Quaternary Structure Sensitive Tyrosine Residues in Human Hemoglobin: UV Resonance Raman Studies of Mutants at α140, β35, and β145 Tyrosine†

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ABSTRACT: Recent studies noted the contribution of α42Tyr to the T−R-dependent UV resonance Raman (UVRR) spectral changes of HbA [Nagai, M., et al. (1996) *J. Mol. Struct. 379*, 65−75; Huang, S., et al. (1997) *Biochemistry 36*, 6197−6206], but the observed UVRR changes of the Tyr residue cannot be fully interpreted with α42Tyr alone. To identify the remaining contributions, the 235 nm-excited UVRR spectra of Tyr mutant Hbs at α140, β35, and β145 were investigated here. The Fe−His stretching mode demonstrated that all of these mutant Hbs take the T structure in the deoxy form under these experimental conditions. The UVRR change of the Trp residue of these mutants upon the T−R transition was the same as that in HbA, indicating that the T−R-dependent UVRR change of β37Trp is not due to stacking with Tyr residues but is due to the formation or destruction of a hydrogen bond. The recombinant Hbs β35Tyr → Phe and β35Tyr → Thr both exhibited UVRR spectra identical with that of HbA, meaning that β35Tyr is not responsible. In the spectra of des(β146His,β145Tyr)Hb with inositol hexaphosphate, the frequency shift of the Tyr RR bands was the same as that in HbA but the intensity enhancement in the CO form was small, suggesting that β145Tyr contributes to a part of the intensity change, but scarcely relates to the frequency shift. In the spectra of Hb Rouen (α140Tyr → His), the frequency shifts of bands at 1617 (Y8a) and 1177 (Y9a) cm^{-1} following ligation were half of those in HbA, while the intensity enhancement was not detected. This result means that α140Tyr is responsible for both the frequency shift and the intensity changes. It is suggested that the frequency shift of the Tyr RR bands upon the T → R transition is due to changes in the hydrogen bonding state of α42- and α140Tyr and that the intensity enhancement is due to changes in the environment of the penultimate Tyr in both α and β subunits (α140 and β145). These alterations in the vibrational spectra clearly demonstrate which tyrosine residues are involved in the T−R transition as a result of modification of their local environments.

The oxygen affinity of hemoglobin (Hb)[1] increases with the occupation number at the four oxygen binding sites. To explain this cooperativity, Hb has been extensively studied. The Hb cooperativity has been interpreted by assuming that there is a chemical equilibrium between two quaternary structures, the T structure with the low oxygen affinity and the R structure with the high oxygen affinity, which are practically close to those of deoxyHb and fully liganded Hb, respectively (*1*−*3*). Although the two quaternary structures have been characterized with numerous techniques, including X-ray diffraction (*4*−*6*), NMR (*7*), circular dichroism (*8*−*11*), and resonance Raman spectroscopy (*12*, *13*), a detailed understanding of Hb cooperativity at the molecular level remains elusive.

Recent technical developments in UV resonance Raman (UVRR) spectroscopy have made it possible to obtain new information about the change of aromatic amino acid residues due to the quaternary structure transition (*14*−*18*). Upon Raman excitation around 230−235 nm, some Raman bands of tyrosine (Tyr) and tryptophan (Trp) are selectively enhanced in intensity, and accordingly, their environmental changes due to the T−R transition can be detected. Both frequency shift and intensity changes were observed for Tyr bands, while only the intensity change was recognized for Trp bands (*14*, *16*). These changes might originate from the alteration of environments and the formation or destruction of hydrogen bonds in the side chain of aromatic amino acid residues primarily located at the α1β2 intersubunit contact.

Studies of Hb Hirose (β37Trp → Ser) (*16*) and Hb Rothchild (β37Trp → Arg) (*14*) have indicated that the

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[1] Abbreviations: CPase A, carboxypeptidase A; Hb, hemoglobin; IEF, isoelectric focusing electrophoresis; IHP, inositol hexaphosphate; rHb, recombinant Hb; UVRR, UV resonance Raman.

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change of Trp UVRR bands was ascribed to β37Trp. On the other hand, the spectral changes of Tyr UVRR bands were interpreted to arise from hydrogen bond formation between α42Tyr and β99Asp upon the R−T quaternary transition (*14*). While this interpretation was compatible with the UVRR studies of recombinant Hb (rHb) (α42Tyr → His) (*17*) and a double mutant rHb (α42Tyr → Asp/β99Asp → Asn) (*18*), some parts of the changes of Tyr bands were observed for these mutant Hbs despite the lack of α42Tyr. Therefore, the UVRR changes of Tyr in HbA could not be attributed to α42Tyr alone (*17*, *18*), while contributions from Tyr residues in the α subunit were demonstrated using isotope hybrid rHb which contains deuterated Tyr (Tyr-d_4) (*19*).

To specify the Tyr residues responsible for the T−R transition, we have examined here the UVRR spectra of mutants at α140-, β35-, and β145Tyr and verified that α140Tyr also contributes to both the frequency shift and intensity change and that β145Tyr contributes to only the intensity change. It became clear that β35Tyr hardly contributes to the T−R spectral changes.

EXPERIMENTAL PROCEDURES

Hemoglobins. HbA was purified from fresh human blood by preparative isoelectric focusing electrophoresis (IEF) using 5% Ampholine (pH 6−9) (*16*). Hb Rouen was purified from patients' hemolysate by preparative IEF (pH 7−8) (*16*). Des-(β146His,β145Tyr)Hb was prepared by the digestion of oxyHbA with carboxypeptidase A (CPase A) (*20*, *21*) and purified by preparative IEF (pH 6−9) (*16*, *22*). Recombinant Hb (β35Tyr → Phe) and rHb (β35Tyr → Thr) were synthesized as a cleavable fusion protein in *Echerichia coli* cells by using an expression vector T7 with cIIFXβ-globin as reported previously (*23*).

