Site-Directed Mutagenesis in Hemoglobin: Functional and Structural Role of the Penultimate Tyrosine in the α Subunit†

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Received August 24, 1993; Revised Manuscript Received November 24, 1993®

ABSTRACT: The penultimate tyrosine in the hemoglobin subunit is considered to be one of the most important residues for the normal structure and function of hemoglobin. To elucidate the functional and structural role of the penultimate residue in the α-subunit, we prepared new artificial mutants; Hb Y140αQ, in which Tyr-140α is replaced by a nonaromatic residue, Gln, and Hb Y140αF, which loses its hydrogen bond to Val-93α by the substitution of Phe for Tyr. HB Y140αQ exhibited a markedly increased oxygen affinity and almost completely diminished cooperativity, whereas Hb Y140αF showed similar but less extensively impaired function, indicating that the aromatic residue at the penultimate position in the α-subunit contributes to the stabilization of the T-quaternary structure as does the corresponding residue in the β-subunit. However, the deoxygenated forms of these mutants bear significant T-state character in their spectroscopic properties observed at high protein concentrations. The tetramer–dimer equilibrium data of the mutants suggested that a significant part of the functional alterations observed for dilute solution appears to result from partial dissociation into $\alpha\beta$ dimers rather than direct destabilization of the T-quaternary structure in the deoxygenated form. Therefore, we can conclude that the penultimate tyrosine in the α-chain plays a key role not only in the stabilization of the T-state but also in the subunit assembly. Such ease of dissociation from tetramer to dimers by amino acid substitution at the penultimate position did not occur in the β-subunit mutant, implying different structural and functional roles of the penultimate tyrosine between the α- and β-subunits.

In all the vertebrate hemoglobins and myoglobins whose primary structures have been elucidated, the penultimate position, HC2, is invariably occupied by tyrosine. An important role has been assigned to this residue by Perutz in his comprehensive model on the relationship between the structure and function of hemoglobin (Perutz, 1970; Perutz, 1989). The penultimate tyrosine residues of the α- and β-subunits participate indirectly, through bound solvent interactions, in a network of polar and charge contacts that include the N-terminus of the opposite subunits (Fermi, 1975; O'Donnell et al., 1979). The natural mutants which have the mutation at the penultimate tyrosine in the β-subunit, Tyr-145β, such as Hb Oslar (Tyr-145β → Asp) (Charache et al., 1975), Hb Bethesda (Tyr-145β → His) (Hayashi & Stamatoyannopoulos, 1972), Hb Rainier (Tyr-145β → Cys) (Hayashi & Stamatoyannopoulos, 1972), and Hb McKees Rocks (Tyr-145β → terminated) (Winslow et al., 1976), fail to retain the T-state conformation in the deoxygenated form, resulting in the extremely high oxygen affinities and absence of cooperativity. Des-(His-146β,Tyr-145β) Hb, in which the terminal histidine and penultimate Tyr are removed by carboxypeptidase, also showed a marked increase in oxygen affinity and greatly diminished cooperativity (Bonaventura et al., 1972). Previously, using the genetic engineering approach, we synthesized a β-chain mutant hemoglobin having the mutation site at the HC2 position (Hb Y145βF; Tyr-145β → Phe) in *Escherichia coli* to investigate the structural and functional role of the penultimate tyrosine (Ishimori et al., 1992). The substitution of Phe for Tyr at the 145β position revealed that the presence of a benzene ring at the 145β site plays a key role in stabilizing the deoxy T-structure and the hydrogen bonds between Tyr-145β and Val-98β stabilized the transition state between the R- and T-states (Togi et al., 1993).

Although the natural and artificial mutants having the amino acid substitution at the HC2 position in the β-subunits have been investigated by a number of researchers, no corresponding α-subunit mutants had been found in more than 500 natural variants until Hb Rouen, in which the penultimate tyrosine is replaced by histidine, was reported (Wajcman et al., 1992). As compared to Hb Bethesda (Tyr-145β → His), a variant with the same amino acid substitution at the penultimate position of the β-subunit (Hayashi & Stamatoyannopoulos, 1972; Bunn et al., 1972), more moderate alterations of the oxygen binding properties for Hb Rouen were observed, suggesting that there are functional and structural differences of the penultimate tyrosine between the α- and β-subunits. However, detailed functional and structural

† This work was supported in part by Research Grants 04225103, 04858073, and 05670043 from the Ministry of Education, Science and Culture of Japan [to I.M., K.I. (Kyoto University) and K.I. (Osaka University)].

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® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

0006-2960/94/0433-2546$04.50/0

roles of the penultimate tyrosine have not yet been clarified since the natural mutant could not be obtained in an amount sufficient for various physicochemical experiments.

In the present study, we report herein two new artificial α-chain mutant hemoglobins which have an amino acid substitution at the 140α(HC2) site and are compared with the corresponding β-chain mutants. The mutants prepared in this study are Hb Y140αF (Tyr-140α → Phe) and Hb Y140αQ (Tyr-140α → Gln).[1] Hb Y140αF has a phenylalanine residue at the penultimate position, and the hydrogen bond between Val-93α and the penultimate residue is missing. We can compare this mutant with the corresponding mutant in the β-subunit, Hb Y145βF, to elucidate the functional and structural difference of the penultimate tyrosine in the α- and β-subunits. In another mutant, Hb Y140αQ, one of the nonaromatic amino acid residues, glutamate, is located at the site to investigate the steric effects of the tyrosine residue. We investigated the functional and structural consequences of these mutations by measuring oxygen binding equilibrium, UV region derivative and difference spectra, resonance Raman scattering, and proton nuclear magnetic resonance spectra.

MATERIALS AND METHODS

Preparation of Mutant Hemoglobins. Hb Y140αF and Hb Y140αQ were synthesized as cleavable fusion proteins in *Escherichia coli* cells by using an expression vector cIIFXβ-globinFXα-globin as described in the previous paper (Nagai & Thøgersen, 1984; Nagai et al., 1985; Tame et al., 1991; Ishimori et al., 1989; Imai et al., 1991; Ishimori et al., 1992). The fraction of methemoglobin contained in oxygenated preparations was 2.2% and 2.8% for Hb Y140αF and Hb Y140αQ, respectively. Hb A and the native β-chain were prepared from human red blood cells as described previously (Ishimori & Morishima, 1986).

