4 ± 0.8	90.6 ± 0.4	2.2	−3.4

[a] $k_{r(H_2O)}$ and $k_{u(H_2O)}$ represent rate constants in water for the major fast phases in the refolding and unfolding reactions, respectively, monitored by CD at 222 nm. $\Delta G^{\ddagger}_{r(H_2O)}$ and $\Delta G^{\ddagger}_{u(H_2O)}$ represent the activation Gibbs energy changes in water for the refolding and unfolding reactions, respectively. $\Delta\Delta G^{\ddagger}_{r(H_2O)}$ and $\Delta\Delta G^{\ddagger}_{u(H_2O)}$ show the differences in the activation Gibbs energy changes of the proline mutant and wild-type α subunits. [b] The values for the wild-type α subunit were calculated from the GuHCl concentration dependences of the log k_{app} values from Ogasahara and Yutani (1994).

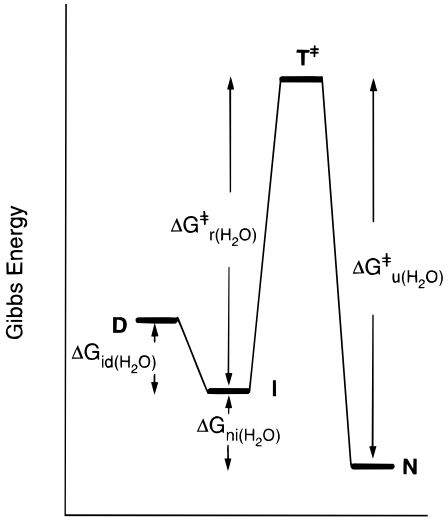

FIGURE 5: Energy profile between the states in the unfolding and refolding process under physiological conditions. D, I, T‡, and N represent the GuHCl-induced denatured, intermediate, transition, and native states, respectively.

In short, the I state in Scheme 1 corresponds to the intermediate state in the equilibrium denaturation. The refolding rate from the D to I states is too fast to follow, and this reaction is lost in the dead time of the apparatus. The major transition (T‡) state, thus, lies between the N and I states.

Scheme 1

$$D \leftrightarrow ... \leftrightarrow I \leftrightarrow N$$

Contribution of the Conserved Proline Residues to the Stability of the α Subunit. The differences in the Gibbs energy change ($\Delta\Delta G_{nd(H_2O)}$, $\Delta\Delta G_{ni(H_2O)}$, and $\Delta\Delta G_{id(H_2O)}$) and the activation Gibbs energy changes ($\Delta\Delta G^{\ddagger}_{r(H_2O)}$ and $\Delta\Delta G^{\ddagger}_{u(H_2O)}$) between each proline mutant and the wild-type α subunit display the following distinct features. (1) $\Delta G_{ni(H_2O)}$ for all proline mutants decreased (Table 1). (2) $\Delta G_{id(H_2O)}$ decreased for P57A, P96A, and P132A, increased for P62A and P207, and was almost insensitive for P28A (Table 1). (3) The changes in $\Delta G_{ni(H_2O)}$ exceeded those in $\Delta G_{id(H_2O)}$ except for P57A, with the result that the decreases in total Gibbs energy changes ($\Delta G_{nd(H_2O)}$) came mainly from the decreases in $\Delta G_{ni(H_2O)}$ (Table 1). (4) For all mutant proteins except for P57A, $\Delta G^{\ddagger}_{u(H_2O)}$ decreased and $\Delta G^{\ddagger}_{r(H_2O)}$ increased (Table 3).