Samples for UVRR measurements were prepared as follows; ca. 0.5 mL of the 400 μM (in heme basis) Hb solution in 0.05 M phosphate buffer (pH 7.0) containing 0.2 M Na_2SO_4 was put into a small rubber-topped tube. The Raman band of SO_4^{2-} ions at 982 cm^{-1} was used as an internal intensity standard for the calculations of difference spectra. The deoxy form was obtained by adding a small amount of sodium dithionite powder to the Hb solution after replacement of the inside air with N_2 gas. The CO form was derived with the same procedure that was used for the deoxy form, but under a CO atmosphere. The 0.1 mL aliquot of the sample was transferred into a spinning cell (diameter of 5 mm) with an airtight microsyringe after the exchange of the inside air of the cell with N_2 for the deoxy form and with CO for the CO form.

Reagents. Inositol hexaphosphate (IHP) (Sigma), Ampholine solution (Pharmacia Biotech Inc.), and suprapure sodium sulfate (Merck) were used as purchased.

Measurements of Visible Resonance Raman Spectra. Visible RR spectra were excited at 441.6 nm with a He/Cd laser (Kinmon Electric, model CD4805R), dispersed with a 1 m single polychromator (Ritsu Oyo Kogaku, model MC-100DG), and detected with a cooled CCD detector (Astromed, model CCD3200). All measurements were carried out at room temperature with a spinning cell (1800 rpm). The laser power at the sample point was 4.0 mW.

Measurements of UV Resonance Raman Spectra. UVRR spectra were excited by a XeCl excimer laser-pumped dye laser system (Lambda Physik, model EMG103MSC and FL2002). The 308 nm line from the XeCl excimer laser (operated at 100 Hz) was used to excite coumarin 480, and the 470 nm output from the dye laser was frequency-doubled with a β-BaB_2O_4 crystal to generate 235 nm pulses. The Raman excitation light (3−4 mJ/cm^2) was introduced into samples from the lower front side of the spinning cell. The scattered light was dispersed with an asymmetric double monochromator (Spex 1404) in which the gratings in the first and second dispersion steps are 2400 grooves/mm (holographic) and 1200 grooves/mm (machine-ruled, 500 nm blaze), respectively, and was detected by an intensified photodiode array (Hamamatsu Photonics, model PCIMD/C5222-0110G) (*24*).

The spinning cell was moved vertically by 1 mm for every spectrum (every 5 min) to shift the laser illumination spot on the sample (*25*). The temperature of the sample solution was kept at 10 °C by flushing with cooled N_2 gas against the cell. The scattered light was collected with Cassegranian optics with f/1.1. One spectrum is composed of the sum of 400 exposures, each exposure accumulating the data for 0.8 s. Raman shifts were calibrated with cyclohexane. The integrity of the sample after exposure to the UV laser light was carefully confirmed by the visible absorption spectra measured before and after the UVRR measurements. If some spectral changes were recognized, the Raman spectrum was discarded. Visible absorption spectra were measured with a Hitachi 220S spectrophotometer.

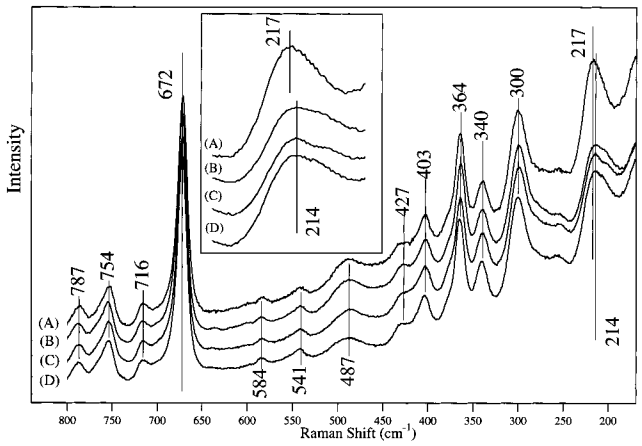

FIGURE 1: Visible resonance Raman spectra of Hb Rouen (A), des-(β146His,β145Tyr)Hb (B), rHb (β35Tyr → Phe) (C), and HbA (D) in the deoxygenated form. The spectra were normalized using the ν_7 peak at 672 cm^{-1}. The spectrum of deoxy-des(β146His,β145Tyr)-Hb was measured in the presence of 5 mM IHP. The Hb concentration was 200 μM (in heme) in 0.05 M phosphate buffer (pH 7.0). Excitation was at 441.6 nm. The inset shows the expansion of the 250−185 cm^{-1} region.

RESULTS

Fe−His Stretch. In advance of specification of the UVRR spectral changes of Tyr residues upon the T → R transition, it is quite important to confirm that the mutant Hbs used in this experiment adopt the T state in the deoxy form and also keep the heme structure similar to that of HbA. For this purpose, we measured the 441.6 nm-excited RR spectra of deoxyHb Rouen (α140Tyr → His), deoxy-rHb (β35Tyr → Phe), and deoxy-des(β146His,β145Tyr)Hb in the presence of IHP. These are compared with that of HbA in Figure 1.

The 441.6 nm excitation wavelength is best to see the stretching mode (ν_{Fe-His}) of the bond between the heme iron and the imidazole ϵ-nitrogen of the proximal histidine in the F-helix (α87 and β92). The intensities of the spectra were normalized using the ν_7 peak at 672 cm^{-1}. No significant difference between HbA and the mutant Hbs was observed in Raman bands for porphyrin vibrational modes above 300 cm^{-1}. The peak at 214 cm^{-1} of HbA has been assigned to the ν_{Fe-His} mode (*26*, *27*). An expanded view of the ν_{Fe-His} bands of other mutant Hbs is given in the inset. The ν_{Fe-His} band is expected to appear around 214−216 and 220−222 cm^{-1} for typical T and R structure deoxyHbs, respectively (*28*), and therefore can be used as a marker for diagnosis. The ν_{Fe-His} frequencies of deoxy-rHb (β35Tyr → Phe) and deoxy-des(β146His,β145Tyr)Hb in the presence of IHP are identical with that of HbA. The ν_{Fe-His} band of Hb Rouen (α140Tyr → His) is upshifted to 217 cm^{-1} and has a greater intensity. This suggests that the ν_{Fe-His} frequencies of the α and β subunits are closer than those in other Hbs, for which the ν_{Fe-His} band of the α subunit is generally weaker and has a lower frequency than that of the β subunit. However, the ν_{Fe-His} frequency of Hb Rouen is within the range of frequencies observed for the T structure.