Fast-Atom-Bombardment Mass Spectrometry. In the endoproteinase Asp N digest of the α-subunit, Tyr-140α is in peptide D9 (Crestfield et al., 1963; Hirs, 1967; Drapeau, 1980). The protonated molecular ion for this peptide of the native α-chain is expected to be observed at m/z 1813.9 (Wada et al., 1989). In Figure 1, the peptide D9 was detected at m/z 1799.0 and at m/z 1780.0 for Hb Y140αF (Figure 1A) and for Hb Y140αQ (Figure 1B), respectively, corresponding to the expected amino acid substitution. The molecular weights of other peptides were normal in both mutations.

Oxygen Equilibrium Experiments. Oxygen equilibrium curves were measured by using an automatic oxygenation apparatus (Imai, 1981, 1982) and analyzed according to the Monod–Wyman–Changeux (MWC) model (Monod et al., 1965). The concentration of the protein was 60 μM on a heme basis, and the buffers used were 50 mM bis-Tris·HCl containing 0.1 M Cl^- for pH 7.4 and 6.9, and 50 mM Tris·HCl containing 0.1 M Cl^- for pH 7.9 and 8.4. All measurements were carried out at 25 °C. The metHb content of the hemoglobin samples as measured immediately after the oxygen equilibrium experiment ranged from 2.6% to 12.7%.

Absorption Spectra. The visible and UV region absorption spectra were recorded on a double-beam spectrophotometer, Model 320L (Hitachi, Tokyo). The UV region derivative spectra were recorded with a first-derivative mode of the spectrophotometer. The UV oxy-minus-deoxy difference spectra were measured as described previously (Imai et al., 1972). Deoxyhemoglobin samples (in 55 μM on a heme basis) were prepared in a versatile tonometer (Benesch et al., 1965) by repeated evacuation and flushing with pure nitrogen (99.995%). The metHb content was determined by using the millimolar absorption coefficient values at 560, 576, and 630 nm (van Assebdelft & Zijlstra, 1975). The metHb contents of the oxyhemoglobin samples were found to be 3.2% for Hb Y140αF and 11.4% for Hb Y140αQ. The deoxy samples contained 14.7% and 11.4% metHb for Hb Y140αF and Hb Y140αQ, respectively.

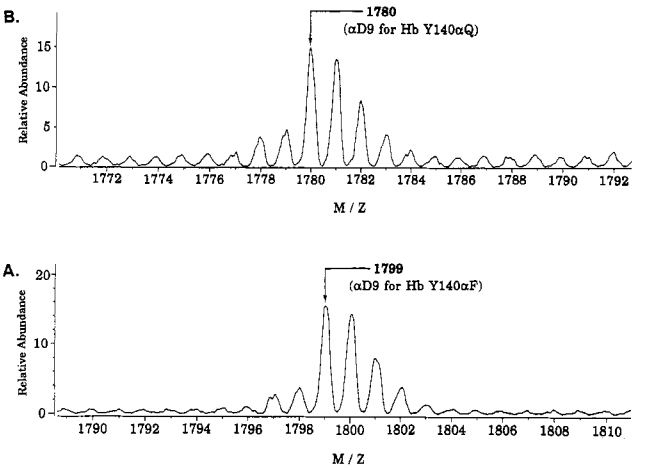

FIGURE 1: Fast-atom-bombardment mass spectrometry of digests of α-chain from mutant Hbs. Ordinate, relative abundance; abscissa, mass to charge ratio. Portions enlarged around the peaks for the D9 peptide of the α-chain are shown. Traces A and B show appearance of peaks at m/z = 1799.0 and 1780.0, which indicates successful replacement of Tyr140α by Phe and Gln, respectively.

Resonance Raman Spectra. Raman scattering was excited by the 441.6-nm line of a He/Cd laser (Kinmon CDR 80 SG, Tokyo, Japan) and was recorded on a JEOL-400D Raman spectrometer equipped with a cooled RCA31034a photomultiplier. The laser power was 60 mW at the sampling point, and the spectral slit width was 150 μm. The measurement was carried out at 20 °C, and the buffer used was 50 mM bis-Tris, pH 7.4, containing 0.1 M Cl^-. The protein concentration was 100 μM on a heme basis. The frequency calibration of the spectrometer was performed with CCl_4 as a standard.

NMR Spectra. ^{1}H-NMR spectra at 500 MHz were recorded on a GE OMEGA 500 spectrometer equipped with a SUN 3 workstation. Hyperfine-shifted NMR spectra were obtained with an 8K data transform of 125 kHz and a 6.7-μs 90° pulse by using a conventional WEFT pulse sequence (180–τ–90 acquire) in order to minimize the strong solvent resonance in H_2O solution. A careful setting of the τ value (typically 120–130 ms) can completely eliminate the H_2O signal under rapid repetition of the sequence. We also used a Redfield 2-1-X pulse sequence with 29.5-μs pulse and 8K data points over a 15-kHz spectral width for recording the exchangeable proton resonances for the subunit interfaces of hemoglobin. The probe temperature was determined as 20 ± 0.5 °C by the temperature control unit of the spectrometer. The concentration and volume of the sample in 50 mM bis-Tris 100 mM Cl^- (pH 7.4) were 1.3 mM and 300 μL, respectively (Ishimori & Morishima, 1986, 1988a,b).

Dimer–Tetramer Equilibrium. Measurements of the dissociation into the dimer in the CO form were made by gel fraction on a Sephacryl S-200 HR column (1.0 × 44 cm) (Valdes and Ackers, 1977). The buffer used was 50 mM Tris 100 mM Cl^- pH 7.4 and saturated with CO. The temperature

[1] Abbreviations: Hb A, human hemoglobin A; Hb Y140αF, Hb (Tyr-140α→Phe); Hb 140αQ, Hb (Tyr-140α→Gln); NMR, nuclear magnetic resonance; IHP, inositol hexaphosphate.

