Proton Nuclear Magnetic Resonance Studies on the Wild-Type and Single Amino Acid Substituted Tryptophan Synthase α-Subunits†

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ABSTRACT: In order to elucidate the effect of single amino acid substitutions on the conformation of the tryptophan synthase α-subunit from *Escherichia coli* in solution, ^{1}H NMR spectra of the wild-type and mutant proteins were measured at various pHs. Two of the four His C2-proton resonances of the α-subunit were assigned to two His residues at positions 92 and 146 by using a mutant protein with Thr substituted for the His at position 92. The replacement did not affect the conformation of the protein significantly. The proton resonances of all the Tyr residues in the aromatic region could be picked up from other resonance peaks, employing the wild-type α-subunit deuteriated at all of the Phe residues. On comparison of the spectra of the wild-type protein with those of the mutant protein with Met substituted for the Glu at position 49, it was concluded that the substitution affects only the residues close to the substituted residue at acidic pH but that a larger part of the protein is affected at alkaline pH. NOE experiments showed that the five Tyr residues, four of which are located in the proximity of position 49, are close to one another. The present results are discussed in the light of the conformational stability of the protein.

Recent advances in molecular biology have allowed the modification of the gene for a protein with virtually no limitations (Ulmer, 1983). By use of mutant proteins, the effect of single amino acid substitutions on the conformational stability and biological function of a protein can be investigated systematically. This is a valuable approach for elucidation of the correlation between the structure of a protein and its stability or specific function.

Mutant proteins derived from the tryptophan synthase α-subunit of *Escherichia coli* have been studied to elucidate the roles of its amino acid residues in protein folding and protein stability (Yutani et al., 1977, 1979, 1980b, 1982, 1984, 1985; Ogasahara et al., 1984, 1985) or to elucidate the mechanism of its function (Hodo et al., 1977; Patterson et al., 1977; Yutani et al., 1987a). Our previous results indicated that single amino acid substitutions at a unique position have a marked effect on the conformational stability of a protein, depending on the nature of the substituted residues (Yutani et al., 1984). Unfortunately, we have not been able to explain those results in detail, since structural data are not available (Ahmed et al., 1985). However, NMR can provide information on the effect of single amino acid substitutions on the protein conformation and dynamics in solution (Wüthrich et al., 1982; Ho & Russu, 1985; Prigodich et al., 1986).

In order to elucidate the conformation in solution of the tryptophan synthase α-subunit from *E. coli*, ^{1}H NMR spectra of this protein and mutant proteins derived from it were measured at various pHs. In this study, we could assign the His C2 protons of the His-92 and His-146 residues, using a mutant protein obtained through site-directed mutagenesis. The aromatic proton signals of all the Tyr residues were also assigned to the respective residue by using the protein specifically deuteriated at all Phe residues. The nature of the Glu residue at position 49 in the α-subunit is discussed on the basis of the results of comparison of the ^{1}H NMR spectra of the wild-type with those of the mutant protein.

Experimental Procedures

Materials. The α-subunit of tryptophan synthase was isolated from the *PW11*, *trpA33*, and *Thr92*[1] strains of *E. coli*. The peak I protein from *PW11* was used as the wild-type α-subunit (Sugino et al., 1980). The *trpA33* strain (Yanofsky & Horn, 1972) was donated by Dr. C. Yanofsky. The mutant gene of the *Thr92* strain was obtained through site-directed mutagenesis using an oligodeoxynucleotide (Yutani et al., 1987a). The *trpA33* strain has Met in place of Glu at position 49 of the α-subunit, and the *Thr92* strain has Thr in place of His at position 92. The purifications were performed as described (Tsunasawa et al., 1983).

For preparation of the α-subunit with phenyl-d_5-alanine substituted at all 12 Phe residues, *E. coli* strain KMBL1788 (*thyA301*, *bio87*, *argA*, *endA101*, *pheA*, *metE72*) was used as a host for the pUC8*PtrptrpA* containing the *trpA* gene (Yutani et al., 1987a) because of its requirement of Phe. The broth used was M9-glucose, to which approximately 0.04 mg/mL each of 19 amino acids other than Phe, 0.1 mg/mL thymine, 0.05 mg/mL biotin, and 0.04 mg/mL phenyl-d_5-alanine (MSD Isotopes) were added. The purifications were performed as described (Tsunasawa et al., 1983).