To explore the role of the individual proline residue at each site in stabilization of the α subunit, the energy profiles for each state in the folding pathway of the wild-type and proline mutant α subunits should be compared. In displaying the energy profiles, we adopted the following postulations: (1) The GuHCl-induced completely denatured (D) state could be suspected to be close to a randomly coiled form, although some proteins in the denatured states have been reported to have residual structures even at high concentrations of denaturants (Shortle & Meeker, 1989; Dill & Shortle, 1991; Neri et al., 1992; Saab-Rincoon et al., 1993, 1996). If so, the D state should be at the same energy level for all proline mutant proteins. (2) The energy levels of the D state for the proline mutants are lower by 5.0 kJ/mol than that for the wild-type protein at 25 °C, if the difference in backbone contribution to the entropy between alanine and proline residues has been estimated to be 4 cal/(mol K) (Nemethy et al., 1966). Figure 6 displays the energy level of each state for the wild-type and mutant proteins when the Gibbs energy level in the D state of the wild-type protein is taken as a reference, and that of each proline mutant is 5.0 kJ/mol lower than that of the wild type. These energy profiles indicated that the energy levels of the I state for the all proline mutants were lower than that of the wild type, but these decreases in the energy level of the I state were almost equivalent to the decrease due to the entropy effect (5.0 kJ/mol) of the alanine replacement for proline as mentioned above for P28A, P57A, P62A, and P132A, although the decrease of P96A was slightly smaller and that of P207A larger than the value of the entropic effect. This means that conformations around the alanines substituted at positions 28, 57, 62, and 132 in the I state are similar to those in the D state.

Table 4: Comparison of Gibbs Energy Changes in Water between the Native and Intermediate States Obtained from Equilibrium and Kinetic Methods of the Proline Mutant and Wild-Type α Subunits of Tryptophan Synthase at pH 7 and 25 °C[a]

	$\Delta G_{ni(H_2O)}$[b] (kJ/mol)	$\Delta G_{ni(H_2O)}$[c] (kJ/mol)	$\Delta\Delta G_{ni(H_2O)}$[d] (kJ/mol)
wild type	17.7 ± 0.8	15.8	1.9
P28A	9.5 ± 0.5	9.3	0.2
P57A	16.7 ± 0.2	15.9	0.8
P62A	14.8 ± 0.7	14.1	0.7
P96A	11.0 ± 0.2	11.0	0
P132A	13.9 ± 0.3	13.2	0.7
P207A	10.2	10.3	−0.1

[a] $\Delta G_{ni(H_2O)}$ represents the Gibbs energy changes in water between the native and intermediate states. [b] Values from the equilibrium method in Table 1. [c] Values obtained from the kinetic parameters [eq 4, $\Delta G_{ni(H_2O)} = \Delta G^{\ddagger}_{u(H_2O)} - \Delta G^{\ddagger}_{r(H_2O)}$] as shown in the energy profile in Figure 5. [d] $\Delta\Delta G_{ni(H_2O)}$ is the difference in $\Delta G_{ni(H_2O)}$ obtained from the equilibrium (footnote *b*) and kinetic (footnote *c*) methods.

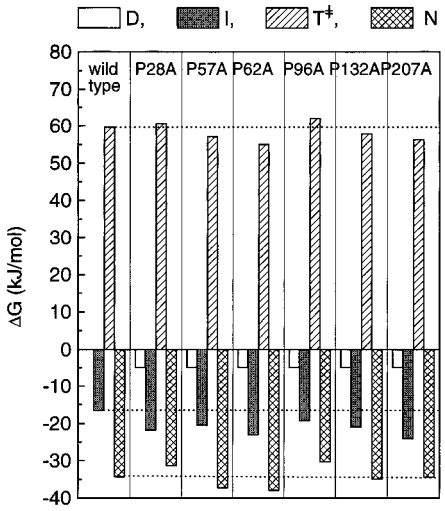

FIGURE 6: Energy levels for states in the folding process under physiological conditions for the proline mutant and wild-type α subunits at pH 7 and 25 °C. The energy level ($\Delta G_{nd(H_2O)}$) of the GuHCl-induced denatured state (D) for the wild-type protein is taken as a reference. The energy levels of D for all proline mutants ($\Delta G_{nd(H_2O)}$) are postulated to be lower by 5 kJ/mol than that of the wild type, which is the Gibbs energy change at 25 °C due to the difference [4 cal/(mol K)] between the backbone entropies of alanine and proline residues (Nemethy et al., 1966). On the bases of the energy levels of D ($\Delta G_{nd(H_2O)}$) for each α subunit, the energy levels of the intermediate (I), native (N), and transition ($T^{\ddagger}$) states were calculated from $\Delta G_{id(H_2O)}$, $\Delta G_{ni(H_2O)}$, and, the average of $\Delta G_u{}^{\ddagger}{}_{(H_2O)}$ and $\Delta G_r{}^{\ddagger}{}_{(H_2O)}$, respectively, from Tables 1 and 3. The dotted lines represent the energy levels for the $T^{\ddagger}$, I, and N states of the wild-type α subunit, respectively.