Table 1: Values of Oxygen Equilibrium Parameters for Native and Mutant Hemoglobins

conditions[a]	P_{50}[b] (mmHg)	P_{50}^{A}/P_{50}^{X}[c]	$P_{50}^{IHP}/P_{50}^{free}$[d]	δH^+[e]	n_{max}[f]	K_T[g] ($mmHg^{-1}$)	K_R[g] ($mmHg^{-1}$)	L_0[h]	L_4[h]
				Hb Y140αQ					
pH 6.9	0.44	17			1.11	1.4	3.0	2.2	1.0×10^{-1}
pH 7.4	0.38	12		−0.18	1.11	1.4	3.3	1.7	5.5×10^{-2}
pH 7.9	0.31	8.4			1.00	3.3	3.0	0.54	7.9×10^{-1}
pH 8.4	0.33	7.0			1.15	2.3	3.1	0.25	7.6×10^{-2}
pH 7.4 + 2 mM IHP	2.8	19	7.4		1.72	0.30	5.2	1.4×10^{4}	1.6×10^{-1}
				Hb Y140αF					
pH 6.9	1.0	7.6			1.79	0.47	4.4	3.0×10^{2}	3.9×10^{-2}
pH 7.4	0.51	8.6		−0.21	1.53	0.66	4.5	2.3×10	1.1×10^{-2}
pH 7.9	0.40	6.5			1.43	0.81	4.2	7.3	1.0×10^{-2}
pH 8.4	0.35	6.6			1.27	1.5	5.0	9.3	7.5×10^{-2}
pH 7.4 + 2 mM IHP	5.0	10	9.8		1.80	0.10	1.5	2.4×10^{3}	4.7×10^{-2}
				Hb A					
pH 6.9	7.6				3.01	0.020	4.6	1.5×10^{6}	5.4×10^{-4}
pH 7.4	4.4			−0.46	3.13	0.028	4.6	1.5×10^{5}	2.1×10^{-4}
pH 7.9	2.6				2.89	0.067	5.7	4.2×10^{4}	8.0×10^{-4}
pH 8.4	2.3				2.99	0.069	6.0	3.1×10^{4}	5.4×10^{-4}
pH 7.4 + 2 mM IHP	52		12		2.42	0.0069	1.3	1.3×10^{7}	1.0×10^{-2}

[a] Other experimental conditions: Hb concentration, 60 μM on a heme basis; in 0.05 M Tris (pH ≥ 7.9) or 0.05 M bis-Tris (pH ≤ 7.4) containing 0.1 M Cl^-; 25 °C. [b] Partial pressure of oxygen at half-saturation (in mmHg). [c] Ratio of P_{50} for Hb A to P_{50} for mutant Hb. [d] Ratio of P_{50} in the presence of 2 mM IHP to P_{50} in its absence. [e] Bohr coefficient ($=(\Delta \log P_{50})/\Delta pH$) measured between pH 7.4 and 7.9. [f] Maximal slope of the Hill plot (the Hill coefficient). [g] Oxygen association equilibrium constant for the T-state (K_T) and R-state (K_R). [h] The allosteric constant of deoxy species ($L_0 = T_0/R_0$) or of fully oxygenated species ($L_4 = T_4/R_4 = L_0(K_T/K_R)^4$).

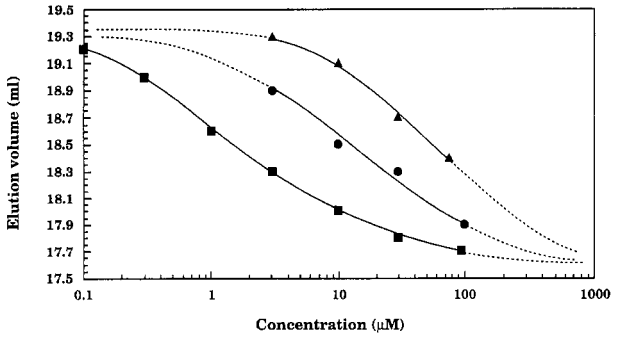

FIGURE 2: Centroid elution volumes of the leading boundaries of hemoglobin and mutants as a function of protein concentration: ■, Hb A; ●, Hb Y140αF; ▲, Hb Y140αQ. The buffer used: 0.05 M bis-Tris 0.1 M Cl^-; 20 °C.

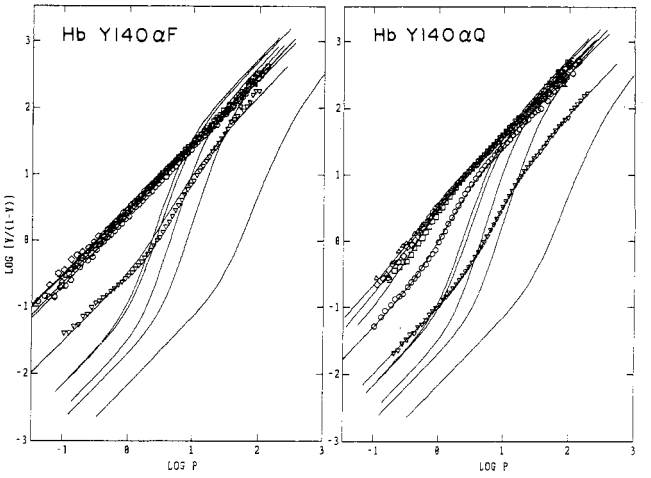

FIGURE 3: Hill plots of oxygen binding by Hb Y140αQ (right) and Hb Y140αF (left): Y, fractional saturation of hemoglobin with oxygen; P, partial pressure of oxygen (mmHg). Symbols express observed points, and lines were calculated from the best-fit MWC parameter values (Table 1): (○) pH 8.4; (□) pH 7.9; (◇) pH 7.4; (△) pH 6.9; (▼) pH 7.4 containing 2 mM IHP. Other experimental conditions are given in the footnote to Table 1. Lines without symbols express the Hill plots for Hb A, and the pH and IHP conditions for each plot from left to right correspond to those given above in the same order.

was regulated at 20 ± 1 °C during experiments. Buffer flow rates were controlled at 32.2 mL/h by a Model SJ1211L peristaltic pump (Atto, Tokyo). The column was calibrated with sperm whale myoglobin, cross-linked hemoglobin, and blue dextran. The monitoring wavelengths were 540 nm at higher protein concentrations (10, 30, and 100 μM on a heme basis) and 420 nm at lower protein concentrations (below 3 μM on a heme basis).

RESULTS

Subunit Dissociation. Figure 2 shows the centroid elution volumes of the leading boundaries of the 140α-mutants and Hb A. The fitting curves of the carbonmonoxy 140α-mutants shift to the right side, indicating that the dissociation into dimers was enhanced in these mutants. The tetramer–dimer dissociation constants, K_D, were estimated as 0.8, 7, and 29 μM for Hb A, Hb Y140αF, and Hb Y140αQ, respectively.

