Site-Directed Mutagenesis in Hemoglobin: Functional and Structural Role of Inter- and Intrasubunit Hydrogen Bonds As Studied with 37β and 145β Mutations

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ABSTRACT: In order to clarify the functional and structural role of intra- and intersubunit hydrogen bonds in human hemoglobin (Hb A), we prepared two artificial β chain mutant hemoglobins by site-directed mutagenesis. The mutant Hb Phe-37β, in which Trp-37β is replaced by Phe to remove the intersubunit hydrogen bond between Asp-94α and Trp-37β at the α1-β2 interface in deoxy Hb A, showed a markedly increased oxygen affinity and almost completely diminished Bohr effect and cooperativity. However, ^{1}H-NMR data indicated that the structure of deoxy Hb Phe-37β is rather similar to that of deoxy Hb A. The enhanced tetramer-to-dimer dissociation previously observed in Hb Hirose (Trp-37β → Ser) together with our observation of the effects of organic phosphate on the structure and function of Hb Phe-37β suggested that a large part of the abnormal properties of Hb Phe-37β observed for dilute solutions appears to result from partial dissociation into $\alpha\beta$ dimers rather than direct destabilization of the T-quaternary structure in the deoxygenated state. Thus, the primary and direct role of the hydrogen bond between Asp-94α and Trp-37β is to stabilize the tetrameric assembly, and thereby this hydrogen bond indirectly contributes to stabilization of the T-quaternary structure. The other mutant Hb Phe-145β has a Phe residue at the 145β site and lacks the intrasubunit hydrogen bond formed between Tyr-145β and the carbonyl group of Val-98β in deoxy Hb A. Although this hydrogen bond has been considered to fix the phenolic group of Tyr-145β in a pocket between the F and H helices, to strengthen the salt bridges formed by His-146β, and thereby to stabilize the T-quaternary structure, Hb Phe-145β exhibited only mild functional and structural alterations. This result led us to conclude that the van der Waals contacts between the benzene ring of Tyr-145β and the tyrosine pocket, rather than the hydrogen bond between Tyr-145β and Val-98β, make a major contribution to the stabilization of the T-quaternary structure. The present NMR spectra of deoxygenated Hb Phe-37β and Hb Phe-145β further showed that the exchangeable proton resonance observed at 6.4 ppm for deoxy Hb A originates from the intersubunit hydrogen bond between Asp-94α and Trp-37β, although it has previously been assigned to the intrasubunit hydrogen bond between Val-98β and Try-145β.

The role of intra- and intersubunit contacts in the allosteric ligand binding function of human hemoglobin (Hb A)[1] has been emphasized by the stereochemical model proposed by Perutz on the basis of crystallographic studies (Perutz, 1970; Baldwin, 1975; Shaanan, 1983). This model provided convincing explanations for functional alterations arising from single amino acid replacements at the subunit interfaces of abnormal human hemoglobins (Valdes & Ackers, 1977; Parkhurst, 1977). However, the usefulness of naturally occurring point mutants is restricted by limited availability and choice of substitution (Imai et al., 1989a). Recent notable advances in protein engineering based on site-directed mutagenesis have made it possible to introduce mutations at desired sites of protein. This technique provides a pinpointing probe for elucidating the functional role of particular hydrogen bonds in hemoglobin by replacing relevant amino acid residue (Nagai et al., 1985, 1987; Ishimori et al., 1989; Imai et al., 1991).

Previously, using this genetic engineering approach, we synthesized two α chain mutant hemoglobins (Hb Phe-42α and Hb His-42α) in *Escherichia coli* to investigate the effects of the hydrogen bonds located at the α1-β2 subunit interface on the tertiary and quaternary structures of hemoglobin (Ishimori et al., 1989; Imai et al., 1991). In the present study, we report herein new two artificial β chain mutant hemoglobins that have an amino acid substitution at the 37β or 145β site. Trp-37β is one of the invariant amino acid residues in hemoglobin and is hydrogen bonded to Asp-94α at the α1-β2 subunit interface. This hydrogen bond is present only in the deoxy quaternary structure and has been considered to serve as one of the key subunit interactions for the allosteric transition of hemoglobin. Two natural mutants (Hb Hirose, Trp-37β → Ser; Hb Rothschild, Trp-37β → Arg) which lack this hydrogen bond have been discovered (Yamaoka, 1971; Sasaki et al., 1978; Gacon et al., 1977). The factor that makes these mutants less useful for elucidating the functional and

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[1] Abbreviations: Hb A, human hemoglobin A; Hb Phe-37β, Hb (Trp-37β → Phe); Hb Phe-145β, Hb (Tyr-145β → Phe); NMR, nuclear magnetic resonance; IHP, inositol hexaphosphate.

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structural roles of this hydrogen bond is the differences in steric hindrance among the amino acid residues occupying the 37β site. Ser and Arg are nonaromatic and nonplanar residues, whereas Trp is an aromatic and planar residue. Thus, we have designed a new mutant hemoglobin, Hb Phe-37β, which has a Phe at the 37β position. Phe is an aromatic and planar residue like Trp, and it cannot form hydrogen bonds to other residues although it is not an isomorphous replacement for Trp. Thus, one can clearly elucidate the roles of this hydrogen bond in the structure and function of hemoglobin.

The other mutant, Hb Phe-145β, lacks the intrasubunit hydrogen bond formed between Tyr-145β and Val-98β in Hb A. The penultimate Tyr at 145(HC2)β is firmly held in the pocket made by the F and H helices in deoxyhemoglobin (Perutz, 1970). When ligands bind to deoxyhemoglobin, the F helix of the liganded subunit moves toward the center of the hemoglobin molecule. Then, the tyrosine pocket becomes narrower and the Tyr side chain is expelled to cause the rupture of its hydrogen bond with Val-98(FG5)β (Perutz, 1970; Baldwin & Chothia, 1979). The natural mutants which have the mutation at the 145β site such as Hb Osler (Tyr-145β → Asp) and Hb McKees Rocks (Tyr-145β → terminated) fail to retain a "T state" conformation in the deoxygenated form, resulting in a high oxygen affinity and low cooperativity (Charache et al., 1975; Winslow et al., 1976). However, it has not yet been clarified with recourse to the natural mutants how the lack of this intrasubunit hydrogen bond causes these functional alterations, because they have the amino acid residue experiencing a steric hindrance quite different from that exerted by Tyr. To overcome these difficulties, we have replaced the Tyr by Phe.

