Site-Directed Mutagenesis in Hemoglobin: Functional and Structural Study of the Intersubunit Hydrogen Bond of Threonine-38(C3)α at the α1–β2 Interface in Human Hemoglobin†

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ABSTRACT: To clarify the functional and structural roles of Thr-38α at the α1–β2 interface, two artificial α-chain mutants, in which Thr-38α is replaced by Ser (Hb T38αS) or Val (Hb T38αV), were prepared. Thr-38α is one of the highly conserved amino acid residues in hemoglobins and forms a hydrogen bond to Asp-99β, which is a crucial residue to stabilize the T state, *via* a water molecule in the deoxygenated form. We investigated their oxygen binding properties together with structural consequences of the mutations by using various spectroscopic probes. Their oxygen equilibrium curves showed small changes in the oxygen binding properties. Structural probes such as ultraviolet-region derivative and oxy-minus-deoxy difference spectra, resonance Raman scattering, and ^{1}H-NMR spectra also indicated that the oxy and deoxy forms of these mutants show spectra characteristic of the R and T states, respectively, and the R–T transition is not very disturbed. The present structural and functional data of the mutants imply that the hydrogen bond between Thr-38α and Asp-99β does *not* play a key role in stabilizing the deoxy T structure, which is in sharp contrast to the role of the hydrogen bond between Tyr-42α and Asp-99β, and suggest that the interactions *via* the intersubunit hydrogen bonds are highly site-specific, depending on the amino acid residue which participates in them.

Extensive studies on hemoglobin over many years have revealed that conformational changes accompanied with the oxygenation play a critical role in the function of hemoglobin (Perutz, 1970; Baldwin, 1975; Shaanan, 1983). The rearrangements of the packing of hemoglobin subunits in oxygenation or deoxygenation, the quaternary structural change, are responsible for cooperative behavior. In spite of the importance of inter- and intrasubunit hydrogen bonds in the packing of hemoglobin subunits, very little is known about the contributions of particular residues participating in these hydrogen bonds.

To identify the key hydrogen bonds for the cooperative oxygen binding, we have focused on the hydrogen bonds located in the α1–β2 subunit interface. In this interface, the α-chain C helix has a large number of conserved residues among mammalian hemoglobin, and the total number of the hydrogen bonds between this C helix and the FG corner of the β subunit is greater than that between other helices (Perutz & Fermi, 1981). Pettigrew et al. (1982) and Baldwin and Chothia (1979) found that the most of the free energy difference between the R and T structures is located in the α1–β2 interface. Among the hydrogen bonds located at the α1–β2 interface, the hydrogen bonds between 42α and 99β have proved to be one of the key interactions to maintain the T state in the deoxygenated form. Several natural mutants having a mutation at the 99β position exhibited remarkable alterations in the oxygen binding properties: high oxygen affinities and absence of cooperativity (Reed et al., 1968; Weatherall et al., 1977; Jones et al., 1967; Rucknagel et al., 1967). Our two artificial mutants, in which Tyr-42α was replaced by Phe or His, revealed that the oxygen binding is similar to that of the natural 99β mutants when the hydrogen bond is lost by the Tyr to Asp replacement, indicating that this hydrogen bond plays a crucial role in stabilizing the deoxy T structure (Ishimori et al., 1989; Imai et al., 1991).

The role of Asp-99β may arise in multiple ways since it is also hydrogen bonded to Thr-38α and Asn-97α *via* a water molecule (Baldwin, 1975). Recently, a natural mutation at Asn-97α, which is one of the conserved amino acid residues, was discovered, and this mutant, Hb Dallas, which has a lysine residue at position 97α, showed a relatively high oxygen affinity and diminished cooperativity, suggesting that the hydrogen bond formed between Asn-97α and Asp-99β is also important for the function and structure of Hb A[1] (Lendaro et al., 1992). Since Thr-38α is also invariant in all known mammals (Dickerson & Geis, 1983) and Anderson (1973) showed that Thr-38α undergoes a significant displacement in going from the deoxy to the oxy quaternary structure, Thr-38α is also

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[1] Abbreviations: Hb A, human hemoglobin A; Hb T38αV, Hb (Thr-38α → Val); Hb T38αS, Hb (Thr-38α → Ser); NMR, nuclear magnetic resonance; IHP, inositol hexaphosphate; P_{50}, partial pressure of oxygen at half-saturation (in mmHg); n_{max}, maximal slope of the Hill plot (the Hill coefficient); K_R, oxygen association equilibrium constant for the R state; K_T, oxygen association equilibrium constant for the T state; L_0, the allosteric constant of deoxy species (T_0/R_0); L_4, the allosteric constant of fully oxygenated species [$L_0(K_T/K_R)^4$].