Sample Preparation for ^{1}H NMR Measurements. Exchangeable protons of the α-subunit were replaced with deuterons by heating a deuterium oxide solution of it (0.5–1 mg of protein/mL) containing 1 mM tetraborate buffer at 70–75 °C at p^{2}H 9.5–9.8 for 2 min. The solution was concentrated with an Amicon 8010 on a YM10 membrane. The concen-

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[1] Abbreviations: wild type (*Glu49*), wild-type tryptophan synthase α-subunit; *Met49*, mutant protein with Met substituted for Glu at position 49 of the wild-type α-subunit; *Thr92*, mutant protein with Thr substituted for His at position 92 of the wild-type α-subunit; *DPhe*, wild-type α-subunit with deuteriated phenyl-d_5-alanine substituted for Phe at all positions; Tyr-1, Tyr-2, Tyr-3, Tyr-4, Tyr-5, Tyr-6, or Tyr-7, Tyr residues of the α-subunit labeled Y1 and Y1′, Y2 and Y2′, Y3 and Y3′, Y4 and Y4′, Y5 and Y5′, Y6 and Y6′, or Y7 and Y7′, respectively, in Figure 5; NOE, nuclear Overhauser effect.

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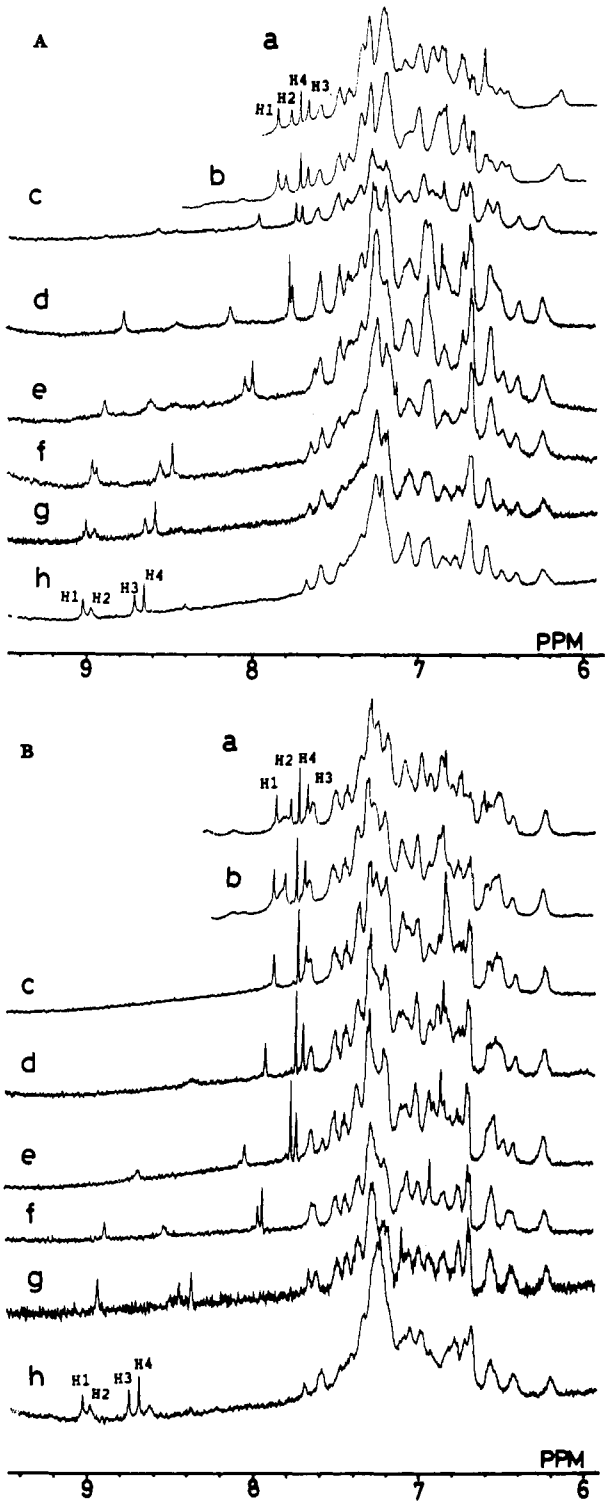

FIGURE 1: Proton NMR spectra of the wild-type (*Glu49*) and mutant (*Met49*) α-subunits in the aromatic region at 30 °C. (A) Wild-type (*Glu49*) α-subunit. Spectra a–h were obtained at p^{2}H 10.45, 9.66, 7.50, 6.97, 6.20, 5.23, 4.83, and 4.32, respectively. Peaks H1–H4 are due to His C2-proton resonances. (B) Mutant (*Met49*) α-subunit. Spectra a–h were obtained at p^{2}H 10.2, 9.53, 8.87, 7.89, 7.34, 6.38, 5.56, and 4.10, respectively. Peaks H1–H4 are due to His C2-proton resonances.

trations of the proteins used for the NMR measurements were in the range of 0.2–1 mM. Each protein solution was titrated with 0.1 N NaO^{2}H or ^{2}HCl. The p^{2}H values reported in the paper are pH-meter readings uncorrected for isotope effects.