Oxygen Equilibrium Curves. Figure 3 shows the Hill plots for Hb A and mutants at four different pH values without phosphate and at pH 7.4 in the presence of 2 mM inositol hexaphosphate (IHP). Each plot for these mutants is shifted markedly toward the left compared to the corresponding plot for Hb A. The maximal slopes of the plots (n_{max}) for Hb Y140αF are decreased compared with those for Hb A, and the plots for Hb Y140αQ without IHP show slopes close to unity. The best-fit MWC values obtained from the oxygen equilibrium curves are listed in Table 1,[3] and the values of overall oxygen affinity (log P_{50}) and n_{max} are plotted against pH values as shown in Figure 4. The overall oxygen affinity of Hb Y140αF is 6.6–10 times higher than that of Hb A, and HbY140αQ showed 7.0–19 times higher oxygen affinity than Hb A. The values of n_{max} are markedly decreased in Hb

[2] Viggiano et al. (1978) assigned the 6.4 ppm resonance to the hydrogen bond between Val-98β and Tyr-145β. It also has been pointed out by them that this assignment is tentative and needs further verification. On the basis of the NMR spectra of artificial mutant hemoglobins such as Hb W37βF (Trp-37β → Phe) and Hb(Y145β → Phe), we concluded that the 6.4 ppm resonance is due to the intersubunit hydrogen bond between Asp-94α and Trp-37β (Ishimori et al., 1992).

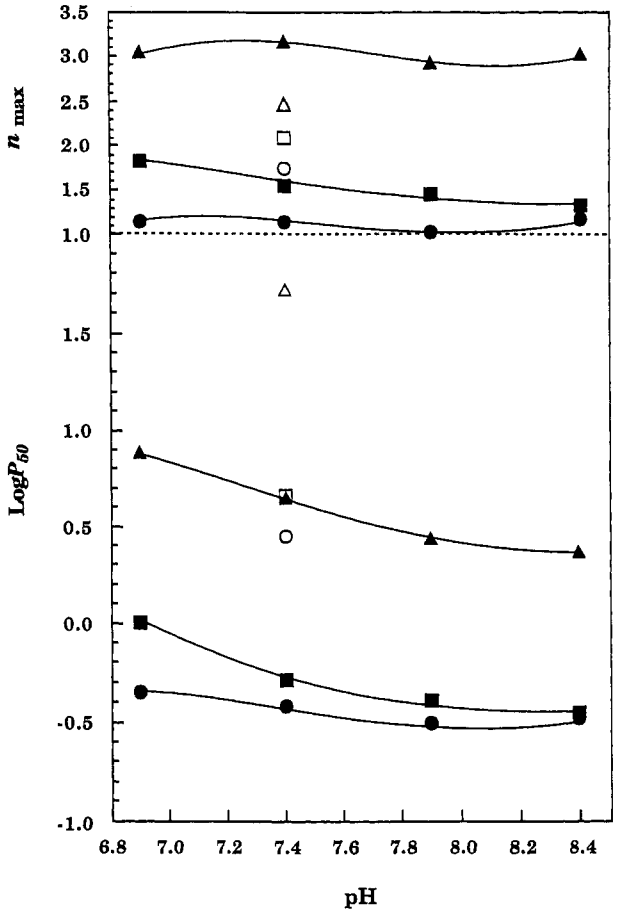

FIGURE 4: pH dependence of overall oxygen affinity (log P_{50}) and the Hill coefficient (n_{max}). The data given in Table 1 are plotted: ■ and □, Hb Y140αF; ● and ○, Hb Y140αQ; ▲ and △, Hb A. Closed symbols, in the absence of IHP; open symbols, in the presence of 2 mM IHP.

Y140αF, and the oxygen binding of Hb Y140αQ without IHP is essentially noncooperative. The Bohr effect (δH^+) at pH 7.4 for Hb Y140αQ and Hb Y140αF is reduced by 61% and 53%, respectively, compared with that in Hb A. However, the IHP effect on the overall oxygen affinity is almost normal in Hb Y140αF as shown in Figure 4. Hb Y140αQ also exhibits a significant IHP effect. The K_T values for Hb Y140αF are roughly 1 order of magnitude larger than those of Hb A without IHP, and Hb Y140αQ shows a 33–70-fold increase in the K_T values compared with Hb A, while these two mutants have K_R values similar to those for Hb A. The L_0 values for the mutants and Hb A become larger with decreases in pH and on the addition of IHP. The L_4 values for both of the mutants are larger than those for Hb A under each set of experimental conditions. The dependence of the L_0 value on IHP is much larger than that of the L_4 value in these mutants.

UV Spectra. Figure 5 illustrates the first derivative of the UV region absorption spectra for the oxy and deoxy forms.

[3] If we use the K_D values for the CO form in place of those for the oxy form, the dimer fractions of the oxygenated hemoglobin samples used for oxygen equilibrium experiments are estimated to be 11%, 29%, and 49% for Hb A, Hb Y140αF, and Hb Y140αQ, respectively. The dimer fractions of the deoxygenated samples would also be increased by mutation, although they would be much smaller than those given above (K_D for deoxy Hb A is approximately 10^6 times smaller than that for oxy Hb A). Under these circumstances, the observed properties of the mutant Hbs biased to the R-state, as indicated by the Monod–Wyman–Changeux (MWC) parameter values, must be attributed partly to the enhanced dimer formation, although it is not easy to separate the effect of the dimer formation from the real destabilization of the T-state.

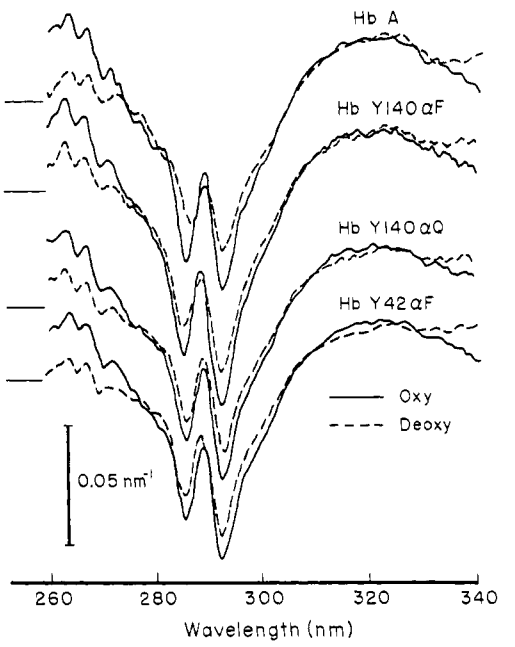

FIGURE 5: UV region derivative spectra of oxy- and deoxyhemoglobin. Experimental conditions: Hb concentration, 55 μM on heme basis; in 0.05 M bis-Tris (pH 7.4) containing 0.1 M Cl^-; 25 °C. The horizontal line attached to each set of oxy and deoxy spectra expresses the base line. The spectra of Hb Y42αF are references of high oxygen affinity Hb.

