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Role of Conserved Proline Residues in Stabilizing Tryptophan Synthase α Subunit: Analysis by Mutants With Alanine or Glycine

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ABSTRACT **To study the role of Pro residues in the conformation and conformational stability of a protein, nine mutant α subunits of tryptophan synthase from *Escherichia coli*, in which Ala or Gly was substituted for each of six Pro residues (positions 28, 57, 62, 96, 132, and 207) that are conserved in 10 microorganisms, were constructed by means of site-directed mutagenesis. The far-ultraviolet (UV) CD spectra of five mutant α subunits with Ala in place of Pro were identical to the spectrum of the wild-type protein, the exception being the mutant at position 207 (P207A). CD values in the far-UV region were less negative for P207A, indicating that the Pro residue at position 207 plays a role in maintaining the intact structure of the α subunit. The negative CD values of the Gly mutants examined (P28G, P96G, and P132G) were also decreased. Calorimetric measurements showed that the two mutants at position 28 (P28G and P28A) gave two peaks in the excess heat capacity curve, whereas the wild type and other Pro mutants had only a single peak. The stability of each mutant protein relative to that of the wild type was about the same for P57A, less for P62A and P132A, and markedly decreased for P96A and P207A, which are substituted at less mobile positions. The changes of denaturation entropy ($\Delta\Delta_d^{\prime}S$) at the denaturation temperature of the wild-type protein (54.1 °C at pH 9.0) were positive for P57A, P62A, and P132A, but negative for P96A, P207A, and P132G. The present results do not indicate that the differences in stability (Δ_dG) among Pro substitutions are caused only by an entropic factor, as might be theoretically expected. The decreases in stability for P96A and P207 were due to the considerable decrease in denaturation enthalpy, although they were partly compensated for by the decrease in entropy. Our results also suggest that Pro-28 stabilizes the interaction between two domains of the α subunit.**

Key words: calorimetry, unfolding of protein, CD spectra, denaturation by guanidine hydrochloride

INTRODUCTION

Studying the effects of single amino acid substitutions on conformational stability using mutant proteins could be a fruitful approach for elucidating the roles of the amino acid sequence in protein folding and protein stability. The stabilities of mutant forms of some proteins[1–3] have been quantitatively estimated.

In the case of the α subunit of tryptophan synthase from *Escherichia coli*, the conformational stabilities of a series of mutant proteins substituted at position 49, which is buried in the interior of the protein, have been compared. The results indicated that 1) the stabilities of the proteins substituted at this position (denaturation Gibbs energy at pH 7 and 25°C) increase by 0.72 to 1.92 times that of the wild-type protein and 2) the increase is proportional to the hydrophobicity of the substituting residues.[4–6] The linear relationship between the stability of a protein and the hydrophobicity of the residues in the interior of a protein has been confirmed for other proteins, i.e., kanamycin nucleotidyltransferase[7] and T4 lysozyme.[8] These results provide clues for understanding the role of the amino acid sequence at *a specific position* in protein stability. To elucidate the relationship between the amino acid sequence and the stability of a protein, moreover, it should also be determined how *each amino acid* plays an important part in the conformational stability at *specific positions* in the spatial structure of a protein.

We have focused on a unique residue, a proline. This residue is expected to decrease the conformational entropy of denaturation[9] and plays a unique role, due to proline imido bond isomerization, with regard to protein folding.[10] The α subunit of tryp-

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Abbreviations: P28A, P57A, P62A, P96A, P132A, and P207A: Ala was substituted for Pro at positions 28, 57, 62, 96, 132, and 207, respectively, in the α subunit of tryptophan synthase; P28G, P96G, and P132G: Gly was substituted for Pro at positions 28, 96, and 132, respectively, in the α subunit of tryptophan synthase; GuHCl, guanidine hydrochloride.