In addition to the structural and functional study of the new β chain mutants, we also intended to reexamine the assignment of the exchangeable proton resonance at 6.4 ppm from H_2O in deoxyhemoglobin. The 6.4 ppm proton resonance in the proton NMR spectrum of deoxy Hb A has been considered to be characteristic of the deoxy T-quaternary structure (Ogawa et al., 1972, 1974; Mayer et al., 1973; Ho et al., 1975; Fung & Ho, 1975). On the basis of a study using the natural mutants described above, Viggiano et al. (1978) assigned the 6.4 ppm signal to the intrasubunit hydrogen bond between the penultimate Tyr-145(HC2) and Val-98(FG5) of the β subunit. In addition, the same research group (Fung & Ho, 1975) pointed out that the intersubunit hydrogen bond between Asp-94α and Trp-37β can be another candidate for that resonance. The mutants described above have now allowed us to reassign the signal to be the intersubunit hydrogen bond between Asp-94α and Trp-37β.

Materials and Methods

Preparation of Hemoglobin Mutants. Hb Phe-37β and Hb Phe-145β were prepared as described previously (Nagai & Thøgersen, 1984, 1988; Nagai et al., 1985, 1987).

The fraction of methemoglobin contained in CO-freed, oxygenated preparations was 1.3% and 6.5% for Hb Phe-37β and Hb Phe-145β, respectively, as determined by spectrophotometry using the millimolar absorption coefficient values at 560, 576, and 630 nm (van Assendelft & Zijlstra, 1975).

We checked whether or not the desired mutation was correctly introduced and no other mutation unexpectedly occurred in the engineered protein by means of fast-atom-bombardment mass spectrometry on tryptic digests (Wada et al., 1989). Figure 1 shows the mass spectra which are enlarged around the peak for the T4β (trace A) and T14β+T15β (trace B) tryptic peptides. They clearly indicate that Trp-37β and Tyr-145β are actually replaced by Phe. No spectral changes

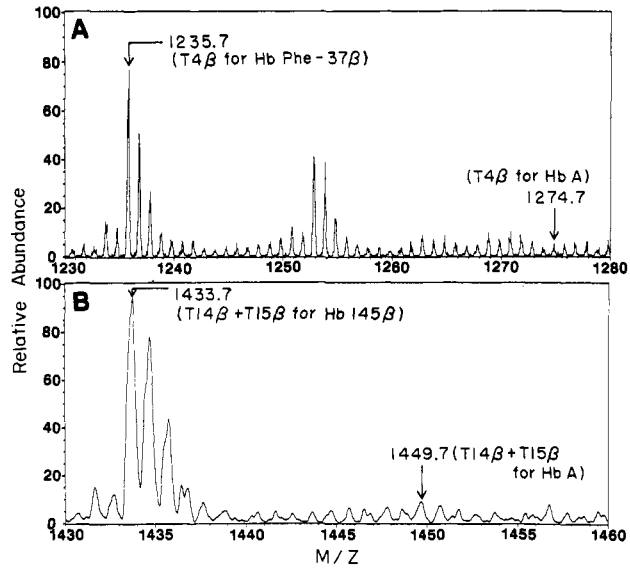

FIGURE 1: Fast-atom-bombardment mass spectra of tryptic digests. Ordinate, relative abundance; abscissa, mass to charge ratio. Portions enlarged around the peaks for the T4β (trace A) and T14β + T15β (trace B) tryptic peptides of the β chain are shown. Trace A shows disappearance of the normal peak at m/z = 1274.7 accompanied by appearance of a new peak at m/z = 1235.7, which indicates replacement of Trp-37β by Phe. Trace B shows disappearance of the normal peak at m/z = 1449.7 accompanied with appearance of a new peak at m/z = 1233.7, which indicates replacement of Tyr-145β by Phe. The peaks around m/z = 1254 have been assigned to T13α, which are also observed for native Hb A.

were detected for other peptide peaks.

Spectrophotomeric Measurements. The visible–UV range absorption spectra were recorded on a double-beam spectrophotometer, Model 320L (Hitachi, Tokyo). The hemoglobin concentration was 55 μM on a heme basis except for 5.5 μM which was used for Soret-band measurements. The UV-region derivative spectra were recorded with a first-derivative mode of the spectrophotometer. UV oxy-minus-deoxy difference spectra were measured as described previously (Imai et al., 1972). Deoxyhemoglobin samples of 55 μM concentration was prepared in Benesch-type versatile tonometer (Benesch et al., 1965) by repeated evacuation and flushing with pure nitrogen (99.9995%), whereas the deoxygenated samples of 5.5 μM concentration were prepared by adding a small amount of sodium dithionite after gentle deoxygenation with nitrogen.

Oxygen Equilibrium Experiments and Analysis. Oxygen equilibrium curves were determined by using an improved version (Imai, 1981a, 1982) of an automatic oxygenation apparatus (Imai et al., 1970). The spectrophotometer used was a Cary Model 118C (Varian Associates, CA). The wavelength of the detection light was 560 nm for 60 μM hemoglobin solutions and 620 nm for 180 μM solutions. The temperature of the hemoglobin sample in the oxygenation cell was maintained constant within ±0.05 °C. The buffers used were 0.05 M Bis-Tris for pH 7.4 and 6.9 and 0.05 M Tris for pHs 8.4 and 8.9. These buffers which contained 0.1 M Cl^- were prepared from 0.05 M Bis-Tris or 0.05 M Tris solutions containing 0.1 M HCl by adjusting the pH value with a concentrated NaOH solution at the same temperature as that for oxygen equilibrium measurements (25 °C). To minimize the autoxidation of hemoglobin during measurement, catalase and superoxide dismutase were added to each sample to a concentration of 0.1 μM (Lynch et al., 1976; Winterbourn, 1976). The methemoglobin content of the hemoglobin samples as measured immediately after oxygen equilibrium experiments ranged from 4% to 12%.