Hb A in both forms shows a characteristic fine structure composed of one maximum at 289 nm and two troughs at 285 and 293 nm. The magnitude of this fine structure around 290 nm is a good indicator of the quaternary structure of hemoglobin (Imai, 1973; Imai et al., 1989). The magnitude of this fine structure measured by the difference between the maximum and either of the minima is halved upon deoxygenation in Hb A. In the two mutants, however, the magnitude of the fine structure for deoxy Hb Y140αF is larger and that for deoxy Hb Y140αQ is much larger than that for deoxygenated Hb A, being close to that for the oxygenated form,[4] although the oxygenated mutants exhibit fine structure of the same magnitude as that for Hb A.

In Figure 6 are shown the UV region oxy-minus-deoxy difference spectra. The spectrum for Hb A has a notch-shaped fine structure with a minimum at 288 nm and a maximum at 290 nm. The magnitude of this fine structure around 290 nm represents the extent of the quaternary structural changes upon oxygenation (Briehl & Hobbs, 1970; Imai, 1973; Imai et al., 1972, 1981; Wajcman et al., 1982). The fine structure for Hb Y140αF was notably decreased in magnitude, and that for Hb Y140αQ almost disappeared, being a small shoulder superimposed on a broad band.

Resonance Raman Spectra. In Figure 7 are presented the low-frequency regions of the resonance Raman spectra for deoxygenated Hbs at pH 7.4 in the presence and absence of 2 mM IHP and at pH 6.9 without IHP. No significant differences between Hb A and the two mutants were observed in Raman lines for porphyrin vibrational modes at or above 300 cm^{-1}. However, at pH 7.4 without IHP, the resonance Raman line of the Fe–Nε stretching in Hb Y140αF (217 cm^{-1}) (trace D) was observed in a higher wavenumber region than that for deoxy Hb A (215 cm^{-1}) (trace A) (Nagai & Kitagawa, 1980; Nagai et al., 1980). Upon pH decreases

[4] In the light of enhanced dimer formation in the mutant Hbs, it is possible that some parts of the observed spectral differences between them and Hb A arose from the difference in dimer fractions.

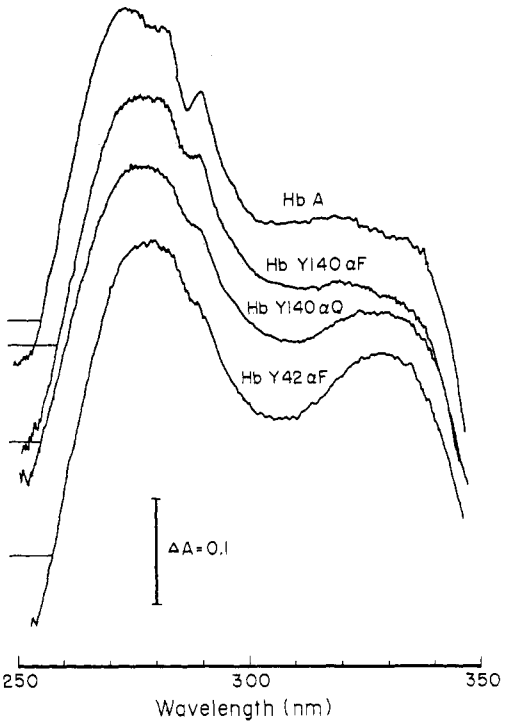

FIGURE 6: UV region oxy-minus-deoxy different spectra. Experimental conditions are as in Figure 5. The left-end horizontal line attached to each spectrum expresses the base line. The spectrum of Hb Y42αF is a reference of high oxygen affinity Hb.

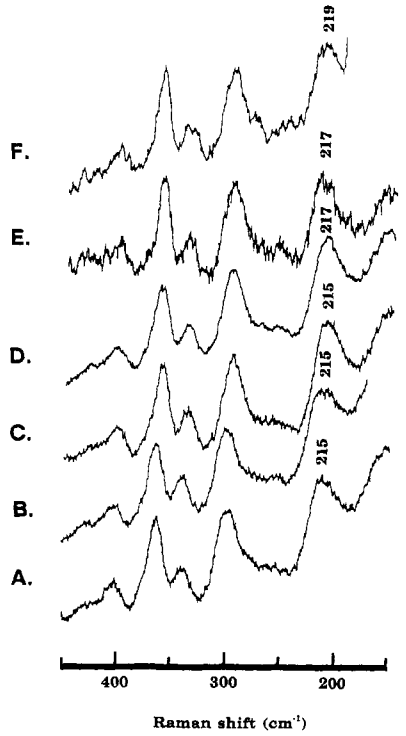

FIGURE 7: Resonance Raman spectra for deoxygenated Hb Y140αF, Hb Y140αQ, and Hb A: A, Hb A at pH 7.4; B, Hb Y140αF at pH 6.9; C, Hb Y140αF at pH 7.4 + 2 mM IHP; D, Hb Y140αF at pH 7.4; E, Hb Y140αQ at pH 7.4 + 2 mM IHP; F, Hb Y140αQ at pH 7.4. Experimental conditions: Hb concentration, 100 μM on a heme basis; in 0.05 M bis-Tris containing 0.1 M Cl^-; 20 °C. Numbers associated with the spectra indicate the wavenumber (cm^{-1}) of each Raman line.

from 7.4 to 6.9, the Fe–Nϵ stretching Raman line exhibited a frequency shift to 215 cm (traces B and D). The Raman line of the stretching mode for Hb Y140αQ (Trace F) was observed at 219 cm^{-1} without IHP, and no significant shift