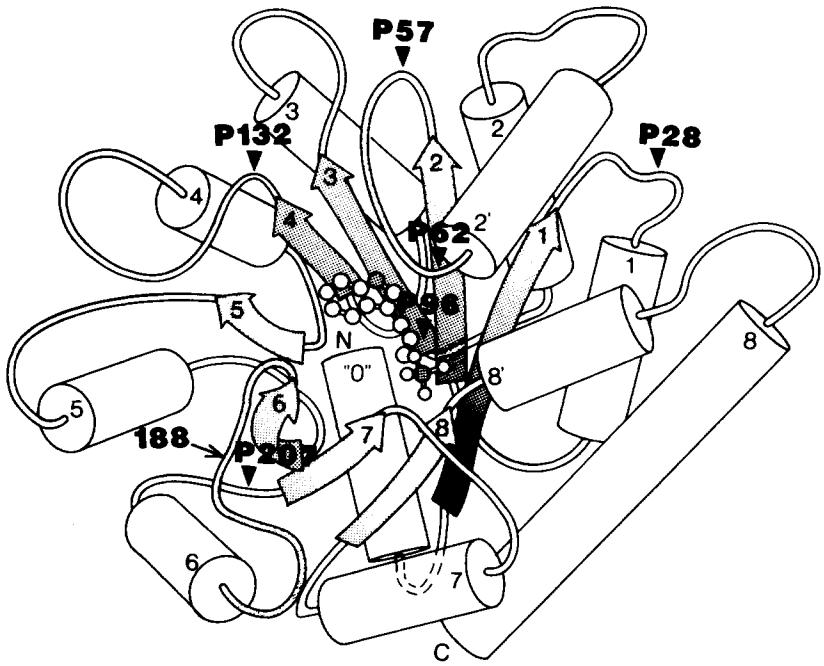

Fig. 1. Locations of the conserved Pro residues in the α subunit of tryptophan synthase from *S. Typhimurium*.[12] Arrowheads indicate the six conserved Pro residues (positions 28, 57, 62, 96, 132, and 207). An arrow at 188 indicates the site susceptible to cleavage by trypsin (Arg-188), which delineates the N- and C-terminal fragments.

tophan synthase from *E. coli* has 19 proline residues. Six of them are conserved in ten microorganisms.[11] Recently, X-ray crystallographic analysis of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* was reported.[12] In this structure, all of the Pro residues are of the trans configuration. The locations of the six conserved Pro residues are shown in Figure 1.[12] Pro-28 is found in the loop between strand 1 and helix 1. Pro-57, -62, -132, and -207 are also in loops, and Pro-96 is at the N-terminal end of strand 3. None of the conserved Pro residues are located in helical regions.

In this paper, changes in the conformations and stabilities of mutant α subunits of tryptophan synthase from *E. coli,* with alanine or glycine substituted for each of six conservative proline residues, were investigated by means of CD and scanning microcalorimetric measurements. The roles of the proline residues in the conformational stability of the protein and the protein folding are discussed.

MATERIALS AND METHODS

Materials

Mutant α subunits of tryptophan synthase from *E. coli,* in which Ala was substituted for each of six conserved Pro residues (positions 28, 57, 62, 96, 132, and 207) and Gly at positions 28, 96, 132, and 207 were constructed as described,[13] using the Amersham oligonucleotide-directed in vitro mutagenesis system. Plasmids (pUC8PtrptrpA) containing the coding sequence *trpA* genes for the mutant α subunits at the Pro residues were prepared. The base substitutions in *trpA* were confirmed by DNA sequencing. The *trpA2* strain of *E. coli*.[30] was transformed with these ligated plasmids. Bacteria were grown on the medium previously described.[13] The purification of mutant and wild-type α subunits was performed as described.[13] However, the mutant α subunit with glycine at position 207 was not obtained in sufficient quantity to measure its physicochemical properties. All the purified mutant proteins gave a single peak on high-performance liquid chromatography (FPLC, Mono Q Pharmacia). Gu-HCl (specially prepared reagent grade) was purchased from Nakarai Chemicals (Kyoto, Japan).

Protein Concentrations

The protein concentrations of the wild-type and mutant α subunits were estimated from the absorbance at 278.5 nm, assuming $E^{1\%}_{1\ cm} = 4.4$.[14]

CD Measurements

The CD spectra were recorded with a Jasco J-500 spectropolarimeter equipped with a data processor for CD (model DP-501). Each memory unit in the computer stored the CD signal for a spectral band of 0.1 nm. Spectra were scanned 16 times at the scan rate of 20 nm/minute, using a 0.25 sec time constant.