^{1}H NMR Measurements. Proton magnetic resonance spectra were taken with a JEOL GX 500s NMR spectrometer at 30 °C. Chemical shifts are shown relative to the internal

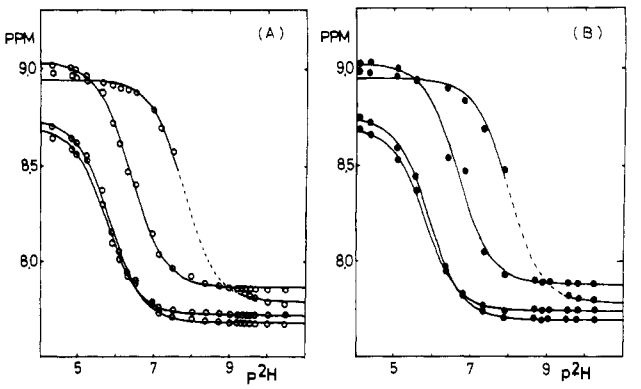

FIGURE 2: p^{2}H dependence of the chemical shifts of the His C2-proton resonances shown in Figure 1A,B. (A) and (B) are the results for the wild type (*Glu49*) and *Met49*, respectively. The solid lines were obtained on nonlinear least-squares fitting; the broken portions indicate regions in which no resonance was observed.

Table I: Comparison of p*K* Derived from His C2-Proton Resonances in the Wild-Type and Mutant α-Subunit with Met Substituted at Position 49 at 30 °C

peak	wild type (*Glu49*)	*Met49*
H1 (His-92)	6.39 ± 0.02	6.67 ± 0.06
H2 (His-146)	7.79 ± 0.03	8.00 ± 0.05
H3	5.86 ± 0.02	5.93 ± 0.02
H4	5.76 ± 0.02	5.81 ± 0.02

TMSPS [3-(trimethylsilyl)propanesulfonic acid sodium salt] signal. The buffer used was 1 mM tetraborate. To observe nuclear Overhauser effects (NOE), an appropriate resonance peak was preirradiated for 0.3 s to allow the buildup of cross-saturation. Normal and NOE spectra were obtained by alternately acquiring 16 transients with off-resonance and on-resonance irradiation, respectively, to minimize the effect of spectrometer drift. The off-resonance free induction decay was subtracted from the on-resonance one to obtain an NOE difference spectrum.

Results

p^{2}H Titration of the Histidine C2-Proton Resonances of Wild-Type and Mutant Proteins. Parts A and B of Figure 1 show ^{1}H NMR spectra of the aromatic regions of the wild-type and mutant α-subunits, respectively, at various p^{2}Hs. Four single-proton resonances (labeled H1–H4 in the spectra) can be seen in the lowest field of the aromatic region. Their chemical shifts are plotted as a function of p^{2}H in Figure 2. The apparent p*K* values for these peaks were estimated by means of nonlinear least-squares fitting (Table I). These four peaks can be ascribed to His C2 protons on the basis of their resonance positions and the p^{2}H dependence of their chemical shifts (Meadows et al., 1968). Since both the wild type (*Glu49*) and *Met49* contain only four histidine residues (His-92, -146, -195, and -244), all the C2 protons of the His residues are observed as separate signals.

The tryptophan synthase α-subunit consists of two domains: α-1, the N-terminal 188 residues, and α-2, the C-terminal 80 residues (Higgins et al., 1979; Miles et al., 1982). Iwahashi et al. (1983) assigned three peaks in a 360-MHz ^{1}H NMR spectrum of the wild type (*Glu49*) at p^{2}H 7.8 to the His C2 protons in the domains of the α-subunit. Peaks 1 and 2 (labeled from the lower field) were assigned to the two His residues in the α-1 domain, and peak 3 (two overlapping signals) was assigned to those in the α-2 domain. In a 500-MHz NMR spectrum, peak 3 had split into two separate peaks, as shown in Figure 1A. From the previous assignments, peaks H1 and H2 in Figure 1A can be ascribed to the two His

