Time-Resolved UV Resonance Raman Study on Bohr Effects of Hemoglobin

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Abstract: Effects of Bohr protons on the quaternary structure dynamics were investigated for photodissociation of carbonmonoxyhemoglobin (COHb) by using time-resolved UV resonance Raman spectroscopy. The 218-nm excited Raman spectra reproduced the quaternary structure-dependent spectral change of the Trp bands reported by us previously (*J. Am. Chem. Soc.* **1990**, *112*, 23). However, in the present study, a newly designed sample-flowing system was used successfully to prevent photodissociated deoxyHb from recombining with CO. This allowed determination of the final point of the change. The intensity of a Trp band at 1011 cm^{-1} relative to a Phe band at 1003 cm^{-1} was used for monitoring the quaternary structure change. At pH 7.4 the intensity change began at 5 μs after photolysis and ended at 20 μs. A two state change was observed and there was no evidence for intermediate state. At pH 5.8, where Bohr protons are attached, the change was significantly faster; it was already finished at 10 μs and remained unchanged after that. The relative intensity values and, therefore, the amount of change were not altered by binding of the Bohr protons, indicating that the structural changes are similar and only the rate is altered by Bohr protons. The likely mechanism of the quaternary structure change, based on the present observations, is discussed.

Hemoglobin (Hb) releases protons upon binding of oxygen. This heterotropic allostery is known as the alkaline Bohr effect[1] and plays an essential role in promoting CO_2/O_2 exchange by Hb in tissues. Since the allosteric effect of Hb can serve as a typical model of general allostery,[2] which is essential to regulation of biological molecular activity, structural analysis of Hb allostery continues to be a subject of intensive study.[3-9] A large body of experimental data on Hb has been satisfactorily explained by assuming a reversible transition between the low affinity (T) and high affinity (R) quaternary structures.[10] Carbonmonoxide-bound Hb (COHb) and deoxyHb adopt the familiar R and T quaternary structures, respectively.[3] Photolysis of COHb has provided a means for studying the dynamical features of the quaternary structure change.[11-13] Structural evolution following the photolysis has been monitored mainly by using the time-resolved variants of optical absorption[13-17] and resonance Raman[18-21] spectroscopies.

The Bohr effect arises from reduction of the pK_a values of a few residues upon ligand binding. Those residues (Bohr residues) were initially thought to be the N and C terminal residues of the α and β subunits,[22] but later were determined, by the tritium exchange technique,[23] to be the α20-, α89-, β143-, and β146-histidines (His). This pK_a lowering can be attributed to the destabilization of the protonated form of these residues which arises as a consequence of increased electrostatic repulsions, or destruction of hydrogen bonds, upon ligand binding.[24] Phenomenologically, acidification of the solution shifts the quaternary equilibrium toward the T structure and thus the oxygen binding equilibrium of Hb toward deoxyHb; the O_2 binding constant is reduced by a factor of 2 for adult human Hb (from 3.31 at pH 7.4 to 1.60 at pH 6.5).[25] Since the oxygen affinity of Hb seems to be controlled by strain exerted by the globin on the Fe–His(F8) bond,[26] it is interesting to examine how the stationary and dynamical features of quaternary structures are influenced by protonation of Bohr residues.

UV resonance Raman (RR) spectroscopy is a powerful technique for probing a structure of proteins.[27-32] Since the intensity of some Raman bands of aromatic residues are enhanced when the excitation wavelength is brought to 240–200 nm,[27] a change of the microenvironment of such residues upon a higher-order structural change of the protein can be revealed. Indeed, the quaternary structure change of Hb from the CO-bound (R) to the deoxy (T) structures was recently pursued with the time-resolved UVRR technique.[33,34] However, at the longer delay times in the experiments reported, rapid recombination within the observation zone (i.e., the illuminating spot of the focused laser) restored the COHb so that the end point of the structural change could not be determined. In order to circumvent this problem,