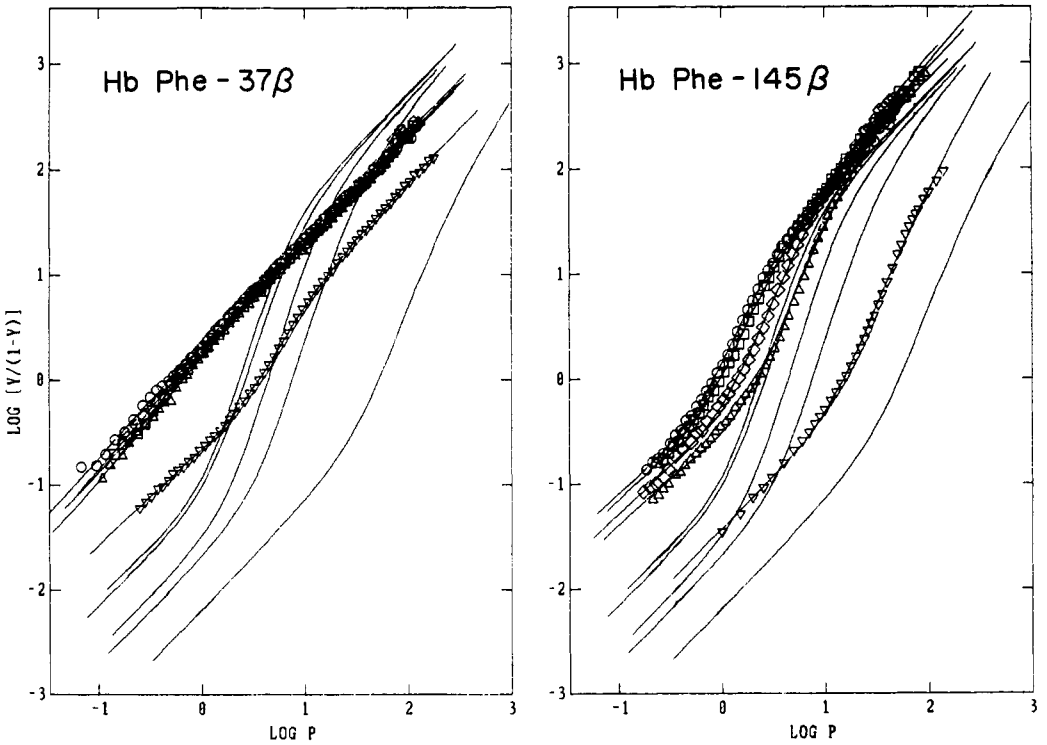

FIGURE 2: Hill plots of oxygen binding by Hb Phe-37β (left) and Hb Phe-145β (right). Y, fractional saturation of hemoglobin with oxygen; P, partial pressure of oxygen (mmHg). Symbols express observed points, and lines were calculated from the best-fit Adair constant values. ○, pH 8.4; □, pH 7.9; △, pH 7.4; ▲, pH 6.8; ▽, pH 7.4 in the presence of 2 mM IHP. Other experimental conditions are as in Table I. Lines without symbols express the calculated Hill plots for Hb A, and the pH and IHP conditions for each plot from the left to the right correspond in the same order given above.

Overall oxygen affinity, magnitude of the alkaline Bohr effect, and cooperativity were expressed by partial pressure of oxygen at half-saturation, P_{50}, the Bohr coefficient δH^+ ($=\Delta \log P_{50}/\Delta pH$), and the Hill coefficient (maximum slope of the Hill plot), respectively. These parameter values were calculated from the best-fit stepwise Adair constants (K_1 to K_4), which were evaluated from the equilibrium curves by a least-squares method (Imai, 1981b).

It has been shown that the normal hemoglobin (Hb A) synthesized by the method of Nagai et al. gives oxygen equilibrium curves that are indistinguishable from those of natural Hb A (Nagai et al., 1987; Tame et al., 1991).

Resonance Raman Spectra. Resonance 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 Model JEOL-400D Raman spectrometer equipped with a cooled RCA31034a photomultiplier. The laser power used was 60 mW at the sample point, and spectral slit width was 150 μm. Sample concentration was 100 μM. Measurements were carried out at 20 °C in the same buffers as those used for oxygen equilibrium experiments. The frequency calibration of the spectrometer was performed with CCl_4 as a standard.

NMR Spectra. ^{1}H-NMR spectra at 300 MHz were recorded on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Hyperfine-shifted NMR spectra were obtained with an 8K data transform of ±36-kHz and a 6.7-μs 90° pulse after the strong solvent resonance in H_2O solution was suppressed by a 500-μs low-power pulse. We used a Redfield 2-1-X pulse sequence with 29.5-μs pulse and 8K data points over a 6-kHz spectral width for recording the exchangeable proton resonances for the subunit interfaces of hemoglobin. The probe temperature was determined as ±0.5 °C by the temperature control unit of the spectrometer. The volume of the NMR sample was 0.3 mL. Proton shifts were referenced with respect to the water signal, which is 4.8 ppm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 23 °C.

Results

Oxygen Equilibrium. Oxygen equilibrium curves were determined at four different pH values without phosphate and at pH 7.4 with 2 mM inositol hexaphosphate (IHP), and they are presented in Figure 2 by means of the Hill plot. Each plot for Hb Phe-37β and Hb Phe-145β is shifted toward the left compared with the corresponding plot for Hb A measured under the same experimental conditions. The plots for Hb Phe-145β are less steep than those for Hb A, whereas the plots for Hb Phe-37β exhibit slopes almost of unity.

Values of oxygenation parameters obtained from these equilibrium curves are listed in Table I. The log P_{50} and n_{max} values are plotted against pH in Figure 3. Overall oxygen affinity of Hb Phe-145β is about 3 times higher and that of Hb Phe-37β is still higher (about 8 times) than that of Hb A. The effect of 2 mM IHP on P_{50} is nearly normal for Hb Phe-145β while it is roughly halved in Hb Phe-37β. The alkaline Bohr effect, which was measured by the slope of the log P_{50} vs pH plot at pH 7.4 (Figure 3), is reduced by 20% in Hb Phe-145β ($\delta H^+ = -0.38$) compared with that of Hb A ($\delta H^+ = -0.48$). The P_{50} of Hb Phe-37β is almost independent of pH ($\delta H^+ = -0.08$). In the absence of IHP, Hb Phe-145β shows moderately decreased cooperativity (n_{max} = 2.0–2.3), while Hb Phe-37β is essentially noncooperative (n_{max} = 1.1–1.2).

Table I also includes the best-fit values of the first and fourth stepwise Adair constants (K_1 and K_4, respectively). In the light of the problem caused by subunit dissociation described below, it would not be particularly meaningful to discuss the values of all the K terms except for K_1 and K_4, which express approximate values for the intrinsic oxygen association constants for the T state (K_T) and the R state (K_R), respectively. The

Table I: Oxygen Equilibrium Parameter Values for Normal and 37β and 145β Mutant Hemoglobins[a]

	P_{50}[b]	P^{A}_{50}/P^{X}_{50}[c]	$P^{IHP}_{50}/P^{free}_{50}$[d]	δH^{+}[e]	n_{max}[f]	K_1[g]	K_4[h]
Hb Phe-37β							
pH 8.4	0.48	4.8			1.12	1.5	2.0
pH 7.9	0.58	4.5			1.24	1.3	2.1
pH 7.4	0.53	8.3		−0.08	1.22	1.2	2.2
pH 6.9	0.65	12			1.22	0.92	1.9
pH 7.4 + 2 mM IHP	3.6	14	6.8		1.57	0.28	0.76
Hb Phe-145β							
pH 8.4	0.83	2.8			2.00	0.92	7.0
pH 7.9	0.96	2.7			2.05	0.77	9.2
pH 7.4	1.4	3.1		−0.35	2.19	0.55	12
pH 6.9	2.2	3.4			2.30	0.44	12
pH 7.4 + 2 mM IHP	15	3.5	11		2.29	0.038	4.1
Hb Phe-42α[i]							
pH 8.4	0.43	5.3			1.15	1.5	3.2
pH 7.9	0.54	4.8			1.11	1.5	2.4
pH 7.4	0.59	7.5		−0.19	1.10	1.3	2.0
pH 6.8	0.79	9.6			1.13	1.1	1.7
pH 7.4 + 2 mM IHP	0.96	54	1.6		1.42	0.74	1.9
Hb A (Trp-37β, Tyr-145β)							
pH 8.4	2.3				2.99	0.072	5.7
pH 7.9	2.6				2.89	0.070	5.2
pH 7.4	4.4			−0.48	3.13	0.027	4.7
pH 6.9	7.6				3.01	0.020	4.6
pH 7.4 + 2 mM IHP	52		12		2.42	0.0064	0.85