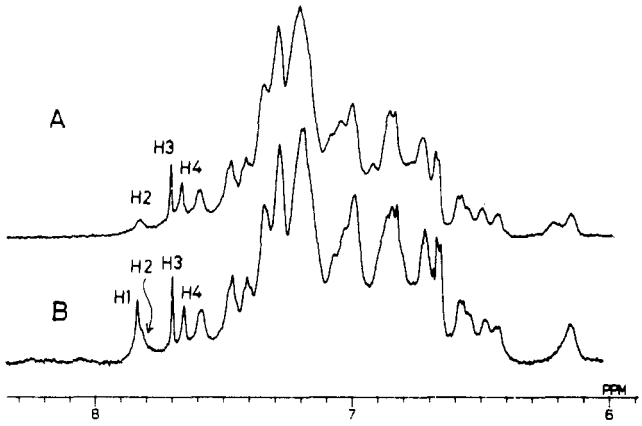

FIGURE 3: Proton NMR spectra in the aromatic region of (B) the wild-type α-subunit at p^2H 9.31 and (A) a mutant protein (His-92 to Thr) at p^2H 9.18 and 30 °C. Peaks H1–H4 are due to His C2-proton resonances.

residues in the α-1 domain (His-92 and -146) and peaks H3 and H4 in Figure 1A to the two His residues in the α-2 domain (His-195 and -244).

To identify the C2-proton signal of His-92, we used a mutant α-subunit (*Thr92*) with Thr substituted for the His at position 92. As shown in Figure 3, the proton NMR spectrum of *Thr92* at p^2H 9.18 was similar to that of the wild type (*Glu49*) at p^2H 9.31 in the aromatic region. The only significant difference in the spectra was the absence of one His C2-proton (labeled H1) peak in the case of *Thr92*. Peak H1 was absent in the spectrum of *Thr92* at all p^2Hs examined. On the basis of this, peak H1 can be unequivocally assigned to the C2 proton of His-92 of the wild type (*Glu49*). Consequently, we can assign peak H2 to His-146, the other His residue in the α-1 domain.

The p^2H titration curves for all the His C2-proton resonances of *Met49* were compared with those in the case of the wild type (*Glu49*) in Figure 2. The pK_a values of two His C2 protons (H1 and H2) of *Met49* were different from those of the wild type (*Glu49*), although the values of the other two His C2 protons (H3 and H4) were similar to each other, as shown in Table I. The effect of substitution of the amino acid at position 49 is much greater in the α-1 domain than in the α-2 domain, as far as His residues are concerned.

Assignments of Tyrosine Residues of the Wild-Type α-Subunit. The tryptophan synthase α-subunit contains 7 Tyr and 12 Phe, but no Trp. For simplification of the spectrum in the aromatic region, we used the wild-type α-subunit after it had been selectively deuteriated at all the phenylalanine aromatic rings (*DPhe*). The spectrum obtained at p^2H 9.00 is shown in Figure 4B, in comparison with the corresponding spectrum for the nondeuteriated protein (Figure 4A). A large number of proton resonances between 6.9 and 7.5 ppm disappeared in the spectrum of *DPhe*, showing that the remaining signals were due to His and Tyr residues. Accordingly, the difference spectrum between *DPhe* and the wild type (*Glu49*) (Figure 4C) can be regarded as the spectrum of the Phe residues of the α-subunit in the aromatic region.

The ^1H NMR spectrum of *DPhe* in the aromatic region showed good resolution at acidic p^2H, as shown in Figure 5. From the results of NOE experiments, all resonances originating from each Tyr residue in the aromatic region could be identified, as shown in Figure 5. By changing the p^2H, we could also assign all peaks of His C4 protons. They are presented in Figure 5 as well. When the chemical shifts of the aromatic ring protons of Tyr were plotted as a function of p^2H, a pH dependence of the four peaks was observed

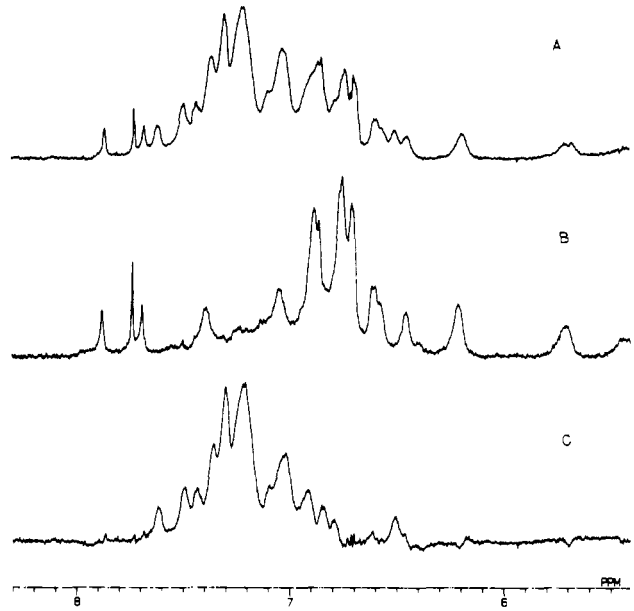

FIGURE 4: Comparison of the proton NMR spectrum in the aromatic region of the normal wild-type α-subunit with that of one deuteriated at all Phe residues and at 30 °C: (A) wild type (*Glu49*) at p^2H 8.98; (B) *DPhe* at p^2H 9.00; (C) difference spectrum between (A) and (B).