The spectra were measured at protein concentrations of 1.5–4.5 mg/ml in 10 mM potassium phosphate buffer (pH 7.0), using a cell of 10 mm (in the region of 250–320 nm) or 0.08 nm (200–260 nm) light-path length at 25°C. For calculation of the mean residue ellipticity, [Θ], the mean residue weight was taken as 107.5 for all the proteins. Denaturation by GuHCl was examined by following the CD values at 222 nm as described.[15]

Calorimetric Measurements

Calorimetric measurements were carried out with a scanning microcalorimeter, DASM4, at the scan rate of 0.5 or 1.0 degrees/minute, equipped with a NEC personal computer. Each memory unit in the computer stored one datum for 5 sec. The protein concentrations used were 0.8–2.3 mg/ml. Just before the measurements, the protein solutions were dialyzed with an Amicon YM10 membrane against 1 mM sodium tetraborate solution containing 1 mM EDTA at various pHs. The pH of the solution was adjusted with 1 N HCl or 1 N KOH. The pH values reported in this paper were measured after calorimetry. Calorimetric and Van't Hoff enthalpies (Δh^{cal} and Δh^{vH}) were calculated by a computer program developed with Kidokoro and Wada.[16]

RESULTS

Comparison of CD Spectra of the Mutant α Subunits

The CD spectra in the region of 200–260 nm of the five mutant α subunits with Ala at positions 28, 57, 62, 96, or 132 in place of Pro were not different from the spectrum of the wild-type protein, suggesting that the secondary structures of these proteins are similar to each other. However, the spectrum of the mutant α subunit with Ala at position 207 (P207A) was different (Fig. 2); the decrease in the negative CD values indicates that the helical content of the mutant protein was reduced by the single amino acid substitution. The helical contents of proteins with Ala in place of Pro might be expected to be increased, in that Pro is a helix breaker and Ala a good helix former. In contrast, the present results seem to show that a Pro residue at position 207 stabilizes the helical structure of the α subunit. That is, Pro-207 plays a role in maintaining the intact structure of the α subunit.

The spectra of the three mutant proteins with Gly at positions 28, 96 and 132 were similar to the spectrum of P207A (Fig. 2). Table I shows the CD values at 222 nm for the wild-type and the mutant α subunits, which indicate that the substitutions of Gly in place of Pro reduced the helical contents of the α subunit.

On the other hand, the CD spectra in the aromatic region (250–320 nm) of the six mutant α subunits with Ala and the three mutants with Gly were not different from the spectrum of the wild-type α subunit, indicating that the six conserved proline residues do not affect the fine structure of aromatic residues.

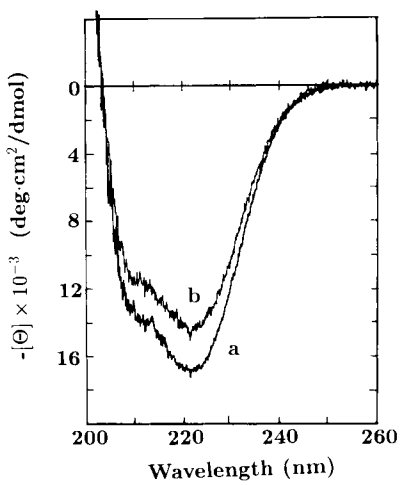

Fig. 2. CD spectra in the far-UV region of mutant α subunit with Ala at positions 62 and 207 in place of Pro at pH 7 and 25°C. a and b show the spectra of P62A and P207A, respectively.

Calorimetric Studies on the Mutant α Subunits

Figure 3A shows a typical excess heat capacity curve, with a single peak for the mutant α subunit, P132A, at pH 9.32. For the wild-type and mutant proteins P57A, P62A, P96A, P207A, P96G, and P132G, the curves showed a single peak, like that of P132A. The excess heat capacity curves of proteins P28A and P28G had more than two peaks, as shown in Figure 3B. The reversibility of the thermal denaturation was confirmed by reheating the protein solution in the calorimeter cell immediately after cooling from the first run. The calorimetric enthalpy and van't Hoff enthalpy were obtained directly from the excess heat capacity curves (Table II). The values of calorimetric enthalpies for the wild-type protein were higher than those reported elsewhere.[17] When the calorimetric enthalpies of hen egg lysozyme were measured with our calorimeter as a standard, the values obtained were the same as those reported elsewhere, within experimental error (less than 5%), indicating that our instrument is correct. We cannot explain the difference between the values at present. As is shown in Table II, the ratios of the two enthalpy values for P57A, P62A, P132A, and P132G were close to unity, indicating that there is no intermediate between the native and denatured states; i.e., it is a two-state denaturation. The ratios for P96A, P96G, and P207A were significantly lower than unity. On the other hand, the ratio for the wild-type protein was higher than unity. These results suggest that substitutions at Pro residues affect the denaturation process of the α subunit.