[a] Experimental conditions: Hb concentration, 60 μM on 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 Hbs. [d] Ratio of P_{50} in the presence of 2 mM IHP to P_{50} in its absence. [e] Bohr coefficient (=Δlog P_{50}/ΔpH). [f] Maximal slope of the Hill plot (Hill coefficient). [g] The first stepwise Adair constant (in $mmHg^{-1}$). [h] The fourth stepwise Adair constant (in $mmHg^{-1}$). [i] From Imai et al. (1991).

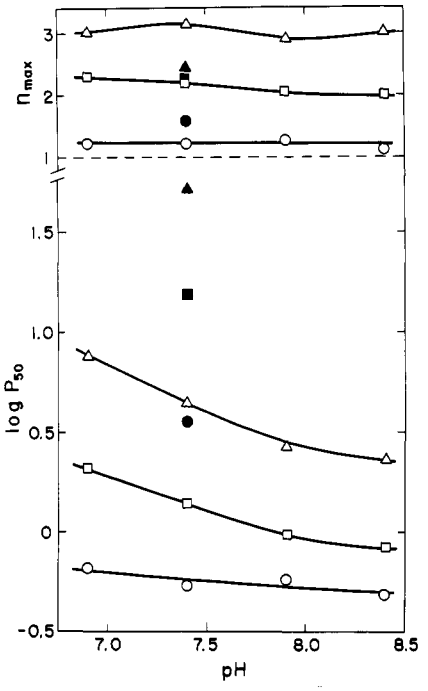

FIGURE 3: pH dependences of overall oxygen affinity (log P_{50}) and the Hill coefficient (n_{max}). The data given in Table I are plotted. ○ and ●, Hb Phe-37β; □ and ■, Hb Phe-145β; △ and ▲, Hb A. Open symbols, in the absence of IHP; closed symbols, in the presence of 2 mM IHP.

K_1 values for Hb Phe-145β are and those for Hb Phe-37β are much more increased compared to those for Hb A, whereas the K_4 values for the two mutant Hbs are not very different from those for Hb A, showing a tendency that the K_4 values for Hb Phe-145β are slightly larger and those for Hb Phe-37β are slightly smaller than those for Hb A.

In the course of the present study, possible enhancement of tetramer-to-dimer dissociation of Hb Phe-37β (described in the Discussion) emerged as a crucial problem which could affect our evaluation of the oxygen equilibrium data. Therefore, we examined the effect of protein concentration on oxygen equilibrium curve by measuring the curves with hemoglobin samples of 180 μM concentration. The P_{50} and n_{max} values, respectively, were 0.52 mmHg and 1.29 at pH 8.4, 0.74 mmHg and 1.37 at pH 7.4, and 4.4 mmHg and 1.57 at pH 7.4 in the presence of 2 mM IHP. Comparison of these values with the corresponding values for 60 μM concentration in Table I indicates that the 3-fold increase in protein concentration definitely lowers the oxygen affinity and increases cooperativity. Further examination more in detail was not possible due to the limited amount of the hemoglobin sample.

Visible–UV Absorption Spectra. No significant difference in the visible and Soret-band spectra at pH 7.4 was noted among oxygenated Hb Phe-37β, Hb Phe-145β, and Hb A. However, the Soret peak at 430 nm for the deoxy form, which was measured by several times repeated scanning immediately after adding sodium dithionite, was lowered in Hb Phe-37β and Hb Phe-145β: the absorbance ratio of the deoxy and oxy Soret peaks, $A^{deoxy}_{430}/A^{oxy}_{415}$, was 0.85, 0.99, and 1.08 for Hb Phe-37β, Hb Phe-145β, and Hb A, respectively. In both the oxy and deoxy forms, the UV-region absorption band for Hb Phe-37β was lowered while that for Hb Phe-145β was normal: the $A^{oxy}_{275}/A^{oxy}_{577}$ value for Hb Phe-37β was 2.02 compared to 2.25 and 2.26 for Hb Phe-145β and Hb A, respectively.

UV Derivative and Difference Spectra. Figure 4 illustrates the first derivative of UV-region absorption spectra for oxy and deoxy forms. In both forms Hb A showed a characteristic fine structure composed of one maximum at 289 nm and two minima at 285 and 293 nm. The magnitude of this fine structure measured by the difference between the maximum and either of the minima was halved upon deoxygenation in native Hb A. Hb Phe-145β also showed a fine structure at the same wavelengths. The magnitude for the oxy form was identical with that for oxy Hb A, whereas the magnitude of the fine structure for deoxy Hb Phe-145β was somewhat larger than that for deoxy Hb A. Oxygenated Hb Phe-37β exhibited a small fine structure, as compared with that for oxy Hb A, and the magnitude of the fine structure for deoxy Hb Phe-37β was almost identical with that for deoxy Hb A; thus, the fine

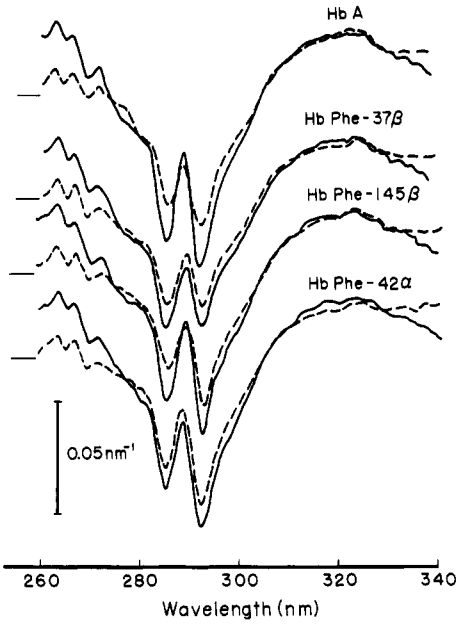

FIGURE 4: UV-region derivative spectra of oxy- and deoxyhemoglobins. —, oxy form; ---, deoxy form. Experimental conditions: Hb concentration, 55 μM on heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 25 °C. The left-end horizontal line attached to each spectrum expresses the base line. The spectra for noncooperative Hb Phe-42α (Imai et al., 1991) are shown for comparison.