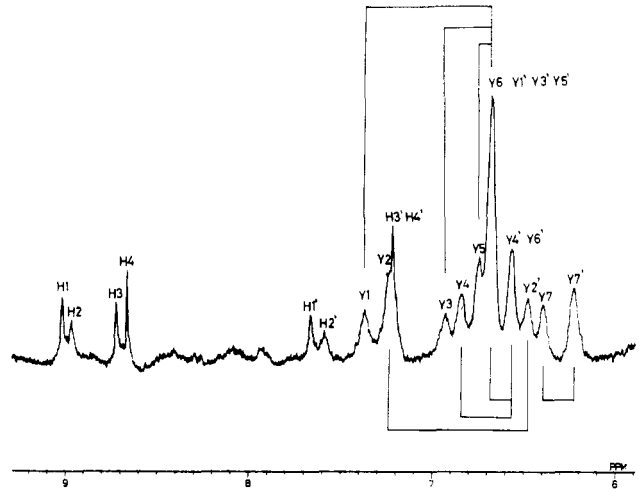

FIGURE 5: Proton NMR spectra in the aromatic region at p^2H 4.64 of the wild-type α-subunit deuteriated at all Phe residues and at 30 °C. The peaks labeled Y1–Y7 and Y1′–Y7′ are due to tyrosine proton resonances. The couples of tyrosine 2,6 and 3,5 protons are also shown with lines in the figure. Peaks H1–H4 are due to His C2-proton resonances. H′ represents the proton resonance due to His C4.

between pH 4 and 10. The transition points of the peaks, Y1, Y2, Y2′, and Y7′, were estimated to be at p^2H 8.9, 7.9, 7.8, and 8.4, respectively.

NOE experiments also showed the proximity relationships between two Tyr residues. On irradiation of Y2, Y4, Y5, Y6, and Y7, some interresidual NOEs were clearly observed in the aromatic region (Figure 6A,B). However, no interresidual NOE was observed in the aromatic region on irradiating resonances Y1 and Y3 (Figure 6C). The proximity relationships between Tyr residues of the α-subunit determined in the NOE experiments are shown in Figure 6D.

Comparison of ^1H NMR Spectra in the Aromatic Region between Wild-Type and Mutant Proteins. Figure 7 shows the ^1H NMR spectra in the aromatic region of the wild type (*Glu49*) and *Met49* at acidic p^2H. The spectrum of the wild type (*Glu49*) agreed with that of *Met49* at lower fields than 7.2 ppm. Some Tyr resonances of the wild type (*Glu49*) (peaks Y4, Y4′, Y6′, Y2′, and Y7′) shifted slightly upfield on sub-