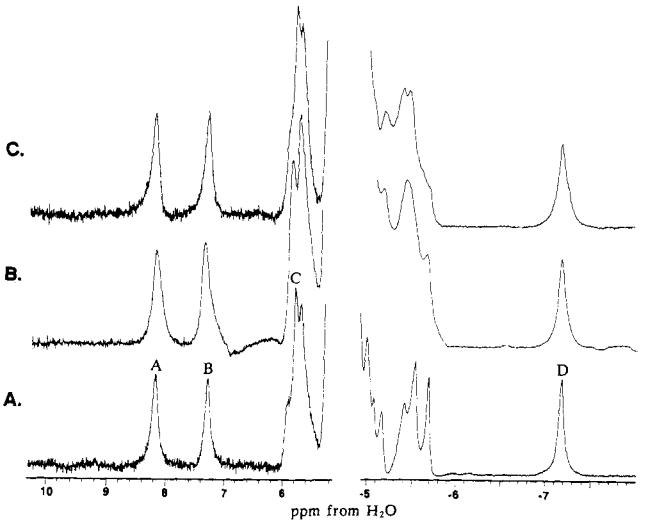

FIGURE 8: Proton NMR spectra (500 MHz) for oxygenated Hb Y140αF, Hb Y140αQ, and Hb A: A, Hb A; B, Hb Y140αF; C, Hb Y140αQ. Experimental conditions: Hb concentration, approximately 1.3 mM on a heme basis; in 0.05 M bis-Tris (pH 7.4) containing 0.1 M Cl^-; 21 °C. Peaks A–D are discussed in the text.

was observed upon pH decreases from 7.4 to 6.9 (data not shown). In the presence of IHP, the stretching modes of Hb Y140αF and Hb Y140αQ appeared at 215 and 217 cm^{-1}, respectively (traces C and E).

NMR Spectra. The NMR spectra in the oxygenated and deoxygenated mutants and Hb A are illustrated in Figures 8 and 9, respectively. The positions of prominent peaks for the mutants and Hb A are compiled in Table 2. As illustrated in Figure 8, the peak at −7.2 ppm (D) in oxygenated Hb A was assigned to the γ_1-methyl group of E11 Val in the α- and β-subunits (Lindstrom et al., 1972), which serves as a marker for the oxy tertiary structure of the heme vicinity. This peak in the mutants was observed at the same position as in Hb A. The resonances around −5.6 ppm in the two mutants were somewhat perturbed.

In the downfield region, the resonances at 8.2 (A) and 7.3 ppm (B) have been assigned to the exchangeable protons at the $\alpha 1\beta 1$ subunit interface, the hydrogen bond between Tyr-35β(C1) and Asp-126α(F9) (Asakura et al., 1976; Russu et al., 1987) and the hydrogen bond between His-103α(G10) and Asn-108β(G10) (Russu et al., 1987), respectively. These resonances were insensitive to the amino acid substitution at the penultimate position in the α-subunit. The exchangeable proton resonance at 5.9 ppm (C), which originated from the hydrogen bond between Asp-94α and Asn-102β (Fung & Ho, 1975) and has been used as an indicator of the R-quaternary structure, also remains almost unchanged upon the amino acid substitution, although the spectral patterns around the 5.9 ppm are a little perturbed by the mutation.

In deoxygenated Hb A (Figure 9), the peaks at 9.4 (I) and 6.4 ppm (L) are the characteristic markers of the deoxy T-quaternary structure. The former resonance has been assigned to the hydrogen bond between Tyr-42α and Asp-99β, and the latter to the hydrogen bond between Asp-94α and Trp-37β^2 (Fung & Ho, 1975; Russu et al., 1987; Ishimori et al., 1992). These resonances reduced their signal intensities upon the amino acid substitution at the position of Tyr-140α. The peaks at 7.4 (K) and 8.2 ppm (J) maintained their signal positions and intensities in these two mutants, although the 7.4 ppm resonance of Hb Y140αF is slightly split.

The hyperfine-shifted resonances at 58.3 and 71.5 ppm from H_2O have been assigned to the $N_\delta H$ proton of the proximal

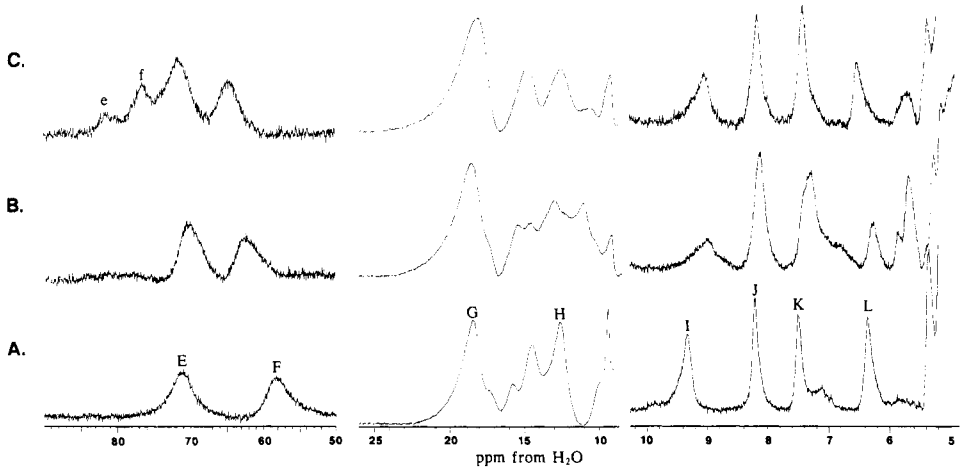

FIGURE 9: Proton NMR spectra (500 MHz) for deoxygenated Hb Y140αF, Hb Y140αQ, and Hb A: A, Hb A; B, Hb Y140αF; C, Hb Y140αQ. Experimental conditions are as in Figure 8. Left: hyperfine-shifted proton resonances of proximal His $N_\delta H$. Middle: hyperfine-shifted proton resonances of heme methyl groups. Right: hydrogen-bonded proton resonances. Peaks E–L, e, and f are discussed in the text.