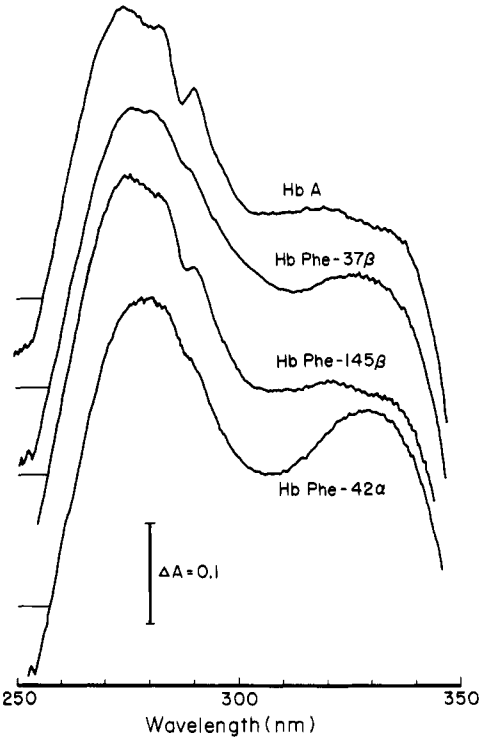

FIGURE 5: UV-region oxy-minus-deoxy difference spectra. Experimental conditions are as in Figure 4. The left-end horizontal line attached to each spectrum expresses the base line. The spectrum for noncooperative Hb Phe-42α (Imai et al., 1991) is shown for comparison.

structure for Hb Phe-37β was insensitive to deoxygenation.

Figure 5 shows 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 superimposed on a broad band with a peak at 275 nm. The fine structure for Hb Phe-145β was decreased in magnitude, and that for Hb Phe-37β almost disappeared, leaving a small shoulder superimposed on the broad band.

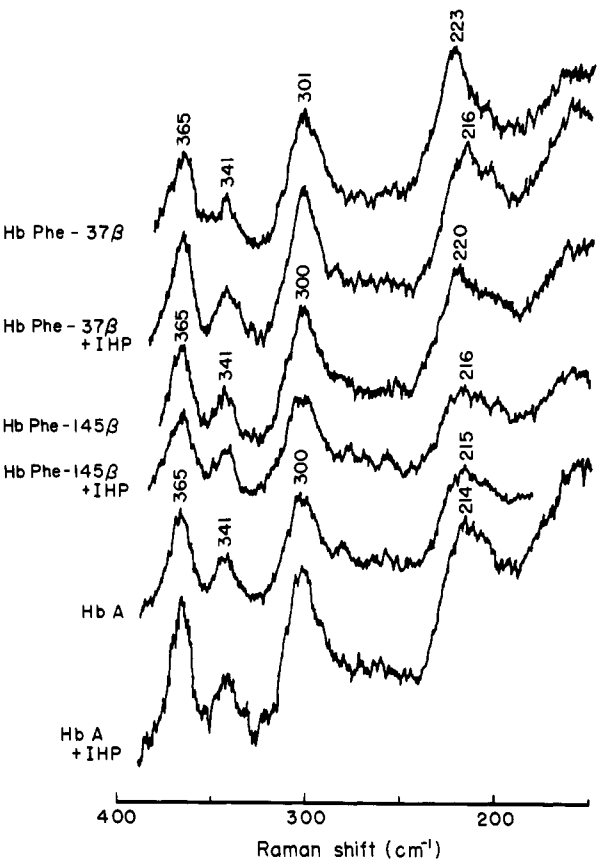

FIGURE 6: Resonance Raman spectra of deoxyhemoglobins. Experimental conditions: Hb concentration, 100 μM on heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 20 °C; in the absence and presence of 2 mM IHP. Numbers attached to the spectra indicate the wavenumber (cm^{-1}) of each Raman line.

Table II: Resonance Raman Line for Fe–Nε (His-F8) Bond of Hb A, Hb Phe-37β, and Hb Phe-145β[a]

hemoglobin	Raman shift (cm^{-1}) IHP free	+2 mM IHP
Hb A	215	214
Hb Phe-37β	223	216
Hb Phe-145β	220	216
Hb Phe-42α[b]	220	220

[a] The Raman shift values were read on the Raman spectra in Figure 6. [b] From Imai et al. (1991).

Resonance Raman Spectra. Figure 6 shows the Raman spectra in the low-frequency region for the deoxy form at pH 7.4 in the presence and absence of 2 mM IHP. No significant difference between Hb A and the mutant hemoglobins was observed in the Raman lines for porphyrin vibrational modes above 300 cm^{-1}. However, the Raman line at 215 cm^{-1} for Hb A, which was previously assigned to the Fe–Nε (His-F8) bond stretching mode and is characteristic of the "T" structure (Nagai et al., 1980; Hori & Kitagawa, 1980), was shifted to larger wavenumbers for the mutant hemoglobins. The Raman line of the Fe–Nε stretching for Hb Phe-145β was observed at 220 cm^{-1}, indicating that the Fe–Nε bond is less stretched. Hb Phe-37β exhibits a Raman line at 223 cm^{-1}, corresponding to the oxy "R" structure (Nagai & Kitagawa, 1980). The addition of 2 mM IHP caused a 7- and 4-cm^{-1} shift for Hb Phe-37β and Hb Phe-145β, respectively, as shown in Table II.

NMR Spectra. In Figure 7 are shown the ^{1}H-NMR spectra of oxygenated mutant Hbs. The positions of prominent peaks

Table III: Resonance Positions (in ppm from the H_2O Signal) of Hb A, Hb Phe-37β, and Hb Phe-145β

	oxygenated form				deoxygenated form								
	exchangeable protons			Val-E11 γ_1-methyl	proximal His N_1H		heme methyl			exchangeable protons			
Hb A	8.2[a]	7.4[b]	5.9[c]	−7.2[d]	72.1[e]	59.5[f]	18.3[g]	12.6[h]	7.6[h]	9.4[i]	8.3[j]	7.6[k]	6.4[l]
Hb Phe-37β	8.2	7.4	5.9	−7.2	72.2	59.8	18.3	12.2	7.8	9.4	8.3	7.6	
Hb Phe-145β	8.2	7.4	5.8	−7.2	70.6	59.5	18.4	12.6	7.9	9.4	8.3	7.6	6.4
peak	a	b	c	d	a	b	c	d	e	f	g	h	i

[a] Hydrogen-bonded proton between Asp-126(H9)α_1 and Tyr-35(C1)β_1 (Russu et al., 1987). [b] Hydrogen-bonded proton between His-103(G10)α_1 and Asn-108(G10)β_1 (Russu et al., 1987). [c] Hydrogen-bonded proton between Asp-94(G1)α_1 and Asn-102(G4)β_2 (Fung & Ho, 1975). [d] γ_1-Methyl group of Val-62(E11)α and Val-67(E11)β (Lindstrom et al., 1972). [e] Proximal His N_1H of the β subunit (Takahashi et al., 1980). [f] Proximal His N_1H 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 Tyr-42(C7)α_1 and Asp-99(G1)β_2 (Fung & Ho, 1975). [j] Hydrogen-bonded proton between Asp-126(H9)α_1 and Tyr-35(C1)β_1 (Russu et al., 1987). [k] Hydrogen-bonded proton between His-103(G10)α_1 and Asn-108(G10)β_1 (Russu et al., 1987). [l] See text.