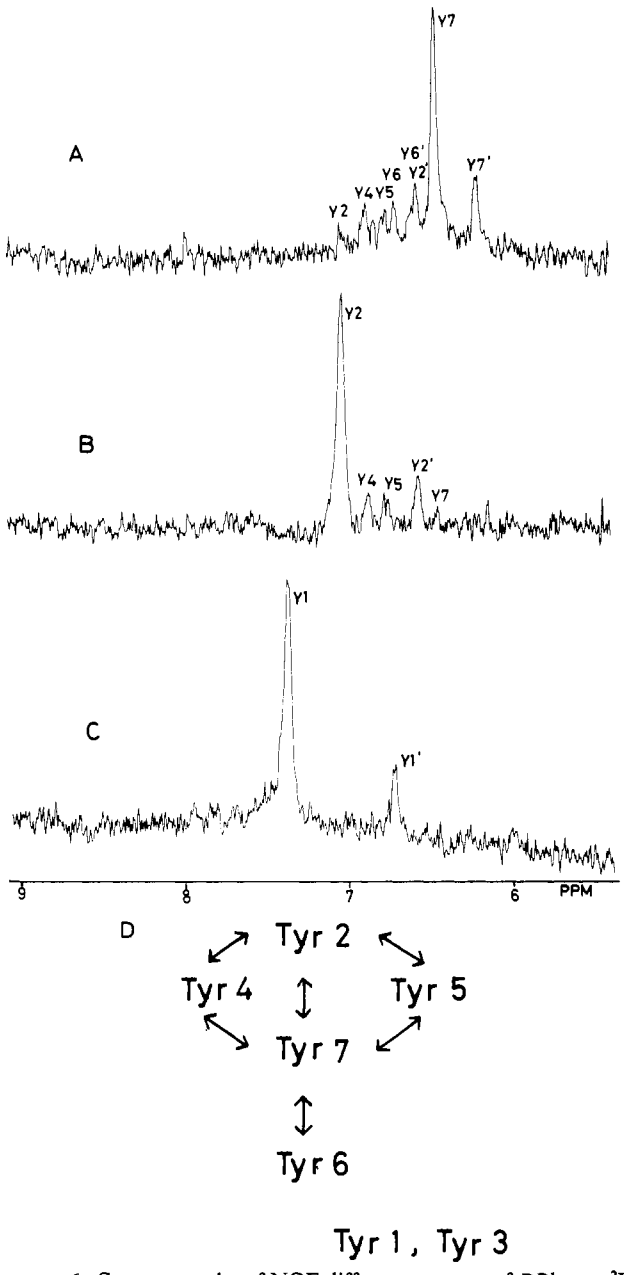

FIGURE 6: Some examples of NOE difference spectra of *DPhe* at p^{2}H 9.00. Y7, Y2, and Y1 were irradiated in (A), (B), and (C), respectively. (D) Proximity relationships between Tyr residues determined in NOE experiments. Arrows indicate Tyr residues affected by irradiation. Tyr-1 to Tyr-7 represent Tyr residues labeled Y1 or Y1′ to Y7 or Y7′, respectively. Tyr-1 and Tyr-3 are not in close proximity to the other Tyr residues.

stitution of Glu by Met at position 49. A change in the signal(s) assigned to the Phe residue at about 7.1 ppm (labeled FB in Figure 7) was also observed.

The pK_a value of Glu at position 49 in the wild type (*Glu49*) has been reported to be 7.5 on the basis of the results of two-dimensional electrophoresis (Yutani et al., 1984). The ^{1}H NMR spectrum in the aromatic region of the wild type (*Glu49*) at p^{2}H 8.99 is compared with that of *Met49* at p^{2}H 8.86 in Figure 8. The Glu residue at position 49 of the wild type (*Glu49*) should be ionized at these p^{2}Hs. In addition to the changes observed at acidic p^{2}H, many resonances due to Phe residues and some due to Tyr residues were affected at p^{2}H 9, indicating that the effect of the substitution propagated over other residues on the ionization of Glu-49. As shown in Figure 9, the p^{2}H dependence of the chemical shifts of one Phe and one Tyr residue of the wild type (*Glu49*) differed from

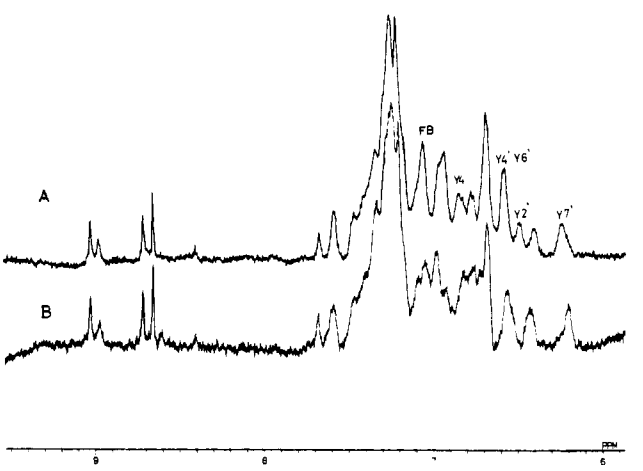

FIGURE 7: Comparison of the proton NMR spectra in the aromatic region of wild-type and mutant α-subunits at acidic p^{2}H: (A) wild type (*Glu49*) at p^{2}H 4.32; (B) *Met49* at p^{2}H 4.36. Peak FB is the Phe proton resonance. Peaks Y4, Y2′, Y4′, Y6′, and Y7′ are due to Tyr proton resonances.