Table 2: Proton Resonance Positions (in ppm from H_2O Signal) of Hb A, Hb Y140αF, and Hb Y140αQ

	oxygenated form				deoxygenated form							
	hydrogen bonds			Val-67α,β γ_1-methyl	proximal His $N_\delta H$		heme methyl		hydrogen bonds			
Hb Y140αQ	8.2	7.3	5.9	−7.2	72.0 (81.5)[n]	65.2 (76.7)[n]	18.2		9.1	8.1	7.4	6.5
Hb Y140αF	8.2	7.3	5.9	−7.2	70.5	62.5	18.7		9.0	8.1	7.3[m]	6.3
Hb A	8.2[a]	7.3[b]	5.9[c]	−7.2[d]	71.5[e]	58.3[f]	18.5[g]	12.7[h]	9.4[i]	8.2[j]	7.4[k]	6.4[l]
peak	A	B	C	D	E (e)[n]	F (f)[n]	G	H	I	J	K	L

[a] Hydrogen-bonded proton between Asp126(H9)α_1 and Tyr35(C1)β_1 (Russu et al., 1987). [b] Hydrogen-bonded proton between Asp94(G1)α_1 and Asn102(G4)β_2 (Fung & Ho, 1975). [d] γ_1-Methyl group of Val62(E11)α and Val67(E11)β (Lindstrom et al., 1972). [e] Proximal His $N_\delta H$ of the β-subunit (Takahashi et al., 1980). [f] Proximal His $N_\delta H$ of the α-subunit (Takahashi et al., 1980). [g] Heme methyl group of the β-subunit (Takahashi et al., 1980). [h] Heme methyl group of the α-subunit (Takahashi et al., 1980). [i] Hydrogen-bonded proton between Tyr42(C7)α_1 and Asn99(G1)β_2 (Fung & Ho, 1975). [j] Hydrogen-bonded proton between Asp126(H9)α_1 and Tyr35(C1)β_1 (Russu et al., 1987). [k] Hydrogen-bonded proton between His103(G10)α_1 and Asn108(G10)β_1 (Russu et al., 1987). [l] Hydrogen-bonded proton between Tyr145(HC2)β and Val98(FG5)β (Viggiano et al., 1978) or Asp94(G1)α_1 and Trp37(C3)β_2 (Ishimori et al., 1992). [m] Split. [n] See text.

His of the α- and β-subunits in deoxy Hb A, respectively (Takahashi et al., 1980). The proximal histidyl $N_\delta H$ resonance of the α-subunit in Hb Y140αF was observed with a substantial downfield bias (F; 62.5 ppm) as compared with Hb A, while the resonance for the β-subunit in the mutant was observed at the same position (E; 70.5 ppm) as that of Hb A. In the spectrum of deoxy Hb Y140αQ, four hyperfine-shifted resonances were observed. Since the dissociation to dimers was highly enhanced in Hb Y140αQ as shown in Figure 4, it is likely that the multiplicity of the resonances of the $N_\delta H$ of the proximal His arises from partial dimer formation in HB Y140αQ.[5] Although the definitive resonance assignments have not yet been made, we can assign the major resonances at 65.2 (F) and 72.0 ppm (E) to the $N_\delta H$ proton of the α- and β-subunits in tetrameric Hb Y140αQ, respectively. The residual resonances at 76.7 (f) and 81.5 ppm (e) could originate from the α- and β-subunits of the dimeric mutants. The resonances of the heme methyl groups were also observed in the 5–20 ppm region (G, H) as shown in Figure 9 and are listed in Table 2. These resonances were also significantly perturbed by the mutation.

DISCUSSION

Oxygen Binding Properties of the Mutants. As known from the parameter values in Table 1, Hb Y140αQ showed a remarkably increased oxygen affinity, a reduced alkaline Bohr effect, and almost completely diminished cooperativity. Hb Y140αF also showed similar but less extensive functional impairments. A natural mutant, Hb Rouen (Tyr-140α → His), shows only mild functional impairments ($P_{50}^{HbA}/P_{50}^{HbRouen}$ = 1.4 and n_{max} = 2.1 in 50 mM bis-Tris 0.1 M Cl^- pH 7.2) (Wajcman et al., 1992). These observations, together with the enhanced dimer formation in Hb Y140αQ, imply that the presence of an aromatic amino acid residue at the penultimate position in the α-subunit stabilizes tetramer formation and the low oxygen affinity state in the deoxy form.

The functional restoration by the aromatic amino acid residue at the penultimate position is also manifested in the β-subunit. The natural mutants, in which Tyr-145β was replaced by nonaromatic amino acid residues, showed extremely high oxygen affinities and absence of cooperativity (Charache et al., 1975; Winslow et al., 1976), whereas only mild functional and structural alterations were observed in an engineered mutant having phenylalanine at the penultimate position (Ishimori et al., 1992). Thus, we can conclude that the interactions between the aromatic ring at the penultimate position and the F or H helices forming the "tyrosine pocket" in the α- and β-subunits actually contribute to cooperative oxygen binding in hemoglobin.

The $P^{IHP}_{50}/P^{free}_{50}$ values in Table 1 indicate that the effect of IHP on oxygen affinity is only mildly decreased in both the 140α-mutants. Previous studies have revealed that the IHP effect is almost completely diminished in hemoglobins with

[5] The K_D values for the CO form of Hb Y140αQ suggest that approximately 14% of the oxy form is dimeric. Although we could not get the K_D value in the deoxygenated state, it is likely that a significant amount of the tetramer dissociates to the dimers in the deoxygenated form.

very high oxygen affinity such as Hb Y42αF (Tyr-42α → Phe) and des-(His-146β,Tyr-145β) Hb. Hb Y42αF showed a very small $P^{IHP}_{50}/P^{free}_{50}$ value (1.6) (Ishimori et al., 1989; Imai et al., 1991). On the other hand, Hb W37βF gave an apparent example of a combination of very high oxygen affinity and a significantly conserved IHP effect. The high affinity and absence of cooperativity of this hemoglobin proved to be due to enhanced dissociation to dimers in the absence of IHP. Upon the addition of IHP, the dimers associated to form a tetramer, resulting in low oxygen affinity and partial restoration of cooperativity. The oxygen equilibrium experiments with higher protein concentrations indicated that the true functional alterations of tetrameric Hb W37βF would be moderate (Ishimori et al., 1992). As illustrated in Figure 2, the dissociation to the dimer in the 140α-mutants is markedly enhanced compared with that of Hb A. The partial functional restoration by IHP in the 140α-mutants implies that some part of the observed functional impairment would be attributed to the subunit dissociation as well as to the destabilization of the low-affinity state.

Tertiary and Quaternary Structure of 140α-Mutants. The NMR spectra of the mutants and Hb A revealed that the tertiary and quaternary structures of the oxygenated mutants are similar to those of oxygenated Hb A, although the dissociation to dimers was enhanced and some NMR resonances were slightly shifted or split as shown in Figure 8 and Table 2. The derivative spectra in the UV region of the oxygenated form are also unaffected by the mutations of the penultimate tyrosine.