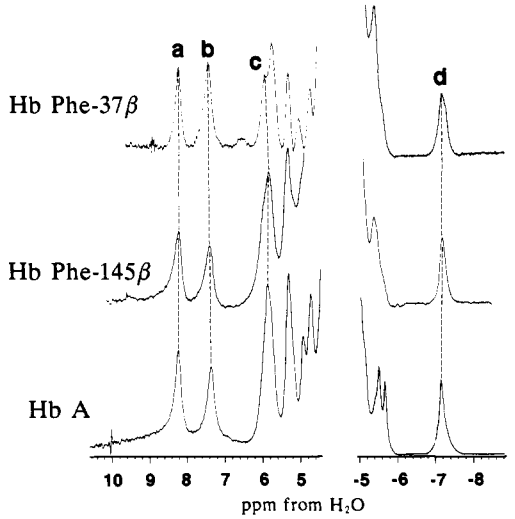

FIGURE 7: Proton NMR spectra (300 MHz) for oxygenated Hb Phe-37β, Hb Phe-145β, and Hb A. Experimental conditions: Hb concentration, approximately 3 mM on heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 23 °C.

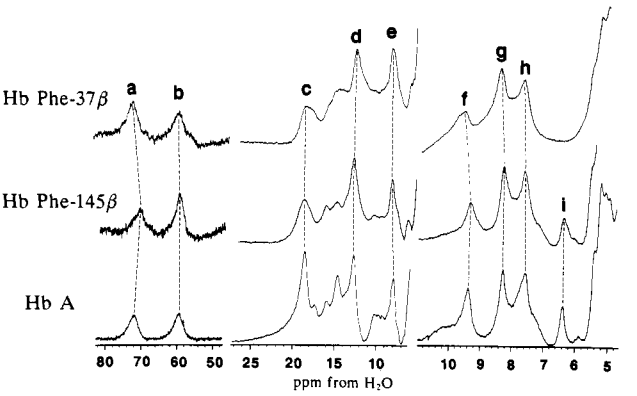

FIGURE 8: Proton NMR spectra (300 MHz) for deoxygenated Hb Phe-37β, Hb Phe-145β, and native Hb A. Experimental conditions are as in Figure 7. Hyperfine-shifted proton resonances of proximal His N_1H (left) and heme methyl groups (middle); hydrogen-bonded proton resonances (right).

are listed in Table III. The ring-current-shifted proton peak at −7.2 ppm (d), which serves as a marker for the oxy tertiary structure in the heme vicinity (Lindstrom et al., 1972), and the exchangeable proton peak at 5.9 ppm (c), which was used as an indicator of the R-quaternary structure (Fung & Ho, 1975), were observed in Hb Phe-37β and Hb Phe-145β at the same positions as those in Hb A. In Hb Phe-37β, a small shoulder was found at the Val-E11 signal at −7.2 ppm (d), which is probably due to the heterogeneity of the heme orientation (Ishimori & Morishima, 1988). Although small changes of other minor resonance peaks are noted, the similarity of the major resonance peaks of the normal and mutant hemoglobins indicates that the tertiary and quaternary structures of the oxygenated mutants are little perturbed by the amino acid substitutions.

Figure 8 shows the NMR spectra in the deoxy state. Resonances f–i arise from exchangeable protons because these resonances were missing in D_2O. The T-state marker signal at 6.4 ppm (i) in deoxy Hb A, which has been assigned to the intrasubunit hydrogen bond between the hydroxy group of Tyr-145β and the carbonyl group of Val-98β (Viggiano et al., 1978), completely disappears in going from native Hb A to Hb Phe-37β. The 6.4 ppm (i) signal is still missing in the presence of IHP (result not shown). However, this exchangeable proton resonance is present in the NMR spectrum of deoxy Hb Phe-145β, in spite of the substitution of Phe for Tyr at the β145 position. It is also worth noting that another T-state marker around 9.4 ppm (f) is observed for these two mutants, indicating that the subunit interfaces of deoxy Hb Phe-37β and Hb Phe-145β are in the T state.

In the hyperfine-shifted region, the resonances at 72.1 (a) and 59.5 ppm (b) of the proximal His N_1H have been assigned to the β and α subunits, respectively, of Hb A (Takahashi et al., 1980). The hyperfine-shifted proton resonances of the heme methyl groups were observed in the 5–20 ppm region. The signal at 18.3 ppm (c) was assigned to the β subunits, and the resonances at 12.6 (d) and 7.6 ppm (e) were assigned to the α subunits (Takahashi et al., 1980). Exchangeable proton resonances are missing in the middle panel because of the rapid pulse repetition for the hyperfine-shifted resonances. The positions of the hyperfine-shifted resonances are also given in Table III. The proximal histidyl N_1H and heme methyl resonances of Hb Phe-37β and Hb Phe-145β are not significantly perturbed by the amino acid substitution of Phe for Trp or Tyr, except for slight broadening of some signals. Thus, although the substitution for Trp or Tyr induces small structural changes in the heme cavity, the tertiary structures of the deoxygenated mutants are almost the same as that of deoxy Hb A.

Discussion

Assignment of the 6.4 ppm NMR Signal. X-ray crystallographic studies (Perutz, 1970; Perutz & Ten Eyck, 1971; Baldwin & Chothia, 1979; Shaanan, 1983) have shown that there are a number of inter- and intrasubunit hydrogen bonds which stabilize the deoxy tertiary and quaternary structures of hemoglobin. The intrasubunit hydrogen bond between Tyr-145(HC2)β and Val-98(FG5)β is one of the most prominent ones in the deoxygenated state. Earlier studies by

Table IV: Oxygen Equilibrium Parameter Values for Natural and Artificial Mutant Hemoglobins[a]

	amino acid residue					
	37β	145β	P_{50} (mmHg)	P^{A}_{50}/P^{X}_{50}	n_{max}	ref
Hb A[b]	Trp	Tyr	4.4		3.1	*f*
Hb A[c]	Trp	Tyr	7.9		3.2	*g*
Hb A[d]	Trp	Tyr	2			*h*
Hb A[e]	Trp	Tyr	9		3.0	*i*
Hb Phe-37β[b]	Phe	Tyr	0.53	8.3	1.2	*j*
Hb Hirose[c]	Ser	Tyr	2.3	3.4	1.5	*g*
Hb Rothschild[d]	Arg	Tyr	3.5	0.57	1.8	*h*
Hb Phe-145β[b]	Trp	Phe	1.4	3.1	2.2	*j*
Hb Nancy[b]	Trp	Asp	0.32	14	1.1	*k*
Hb Bethesda[e]	Trp	His	0.30	30	1.2	*i*
Hb Rainier[e]	Trp	Cys	0.34	26	1.1	*i*