Similar comparisons were carried out for Raman bands of deoxy and CO forms in a high-frequency region (1700−1200 cm^{-1}) (data not shown), but no significant difference between the mutant Hbs and HbA was recognized, indicating that environments surrounding the heme in these mutant Hbs maintain normal conditions.

α140 Tyrosine. Hb Rouen (α140Tyr → His), which is a variant found in a French family (*29*), has a moderately high oxygen affinity and a decreased level of heme−heme interaction ($n = 2.1$). The UVRR spectra of deoxy and CO forms of Hb Rouen are compared with those of HbA in Figure 2, where the observed spectra of HbA (A), its deoxy-minus-CO difference spectrum (B), the observed spectra of Hb Rouen (C), and its deoxy-minus-CO difference spectrum (D) are displayed. The RR bands of Tyr in deoxyHbA are observed at 1617 (Y8a), 1207 (Y7a), 1177 (Y9a), and 851 cm^{-1} (Y1). The frequency downshift and the intensity enhancement for Y8a and Y9a RR bands in the CO form are distinctive of HbA. The RR bands of Trp in deoxyHbA are observed at 1556 (W3), 1358 and 1340 (W7, Trp doublet), 1009 (W16), 875 (W17), and 754 cm^{-1} (W18). The intensities of these Trp RR bands are appreciably reduced in the CO form. The spectral changes of Tyr and Trp residues in HbA due to the T → R transition (Figure 2B) are in agreement with those reported previously (*14*, *16*, *30*, *31*).

In the spectra of Hb Rouen (Figure 2C), the intensities of all Tyr bands relative to those of Trp bands are decreased by ∼20% in both the deoxy and CO forms on account of deletion of one of six Tyr residues per αβ dimer unit. The intensity changes of Trp RR bands on ligand binding are similar to those of HbA (Figure 2D). On the other hand, Tyr RR bands of deoxyHb Rouen are seen at 1615 (Y8a), 1207 (Y7a), 1176 (Y9a), and 849 cm^{-1} (Y1), which are lower frequencies than those of deoxyHbA by 1−2 cm^{-1} except for that of Y7a.

The changes of Tyr RR bands of Y8a and Y9a for HbA are compared with those of Hb Rouen in greater detail in Figure 3. The Y8a band of deoxyHbA is shifted to a lower frequency by 2 cm^{-1}, and its intensity increases in the CO

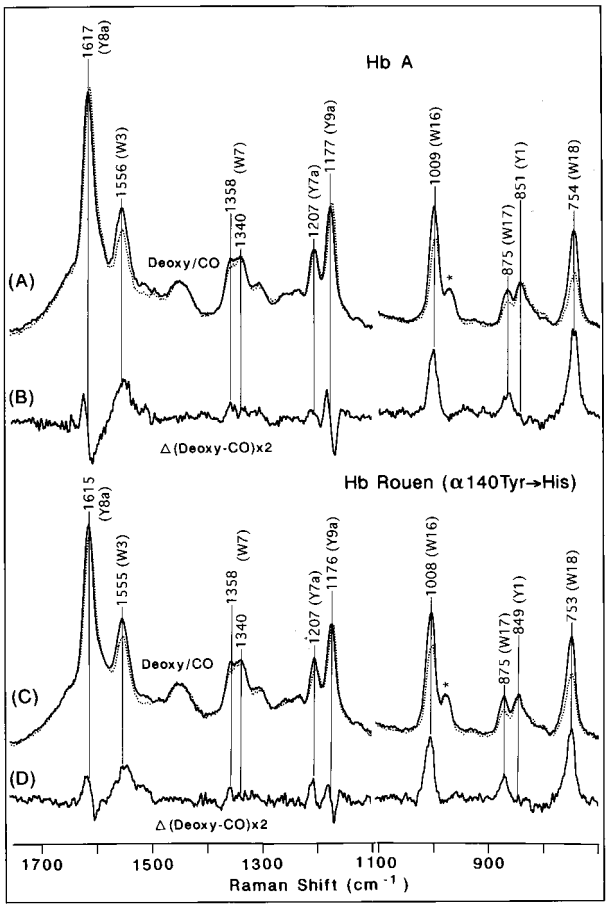

FIGURE 2: 235 nm-excited UVRR spectra of deoxyHb (solid line) and COHb (dotted line) of HbA (A) and Hb Rouen (C) and the deoxy-minus-CO difference spectra of HbA (B) and Hb Rouen (D). The difference spectra are multiplied by a factor of 2. The Hb concentration was 400 μM (in heme) in 0.05 M phosphate buffer (pH 7.0) containing 0.2 M Na_2SO_4. The peak marked by an asterisk denotes the Raman band of SO_4^{2-}. The difference spectra have been obtained so that the Raman band of SO_4^{2-} could be abolished. Each spectrum is an average of 20 spectra.

form (Figure 3A). In contrast, in the spectra of Hb Rouen, the shift of Y8a is evident but small ($\Delta = -1$ cm^{-1}) and no intensity enhancement is observed. The Y9a band of deoxy-HbA is downshifted by 3 cm^{-1} and enhanced in intensity in the CO form. The frequency shift of Y9a on CO binding is clearly observed for Hb Rouen, but the magnitude of the shift is also small ($\Delta = -1.6$ cm^{-1}) with no obvious increase in intensity (Figure 3B). Since the small frequency shift and no intensity increase of Tyr RR bands in Hb Rouen are due to the substitution of His for Tyr at α140, it is evident that α140Tyr is responsible for the frequency shifts of Y8a and Y9a and for intensity enhancement of HbA upon the T → R transition. The remaining frequency shift in Hb Rouen is probably due to the alteration of the hydrogen bonding state for α42Tyr which forms a hydrogen bond with β99Asp in the deoxy form but not in the CO form, owing to rearrangements in the subunit interface (*4*). On the other hand, the Trp RR bands of Hb Rouen were the same as those of HbA.