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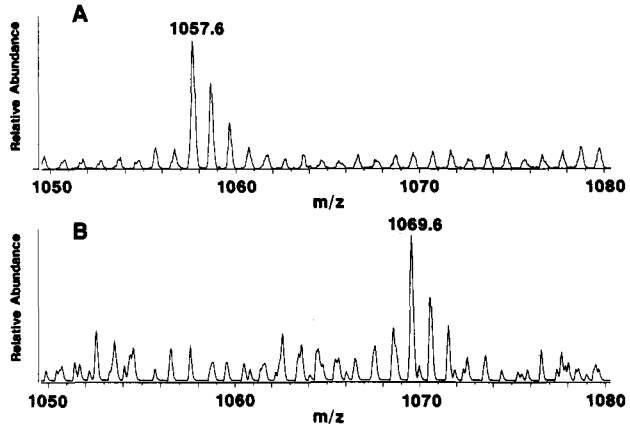

FIGURE 1: Fast-atom-bombardment mass spectrometry of tryptic digests of the α-chain from mutant Hbs. Ordinate, relative abundance; abscissa, mass to charge ratio. Portions enlarged around the peaks for the T5 peptide of the α-chain are shown. Traces A and B show disappearance of the normal peak at m/z 1071.5 accompanied with appearance of the new peak at m/z 1057.6 and 1069.6, which indicates successful replacement of Thr-38α by Ser and Val, respectively.

considered to be important for the oxygen binding property of Hb A. Since, no spontaneous mutations have been discovered at the Thr-38α position, we intended to examine the importance of this residue by studying two artificial mutants, Hb T38αV (Thr-38α → Val) and Hb T38αS (Thr-38α → Ser), in which the hydrogen bond between Thr-38α and Asp-99β is broken and perturbed, respectively. We investigated the functional and structural consequences of these mutations by measuring the oxygen binding equilibrium and CO rebinding kinetics, UV-region derivative and difference spectra, resonance Raman scattering, and proton nuclear magnetic resonance (NMR) spectra.

MATERIALS AND METHODS

Preparation of Mutant Hemoglobins. Hb T38αS and Hb T38αV were prepared as described previously (Nagai & Thøgersen, 1984; Nagai et al., 1985; Tame et al., 1991). The fraction of methemoglobin contained in oxygenated preparations was 0.75% and 6.6% for Hb T38αS and Hb T38αV, respectively. Native human Hb A and the native β-chains were prepared from human red blood cells as described previously (Ishimori & Morishima, 1988).

Fast-Atom-Bombardment Mass Spectrometry. In the tryptic digest of the α subunit, the Thr-38α residue is in peptide T5. The protonated molecular ion for this peptide of the native α-chain will be observed at m/z 1071.6 (Wada et al., 1989). In Figure 1, the peptide T5 was detected at m/z 1057.6 and at m/z 1069.6 in Hb T38αS and Hb T38αV, respectively, corresponding to the expected amino acid substitution. The molecular weights of other peptides were normal in both mutants.

Oxygen Equilibrium Experiments. Oxygen equilibrium curves were measured by using the automatic oxygenation apparatus (Imai, 1981, 1982, 1983) and analyzed according to the Monod–Wyman–Changeux (MWC) method (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 oxygen equilibrium experiment ranged from 2.6% to 12.7%. Overall oxygen affinity was expressed by partial pressure of oxygen at half-oxygen saturation, P_{50} (in millimeters of mercury: 1 mmHg = 133.3 Pa). Oxygen affinity in the R and T state was expressed by the MWC constants, K_R and K_T (in mm Hg^{-1}), respectively. Cooperativity in oxygenation was expressed by the maximal slope of the Hill plot, n_{max}. The allosteric constant of deoxy species, L_0, and the allosteric constant of fully oxygenated species, L_4, are given as

$$L_0 = (T_0/R_0) \qquad L_4 = [L_0(K_T/K_R)^4]$$

where T_0 and R_0 are the fully deoxygenated T and R states, respectively.

Spectroscopic Measurements. The visible–UV region absorption spectra were recorded on a double-beam spectrophotometer, Model 320L (Hitachi, Tokyo). MetHb content was determined by using the millimolar absorption coefficient values at 560, 576, and 630 nm (van Assebdelft & Zijlstra, 1975). 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 (99.9995%).

Measurements of CO Rebinding Constants. The CO rebinding constants for the α and β subunits in the R-state hemoglobin and overall rebinding constants for the T-state hemoglobin were determined by laser photolysis techniques as described previously (Sawicki & Gibson, 1976; Unno et al., 1990; Togi et al., 1993). The degree of CO dissociation was less than 10% in partial photolysis experiments and more than 50% in full photolysis experiments. The former and latter experiments were performed to measure the rate constants of the R and T states, respectively.

The protein concentration for the kinetic measurement was 40 μM on a heme basis (in 50 mM Bis-Tris containing 0.1 M Cl^-, pH 7.4). The monitoring wavelength was 436 nm, which is an isosbestic point for the R- and T-state deoxyhemoglobin (Sawicki & Gibson, 1976). The temperature of the sample cell was controlled at 20.0 ± 0.1 °C.

In the measurement of the R-state rate constants (partial photolysis), the time courses of 436-nm absorption change were fitted to a two-exponential expression (eq 1) following the bimolecular ligand recombination in the time range 0.5–4 ms. In the measurement of the T-state rate constant (full photolysis) they were fitted to a single exponential expression in the time range 5–15 ms (eq 2).

$$\Delta A_t = (0.5)(\Delta A_0)[\exp(-k_f t) + \exp(-k_s t)] \qquad (1)$$

$$\Delta A_t = (\Delta A_0)\exp(-k_T t) \qquad (2)$$

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 JEOL-400D Raman spectrometer equipped with a cooled RCA31034a photomultiplier. The laser power was 60 mW at the sample point, and the spectral slit width was 150 μm. Measurements were carried out at 20 °C, and the buffers used were the same as those used for the measurement of CO rebinding constants. Sample concentration was 150 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 of 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