[a] The definitions of parameters are as in Table I. The parameter values for each mutant hemoglobin should be compared to those for Hb A obtained under the same experimental conditions. [b] In 0.05 M Bis-Tris (pH 7.2–7.4) containing 0.1 M Cl^-; 25 °C. [c] In 0.1 M phosphate (pH 7.4); 20 °C. [d] In 0.05 M Bis-Tris (pH 7.1); 25 °C. [e] In 0.1 M potassium phosphate (pH 7.14); 20 °C. [f] Imai et al., unpublished data. [g] Sasaki et al., 1978. [h] Gacon et al., 1977. [i] Hayashi and Stamatoyannopoulos, 1972. [j] This work. [k] Charache et al., 1975.

Viggiano et al. (1978) of the exchangeable proton resonances using two 145β natural mutants, Hb Osler [Tyr-145(HC2)β → Asp] and Hb McKees Rocks [Tyr-145(HC2)β → terminated], indicated that the 6.4 ppm resonance was missing in the deoxygenated spectra of these mutants regardless of whether IHP was present or absent. They assigned the exchangeable proton resonance at 6.4 ppm in the spectrum of deoxy Hb A to the intrasubunit hydrogen bond between Tyr-145(HC2)β and Val-98(FG5)β. However, since a new resonance appeared at about 6.2 ppm in deoxygenated Hb Osler and Hb McKees Rock (Viggiano et al., 1978), it was possible that the 6.4 ppm resonance in deoxy Hb A was shifted upfield by some structural perturbation in these mutant hemoglobins. There has been no further reexamination of the ambiguous assignment of the 6.4 ppm resonance.

In the present study, the 6.4 ppm resonance appeared with the normal intensity and position in deoxy Hb Phe-145β, in which the hydrogen bond between Tyr-145β and Val-98β is ruptured. Obviously, this is *not* what we expect when the 6.4 ppm resonance actually originates from this hydrogen bond. The absence of the 6.4 ppm resonance in deoxy Hb Phe-37β, even in the presence of IHP, clearly indicates that this resonance comes from a hydrogen bond involving Trp-37β, not Tyr-145β. X-ray diffraction studies of Hb A have shown that there is an intersubunit hydrogen bond between Asp at $94\alpha_1$ and Trp at $37\beta_2$. The previous paper (Fung & Ho, 1975) also suggested that the NH proton of the Trp could be a candidate for the 6.4 ppm resonance because some exchangeable protons from Trp in hen egg white lysozyme give resonances in the region between 5 and 7 ppm from H_2O (Glickson et al., 1969) and the Trp at 37(C3)β located in the α1–β2 subunit interface is sensitive to the quaternary structure change (Perutz & Ten Eyck, 1971). Hence, we can assign the 6.4 ppm resonance to the imino proton of Trp at 37(C3)β, which is hydrogen bonded to the carbonyl of Asp at 94(G1)α.

Structure and Function of Phe-37β Mutants. Oxygen binding properties of normal and 37β and 145β mutant hemoglobins are compared in Table IV. The effects of the natural mutation at Trp-37β on the oxygen binding properties are not simple: Hb Hirose shows a high oxygen affinity and low cooperativity whereas the oxygen affinity of Hb Rothschild is low and its cooperativity is higher than that of Hb Hirose. The artificial mutant Hb Phe-37β exhibits a markedly increased oxygen affinity and almost completely diminished alkaline Bohr effect and cooperativity. The oxygen binding properties of Hb Phe-37β clearly indicate that lack of the proton donor group at the 37β position causes a drastic change in the function of hemoglobin. The present resonance Raman scattering data (Figure 6) also suggest that the Fe–Nε (proximal His) bond in deoxy Hb Phe-37β is less stretched than in deoxy Hb A due to a decrease in the restraints that the globin experiences (Perutz, 1980). In the light of an empirical correlation between the spectral and oxygen binding properties observed in many mutant and chemically modified hemoglobins [see Imai et al. (1989b) for examples], the reduced peak height of the Soret band for deoxy Hb Phe-37β suggests that this hemoglobin remains in the R structure upon deoxygenation. These spectral data appear to support their quaternary structure in favor of the R state in deoxy Hb Phe-37β.

However, the NMR spectrum of deoxygenated Hb Phe-37β is little different from that of deoxy Hb A, except for the lack of the 6.4 ppm resonance. The hyperfine-shifted resonances from the proximal His and heme methyl groups and the exchangeable proton resonance at 9.4 ppm clearly indicate that deoxy Hb Phe-37β is in the T state.

It seems very important here to remember that the natural β37 mutant, Hb Hirose, has an abnormal dimer–tetramer equilibrium (Sasaki et al., 1978). Carbonmonoxy Hb Hirose is completely dissociated into αβ dimers under the condition where liganded Hb A is tetrameric. The tetramer–dimer dissociation constant of deoxy Hb Hirose is 1.2 μM, 10^6 times greater than that of deoxy Hb A. The oxygen binding properties of Hb Phe-37β are rather similar to those of Hb Hirose. Hb Hirose exhibits a small alkaline Bohr effect ($\delta H^+ = -0.17$), whereas the organic phosphate effect on P_{50} of Hb Hirose does not differ very much from that of Hb A. The alkaline Bohr effect of Hb Phe-37β is also small ($\delta H^+ = -0.08$) as shown in Table I. The IHP effect on P_{50} in Hb Phe-37β is not very much impaired compared to other high-oxygen-affinity mutants such as Hb Phe-42α (Table I). As shown in Table II, IHP induces a 7-cm^{-1} shift of the Raman line for Hb Phe-37β, while it induces no shift in Hb Phe-42α, and the former exhibits an almost normal Raman shift (216 cm^{-1}) compared to that for deoxy Hb A (214 cm^{-1}) in the absence of IHP. Since the protein concentrations of Hb Phe-37β solutions used for the measurements of oxygen equilibrium curves and resonance Raman spectra were dilute (60–100 μM), whereas for Hb Phe-37β solutions 2–5 mM was required to measure the NMR spectra, it is likely that Hb Phe-37β was extensively dissociated into αβ dimers under the conditions for the oxygen equilibrium and resonance Raman experiments. Our preliminary gel filtration experiments actually showed that the dissociation of Hb Phe-37β is markedly enhanced compared to that of Hb A.[2] Therefore, a large part of the markedly increased oxygen affinity, and the less stretched Fe–Nε bond and lowered Soret peak in deoxy Hb Phe-37β, appears to be attributed to the subunit dissociation rather than to the stabilization of the R-state quaternary structure in the deoxygenated form. We therefore conclude that the direct role of the hydrogen bond between Asp-94α and Trp-37β is to stabilize the tetrameric assembly and the stabilization of the T state in the deoxygenated form is a secondary, indirect role of that bond. The oxygen equilibrium data for 180 μM samples indicate that the true oxygen binding properties of tet-

[2] Our preliminary analysis of the dimer–tetramer equilibrium suggests that more than 90% of carbonmonoxy Hb Phe-37β dissociates to the dimer.

rameric Hb Phe-37β would not be so drastically altered as observed for 60 μM samples.