The RR spectrum of α140Tyr in the deoxyHbA can be provided by the difference calculations between the spectra of deoxyHbA and deoxyHb Rouen as shown in Figure 4B. The spectrum of α140Tyr in COHbA can be similarly obtained as shown in Figure 4D. In these difference spectra digitally obtained on the basis of the intensity of the 982

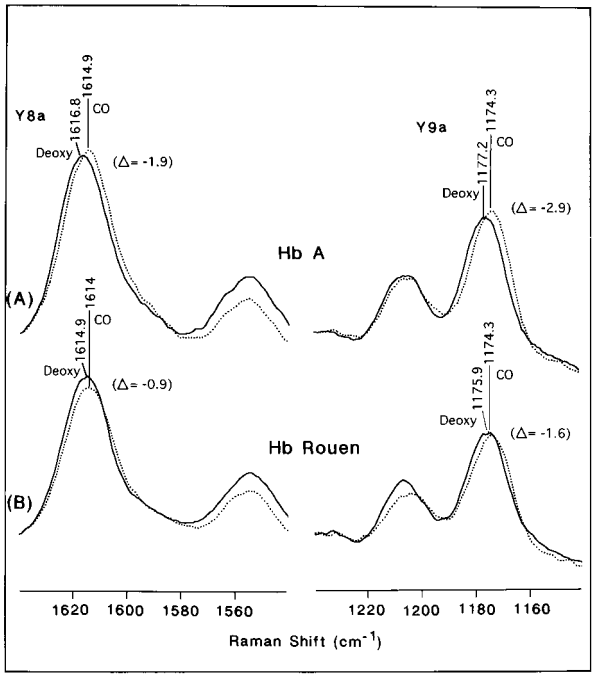

FIGURE 3: Expanded spectra in the 1640−1540 and 1240−1140 cm^{-1} regions of Figure 2 for HbA (A) and Hb Rouen (B). The solid line and the dotted line denote the deoxy and CO forms, respectively. The frequency difference (△) was obtained directly from the band maximum by reading in the deoxyHb and COHb spectra.

cm^{-1} band of SO_4^{2-} incorporated as an internal intensity standard, the Trp RR bands are completely canceled. This means that the environments of β37Trp in Hb Rouen are the same as that for HbA in both deoxy and CO forms. It is noted that the Y8a and Y9a frequencies of α140Tyr are smaller in the CO form (Figure 4D) than those in the deoxy form (Figure 4B). This is depicted in Figure 5.

Figure 5 shows the expanded view of the selected bands of α140Tyr in the deoxyHbA (A) and COHbA (B) and their difference (C). The Y8b band could not be distinguished from Y8a in the original spectra of HbA (Figure 2A), but is separated from Y8a in the extracted spectra of α140Tyr (Figure 5) for both deoxy and CO forms. The RR bands of Y8a at 1622 cm^{-1} and of Y8b at 1605 cm^{-1} in the deoxy form are shifted to 1617 and 1599 cm^{-1}, respectively, in the CO form. The band at 1181 cm^{-1} (Y9a) was also downshifted to 1175 cm^{-1}, but the 1207 cm^{-1} (Y7a) band remained unshifted. These changes are more clearly demonstrated in the double-difference spectrum (Figure 5C), which exhibits a differential pattern for Y8a and Y9a. The change of Y8b appears obscure in the double-difference spectrum because it overlaps with the change of Y8a. In the presence of IHP, these spectral features of Hb Rouen remained unaltered, although the oxygen affinity was slightly decreased without exhibiting any change in cooperativity.

β145 Tyrosine. To examine the contribution of β145Tyr to the UVRR spectral changes, we measured the UVRR spectra of des(β146His,β145Tyr)Hb prepared by CPase A digestion of HbA (*32*). Des(β146His,β145Tyr)Hb alone shows a high oxygen affinity and no cooperativity, but the normal function can be partially restored by the addition of IHP (*22*). Figure 6 shows the UVRR spectra of deoxy and CO forms of des(β146His,β145Tyr)Hb in the presence of

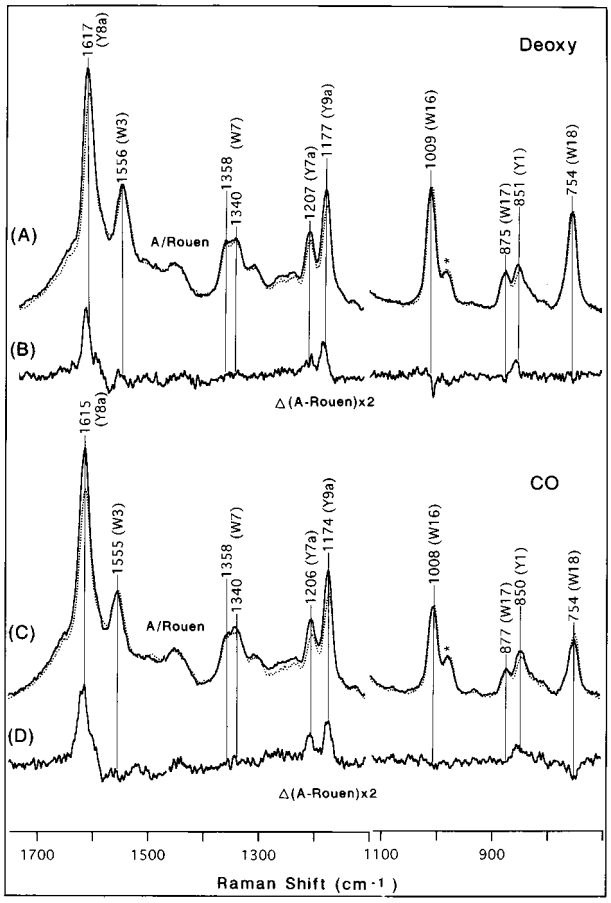

FIGURE 4: 235 nm-excited UVRR spectra of deoxy (A) and CO forms (C) of HbA (solid line) and Hb Rouen (dotted line) and the difference spectra between HbA and Hb Rouen in deoxy (B) and CO forms (D). The ordinate scale of the difference spectra is expanded by a factor of 2. The peak marked by an asterisk denotes a Raman band of SO_4^{2-}. The difference spectra have been obtained so that the Raman band of SO_4^{2-} could be abolished.