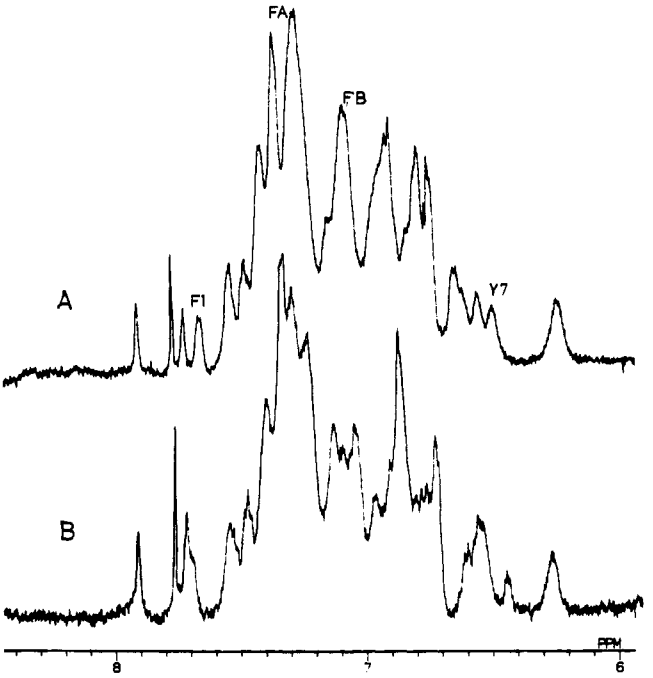

FIGURE 8: Comparison of the proton NMR spectra in the aromatic region of wild-type and mutant α-subunits at alkaline p^{2}H: (A) wild type (*Glu49*) at p^{2}H 8.99; (B) *Met49* at p^{2}H 8.86. Peaks F1, FA, and FB are due to Phe proton resonances. Peak Y7 is due to the Tyr proton resonance.

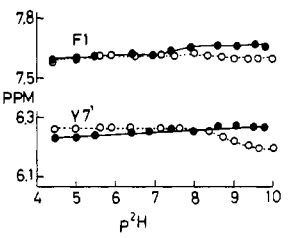

FIGURE 9: p^{2}H dependence of one Phe resonance and one Tyr resonance in the aromatic region of the wild type (*Glu49*) and *Met49*. Open and filled circles indicate the wild type (*Glu49*) and *Met49*, respectively. F1 represents the proton resonance assigned to the Phe in the lowest field in the aromatic region. Y7′ represents a Tyr proton resonance, as shown in Figure 5.

those of *Met49*. The transition point of F1 of *Met49* was at about p^{2}H 7.5, although F1 of the wild type (*Glu49*) did not show p^{2}H dependence. On the other hand, Y7′ of *Met49* did

not change, as shown in Figure 9, and the transition point of Y7′ of the wild type (*Glu49*) was at about p^{2}H 8.8. These differences should be due to the substitution of Glu at position 49 by Met.

Discussion

Assignments of ^{1}H NMR Resonances of a Protein by Single Amino Acid Substitutions. Ho et al. (1986) succeeded in assigning the resonance of Trp-384 of lactate dehydrogenase, using a mutant protein with Tyr substituted for the Trp at position 384. We also used a mutant protein obtained by site-directed mutagenesis to assign the ring-proton signals of His-92 and -146. In order to use a mutant protein for such an assignment, the substitution of an amino acid should not affect the spectrum significantly except for the signals of the substituted residue. Since signal H1 in Figure 1A has a normal pK_a for a His residue, the residue responsible for H1 should preferably be substituted for the assignment. Thr was chosen as the amino acid to be substituted because it may cause the smallest structural and electrostatic perturbations. Fortunately, H1 disappeared without any significant spectral change when His-92 was replaced by a Thr residue. The effect of the substitution on the spectrum in the aromatic region was slight, as had been anticipated. The same method should work for the assignments of peaks H3 and H4. The aromatic signals of Tyr and Phe of the α-subunit overlapped, and thus it is difficult to assign their resonances to individual amino acid residues. In such a case, deuteriation at a specific amino acid residue was shown to be useful. Proton resonance signals in the aromatic region of the α-subunit deuteriated at the phenylalanyl rings (*DPhe*) showed good resolution at acidic p^{2}H (Figure 5). Now it is possible to apply single amino acid substitutions for the assignment of some of these resonances to specific Tyr residues in the sequence.

Effect of Substitution at Position 49 on the Proton Resonances of the α-Subunit. The substitution by Met at position 49 had significant effects on ^{1}H NMR resonances in the aromatic region. That is, (1) at acidic p^{2}H, four Tyr residues (five resonances) and more than one Phe resonance were affected by the substitution. The four Tyr residues were Tyr-2, Tyr-4, Tyr-6, and Tyr-7, which are close to one another, as found in NOE experiments (Figure 6D). (2) At alkaline p^{2}H, the effect of the substitution was much more significant than that at acidic p^{2}H. In particular, many resonances of Phe residues were affected in addition to the changes at acidic p^{2}H. This seems to be due to the ionization of Glu at position 49 of the wild-type α-subunit. (3) The substitution affected the pK_a values of two His C2 protons and the p^{2}H dependence of at least one Phe and one Tyr proton resonance. The two His residues affected are located in the same domain (α-1) that contains the substituted position. These results suggest that the effect of the substitution is restricted to the neighborhood of position 49 at acidic p^{2}H, but affects a larger part of the protein at alkaline p^{2}H.