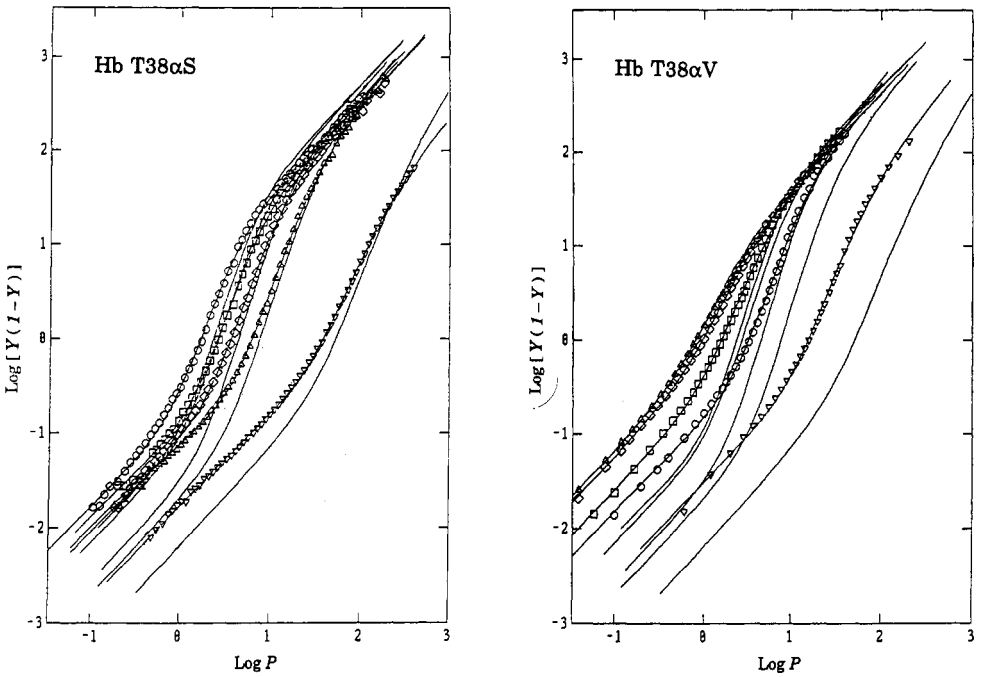

FIGURE 2: Hill plots of the oxygen binding by Hb T38αS (left) and Hb T38αV (right). Y, fractional saturation of hemoglobin with oxygen; P, partial pressure of oxygen (mmHg). Symbols express observed points, and lines were calculated from the MWC parameter values (Table I). (○) pH 8.4; (□) pH 7.9; (◇) pH 7.4; (△) pH 6.9; (▽) pH 7.4 containing 2 mM IHP. Other experimental conditions are given in the footnote to Table I. 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.

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 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 21 ± 0.5 °C by the temperature control unit of the spectrometer. Sample concentration was about 2 mM in 50 mM Bis-Tris containing 0.1 M Cl^-, pH 7.4, and the volume of the sample was about 300 μL (Ishimori & Morishima, 1986, 1988).

Tetramer–Dimer Equilibrium. The dimer–tetramer equilibrium in CO form was examined by a gel-filtration method (Sasaki et al., 1978; Ip et al., 1977). The dissociation from tetramer to dimer was not enhanced by the mutation at Thr-38α (data not shown).

RESULTS

Oxygen Equilibrium Curves. Oxygen equilibrium curves (OEC) 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. The plots for the mutants are less steep than those for Hb A. Values for oxygenation parameters obtained from these plots are listed in Table I. The log P_{50} and n_{max} are plotted against pH in Figure 3. The overall oxygen affinity of Hb T38αV is about 2 or 3 times higher than that of Hb A, whereas Hb T38αS shows a slightly higher affinity. The values of n_{max} are moderately decreased in these two mutants. The effect of 2 mM IHP on P_{50} is nearly normal, and the alkaline Bohr effect [$\delta H^+ = C(\log P_{50})/C(pH)$] is also substantially conserved in these mutants. The K_T values for Hb T38αV (Table I) are roughly 1 order of magnitude larger than those for Hb A, while the K_R values for these mutants are similar to those for Hb A. The L_0 values for Hb T38αV 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. In all the three hemoglobins, the dependence of the L_4 value on pH and IHP conditions is much smaller than that of the L_0 value. The values of i_s (switchover point), which is a marker for the ligation step of the transition from the T to the R state, indicate that the T–R transition in these two mutants occurs at some later oxygen binding step than that in Hb A.

UV Spectra. The ligand-induced changes are in the narrow-banded spectrum around 290 nm, a good indicator of the quaternary state and quaternary structural changes of Hb (Imai, 1973a; Imai et al., 1972, 1981; Wajcman et al., 1982). The first derivative of UV-region absorption spectra for the oxy and deoxy mutants and native Hb are illustrated in Figure 4. In both forms, Hb A showed a characteristic fine structure composed of one peak at 289 nm and two troughs at 285 and 292.5 nm. The mutants showed fine structures at the same wavelength, and their magnitude was nearly identical to that of Hb A.

Figure 5 shows UV-region oxy-minus-deoxy difference spectra. For Hb A, the spectrum had 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 an extent of the oxygenation-induced changes of the quaternary structure (Imai, 1973a; Imai et al., 1972, 1981; Wajcman et al., 1982). The fine structure is considered to result from Tyr-42α (Perutz, 1970) and/or Trp-37β (Briehl & Hobbs, 1970; Imai, 1973a; Ishimori et al., 1992) at the α1–β2 interface. The fine structures for both the mutants are in the same magnitude as that in Hb A.