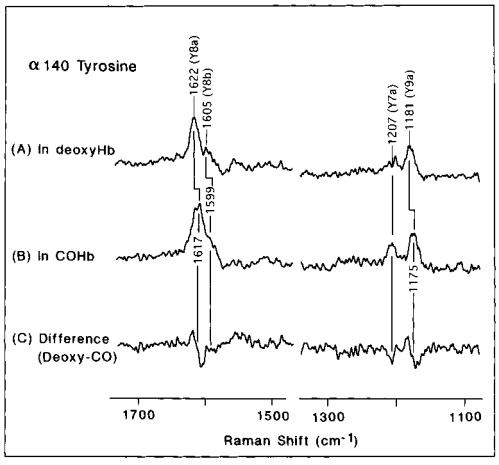

FIGURE 5: Extracted UVRR spectra of α140Tyr in deoxyHbA (A) and COHbA (B) and their difference spectrum (C). These were digitally obtained by subtraction of UVRR spectra of Hb Rouen from that of HbA in the deoxy and CO forms, respectively.

IHP. The RR bands at 1618 (Y8a) and 1176 cm^{-1} (Y9a) of deoxy-des(β146His,β145Tyr)Hb are downshifted by 2 cm^{-1} upon ligation. Significant intensity reduction of Trp RR bands was observed in the CO form. The difference spectrum between deoxy and CO forms of des(β146His,β145Tyr)Hb is compared with that of HbA in Figure 7. The intensities of

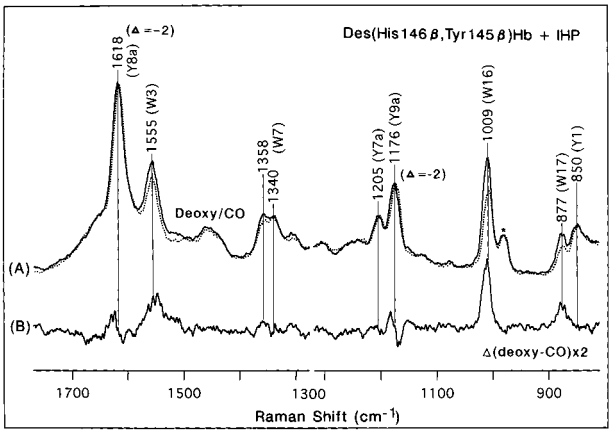

FIGURE 6: 235 nm-excited UVRR spectra of deoxy-des(β146His, β145Tyr)Hb (A, solid line), CO-des(β146His,β145Tyr)Hb (A, dotted line), and the deoxy-minus-CO difference spectrum (B). The spectra of des(β146His,β145Tyr)Hb were measured in the presence of 5 mM IHP. The difference spectrum is multiplied by a factor of 2. The other conditions were the same as those described in the legend of Figure 2. The peak marked by an asterisk denotes the Raman band of SO_4^{2-}. The difference spectra have been obtained so that the Raman band of SO_4^{2-} could be abolished.

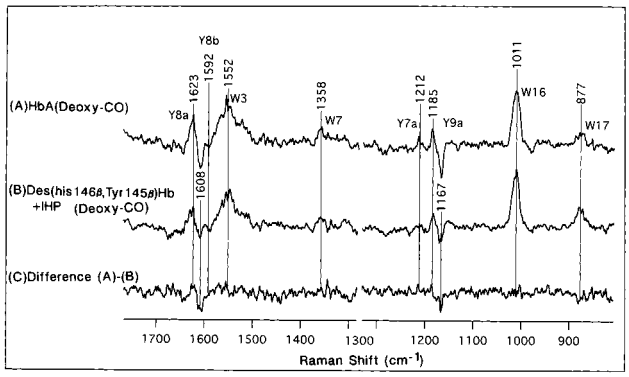

FIGURE 7: Deoxy-minus-CO difference spectra for HbA (A) and des(β146His,β145Tyr)Hb (B). Spectrum C depicts the double difference between spectra A and B.

Trp bands at 1552, 1358, 1011, and 877 cm^{-1} are similar between spectra A and B, and accordingly, the double-difference spectrum between HbA (deoxy-minus-CO) and des(β146His,β145Tyr)Hb (deoxy-minus-CO) (Figure 7C) yields no peak for Trp RR bands, indicating that the same change as that in HbA occurs with Trp residues in des-(β146His,β145Tyr)Hb upon ligation. The change of Tyr RR bands in des(β146His,β145Tyr)Hb looks to be similar to that of HbA, but actually there are small differences in intensities of Y8a and Y9a bands.

The spectra of single β145Tyr in deoxyHbA and COHbA, deduced from the difference calculations between the spectra of HbA and des(β146His,β145Tyr)Hb, are displayed in Figure 8. Besides Y8a, the Y8b band is clearly observed in both deoxy and CO forms. The Tyr RR bands of β145Tyr in deoxyHbA are seen at 1618 (Y8a), 1598 (Y8b), 1207 (Y7a), and 1175 cm^{-1} (Y9a). No frequency shift upon ligation is detected for β145Tyr, but the intensity of the Y8b and Y9a bands seems to increase upon ligation. This is more clearly demonstrated by the double-difference spectrum (Figure 8C). This result means that β145Tyr is not responsible for the frequency shift on the T → R transition but partly contributes to intensity enhancement.

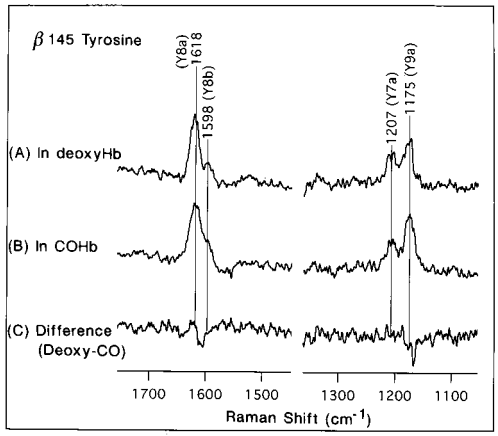

FIGURE 8: Extracted UVRR spectra of β145Tyr in deoxyHbA (A) and COHbA (B) and the difference (C) between spectra A and B. The spectrum of β145Tyr was digitally obtained by subtraction of the UVRR spectrum of des(β146His,β145Tyr)Hb from that of HbA for the deoxy and CO forms, respectively.