The oxygenation-induced change in the fine structure around 290 nm in the UV derivative spectrum of hemoglobin is attributed to perturbations of aromatic amino acid residues, most probably Trp-37(C3)β and/or Tyr-42(C7)α, which are located at the α1–β2 interface and undergo environmental changes upon oxygenation. The magnitude of this fine structure is a good indicator of the quaternary state of hemoglobin as demonstrated in many modified and mutant hemoglobins (Imai, 1973; Imai et al., 1989b, 1991). In all the hemoglobins studied so far, the magnitude of the fine structure for the oxy form was identical with that for oxy Hb A, suggesting that these hemoglobins assume a common R structure after complete oxygen binding. The small magnitude of the fine structure for oxy Hb Phe-37β and its insensitiveness to deoxygenation (Figure 4) are consistent with the idea that this fine structure mostly originates from Trp-37β. The previous studies by Imai et al. further showed that the magnitude of the fine structure around 290 nm in the oxy-minus-deoxy difference spectrum represents the extent of the deoxygenation-induced change of quaternary structure. The complete disappearance of this fine structure in the difference spectrum of Hb Phe-37β (Figure 5) is also consistent with the idea described above. However, this assignment of the narrow-banded spectrum is not conclusive since a major part of the spectral changes observed for Hb Phe-37β may have come from its extensive dissociation into $\alpha\beta$ dimers by cleavage along the α1–β2 interface.

The reduction of the 275-nm peak height in Hb Phe-37β is attributed to the loss of the contribution by Trp-37β which is present in Hb A.

Structure and Function of Phe-145β Mutants. The side chains of the penultimate Tyr residues at HC2α and HC2β occupy the pockets made by helicies F and H in the deoxy form, and the phenolic hydroxyl is hydrogen bonded to the carbonyl group of Val at FG5. In the R form, this Tyr is expelled out of the pocket so that the hydrogen bond is not formed (Perutz, 1970; Shaanan, 1983). This hydrogen bond has been considered to play an important role to maintain the low-oxygen-affinity state (T state) in deoxy Hb A. One would expect that the removal of the hydrogen bond between Val-98β and Tyr-145β destabilizes the T state in the deoxygenated form, resulting in high-affinity and noncooperative oxygen binding. In fact, 145β natural mutants exhibit extremely high oxygen affinities and loss of cooperativity as shown in Table IV.

Surprisingly, however, the replacement of the Tyr with Phe did not drastically affect the function and structure of hemoglobin. In spite of the substitution of Phe for Tyr, two exchangeable proton resonances were observed around 9 and 6 ppm where the T-state marker resonances are expected to appear for Hb A. The positions of two histidyl N_1H signals in the hyperfine-shifted region were not significantly perturbed by the amino acid substitution. The heme methyl signals around 7, 13 and 19 ppm were also unperturbed (Table III). The resonance Raman line arising from the Fe–Nϵ (proximal His) bond was observed at 220 cm^{-1}, at a smaller wavenumber than that of Hb Phe-37β. The lowered Soret peak for Hb Phe-145β and the present UV difference spectral data (Figure 5) together with the UV derivative spectra (Figure 4) indicate that the R to T transition of quaternary structure upon deoxygenation is restricted to some extent in Hb Phe-145β, but not so extensively as found for Hb Phe-42α (Imai et al., 1991). Thus, the tertiary and quaternary structures of Hb Phe-145β are only a little favored to the R state compared to those of Hb A, and the hydrogen bond between Val-98β and Tyr-145β is *not* very important for stabilizing the T state in deoxy Hb A.

It is also of interest that all of the natural 145β mutants show extremely high oxygen affinities and hyperbolic oxygen equilibrium curves (n_{max} = 1.1–1.2) (Table IV). X-ray studies of Hb Nancy show that the carboxy-terminal tetrapeptide of the β chain is severely disordered, and as a result His-146(HC2)β does not form the intrasubunit hydrogen bond (Arnone et al., 1976). In contrast to the natural mutants, Hb Phe-145β exhibits less drastic functional and structural impairments. These results lead us to the proposal that the tyrosine pocket formed by the F and H helices in deoxy Hb A fits to the benzene ring of the Tyr or Phe and the conformation of Tyr-145β can be stabilized mostly by van der Waals contacts around the phenolic ring and additionally by the hydrogen bond to Val-98β. In other words, the steric hindrance of the benzene ring which is accommodated in the tyrosine pocket stabilizes the T state in deoxy Hb A.

From the oxygen binding parameters, we can estimate the effects of the hydrogen bond on some oxygen binding properties (Wolfenden & Kati, 1991). Using $\Delta G(K_i) = 2RT \ln [K_i(\text{Hb Phe-145}\beta)/K_i(\text{Hb A})]$ and $\Delta G(P_{50}) = -2RT \ln [P_{50}(\text{Hb Phe-145}\beta)/P_{50}(\text{Hb A})]$, the effect of the hydrogen bond (ΔG) on the first (K_1) and fourth stepwise Adair constants (K_4) and the overall oxygen affinity (P_{50}) amounts to 3.5, 1.1, and 1.3 kcal, respectively. The preferential effect of the hydrogen bond on the first stepwise Adair constant (K_1) suggests that the cleavage of this hydrogen bond occurs in the initial state of the oxygen binding to hemoglobin.

In conclusion, removal of the hydrogen bond between Asp-94α and Trp-37β via site-directed mutagenesis of Trp-37β to Phe made clear the primary role of that hydrogen bond to be the stabilization of the tetrameric assembly and the secondary role to be the stabilization of the low-oxygen-affinity T structure. The substitution of Phe for Tyr at 145β revealed that the presence of a benzene ring at the 145β site plays a key role in stabilizing the deoxy T structure. From the present NMR study, we also concluded that the 6.4 ppm resonance observed for deoxy Hb A originates from the intersubunit hydrogen bond between Asp-94α and Trp-37β, not from the intrasubunit hydrogen bond between Val-98β and Tyr-145β.

Registry No. Hb A, 9034-51-9; Trp, 73-22-3; Tyr, 60-18-4; Phe, 63-91-2; Asp, 56-84-8; O_2, 7782-44-7.

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