TABLE I. CD Values at 222 nm for the Wild-Type and Mutant α Subunits of Tryptophan Synthase Substituted at Each of the Conserved Pro Residues at pH 7.0 and 25°C ([Θ] in deg · cm²/dmol)

Proteins	[Θ]
Wild-type	−16,700
P28A	−16,400
P57A	−17,000
P62A	−17,000
P96A	−17,100
P132A	−16,600
P207A	−15,100
P28G	−14,900
P96G	−15,200
P132G	−15,800

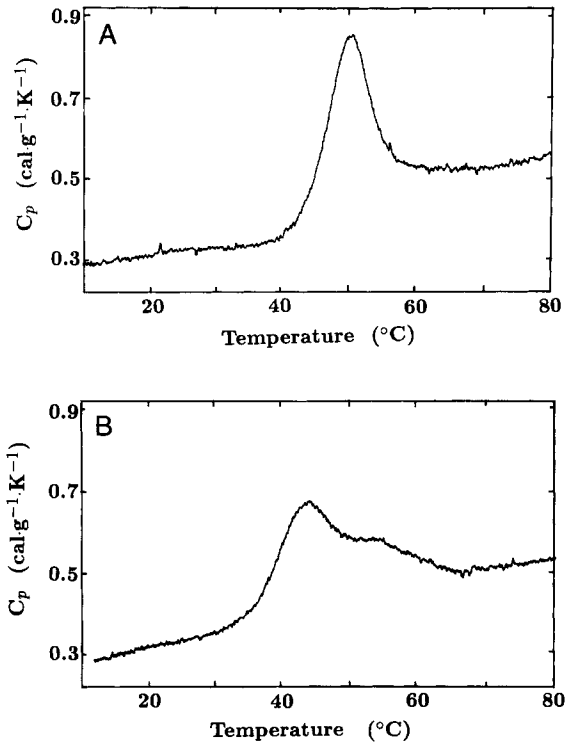

Fig. 3. Typical excess heat capacity curves of two mutant α-subunits. Calorimetric recordings for P132A at pH 9.32 **(A)** and P28G at pH 9.49 **(B)**.

Figure 4 shows the pH dependence of the denaturation temperatures of the five mutant proteins with Ala, except for the mutant at position 28. These denaturation temperatures (T_d) correspond to the peak temperatures observed in excess heat capacity curves. Figure 4 shows that the T_ds of all the mutant proteins decreased with increasing pH. The T_d of P57A was the highest at each pH, compared with those of the other mutant proteins. The results for P57A were similar to those for the wild-type α subunit (not shown in Fig. 4). P96A and P207A are the least stable, P62A and P132A of medium stability. The pH dependence of P132G was similar to that of P132A (not shown in Fig. 4).

Figure 5 shows the denaturation calorimetric specific enthalpies ($\Delta_d h^{cal}$) for the five mutant proteins with Ala, except for the mutant at position 28, as a function of T_d. The $\Delta_d h^{cal}$ values were taken from Table II. The straight line (line 1) presents a least-square fit of seven experimental points of $\Delta_d h^{cal}$ for the wild-type protein (Table II). The $\Delta_d h^{cal}$ values for four mutant proteins (P57A, P62A, P96A, and P132A) seem to be the same linear function of T_d (Fig. 5). The $\Delta_d h^{cal}$ values for P207A were also a linear function of T_d (line 2 in Fig. 5) but were lower than those of other proteins at the same temperature.

Denaturation by GuHCl

It has been considered, on analysis of denaturation curves obtained with GuHCl and the wild-type and the mutant α subunits at position 49, that the α subunit of tryptophan synthase from *E. coli* consist of two domains, α_1 (N-terminal 188 residues) and α_2 (C-terminal 80 residues).[18–20] In the first step of the denaturation by GuHCl, only the α_2 domain unfolds, the α_1 domain remaining in the native state, which results in a stable intermediate form; in the second step, the α_1 domain also unfolds. On the other hand, thermal denaturation of the wild-type and mutant α subunits at position 49 gives no evidence for an intermediate during the denaturation process.[21,22] The present results, on thermal denaturation of mutants at Pro residues, indicated no intermediate, except for in the case of the mutant at position 28, because the excess heat capacity curves had a single peak, and the ratios of two kinds of enthalpies were close to unity.