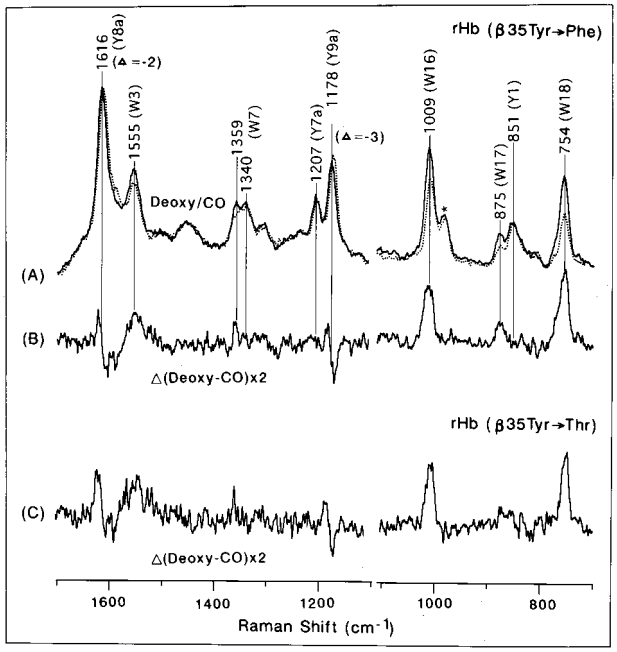

FIGURE 9: 235 nm-excited UVRR spectra of the deoxy (solid line) and CO forms (dotted line) of rHb (β35Tyr → Phe) (A) and the deoxy-minus-CO difference spectrum (B). Spectrum C is the deoxy-minus-CO difference spectrum of rHb (β35Tyr → Thr). The Hb concentration for rHb (β35Tyr → Phe) was 350 μM in 0.05 M phosphate buffer (pH 7.0) containing 0.175 M Na_2SO_4, and that for rHb (β35Tyr → Thr) was 200 μM in 0.05 M phosphate buffer (pH 7.0) containing 0.1 M Na_2SO_4. The peak marked by an asterisk denotes the Raman band of SO_4^{2-}. The difference spectra have been obtained so that the Raman band of SO_4^{2-} could be abolished. Each spectrum is an average of eight spectra. The difference spectrum is multiplied by a factor of 2.

β35 Tyrosine. The β35Tyr is a residue contributing not only to the α1β1 contact but also to the α1β2 contact by interacting with α141Arg which is spatially adjacent to β37Trp at the α1β2 contact (*5*). A mutant Hb at β35Tyr, rHb (β35Tyr → Phe), exhibits normal oxygen affinity and normal cooperativity ($n = 2.7$) (*33*). The UVRR spectra of rHb (β35Tyr → Phe) are shown in Figure 9A. The frequencies of RR bands for Tyr and Trp residues in the deoxy form are very close to those of deoxyHbA. Upon binding of CO, Tyr RR bands at 1616 (Y8a) and 1178 cm^{-1} (Y9a) were

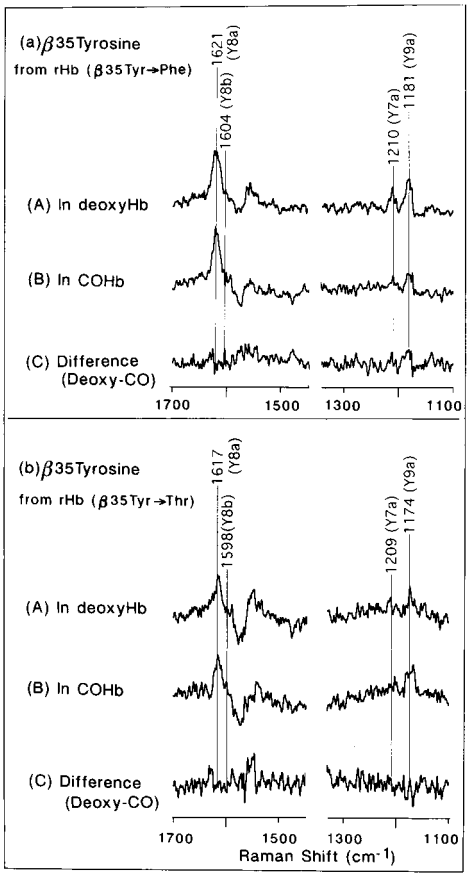

FIGURE 10: Extracted UVRR spectra of the β35Tyr residue in deoxyHbA (A) and COHbA (B) obtained with the spectra of rHb (β35Tyr → Phe) (a) and those of rHb (β35Tyr → Thr) (b) and the difference (C) between spectra A and B. The extracted spectra of β35Tyr were digitally obtained by the subtraction of UVRR spectrum of rHb (β35Tyr → Phe) or rHb (β35Tyr → Thr) from that of HbA for the deoxy and CO forms, respectively.

downshifted by 2 and 3 cm^{-1}, respectively. RR bands of Trp of deoxy-rHb (β35Tyr → Phe) were also greatly reduced in intensity by CO ligation. Consequently, the difference spectrum between deoxy and CO forms of this mutant Hb (Figure 9B) is nearly identical with that of HbA, indicating that all the spectral changes of Tyr and Trp RR bands in rHb (β35Tyr → Phe) are the same as those of HbA. In Figure 9C, the difference spectrum between deoxy and CO forms of another mutant Hb at β35Tyr, rHb (β35Tyr → Thr), is also shown. Since the Thr mutation is more disruptive from the native structure, rHb (β35Tyr → Thr) exhibits moderately high oxygen affinity and decreased cooperativity ($n = 2.1$) (T. Nakatsukasa et al., unpublished results). However, the deoxy-minus-CO difference spectrum of rHb (β35Tyr → Thr) is very similar to those of rHb (β35Tyr → Phe) and HbA. In other words, β35Tyr is not directly involved in the T → R structure transition. In a comparison of the UVRR spectra of the rHbs with that of HbA, intensities of all Tyr bands are weaker than those of HbA, owing to deletion of one tyrosine residue. Panels a and b of Figure 10 show the difference spectra between HbA and rHb (β35Tyr → Phe) and between HbA and rHb (β35Tyr → Thr), respectively, which reveal the spectra of a single β35Tyr residue in deoxy (A) and CO forms (B), respectively. The frequencies and intensities of the Y8a and Y9a bands of β35Tyr in deoxyHbA are nearly identical to those in COHbA. Accordingly, the double difference between them gives no clear peaks (trace C in Figure 10a,b).