Significant changes in the aromatic region of the ^{1}H NMR spectrum agreed with the report that circular dichroism (CD) spectra in the aromatic region of mutant proteins at position 49 of the tryptophan synthase α-subunit are considerably different from that of the wild-type one at pH 7.0 (Yutani et al., 1980a). On the other hand, CD spectra in the region of 200–250 nm of the mutant proteins are indistinguishable from that of the wild type (*Glu49*) (Yutani et al., 1978), indicating that the backbone conformations of the mutant and wild-type proteins are similar.

Our conclusions from NMR results about the spatial relationships between Glu-49 and tyrosyl residues in the α-subunit are supported by recent preliminary results obtained from X-ray crystallographic analysis of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* [Hyde et al. (1987) and personal communication]. The comparison of the α-subunits from *E. coli* and *S. typhimurium* appears to be valid since the sequences of both enzymes are similar and since all seven tryosyl residues and Glu-49 are in an α-subunit from both species. The X-ray analysis shows that Glu-49 is located near the center of an 8-fold α/β barrel and close to Tyr-173 and Tyr-175. The side-chain atoms of Glu-49 may be found to form van der Waals contacts with both Tyr-173 and Tyr-175 upon further refinement of the structure. A third tyrosine, Tyr-4, contacts Tyr-173. Tyr-102 and Tyr-115 are located in the hydrophobic interior. Two other tyrosines, Tyr-169 and Tyr-203, appear to be exposed to solvent at the surface of the α-subunit.

Effect of the Ionization of Glu-49 on the Proton Resonances in the Aromatic Region. The difference in p^{2}H dependence of the chemical shifts of the F1 and Y7′ peaks between the two proteins (Figure 9) should be associated with the ionization of Glu-49 of the wild type (*Glu49*). The transition point of the chemical shift of F1 of *Met49* was at about 7.5, which is close to the pK_a of Glu-49 of the wild type (*Glu49*). On the other hand, F1 for the wild type (*Glu49*) showed little p^{2}H dependence. This may mean that the Phe residue of *Met49* is affected by the ionization of some other residue (presumably His-146), and the effect on the Phe residue of the wild type (*Glu49*) is apparently suppressed by the several shifts induced by the ionization of Glu-49. On the other hand, the Y7′ peak of the wild type (*Glu49*) showed a transition point at p^{2}H 8.8, but that of *Met49* was pH independent (Figure 9). This indicates that the ionization of Glu-49 lowers the pK_a of another residue, which is higher than p^{2}H 10 for *Met49*.

Role of Glu Residue at Position 49 in the Conformational Stability of the Wild-Type α-Subunit. Nineteen mutant proteins, with substitutions at position 49 of the α-subunit, have been prepared, and the enzymatic activities of all these mutant proteins have been examined (Yutani et al., 1987a). All of them lost their catalytic activity in the process of the formation of Trp from indole-3-glycerol phosphate, indicating that the Glu residue at position 49 of the α-subunit plays a crucial role in the catalysis. It has been also reported that the conformational stability of mutant proteins at position 49 increases linearly with the hydrophobicity of the substituting residues (Yutani et al., 1984, 1987b). Position 49 must play an important role in both the function and stability of the protein.

Although the conformational stability of the wild type (*Glu49*) is similar to that of *Met49* in the acidic region, that of the wild type (*Glu49*) becomes remarkably less stable than that of *Met49* in the alkaline region [Yutani et al. (1980b) and Table I in Yutani et al. (1982)]. This is in good agreement with the present results. The difference in the NMR spectra in the aromatic region between the wild type (*Glu49*) and *Met49* was restricted to the neighborhood of position 49 in the acidic region, but a larger part of the protein was affected in the alkaline region. A conformational change induced at an alkaline p^{2}H would destabilize the wild type (*Glu49*). Actually, the unfolding Gibbs energy of this protein in water is 8.8 kcal/mol at pH 7.0 and 4.9 kcal/mol at pH 9.0 (Yutani et al., 1982). This destabilization would be associated with the ionization of Glu-49 (pK_a = 7.5). As the Glu residue is buried in the interior of the molecule (Ogasahara et al., 1980), the ionization of the buried residue must destablize the protein. However, the only Tyr residue that was apparently perturbed in the same p^{2}H region was Y2. Further changes took place

through the protonation of other residue(s), which was induced by the ionization of Glu-49. Accordingly it is suggested that the destabilization of the wild type (*Glu49*) in the alkaline region was the result of the ionization and/or deionization of specific residues, which was initiated by the ionization of Glu-49.

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Registry No. His, 71-00-1; Tyr, 60-18-4; Met, 63-68-3; Glu, 56-86-0; tryptophan synthase, 9014-52-2.

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