On the other hand, the excess heat capacity curves of P28A and P28G showed two peaks. To elucidate the denaturation mechanism for these mutants, GuHCl denaturation of P28G and P28A was compared with that of other proteins. The denaturation curves were followed by measuring the CD values at 222 nm with various concentrations of GuHCl. Figure 6 shows the denaturation curves of P28G and P132G, which indicate that the first step of the denaturation of P28G occurs at lower concentrations of GuHCl than that of P132G. The denaturation curves were analyzed by assuming that there is one stable intermediate in the denaturation process as described.[15] The denaturation Gibbs energy values in water in the first step of denaturation ($\Delta_d G_{ni}$) were estimated to be 2.3 and 3.6 kcal · mol^{-1} for P28G and P132G at pH 7 and 25°C, respectively, which indicate that the α_2 domain of P28G was less stable than that of P132G (Table III). On the other hand, the denaturation Gibbs energy values in water in the second step of denaturation ($\Delta_d G_{id}$) were 4.9 and 4.6 kcal · mol^{-1} for P28G and P132G, respectively, indicating

TABLE II. Thermodynamic Parameters Obtained by Calorimetry of Mutant α Subunits of Tryptophan Synthase Substituted With Ala or Gly at Each of the Conserved Pro Residues

Proteins	pH	T_d (°C)	$\Delta_d h^{cal}$ (cal/g)	$\Delta_d H^{vH}$ (cal/g)	Ratio $\Delta_d h^{cal}/\Delta_d h^{vH}$	Average
Wild	9.75	50.4	3.63	2.99	1.21	1.25 ± 0.09
	9.64	50.2	3.53	3.15	1.12	
	9.32	53.3	4.04	3.06	1.32	
	8.98	54.2	4.09	3.15	1.28	
	8.47	55.6	4.45	3.52	1.37	
	8.23	57.0	4.66	3.59	1.30	
	7.45	58.8	4.92	4.27	1.15	
P57A	10.33	43.2	2.28	2.59	0.88	1.02 ± 0.08
	9.69	49.7	3.58	3.41	1.05	
	9.23	52.6	4.12	3.78	1.09	
	8.95	53.8	4.37	3.90	1.12	
	8.50	55.6	4.27	4.23	1.02	
	8.05	56.7	4.65	4.31	1.08	
	7.57	58.2	4.73	5.09	0.93	
P62A	10.15	43.4	2.48	2.53	0.98	1.06 ± 0.05
	9.74	47.7	3.37	3.01	1.12	
	9.42	50.5	3.58	3.49	1.03	
	8.84	52.6	3.98	3.62	1.10	
	8.42	54.0	4.16	3.85	1.08	
	7.56	57.4	4.99	4.66	1.07	
P96A	9.89	40.1	1.92	3.32	0.58	0.80 ± 0.10
	9.50	43.1	2.63	3.23	0.81	
	9.51	43.0	2.90	3.14	0.92	
	9.52	44.5	2.84	3.23	0.88	
	9.48	45.0	2.77	3.68	0.75	
	9.34	45.3	2.56	3.74	0.68	
	9.08	46.7	2.99	3.69	0.81	
	8.67	48.0	3.15	3.94	0.80	
	8.38	49.4	3.51	3.86	0.91	
	7.79	51.5	3.55	4.38	0.81	
	7.59	53.5	3.79	4.74	0.80	
P96G	9.00	40.0	1.99	3.06	0.65	
P132A	9.68	47.4	3.18	3.28	0.97	1.02 ± 0.07
	9.32	49.7	3.55	3.45	1.03	
	9.04	51.3	3.48	3.74	0.93	
	8.70	52.3	3.86	3.68	1.05	
	7.73	56.1	4.66	4.17	1.12	
P132G	9.53	49.2	3.09	3.47	0.89	1.05 ± 0.09
	9.42	50.2	3.33	3.23	1.03	
	9.26	51.1	3.74	3.31	1.13	
	9.01	51.9	3.69	3.35	1.10	
	8.78	53.1	3.76	3.50	1.11	
P207A	8.82	47.6	2.20	2.72	0.81	0.82 ± 0.07
	8.95	48.4	2.21	2.57	0.86	
	8.37	48.7	2.53	2.81	0.90	
	7.21	53.8	3.25	4.64	0.70	
P28G	9.41	45.3	2.44			
	9.39	45.8	2.72			
	9.26	45.9	2.71			
P28A	9.25	45.9	2.40			
	9.21	45.5	2.49			

that their α_1 domains had similar stability. $\Delta_d G_{id}$ values for all the mutant proteins examined were similar to (or higher than) the value for the wild type, whereas the values in the first step ($\Delta_d G_{ni}$) for the mutant proteins were lower than that for the wild-type protein (Table III). The $\Delta_d G_{ni}$ values for mutants at positions at 28 and 207 were remarkably decreased, indicating that the α_2 domains of these mutants were strongly destabilized by the substitutions. Position 207 is in the α_2 domain, but position 28 is not. These results suggest that the substitutions at position 28 (P28A and P28G) destabilize the interaction between the α_1 and α_2 domains in the α subunits.