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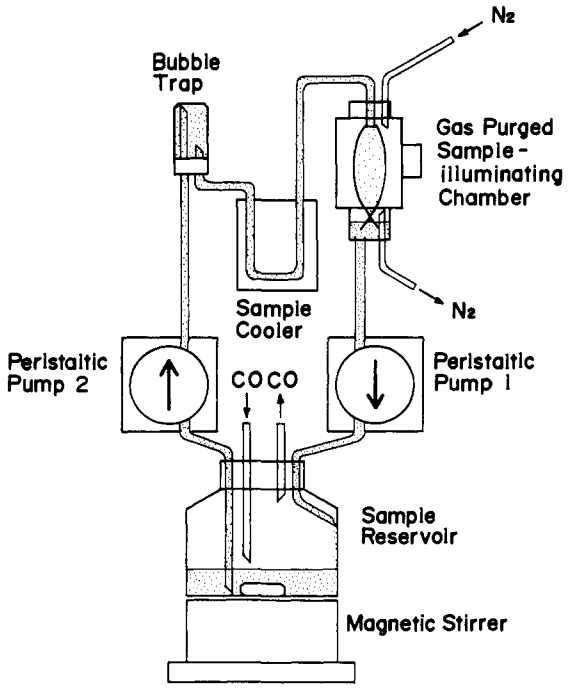

Figure 1. Schematic diagram of the sample flowing system used in this study. The sample illuminating chamber is the same as that in ref 34 (Figure 2) except for N_2 gas flowed.

we have devised a new sample illuminating system and use it in this study to investigate the dynamical aspects of the alkaline Bohr effect. It is found that the structural changes of the globin which occur upon photodissociation of COHb are similar at pH 5.8 and 7.4, but that the rate is much faster at pH 5.8 where Bohr protons are attached.

Experimental Procedures

Sample Circulation. The system for measuring the time-resolved UVRR spectra was described previously.[34] In order to prevent the photodissociated Hb from recombining with CO within the illumination spot, the sample circulation system was redesigned as illustrated in Figure 1. The sample illuminating chamber is similar to the previous one (Figure 2 of ref 34), but it is filled with pure N_2 gas (99.999%) in this study instead of pure CO gas as in the previous experiments. Since it is possible that CO could dissociate in an N_2 atmosphere before laser illumination, it is crucial to reduce the distance between the laser illuminating spot and initial point of the wire-guided flow device where the COHb solution is first exposed to the N_2 atmosphere.

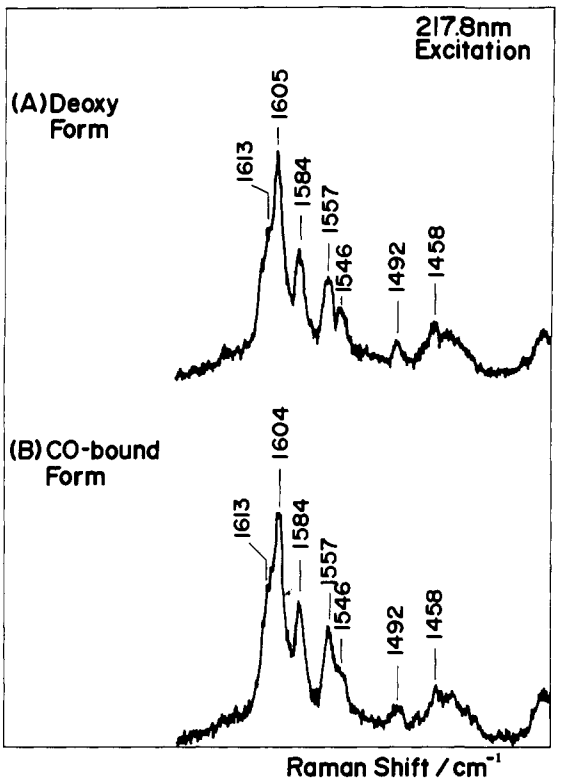

Figure 2. The 217.8 nm excited RR spectrum of deoxyHb (A) and COHb (B) in the 1700–1350 cm^{-1} region. Both were obtained with the flow apparatus with N_2 gas (A) and CO gas (B) in the sample chamber. For the measurements of spectrum A, the second harmonic of a Nd:YAG laser was used as a pump beam.

The sample solution is led to a CO-purged sample reservoir wherein it is stirred by a Teflon-coated magnetic stirrer to ensure equilibrium with the CO (99.999%) atmosphere. The flow rate and CO pressure are suitably adjusted to ensure complete CO binding. In order to maintain a constant level of solution at the bottom in the sample illuminating chamber, where the wire-guide is in touch with the solution, it was necessary to add a second peristaltic pump (Tokyo Rikakikai Corp. MP-3, peristaltic pump 1 in Figure 1), whose flow rate was matched with that of peristaltic pump 2. The Hb solution was replaced with fresh one every 20 min.