For P57A and P62A substituted at the flexible positions exposed to solvent, the decreases in the energy levels of the I, N, and $T^{\ddagger}$ states were nearly equivalent to the decrease in the energy level of the D state (5 kJ/mol) (Figure 6), resulting in little effect on the stability shown as the difference between the energy levels of the two states (Table 1). This means that the conformations around prolines 57 and 62 in the I, N, and $T^{\ddagger}$ states are similar to each other and to those of the D state. Replacement of cis proline 55 in RNase T_1, which is exposed to solvent, has led to only a small decrease in the stability of this protein (Kiefhaber et al., 1990).

In the case of P28A, $\Delta G_{ni(H_2O)}$ was reduced by 8.2 kJ/mol relative to that of the wild-type protein, but the change in $\Delta G_{id(H_2O)}$ was slight (Table 1). Figure 6 suggests that the origin for the decrease in the stability of P28A comes from both an increase in the energy level of the N state and a decrease in that of the I state. The decrease in the energy level of the I state was comparable to that of the energy level of the D state (5 kJ/mol). Therefore, proline 28 contributes to stabilization of the α subunit by stabilizing the N state by 3.2 kJ/mol and by destabilizing the I state by 5 kJ/mol. Proline 28, which is in turn on a loop connecting helix 1 and long β sheet 1, might strengthen these secondary structures, and both helix 8 and helix 8′ hydrophobically interacted with β sheet 1 in the N state (Yutani et al., 1991). It has been reported that the drastic destabilization (about 20 kJ/mol) of cis proline mutants in RNase T1 (Mayr et al., 1993) and carbonic anhydrase II (Tweedy et al., 1993) are attributed mainly to the unfavorable cis alanine peptide bond retained after substitutions (Mayr et al., 1994; Tweedy et al., 1993). The same event may not occur in the cis-P28A of the α subunit, because the decrease in $\Delta G_{ni(H_2O)}$ for P28A was smaller than those of the above cis proline proteins.

For P96A, $\Delta G_{ni(H_2O)}$ and $\Delta G_{id(H_2O)}$ were decreased by 6.7 and 2.3 kJ/mol, respectively, compared with those of the wild-type protein (Table 1). The energy levels of the I and N states are decreased and increased, respectively, as in the case of P28A (Figure 6). However, the decrease in the energy level of the I state of P96A was smaller than that in the D state (5 kJ/mol), resulting in the decreases in both $\Delta G_{ni(H_2O)}$ and $\Delta G_{id(H_2O)}$. Thus, it is revealed that proline 96 acts to stabilize the α subunit by stabilizing the N state and by destabilizing the I state. The energy level of the I state for P96A was higher than that of P28A and lower than that of the wild-type protein. This suggests the presence of some conformation retained around proline 96 in the I state, whose conformation is different from that in the D state. Less mobile proline 96 in β sheet 3 plays an important role in tightly maintaining the β strand in the N state, although a propensity for formation of a β sheet for a proline is lower than those for other residues (MacArthur & Thornton, 1991). The decrease in ΔG of the heat stability of P96A relative to the wild-type one is attributed to the decrease in enthalpy change by calorimetric study (Yutani et al., 1991). This agrees with the present result that proline 96 plays an important role in stabilizing the N state. On the other hand, replacements of prolines in the β strands of nuclease change the Gibbs energy by only a small amount (Green et al., 1992).