In the deoxygenated form, however, some significant differences from Hb A were observed in the tertiary and quaternary structures of the mutants. The shift of the Fe–Nε stretching mode in the two mutants (Figure 7) indicates that the Fe–Nε bond in the deoxy mutants is less stretched than in Hb A. In the resonance Raman spectra, it is not easy to distinguish between the effect of dimer formation and that of structural perturbations in the deoxygenated tetramer. Instead, the structural perturbations in the deoxygenated tetramer are manifested in the NMR spectra. In the NMR spectra of Hb Y140αQ, we could observe the two sets of $N_\delta H$ signals from the dimer and tetramer. For Hb Y140αF, the dimers associate to the tetramer due to the high concentration, resulting in one set of signals of $N_\delta H$ of the tetramer. The downfield bias of these $N_\delta H$ contact shifts in the α-subunit of the tetrameric mutants implies that a strain imposed on the bond between the heme iron and the proximal His is released in favor of electron spin delocalization from the iron to the imidazole (Nagai et al., 1982; La Mar et al., 1977). These spectral changes concur with the results of high oxygen affinity hemoglobins in the deoxygenated state. In the hydrogen-bonded proton region, however, the two characteristic markers of the T-state, the resonances at 9.4 and 6.4 ppm, appeared in the NMR spectra of the mutants although their intensities were reduced and the resonance positions were shifted. The decrease in intensity of these T-state markers might be due to partial dissociation to dimers, and the shifts of these signals suggest that some structural changes have occurred around the hydrogen bonds. It should be noted also that the $N_\delta H$ signal arising from the unmodified β-subunit was insensitive to the amino acid substitution in the α-subunit. These spectroscopic data of the present mutants are different from those of the typical high oxygen affinity Hbs such as Hb Y42αF or des-(His-146β,Arg-141α) Hb (Ishimori et al., 1989; Imai et al., 1991; Nagai et al., 1982), which are considered to retain the R-structure upon deoxygenation. In the NMR spectra of deoxygenated Hb Y42αF and des (His-146β,Arg-141α) Hb, these T-state markers completely disappeared. Thus, the structural alteration of the present mutants in the deoxygenated state is localized around the mutation site, i.e., the C-terminal of the α-subunit, and we could consider that these mutants are in a perturbed T-state, at least a "T-like state", under deoxygenated conditions.

It is quite interesting that the stretching mode of the Fe–Nε bond in the deoxygenated Hb Y140αF shifted from 217 to 215 cm^{-1} in going from pH 7.4 to 6.9. The position of the Raman line from the Fe–Nε bond in Hb Y140αF at pH 6.9 was identical to that in Hb A, indicating that at acidic pH a strain of the same magnitude as that in deoxy Hb A was imposed on the Fe–Nε bond in deoxy Hb Y140αF. On the other hand, no significant shifts of the stretching modes were detected in the resonance Raman spectra of deoxygenated Hb Y140αQ at low pHs (data not shown). The downfield bias of the $N_\delta H$ resonance in the NMR spectrum of Hb Y140αF was also smaller than that in Hb Y140αQ. These spectroscopic data indicate that Hb Y140αF has more T-structure character than Hb Y140αQ, being consistent with the changes in their oxygen binding properties. These data also support the role of the aromatic ring at the penultimate position in stabilization of the T-state hemoglobin and agree with our previous conclusion in case of the β-subunit (Ishimori et al., 1992).

Different Role of the Penultimate Tyrosine between the α- and β-Subunits. As discussed above, the substitution of the amino acid at the penultimate position in the α-subunit enhanced the subunit dissociation from the tetramer to dimers. Such an enhancement of the dissociation to dimers was not experienced in the corresponding β-mutant, Hb Y145βF (Ishimori et al., 1992). The penultimate tyrosine of the α-chains is located near Trp-37β, which is one of the crucial residues stabilizing the tetrameric assembly of hemoglobin, and there is a van der Waals contact between Tyr-140α and Trp-37β in the $\alpha_1\beta_2$ subunit interface. Upon the replacement of the penultimate tyrosine of the α-chains, the van der Waals contact with the tryptophan is likely perturbed or lost, which in turn would cause the enhancement of the dissociation from the tetramer to dimers. On the other hand, the penultimate tyrosine of the β-subunit interacts with Thr-41α and, in the 35α–41α sites of the C helix, there are no aromatic amino acid residues which are expected to have important roles in the subunit assembly. Our recent paper (Hashimoto et al., 1993) also showed that the replacement of Thr-38α, which is spatially equivalent to Trp-37β in the β-subunit, caused no significant enhancement of the subunit dissociation, and functional and structural alterations accompanied by the mutation were also small.

The nonequivalent features of the penultimate tyrosine residues of the α- and β-subunits were also found in some natural mutants. In Hb Rouen (Tyr-140α → His), the alteration of the oxygen-binding properties is considerably less dramatic than in Hb Bethesda (Tyr-145β → His), whose oxygen-binding parameters are similar to those of the isolated subunits. The crystallographic and functional data (Wajcman et al., 1992) suggested that the side chain of the histidine at the penultimate position in the β-subunit is too short to make a hydrogen bond with the carbonyl group of valine at FG5, whereas the distance may be slightly shorter in the α-subunit, allowing a nearly normal structure by some interaction between the histidine and the residues forming the "tyrosine pocket". Thus, we can also suggest that the different roles of the penultimate tyrosine in the two subunits arise from the

differnce in the amino acid residues with which the tyrosine interacts in the $\alpha_1\beta_2$ subunit interface.

In summary, the aromatic amino acid residue of the penultimate position in the α-subunit has a role in stabilization of the deoxy T-state in hemoglobin as well as that in the β-subunit. However, the substitution of the penultimate tyrosine in the α-subunit also caused remarkable enhancement of the subunit dissociation from tetramer to dimers in hemoglobin, resulting in an extremely high oxygen affinity and almost completely diminished cooperativity. On the other hand, in the mutant having the mutation at the 145β position, such an enhancement of the dissociation did not occur, probably due to the difference of the amino acid residues located in the $\alpha_1\beta_2$ subunit interface. Thus, we can conclude that the penultimate tyrosine in the α-subunit plays a key role not only in the stabilization of the T-state but also in the subunit assembly.

ACKNOWLEDGMENT

We thank Professor Teizo Kitagawa (Institute of Molecular Science) for his cooperation in resonance Raman spectral measurements.

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