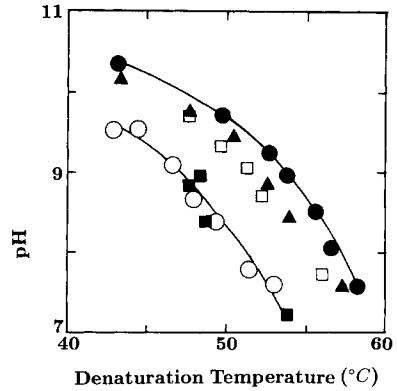

Fig. 4. Denaturation temperatures of P57A (●), P62A (▲), P96A (○), P132A (□), and P207A (■) at various pHs.

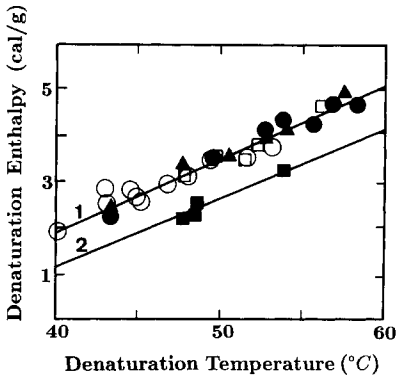

Fig. 5. Denaturation calorimetric specific enthalpy for the mutant α subunits with Ala as a function of the denaturation temperature. The symbols are the same as in Figure 4. Line 1 was obtained by a least-squares fit of seven experimental points for the wild-type α subunit and line 2 by four points for P207A.

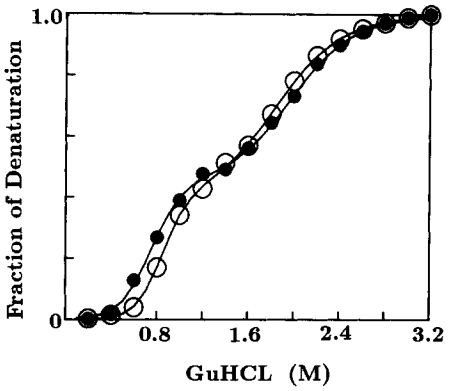

Fig. 6. GuHCl denaturation of P132G (○) and P28G (●) at pH 7.0 and 25°C. The fractions of denaturation (F) were calculated with the following equation: $F = [([\Theta]_n - [\Theta])/([\Theta]_n - [\Theta]_d)]$ where $[\Theta]$ represents the CD values at 222 nm at a given concentration of GuHCl and $[\Theta]_n$ and $[\Theta]_d$ the CD values at 222 nm in the absence and the presence of 3.2 M GuHCl, respectively. The solid lines are best fit curves according to equation 9 of Yutani et al.[15]

DISCUSSION

Effect of Conserved Pro Residues on the Conformation of the α Subunit

Judging from CD spectra in the far-UV region (200–260 nm), the secondary structures of the five mutant proteins with Ala in place of the conserved Pro residues were not affected, the exception being P207A, which had less negative CD values (Fig. 1, Table I). The α-helical contents of the wild type and P207A were estimated to be 54.5% and 48.0%, respectively, from CD data using the standard CD values obtained by Chen et al.[23] Furthermore, the negative CD values in the far-UV region of the mutant proteins with Gly (P28G, P96G, and P132G) were also decreased, and similar to that of P207A (Table I). The mutant protein with Gly at position 207 could not be obtained in sufficient quantity to measure its physicochemical properties; its conformation might be significantly destabilized by the substitution. These results suggest that the Pro residue at position 207, which is located in a loop, plays an important role in maintaining the helical structure of the α subunit. The loop containing Pro-207 follows helix 6 (Fig. 1). The N-terminal residue of the helix 6 connects with a highly mobile surface loop.[12] When Pro-207 is substituted with a more flexible residue, Ala or Gly, both sides of the helix 6 may become so mobile that the helix is destroyed. If helix 6 were destroyed by the substitution at position 207, Tyr-203 among seven Tyr residues in the protein might be affected. However, the CD spectrum in the aromatic region of P207A was not affected. This means that the fine structure of Tyr-203 remains unchanged by the substitution, because Tyr-203 in the wild-type protein is completely exposed to the solvent.[12] It has also been reported that, in the case of a mutant adenylate kinase with Ser in place of a Pro that is located in a loop, the helical content of the protein is decreased.[24]

Effect of the Conserved Pro Residues on the Conformational Stability of the α Subunit