DISCUSSION

Changes of Tyr UVRR Bands. The 235 nm-excited UVRR spectra reveal spectral changes of Tyr and Trp residues characteristic of the T−R transition of hemoglobin. Tyr RR bands of deoxyHbA at 1617 (Y8a) and 1177 cm^{-1} (Y9a) were downshifted by 2−3 cm^{-1} and increased in intensity upon ligand binding. These changes are due to the quaternary structure transition but not to the tertiary structural change because similar spectral changes are also induced in metHb by addition of IHP (*15*, *30*). Rodgers et al. (*14*) reported that the frequency shift probably originates from alteration of the hydrogen bond of α42Tyr which forms a hydrogen bond with β99Asp in deoxyHb but not in COHb on account of rearrangements of residues at the α1β2 subunit contact (*4*). In fact, the changes of Tyr RR bands in the mutant Hbs caused by replacing α42Tyr with His or Asp were different from those of HbA (*17*, *18*). However, distinct spectral changes of Tyr RR bands were observed in these mutant Hbs, suggesting that other Tyr residues are also responsible for the T−R transition.

There are four Tyr residues at the α1β2 subunit contact: α42, α140, β35, and β145. In this paper, we have presented the UVRR spectra of mutant Hbs at α140, β35, and β145Tyr. Since rHb (β35Tyr → Phe) and rHb (β35Tyr → Thr) gave spectra similar to that of HbA in both the deoxy and CO forms, there is no contribution of β35Tyr on the T−R transition. Des(β146His,β145Tyr)Hb exhibited frequency shifts similar to those of HbA upon CO binding with regard to Y8a and Y9a, but the intensity enhancement of these Tyr RR bands was small, suggesting that β145Tyr is partly responsible for the intensity changes in HbA on the T−R transition.

In the UVRR spectra of Hb Rouen (α140Tyr → His), frequency shifts of Y8a and Y9a were present but the magnitude was one-half of the normal case, and moreover, the intensity of these RR bands was not altered by ligation. The frequency shift observed in the spectra of Hb Rouen is probably due to the change of the hydrogen bonding state of α42Tyr. From these results for Hb Rouen, it is strongly suggested that α140Tyr is also responsible for both the frequency shift and the intensity enhancement of Y8a and Y9a upon the T → R transition. The sizes of frequency shifts of Y8a and Y9a of α140Tyr are similar to those of α42Tyr. Therefore, the frequency shift of Tyr RR bands in HbA is derived from two Tyr resides in the α subunit (α42Tyr and α140Tyr). This result is consistent with the recent UVRR study of the isotopic hybrid Hbs, in which the frequency shift of Tyr RR bands was observed only for those in the α subunit (*19*). As shown in the UVRR spectra of Hb Rouen, the change of α42Tyr upon the T−R transition was a frequency shift without an intensity change. On the other hand, both the frequency shifts and intensity enhancement were displayed by α140Tyr. Then a question arises. What is the origin for the different features between α42Tyr and α140Tyr?

It was clarified from the studies of model compounds, such as *p*-cresol and *N*-acetyltyrosine ethyl ester dissolved in various solvents, that the formation of hydrogen bonds in

basic solvents (such as a hydrogen acceptor) causes frequency downshifts of Y8b and Y9a in addition to the intensity enhancement (*34*−*36*). The intensity of Y8a and Y9a of these model compounds is also influenced by the hydrophobicity of its surroundings (*37*−*39*). However, it is difficult to separate the two solvent effects of hydrogen bond formation and hydrophobicity. We suggest that the frequency shift of Tyr RR bands is due to the formation or destruction of hydrogen bonds and the intensity enhancement is due to environmental alteration from less polar (hydrophobic) to polar (hydrophilic). If the hydrophobicity remains unchanged after cleavage of hydrogen bonds, only frequency shifts would be expected as in the case of α42Tyr. In fact, since α42Tyr stays inside of the globin molecule, it is possible for α42Tyr to undergo little alteration in hydrophobicity between the deoxy and CO forms. Although the X-ray study suggests that α140Tyr forms an intramolecular hydrogen bond with α93Val in both deoxy and CO forms (*40*), the present UVRR data indicate that α140Tyr forms a hydrogen bond in the deoxy form but not in the CO form. The occurrence of an intensity change for α140Tyr means that the environment around the side chain of α140Tyr, the penultimate Tyr of the α subunit, may change from a less polar to a polar environment (*37*, *38*). A similar environmental change might occur in the case of β145Tyr, the penultimate Tyr of the β subunit, because intensity enhancement in CO-des(β146His,β145Tyr)Hb is less distinct than that in COHbA. An intramolecular hydrogen bond between β145Tyr and β98Val seems to remain intact in the CO form on the basis of X-ray study (*40*), and no frequency shift takes place upon the T−R transition. In fact, the X-ray studies indicate that the C-terminal residues of both the α and the β subunits are much more disordered in the R state than in the T state. That is, the C termini of α and the β subunits may be "locally unfolded" to a large degree in the R state. Therefore, the X-ray diffraction data and the present UVRR data may be in agreement if the UVRR results are consistent with the loss of the α140Tyr hydrogen bond in most but not all of the hemoglobin tetramers in solution. Consequently, α42Tyr and α140Tyr are considered to contribute to the frequency shift, and the penultimate Tyr residues (α140 and β145) are responsible for the UVRR intensity enhancement on the T → R transition.

Changes of Trp RR Bands. The main spectral changes of Trp RR bands on the T−R transition are seen with regard to intensity. The RR bands for Trp residues of deoxyHbA at 1556 (W3), 1009 (W16), 875 (W17), and 754 cm^{-1} (W18) lose intensity significantly upon binding of a ligand, and as a result, prominent peaks appear in the deoxy-minus-CO difference spectrum. The major changes of these Trp RR bands were assigned to β37Trp from the studies of Hb Hirose (β37Trp → Ser) (*16*) and Hb Rothchild (β37Trp → Arg) (*14*).