Raman Measurements. The probe beam (217.8 nm) was obtained as the H_2 second anti-Stokes Raman-shifted line of the fourth harmonic (266 nm) of a Nd:YAG laser (Quanta Ray, DCR-1A). The power of the 218 nm line was 100–120 μJ/pulse at the sample point, and it was illuminated to the area of 0.8 × 5 mm^2. Therefore, the photon flux density is as low as 3 mJ/(cm^2 pulse). For the time-resolved measurements, the pump beam for photodissociation of COHb was generated by a nitrogen laser-pumped dye {$(MS)_2B$ [*p*-bis(*o*-methylstyryl)benzene]} laser (Molectron UV24-DLII system) which was operated at 419 nm at a power of 180 μJ/pulse at the sample point. The two beams were spatially overlapped on the wire-guided flowing protein solution, but in order to acquire strict overlap, the upstream sides of both beams were masked by a single metal block. The delay time (Δt_d) of the probe pulse from the pump pulse was controlled by a pulse generator system[34] and monitored by detecting the partially reflected light of the two pulses with photodiodes. The uncertainty of Δt_d was 1–2 μs.

The CO-bound form of the protein was measured both with an air tight spinning cell (60 rpm) in which CO occupied the gas phase and also for the flowing sample (in the CO atmosphere) in the absence of the pump beam. Both methods yielded identical spectra. The UVRR spectra of the equilibrium deoxyHb could not be satisfactorily observed with the spinning cell since photoaggregation of Hb took place during the measurements (such a phenomenon is not seen upon visible excitation). Therefore, it was necessary to use the pump/probe technique and the flowing sample design. However, in this case, higher energy (1 mJ) pulses of the second harmonic (532 nm) were spatially expanded (to 5 × 20 mm^2) and used as the pump beam (in the N_2 atmosphere), and the delay time of the probe beam was extended to the maximum value possible with the present apparatus (500 μs).

The scattered light was dispersed with a 1.2 m single monochromator (Spex 1269) equipped with a 2400 groove/mm holographic grating and

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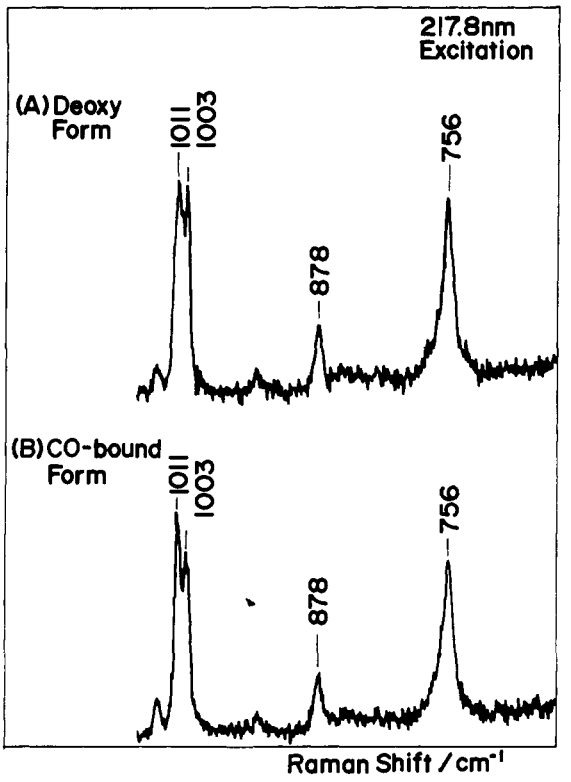

Figure 3. The 217.8 nm excited RR spectrum of deoxyHb (A) and COHb (B) in the 1050–650 cm^{-1} region. The spectra of deoxyHb and COHb were measured alternatively and 20 spectra of each were averaged. The data were collected for 3.2 s at every 0.8 cm^{-1}. Other experimental conditions used were the same as those for Figure 2.

detected by a UV-sensitive photomultiplier (Hamamatsu R-166UH). The grating was used in the second order. The output from the photomultiplier was averaged by a boxcar integrator (NF BX-531) and transferred to a microcomputer (NEC PC-9801 VM). The Raman shifts were calibrated with acetone, acetonitrile, and cyclohexane as standards.

Hb was isolated from fresh human adult blood by the usual method.[35] Minor components were removed by passage through the ion exchange columns (Pharmacia CM-23 and DE-23). The purified Hb was stored at 7 °C (as COHb), and just before Raman experiments it was diluted to 400 μM (heme) with 50 mM Tris/HCl buffer at pH 7.4 or with bis-Tris/HCl buffer at pH 5.8.