Enthalpy, entropy, and Gibbs energy changes on thermal denaturation as a function of temperature can be calculated with the following equations, if the denaturation heat capacity change (Δ_dC_p) does not depend on temperature[25]:

$$\Delta_dH(T) = \Delta H_d - \Delta_dC_p(T_d - T) \quad (1)$$

$$\Delta_dS(T) = \Delta H_d/T_d - \Delta_dC_p\ln(T_d/T) \quad (2)$$

$$\Delta_dG(T) = \Delta_dH(T) - T\Delta_dS(T), \quad (3)$$

where ΔH_d is the enthalpy change at the denaturation temperature (T_d). Table IV shows the thermodynamic parameters of denaturation for the wild-type and mutant proteins at the denaturation temperature of the wild-type protein (54.1 °C at pH 9). The Δ_dH values for the mutant proteins were

TABLE III. Unfolding Gibbs Energies in Water Obtained on Analysis of the Denaturation Curves With GuHCl for Mutant α Subunits at the Conserved Pro Residues*

Proteins	Δ_dG_{ni}	Δ_dG_{id}	Δ_dG_{nd}
Wild-type	4.6	4.2	8.8
P28G	2.3	4.9	7.2
P28A	2.2	4.1	6.3
P132G	3.6	4.6	8.2
P207A	2.4	4.6	7.0

*Δ_dG_{ni} and Δ_dG_{id} represent the denaturation Gibbs energies (kcal · mol^{-1}) in water in the first and the second steps, respectively, of denaturation by GuHCl at 25°C and pH 7. Δ_dG_{nd} represents the summation of the Gibbs energies in the two steps.

estimated by a linear least squares fit of the experimental data for each protein (Table II). The stability (Δ_dG) of P57A was similar to that of the wild type. The other mutant proteins were destabilized by the substitutions. The stabilities of the mutants with Ala at positions 96 and 207 were especially decreased. The mutant protein with Gly at position 96 was less stable than that with Ala (not shown in Table IV). The stabilities of mutants at positions 62 and 132 were slightly lower than that of the wild-type protein.

The pyrrolidine ring of proline restricts the number of conformations it can take on, compared to those possible for the other amino acids. On the other hand, glycine lacks a β-carbon and exhibits more conformational flexibility of the backbone. The backbone contributions to the entropy of denaturation of Ala and Gly relative to Pro have been estimated to be 4 and 6.5 cal · mol^{-1} · K^{-1}, respectively,[9] suggesting that the stabilities of the mutant proteins substituted with Ala or Gly in place of Pro might be decreased due to the entropy gain in the denatured state. The values of $\Delta\Delta_dS$ of three mutant proteins, P57A, P62A, and P132A, were positive, indicating that they are destabilized by the entropy gain. However, the other mutant proteins do not seem to be destabilized by entropic factors, because their values were negative.

The crystallographic thermal factor (B values) is related to the mobility of atoms. The B values of conserved Pro residues, obtained from X-ray analysis, are considerably different from each other, with mean values of the main chain and the side-chain atoms for Pro-62, Pro-96, Pro-132, and Pro-207 of 34.3, 7.5, 7.9, and 14.1 Å^2, respectively; Pro-57 is not determined to be mobile.[12] The stabilities of the mutant proteins (P96A and P207A) substituted at less mobile positions were remarkably decreased, whereas those of P57A and P62A substituted at mobile positions were only slightly decreased (Table IV). The stability of P132A did not decrease in parallel to the decrease in mobility. This might be caused by underestimation of the thermal factor at position 132, because this value was obtained by the crystallographic data for $\alpha_2\,\beta_2$ complex; the proline residue at position 132 is at the α/β contact surface but would be on the surface of the α-subunit monomer measured here. The present results suggest that proline residues in less mobile region play an important role in stabilizing the α subunit and that the destabilization effect by the proline substitutions is caused mainly by enthalpy.

It has been reported that the enhancement of the thermal stability for T4 lysozyme with Pro in place of Ala is due to a decrease in entropy rather than a change in enthalpy.[26] On the other hand, it has been found that the unfolding enthalpy of cross-linked lysozyme is only slightly larger than that of the native one, and the unfolding entropy of the cross-linked one is nearly equal to that of the native one, when both are compared at the same temperature.[27]

Stability of Domains of the Tryptophan Synthase α Subunit

It has been reported that there is a stable intermediate in the processes of denaturation by GuHCl[15,19,20] and urea[28] for the wild-type and mutant α subunits of tryptophan synthase. The stability of each domain of the mutant α subunits was estimated from the GuHCl denaturation (Table III). At pH 7, the substitutions at positions 28 and 207 greatly affected the stability of the α_2 domain. The Pro residue at position 207, which is located in the α_2 domain, directly plays an important role in maintaining the intact structure of the α_2 domain. Since the excess heat capacity curve of P207A had a single peak and the ratio for the two enthalpies was close to unity, it seemed that the thermal denaturation of P207A proceeded as a single cooperative unit, although the stabilities of two domains against GuHCl differed.