Laser Photolysis Measurements of CO Rebinding. The CO association rate constants for the mutant and native Hbs were obtained by laser photolysis measurements, and the results are shown in Table II. The values listed there are consistent with those reported by previous papers (Philo et al., 1988;

Table I: Oxygen Equilibrium Parameter Values for Native and Thr-38α Mutant Hemoglobins[a]

pH[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]	i_s[i]
Hb T38αS										
6.9	6.2	1.2			2.67	0.065	6.0	7.5×10^5	1.0×10^{-2}	2.99
7.4	3.6	1.2		−0.44	2.67	0.087	4.9	6.1×10^4	6.1×10^{-3}	2.73
7.9	2.6	1.0			2.64	0.11	4.7	1.6×10^4	4.8×10^{-3}	2.58
8.4	1.7	1.4			2.49	0.15	4.4	2.4×10^3	3.2×10^{-3}	2.30
7.4 + IHP[j]	38	1.4	1.3		2.06	0.014	0.30	7.9×10^3	3.7×10^{-2}	2.93
Hb T38αV										
6.9	3.2	2.4			2.41	0.14	11	7.7×10^5	2.0×10^{-2}	3.11
7.4	1.6	2.8		−0.41	2.16	0.26	7.8	1.4×10^4	1.7×10^{-2}	2.81
7.9	0.95	2.8			1.78	0.55	6.8	1.0×10^3	4.3×10^{-2}	2.75
8.4	0.80	2.9			1.71	0.63	6.2	5.0×10^2	5.3×10^{-2}	2.72
7.4 + IHP[j]	16	3.3	10		2.32	0.030	1.2	5.6×10^4	2.2×10^{-2}	2.96
Hb A										
6.9	7.6				3.01	0.020	4.6	1.5×10^6	5.4×10^{-4}	2.62
7.4	4.4			−0.48	3.13	0.028	4.6	1.5×10^5	2.1×10^{-4}	2.34
7.9	2.7				2.89	0.067	5.7	4.2×10^4	8.0×10^{-4}	2.40
8.4	2.3				2.99	0.069	6.0	3.1×10^4	5.4×10^{-4}	2.32
7.4 + IHP[j]	52		12		2.42	0.0069	1.3	1.3×10^7	1.0×10^{-2}	3.13

[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 Hbs. [d] Ratio of P_{50} in the presence of 2 mM IHP to P_{50} in its absence. [e] Bohr coefficient [$=\Delta(\log P_{50})/\Delta pH$]. [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 ($L_0 = T_0/T_0$) or of fully oxygenated species [$L_4 = T_4/R_4 = L_0(K_T/K_R)^4$]. [i] Switchover point in the T-to-R transition, which was calculated as $-\log L_0/\log (K_R/K_T)$. [j] Containing 2 mM IHP.

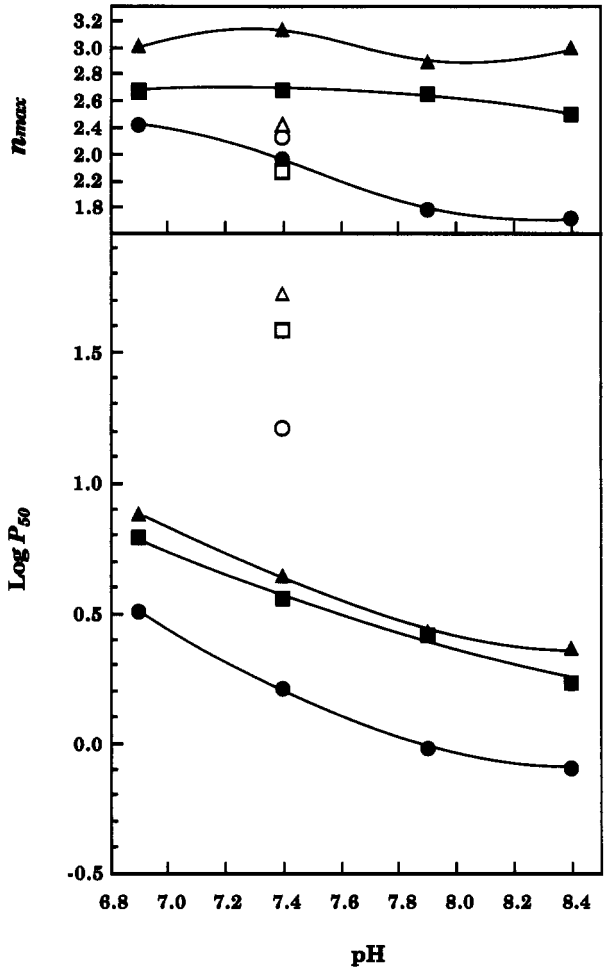

FIGURE 3: pH dependences of overall oxygen affinity (log P_{50}) and the Hill coefficient (n_{max}). The data given in Table I are plotted. ■ and □, Hb T38αS; ● and ○, Hb T38αV; ▲ and △, Hb A. Closed symbols, in the absence of IHP; open symbols, in the presence of 2 mM IHP.

Togi et al., 1993). No significant differences are noted in the mutant and native Hbs in both the R and T states.