For P207A, $\Delta G_{ni(H_2O)}$ and $\Delta G_{id(H_2O)}$ were decreased by 7.5 kJ/mol and increased by 2.6 kJ/mol, respectively (Table 1). The energy level of the I state decreased more than the decrease due to the entropic effect of the D state, but the energy level of the N state was unchanged (Figure 6), leading to a large decrease in $\Delta G_{ni(H_2O)}$ and an increase in $\Delta G_{id(H_2O)}$. This indicates that proline 207 plays a role in stabilization of the α subunit by largely destabilizing the I state. The decrease in the stability of this mutant protein is also attributed to the decrease in enthalpy change (Yutani et al., 1991).

On the other hand, for P132A, the energy level of the N state was unchanged, but that of the I state decreased by the same amount as the decrease in the D state, indicating that proline 132 plays a role in stabilizing the α subunit by destabilizing the I state due to entropic effect. Calorimetric study has shown that this mutant protein is destabilized due to entropic effect (Yutani et al., 1991).

The energy levels of the I states for P96A and P207A were far from those of the entropic effect due to the substitution of proline with alanine (Figure 6). This means that those proline residues affect the stability of the molten globule state of the α subunit. It has been reported that the molten globule state in α-lactalbumin is stabilized by the amino acid substitutions which raise the hydrophobicity of the residues (Uchiyama et al., 1995).

Role of the Conserved Proline Residues in Folding of the α Subunit. In all the proline mutant proteins, the amplitudes of the fast and slow phases in the refolding kinetics in the native region were similar to those of the wild-type protein (Table 2), and the CD values for the early folding intermediate obtained from the amplitudes of the undetectable phase, which were almost independent of GuHCl concentration, were also similar to those of the wild type. The rate constants for the unfolding and refolding reactions monitored by peptidyl CD and aromatic fluorescence were almost the same for the mutant proteins examined (Figure 4). The rate constant of the slow phase of the refolding appearing in the

native region was similar for all mutant and wild-type proteins, which is assumed to be due to the proline isomerization reaction. The results indicate that the substitutions of the conserved proline residues do not affect the folding pathway itself of the α subunit but change the rate constants of the major unfolding and refolding reactions (Figure 4).

The unfolding and refolding reactions in water of all mutant proteins were more rapid except for P57A and slower, respectively, than those of the wild-type protein, resulting in the decreases in $\Delta G^{\ddagger}_{u(H_2O)}$ and increases in $\Delta G^{\ddagger}_{r(H_2O)}$ (Table 3). These changes in $\Delta G^{\ddagger}_{u(H_2O)}$ and $\Delta G^{\ddagger}_{r(H_2O)}$ in the opposite direction are attributed to decreases in $\Delta G_{ni(H_2O)}$ (eq 4). The decreases in $\Delta G^{\ddagger}_{u(H_2O)}$ and increases in $\Delta G^{\ddagger}_{r(H_2O)}$ were especially large in P28A, P96A, and P207A (Table 3), which were less stable than the other mutant proteins (Table 1). This indicates that prolines 28, 96, and 207 play a crucial role in raising the activation energy from N to the D states and in accelerating the refolding rate by destabilizing the I state.

As postulated that the energy level of the D state of each mutant protein is lower by 5 kJ/mol than that of the wild-type protein (Figure 6), the energy levels of the $T^{\ddagger}$ state for P57A, P62A, P132A, and P207A decreased comparably with the decreasing energy level of the D state. However, the energy level of the $T^{\ddagger}$ state for P96A was higher than those for the other mutant and wild-type proteins, and that for P28A was similar to that of the wild-type protein, although the energy levels of the N states for P96A and P28A were similar to each other but higher than that of the wild type (Figure 6), indicating destabilization of the $T^{\ddagger}$ state for P96A. It has been reported that the conformation of the transition state in the folding pathway for chymotrypsin inhibitor 2 is affected by amino acid replacements, using many mutants (Itzhaki et al., 1995).