In the case of mutants at position 28, a marked decrease in stability of the α_2 domain and two peaks in excess heat capacity curves were found. It has been reported that the first step of denaturation of the α subunit nicked by trypsin at position 188, which is the boundary between the two domains, was remarkably decreased, suggesting that the α_2 domain is stabilized by its covalent attachment to the α_1 domain in the intact α subunit.[20] This means that the interaction between the two domains of the nicked protein is destabilized.[29] By analogy, the Pro residue at position 28 may play an important role in stabilizing the interaction between the two domains. Pro-28 is in a loop between helix 1 and strand 1 that hydrophobically interacts with helix 8 and helix 8′ in the α_2 domain (Fig. 1).[12] The hydrophobic interaction might have been weakened when the residue at position 28 became more flexible by the substitution of Ala or Gly in place of Pro, resulting in destabilization of the α_2 domain and an appearance of two peaks in the excess heat capacity curve.

TABLE IV. Thermodynamic Parameters Obtained on Calorimetry of Mutant α Subunits Substituted at the Conserved Pro Residues at the Denaturation Temperature, 54.1°C, for the Wild-Type Protein at pH 9*

Proteins	T_d	ΔT_d	$\Delta_d C_p$	$\Delta_d G$	$\Delta_d H$	$\Delta\Delta_d H$	$\Delta_d S$	$\Delta\Delta_d S$
Wild-type	54.1	0	4.6	0	120.4	0	367.9	0
P57A	54.0	−0.1	4.7	−0.04	121.1	0.7	370.3	2.4
P62A	52.7	−1.4	4.9	−0.52	123.5	3.1	378.8	10.9
P132A	51.5	−2.6	4.8	−0.92	121.2	0.8	373.0	5.1
P132G	51.8	−2.3	5.1	−0.78	116.6	−3.8	358.6	−9.3
P96A	47.8	−6.3	3.6	−2.00	113.2	−7.2	352.0	−15.9
P207A	47.4	−6.7	4.9	−1.64	95.0	−25.4	295.3	−72.6

*T_d and ΔT_d in °C, $\Delta_d G$ in kcal · mol^{-1}, $\Delta_d H$ in kcal · mol^{-1}, $\Delta_d S$ and $\Delta\Delta_d S$ in cal · mol^{-1} · K^{-1}, and $\Delta_d C_p$ in kcal · mol^{-1} · K^{-1}. T_d at pH 9 was estimated from the pH dependence on the denaturation temperature (Fig. 4). The $\Delta_d H$ values at T_d and $\Delta_d C_p$ were obtained by a linear least-squares fit of the experimental data for each protein (Table II). Thermodynamic parameters at 54.1°C were calculated by using equations 1–3.

CONCLUSIONS

The present results indicate that the conserved Pro residues of the tryptophan synthase α subunit play roles in the conformational stability and protein folding, among other significant functions. Proline residues in less mobile regions play an especially important role in stabilizing the α subunit.

When Pro-28 was substituted with Ala or Gly, the α_2 domain of the α subunit was remarkably destabilized, suggesting that Pro-28 stabilizes the interaction between the two domains.

The mutants at Pro-57, Pro-62, and Pro-132, which are located in loops (mobile regions), did not remarkably decrease the stabilities. The decreases in stability for the mutants were caused by entropic factors, as was theoretically expected.

The substitution (P96A) at position 96 (less mobile region) in a β strand remarkably decreased the stability due to a decrease in denaturation enthalpy, although this was partly compensated for by the decrease in entropy, this suggests that a Pro residue in a β strand plays an important role in conformational stability of a protein.

The Ala mutant at position 207 had a decreased helical content. The mutation markedly decreased the stability due to a decrease in denaturation enthalpy, although this was partly compensated for by the decrease in entropy. The Gly mutant at this position could be not obtained in a sufficient quantity to measure its physicochemical properties, suggesting that its conformation was significantly destroyed. Pro-207 in a loop and less mobile region plays a role in maintaining the intact structure of the α subunit.

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