The residuals in the two-exponential (eq 1) and the single-exponential (eq 2) fitting exhibited a random distribution (data

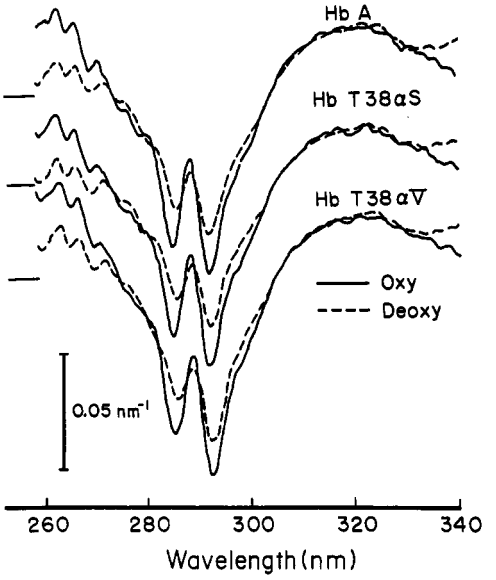

FIGURE 4: UV-region derivative spectra of oxy- and deoxyhemoglobin. Experimental conditions: Hb concentration, 55 μM on a heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 25 °C. The horizontal line attached to each set of oxy and deoxy spectra expresses the base line.

are not shown), indicating that the observed time courses can be well expressed by these equations.

Resonance Raman Spectra. As shown in Figure 6, the low-frequency region Raman spectra for deoxy Hbs at pH 7.4 were measured in the presence or absence of IHP. No significant differences between Hb A and the two mutant Hbs were observed in Raman lines for porphyrin vibrational modes at or above 300 cm^{-1}. The Fe–Nε stretching mode of the mutants appeared at 215 cm^{-1} (in Hb T38αS and Hb T38αV), which corresponds to that of the T state in Hb A (Nagai et al., 1980; Hori & Kitagawa, 1980). Addition of IHP exerted no significant influence on the Raman line for the Thr-38α mutants.

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

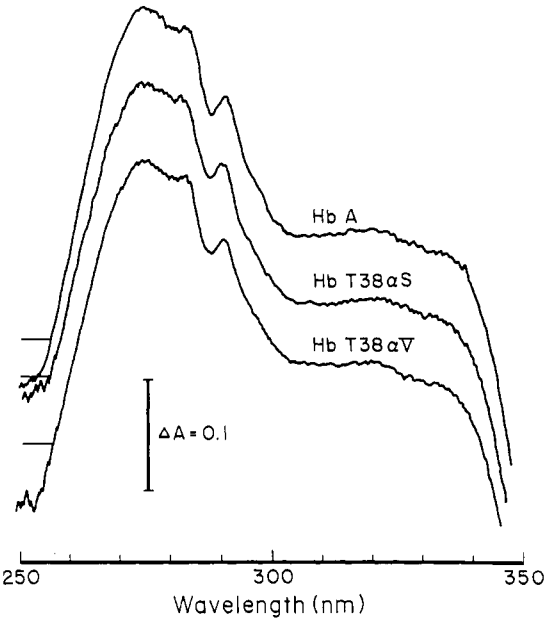

FIGURE 5: UV-region oxy-minus-deoxy difference spectra. Experimental conditions are as in Figure 4. The left-end horizontal line attached to each spectrum expresses the base line.

Table II: Value of Rate Constants for Bimolecular CO Association Reaction (10^{-6} M^{-1} s^{-1})[a]

	R state		
	α subunit (k_s)	β subunit (k_f)	T state (k_T)
Hb T38αS	4.0 ● 0.45	11 ± 2.2	0.25 ± 0.054
Hb T38αV	4.0 ± 0.73	12 ± 1.4	0.21 ± 0.044
Hb A	4.5 ± 0.23	12 ± 1.2	0.20 ± 0.022

[a] Experimental conditions: Hb concentration, 40 μM on a heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 20 °C.

native Hb A, the ring-current-shifted proton peak at −7.2 ppm (peak D) from H_2O, which was assigned to the γ_1-methyl group of E11 valine, serves as a marker for the oxy tertiary structure of the heme vicinity in oxygenated Hb A (Lindstrom et al., 1972). In the two mutants the peak from Val E11 was observed at the same position as in Hb A. Small changes of other peaks were noted in the region from −5 to −6 ppm.

The exchangeable proton peak at 5.9 ppm (C) in the downfield region, which arises from the hydrogen bond between Asp-94α and Asn-102β, has been used as an indicator of the R quaternary structure (Ho et al., 1975). The corresponding peak for Hb T38αV and Hb T38αS was observed at the same positions, and only a small spectral change was found at the 8.3 ppm resonance (A), showing that the quaternary structures of the oxygenated mutants are little perturbed by the amino acid substitutions.

The NMR spectra in the deoxygenated form are shown in Figure 8, and the position of prominent peaks are also listed in Table III. In deoxygenated Hb, the 7.6 (I) and 8.4 (H) ppm resonances have been assigned to the α1–β1 intersubunit hydrogen bonds His-103(G10)α–Asn-108(G10)β and Asp-126(H9)α–Tyr-35(C1)β (Russu et al., 1987), respectively (Table III). The peaks at 6.4 (J) and 9.4 (G) ppm arise from the characteristic intersubunit hydrogen bonds of the T state: Asp-94(G1)α–Trp-37(C3)β (Ishimori et al., 1992) and Tyr-42(C7)α–Asp-99(G1)β (Fung et al., 1975), respectively, which are located in the "switch region" of the α1–β2 interface. Since these T-state markers also appeared in the NMR spectra of the two deoxygenated mutants, large conformational perturbations in the deoxygenated state are unlikely to be