In the unfolding kinetics for less stable P28A, P96A, and P207A, the minor slow phase appearing in the wild-type protein in the GuHCl concentrations above 1.8 M was not observed (Figure 4). It has been reported that the biphasic unfolding of RNase A may arise from the presence of two interconvertible native species (Sugawara et al., 1991). It has been discussed that the molecular origin of the slow phase in the unfolding kinetics of the wild-type α subunit may also come from two interconvertible native species (Ogasahara & Yutani, 1994). However, this interpretation seems to be inconsistent with the disappearance of the slow phase in the three mutant proteins. Because of the disappearance in the less stable mutant proteins, this suggests that the slow phase may come from some small structure such as a core, which is easily disrupted in the labile mutant proteins or may be related to the residual structure at higher urea concentration reported by Saab-Rincon et al. (1993, 1996). Mutant α subunits in which phenylalanine is substituted at position 139 or 258 with tryptophan show two phases in the urea unfolding reaction, but the origin of the minor slow phase is not discussed (Choi & Hardman, 1996).

Cis Proline at Position 28 Is Not the Origin of the Slow Phase in the Refolding Reaction of the α Subunit. Garel and Baldwin (1973) and Brands et al. (1975) have shown that a very slow phase due to cis−trans isomerization of proline is often observed with rate constants of $10^{-2}-10^{-3}$ s^{-1} in the protein folding reaction. Replacements of cis prolines have eliminated the slow phase in refolding reactions or simplified the kinetic curves for RNase A (Kelley & Richards, 1987; Schultz et al., 1992), RNase T1 (Kiefhaber et al. 1990), and nuclease (Kuwajima et al., 1991). For refolding reactions of the wild-type and proline mutant α subunits, the rates of the slow phase observed in the native region were of the order of 10^{-3} s^{-1} and independent on GuHCl concentration (Figure 4). The activation enthalpy of the slow phase of the α subunit has been similar to that of the proline isomerization reaction, whose rate constants have not depended on urea concentration and pH (Crisanti & Matthews, 1981). It was expected that the slow phase in the refolding of the α subunit comes from the isomerization of cis proline at position 28. However, the replacement of proline at position 28 did not eliminate the slow phase in refolding kinetics and did not reduce the relative amplitude of the slow phase (Figure 4 and Table 2). This indicates that the slow phase in the refolding kinetics of the α subunit does not originate from cis proline 28.

There are some possible explanations for the fact that the slow phase does not come from cis proline at position 28 in the α subunit. The first possibility is that some trans proline residues in the native state convert to the cis form in the denatured state. If so, the isomerization of cis to trans forms occurs in the folding process, resulting in the appearance of the slow phase. It has been suggested that prolines in a random chain might possess something on the order of 10−30% cis character (Torchia, 1972; Evans & Rabenstein, 1974; Keim et al., 1974; Brandts et al., 1975; Raleigh et al., 1992). This possibility could be greater than the following two possibilities, because the α subunit contains 19 prolines. In fact, recently, Saab-Rincon et al. (1993, 1996) have suggested that the His92−Pro93 peptide bond of the α subunit is present in both trans and cis isomers in higher concentrations of urea, where the secondary structure is entirely disrupted and the tyrosine residues are exposed to the solvent based on NMR studies. The second possibility is that proline is involved in an equilibration of cis−trans isomers in the native state. The existence of two native species of cis and trans isomers has been suggested by NMR studies on nuclease (Markley & Jardetzky, 1970; Fox et al., 1986; Evans et al., 1989; Alexandrescu et al., 1990) and Calbindin D_{9k} (Chazin et al., 1989). The third is that alanine 28, after replacement of cis proline 28 of the α subunit, may retain a cis conformation in the native state. The non-proline cis peptide bond is retained after replacement of cis proline with alanine in RNase T1 (Mayr et al., 1994) and carbonic anhydrase II (Tweedy et al., 1993). The mutant RNase T1 involves a slow phase due to isomerization of the cis-Ala peptide bond (Mayr, et al., 1993). On the other hand, RNase H containing two cis prolines shows only a single phase in the refolding kinetics (Yamasaki et al., 1995).

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