We have suggested that the intensity reduction of β37Trp in the CO form might be due to a hypochromic ring stacking between β37Trp and one of the nearby Tyr residues (*16*). Candidates for the counterpart residue can be β35-, α42-, and α140Tyr. In the UVRR spectra of rHb (α42Tyr → His), the changes of Trp RR bands influenced by ligand binding were identical to those of HbA, indicating that α42Tyr is not involved in the intensity change of β37Trp (*17*). Here we have shown that the UVRR spectra of the other mutant Hbs in which β35 or α140Tyr was replaced by a nonaromatic amino acid residue gave rise to spectral changes identical with that of HbA. Consequently, we are obliged to withdraw our proposal of the stacking mechanism as the origin of the intensity reduction of Trp bands in COHbA.

There are two other factors accounting for the intensity change; one is due to the formation of a hydrogen bond with α94Asp which would be cleaved in the liganded form, and the other is due to the environmental change of the side chain of β37Trp upon the T−R transition. Studies of solvent effects on the model compound of Trp have demonstrated that the Raman bands in resonance with the B_b electronic transition such as W3, W16, W17, and W18 would yield higher intensities when the indole ring interacts with hydrophobic surroundings and the indole NH site is hydrogen bonded with a proton acceptor (*14*, *39*). It was desirable to separate the effect of hydrogen bond formation from the change of hydrophobicity. Matsuno and Takeuchi (*41*) recently succeeded in its separation in the studies of solvent effects, pointing out that Trp RR bands in resonance with the B_b band (235 nm) are greatly intensity-enhanced by hydrogen bond formation under identical hydrophobic environments. According to Strickland et al. (*42*), the UV absorption band of 2,3-dimethylindole is red-shifted by 3−10 nm when it forms hydrogen bonds in a hydrogen acceptor solvent. When the absorption maximum of Trp at 220 nm is red-shifted by hydrogen bond formation (*43*), the intensity of the Trp RR band excited at 235 nm is expected to increase significantly owing to the resonance effect. Therefore, it is most likely that the enhancement of Trp RR bands in deoxyHbA is due to the formation of hydrogen bonds resulting in a red shift of the Raman excitation profile as suggested by Hu and Spiro (*19*).

Structural and Functional Role of Tyr Residues at the α1β2 Interface. It is well-known that the hydrogen bonding between α42Tyr and β99Asp in deoxyHbA is essential for stabilization of the T structure (*6*). However, other Tyr residues at the α1β2 contact also seem to be important for the communication pathway that links ligand binding to the quaternary structure transition.

In the T structure, α140Tyr forms intersubunit contacts less than 4 Å from β37Trp and β36Pro as well as intrasubunit contacts with α87His, α88Ala, α92Arg, α93Val, α95Pro, α98Phe, α136Leu, α137Thr, and α138Ser (*44*). An intrasubunit hydrogen bond with the carbonyl group of α93Val is also formed (*40*). These residues form a tight pocket that constrains the side chain of α140Tyr and reduces its mobility. According to the stereochemical mechanism of Perutz (*1*), oxygen binding to the α heme in the T structure results in expulsion of α140Tyr from its pocket. A more recent high-resolution crystallographic study (*40*) shows that the penultimate Tyr remains within the F−H pocket, while the Tyr residue does appear to be more disordered. This is suggestive of a weakening or disruption of the hydrogen bond to the FG corner α93Val, as indicated in this study.

The replacement of the penultimate Tyr of the α subunit by Gln or Phe caused remarkable enhancement of subunit dissociation from tetramers to dimers, resulting in a high oxygen affinity and diminished cooperativity (*45*). On the other hand, in Hb Rouen (α140Tyr → His) (*29*), alteration of the oxygen binding properties is considerably less dramatic than that in rHb (α140Tyr → Gln) and rHb (α140Tyr →

Phe) (*45*). The functional data suggest that the side chain of the His at the penultimate position is too short to make a hydrogen bond with the carbonyl group of α93Val, allowing a nearly normal structure by some interaction between the His and the residues forming the "tyrosine pocket" (*29*). The ν_{Fe-His} frequency of deoxyHb Rouen is upshifted (217 cm^{-1}) compared to that of deoxyHbA (214 cm^{-1}). This means that the constraints on the F-helix by substitution of His for α140Tyr have been reduced, and thus the tension exerted on the Fe−N$^{\epsilon}$His(F8) bond within the deoxy-T structure has also been reduced.

Although the penultimate Tyr of the β subunit interacts with α41Thr and amino acid residues α37−α40 of the C-helix (*4*), there is no aromatic residue which is expected to have an important role in the subunit assembly. The replacement of the β145Tyr by Asp in Hb Nancy and Hb Osler caused high oxygen affinity and a lack of cooperativity, but the Hill constant for Hb Nancy increased from 1.1 to 2.0 with the addition of IHP (*46*, *47*). The NMR spectrum of Hb Osler in the presence of IHP becomes similar to that of deoxyHbA (*47*). The NMR studies of deoxyHb Mckees Rocks (β145Tyr → terminated) indicate that this Hb is predominantly in the R quaternary structure, but changes into the T quaternary structure upon addition of IHP (*47*). Removal of β145Tyr and β146His from HbA produces a molecule with high oxygen affinity and no cooperativity, but the normal function is partially restored by addition of IHP (*22*). These facts mean that the structural disorder caused by removal or substitution of the penultimate Tyr in the β subunit could be restored by the addition of IHP.

In HbA, β35Tyr is located mainly in the $\alpha1\beta1$ contact region and the phenolic hydroxyl group of β35Tyr is hydrogen bonded to the carboxyl group of α126Asp in both the oxy and deoxy forms (*48*). Therefore, one would have expected the substitution of β35Tyr to have little effect on the allosteric equilibrium. The β35Tyr also forms intersubunit contact with α141Arg less than 4 Å away and spatially adjacent to β37Trp at the $\alpha1\beta2$ contact (*5*). Although the naturally occurring β35Tyr mutant Hb, that is, Hb Philly (β35Tyr → Phe), showed an impaired function (*49*), very minor changes in the functional properties were detected in rHb (β35Tyr → Phe) and rHb (β35Tyr → Ala) (cited in ref *44*). The rHb (β35Tyr → Phe) used in this study also showed normal oxygen affinity and cooperativity ($n = 2.7$) (*33*). Therefore, Hb Philly may have another unknown structural factor.

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