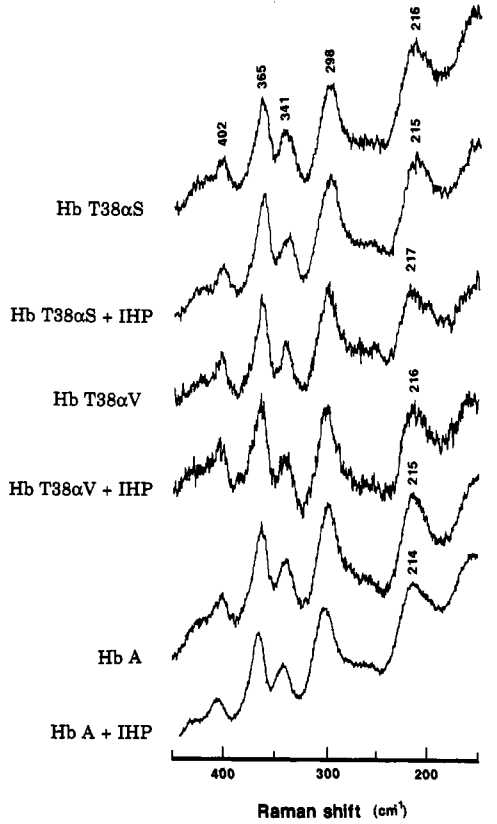

FIGURE 6: Resonance Raman spectra for deoxygenated Hb T38αS, Hb T38αV, and Hb A. Experimental conditions: Hb concentration, 150 μM on a heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 20 °C; in the absence and presence of 2 mM IHP. Numbers attached to the spectra indicate the wavenumber (cm^{-1}) of each Raman line.

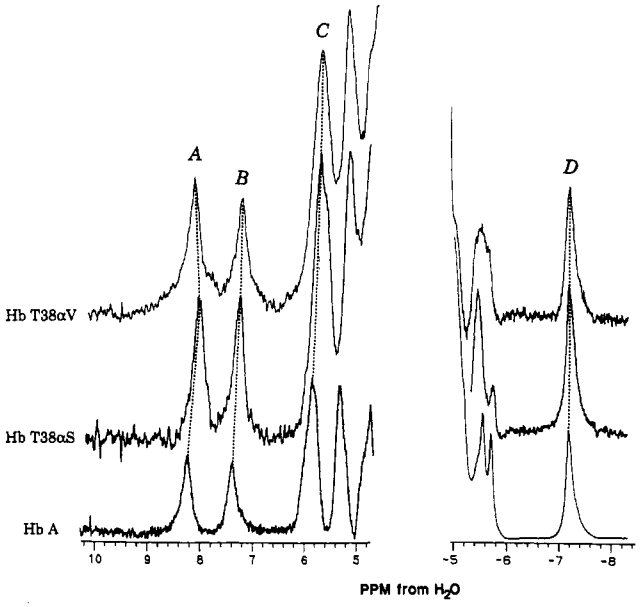

FIGURE 7: Proton NMR spectra (300 MHz) of oxygenated Hb T38αS, Hb T38αV, and Hb A. Experimental conditions: Hb concentration, approximately 2 mM on a heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 21 °C.

induced by the mutation. In Hb T38αV, however, the exchangeable proton peak (G) is more shifted to downfield than that in Hb A.

The hyperfine-shifted resonance peaks arising from heme methyl groups (7.9, 12.6, and 18.3 ppm from H_2O in Hb A) (Takahashi et al., 1980) were not significantly affected by the mutations (figure not shown). The two resonances observed

Table III: Resonance Positions (in ppm from H_2O Signal) of Hb A, Hb T38αS, and Hb T38αV

	oxygenated form				deoxygenated form								
	exchangeable protons			Val-E11 γ_1-methyl	proximal His NεH		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]	7.9[h]	9.4[i]	8.4[j]	7.6[k]	6.4[l]
Hb T38αS	8.2	7.4	5.8	−7.2	72.0	59.3	18.0	12.5	7.4	9.5	8.4	7.6	6.4
Hb T38αV	8.2	7.4	5.8	−7.2	72.5	60.0	18.2	12.7	7.9	9.8	8.2	7.5	6.3
Hb A + IHP					71.7	59.5	18.4	12.5	7.8	9.4	8.2	7.6	6.4
Hb T38αS + IHP					72.0	59.5	18.5	12.6	7.5	9.4	8.3	7.6	6.4
Hb T38αV + IHP					71.0	58.0	18.3	12.8	8.1	9.7	8.2	7.4	6.2
peak	A	B	C	D	E	F				G	H	I	J

[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-162(E11)α and Val-67(E11)β (Lindstrom et al., 1972). [e] Proximal His NεH of the β subunit (Takahashi et al., 1980). [f] Proximal His NεH 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] Hydrogen-bonded proton between Asp-94(G1)α_1 and Trp-37(C3)β_2 (Ishimori et al., 1992).

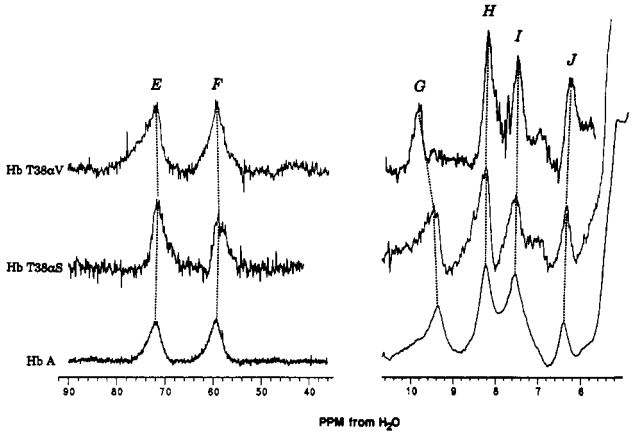

FIGURE 8: Proton NMR spectra (300 MHz) for deoxygenated Hb T38αS, Hb T38αV, and Hb A. Experimental conditions are as in Figure 7. Left, hperfine-shifted proton resonances of the proximal His $N_\delta H$, and right, the hydrogen-bonded proton resonances.

at 59.5 (F) and 72.1 (E) ppm in Hb A have been assigned to the $N_\delta H$ of the proximal histidyl residues of the α- and β-chains in deoxygenated Hb A, respectively (La Mar et al., 1977; Takahashi et al., 1980). In the two mutants, they are also insensitive to the amino acid substitution as listed in Table III.

DISCUSSION

Oxygen Binding Properties of Thr-38α Mutants. In spite of the complete loss of the hydrogen bond that originally existed between Thr-38α and Asp-99β, the oxygen affinity of Hb T38αV is moderately higher than that of Hb A and the cooperativity is also mildly decreased. This indicates that this hydrogen bond is not crucial to the oxygen binding function of hemoglobin. The best fit MWC parameter values (Table I) would provide further insight into the role of this hydrogen bond.

The K_R values for the Thr-38α mutants are similar to those for Hb A; that is, the oxygen affinity in the R state of the mutants is little influenced by the mutations. However, the mutants exhibited 10- to 100-fold lower L_4 values than those for Hb A, indicating that the T–R equilibrium in the fully oxygenated state was shifted from the R state to the T state. Differences in the free energy between the R and T states in the oxygenated form are estimated[2] as about 13 kJ mol^{-1} for Hb T38αS, 10 kJ mol^{-1} for Hb T38αV, and 21 kJ mol^{-1} for Hb A at pH 7.4, 25 °C, resulting in about 9 and 11 kJ mol^{-1} destabilization in the R state, respectively. The increased i_s values also indicate that the transition from the T to the R state occurs at a later oxygen binding step than that in Hb A.

In the deoxygenated state, however, the variations of L_0 caused by mutation were rather smaller than those of L_4 and the destabilization of the T state in the deoxygenated mutants amounts to 2.2 kJ mol^{-1} for Hb T38αS and 5.8 kJ mol^{-1} for Hb T38αV at pH 7.4, 25 °C. One of the high-oxygen-affinity Hbs, Hb Y42αH, in which Tyr-42α is replaced by His, shows about 17 kJ mol^{-1} destabilization in the deoxy T state (Togi et al., 1993). The K_T values for the Thr-38α mutants are, at most, 10 times as large as those for Hb A, whereas K_T for high-oxygen-affinity Hbs such as des-Tyr-His Hb or Hb Y42αF (Tyr-42α → Phe) show about 16–26 times increase compared to K_T for Hb A (Imai, 1973b; Imai et al., 1991). These observations imply that the hydrogen bond between Thr-38α and Asp-99β contributes to the stabilization of the oxy R state rather than that of the deoxy T state and modulates the T–R equilibrium in the oxy form, although no remarkable changes in their oxygen affinity and structure were able to be detected in the oxygenated state.

Effects of Thr-38α Mutation on Equilibrium and Dynamic Structure. The present structural data indicate that the 38α mutants hold the normal tertiary and quaternary structure. Although no extensive structural alterations were found for the Thr-38α mutants, some local conformational changes were detected. Deoxygenated Hb T38αV showed a proton resonance from the intersubunit hydrogen bond between Tyr-42α and Asp-99β at 9.8 ppm, which is 0.4 ppm downfield shifted from that of Hb A at 9.4 ppm. Such shifts of the resonance at 9.4 ppm also occur in some mutant and hybrid Hbs. These shifts may have arisen from local conformational changes around the hydrogen bond (Miura & Ho, 1984; Ishimori & Morishima, 1988). Since Thr-38α is close to the hydrogen bond between Tyr-42α and Asp-99β, the substitution of Val for Thr induces not only the cleavage of the hydrogen bond between Thr-38α and Asp-99β but also some environmental

[2] The difference in the free energy between the R and T states was determined from the allosteric constant L_i (i denotes the number of the bound oxygen molecules):

$$\Delta G_{R-T} = -RT \ln L_i$$

where R is the gas constant and 298.15 K was used for the absolute temperature, T.

Table IV: Oxygen Equilibrium Parameter Values for Native and Several Mutant Hemoglobins

	amino acid residue								
	42α	97α	P_{50} (mmHg)	P_{50}^{A}/P_{50}^{X}	n_{max}	K_T (mmHg^{-1})	K_R (mmHg^{-1})	L_0	ref
Hb Y42αH[a]	His	Asn	1.4	3.1	1.95	0.29	3.8	7.0×10^2	Imai et al. (1991)
Hb Y42αF[a]	Phe	Asn	0.59	7.5	1.10	0.91	2.1	1.7	Imai et al. (1991)
Hb A[a]	Tyr	Asn	4.4		3.13	0.028	4.6	1.5×10^5	Imai et al. (1991)
Hb Dallas[b]	Tyr	Lys	1.0	3.2	1.7	0.59	16	4.7×10^4	Lendaro et al. (1992)
Hb A[b]	Tyr	Asn	3.2		2.8	0.042	16	1.0×10^6	Lendaro et al. (1992)

[a] Experimental conditions: Hb concentration, 60 μM on a heme basis; in 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl$^-$; 25 °C. [b] Experimental conditions: Hb concentration, 500 μM on a heme basis; in 0.1 M Bis-Tris (pH 7.4) containing 0.1 M NaCl; 20 °C.

changes around the hydrogen bond between Tyr-42α and Asp-99β by increasing hydrophobicity at the 38α position.

Some mutant Hb shows apparent changes in dynamic state even if no obvious alterations are observed in the equilibrium state. In CO rebinding reaction experiments, Togi et al. (1993) observed the destabilization of the transition state in the R–T transition in a 145β mutant which exhibits almost normal oxygen binding property and structure. The transition rate of the 145β mutant from the R and T state was decreased by 20-fold compared with that of Hb A. In the present mutants, Hb T38αS and Hb T38αV, no significant differences in the time course from that of Hb A were observed. It can be concluded that the destabilization of the transition state is not induced by the 38α mutation.

According to the X-ray crystallography data, the position of Thr-38α is equivalent to that of Trp-37 in the β subunit. This tryptophan residue is hydrogen-bonded to Asp-94α in the T state. An artificial mutant prepared to remove this intersubunit hydrogen bond, Hb W37βF (Trp-37β → Phe), showed a very high oxygen affinity and diminished cooperativity owing to extensive dissociation into $\alpha\beta$ dimers (Ishimori et al., 1992). The natural mutant, Hb Hirose (Trp-37β → Ser), also shows enhanced tetramer-to-dimer dissociation (Yamaoka, 1971; Sasaki et al., 1978). However, the present amino acid substitution caused no enhancement of the dissociation, suggesting that Thr-38α does not contribute to the tetramer formation in carbonmonoxyhemoglobin.

Comparison to Other Hydrogen Bonds near Thr-38α. It is quite interesting that the hydrogen bond between Asp-99β and Tyr-42α is located near the hydrogen bond between Thr-38α and Asp-99β in the deoxygenated state and Asp-99β is the common amino acid residue for these two hydrogen bonds. As previously reported (Ishimori et al., 1989; Imai et al., 1991), the cleavage of the hydrogen bond between Tyr-42α and Asp-99β caused serious functional and structural defects: extremely high oxygen affinity and absence of cooperativity (Table IV). NMR, resonance Raman, and absorption spectra have also disclosed that the structure of the deoxygenated 42α mutant is quite different from that of deoxy Hb A and rather resembles the oxygenated state of Hb A (Ishimori et al., 1989; Imai et al., 1991). On the basis of the functional and structural data, we have concluded that the hydrogen bond between Tyr-42α and Asp-99β is crucial for the stabilization of the deoxy T state.

On the other hand, the present study clearly shows that the perturbation of the hydrogen bond between Thr-38α and Asp-99β in the deoxygenated state does *not* cause marked structural and functional alterations. Among the MWC parameters, the most remarkable difference between the 42α and 38α mutants are found in K_T and L_0. The 42α mutants exhibit markedly large K_T and extremely high L_0 values, whereas the variations in the K_T and L_0 values of the 38α mutants from those of Hb A are rather small. It is, therefore, likely that the contribution to the oxygen affinity and stabilization of the deoxygenated state is quite different between these two hydrogen bonds. Such a different contribution suggests that the subunit interactions through the hydrogen bond are "pin point" interactions, which is highly site-specific and depends on the amino acid residues forming the hydrogen bonds.

It should be also noted that Asp-99β is hydrogen-bonded to Asn-97α *via* a water molecule in the α1-β2 intersubunit as well as to Thr-38α and Tyr-42α. The oxygen binding parameters for Hb Dallas (Asn-97α → Lys) are also listed in Table IV. Hb Dallas exhibited an increased oxygen affinity, a reduced cooperativity, and reduced Bohr effect. K_T and L_0 are affected by this mutation, although the deviations from Hb A are not so large as those of the 42α mutants. The computer simulation showed that a rearrangement of the C- and G-loops in the α and β subunits is induced (Lendaro et al., 1992). However, deoxy Hb Dallas was shown to maintain the hydrogen bond between position α97(G4) and position 99β(G1) as found for deoxy Hb A; this may explain the moderate functional alterations. Since the substituting amino acid residues are different, it may not be easy to make a direct comparison of the contribution of these hydrogen bonds to oxygen binding property. It would be safe to say that the hydrogen bond between Asp-97α and Asp-99β is more important for the function of Hb than the hydrogen bond between Thr-38α and Asp-99β. The discussion made above leads us to the conclusion that the subunit interactions through the hydrogen bond are highly site-specific and the contributions of the hydrogen bond between Asp-99β and Thr-38α to the function and structure of Hb is not very important compared to those of the hydrogen bonds close to Thr-38α.

The Thr-38α residue has been considered to play a crucial role in the function and structure of Hb A, since this residue is located near the hydrogen bond between Tyr-42α and Asp-99β, which is one of the most essential hydrogen bonds at the α1–β2 interface. This idea has not been proved experimentally since no natural mutation at this position has occurred. Although Thr-38α has a small but definite effect on stabilizing the R state in the oxygenated form, the present study proved that the contribution of the hydrogen bond between Thr-38α and Asp-99β to the function and structure of Hb is not very important, being quite different from the hydrogen bond between Tyr-42α and Asp-99β. It is also concluded that the subunit interactions through intersubunit hydrogen bonds are highly site-specific, which depends on the kind of the participating amino acid residue.

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