Results

The 218 nm excited RR spectra of COHb and deoxyHb (both in an equilibrium state) are displayed in Figure 2 (1700–1350 cm^{-1}) and Figure 3 (1050–650 cm^{-1}). The single color excited spectrum of COHb observed with the spinning cell was the same, within experimental error, as that observed for the flowing sample. Previously, we noted that, for the T and R structures of metHbF's, the intensity of a Trp band at 1011 cm^{-1} relative to that of a Phe band at 1003 cm^{-1} was altered and that the frequency of a Trp band at 878 cm^{-1} was shifted to 883 cm^{-1} by the quaternary structure change.[34] In order to examine the spectra of deoxyHb and COHb observed under the exactly same conditions, the spectra of deoxyHb and COHb were measured alternatively (one scan/25 min) and 20 spectra of each species were averaged. As shown in Figure 3, the relative intensity change of the Trp band is observed for the spectra of COHb (R structure) and deoxyHb (T structure), but the frequency of the Trp band at 878 cm^{-1} is the same between deoxyHb and COHb.

It may be argued that the intensity difference arises from saturation effects.[27d,36] If that were the case, the relative intensity would be expected to vary when the laser power is changed. We have confirmed, however, that the relative intensity remains unaltered when the laser power (probe beam) is raised to 180 and 250 μJ/pulse and also when it was reduced to 60 μJ/pulse. Since the photon flux density in the last measurement is 1.5 mJ/(cm^2 pulse), it is sufficiently lower than that at which the saturation effect appears.[36b,c] Therefore, the saturation effects,[27d,36] as well as photochemical damage of the protein[28b] are negligible at the level of 120 μJ/pulse (3 mJ/cm^2 pulse) used in this study.

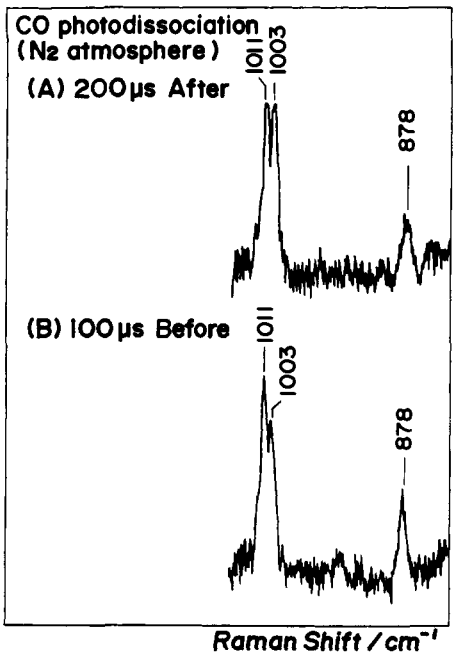

Figure 4. Time resolved resonance Raman spectra of COHb; the pump beam is 419 nm and probe beam is 217.8 nm. The delay times are +200 μs (A) and −100 μs (B).

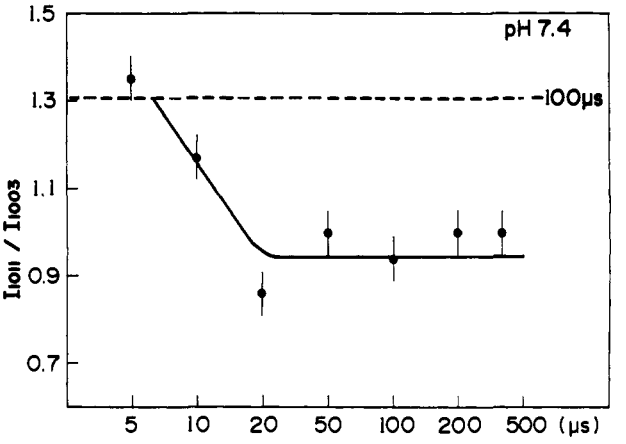

Figure 5. The quaternary structure change of COHb at pH 7.4 after photolysis. The intensity of a Trp band at 1011 cm^{-1} relative to that of a Phe band at 1003 cm^{-1} observed for the COHb solution at pH 7.4 (50 mM Tris/HCl) are plotted against the delay time. The broken line denotes the value for $\Delta t_d = -100$ μs. The solid line represents a smooth curve that passes the observed points.

Figure 4 depicts the time-resolved RR spectra in the frequency region between 1050 and 800 cm^{-1} for $\Delta t_d = +200$ μs (A) and −100 μs (B). Spectrum B is essentially the same as that of COHb observed without the pump beam in the CO atmosphere (Figure 3). It demonstrates that the present sample circulation system does not induce significant perturbations of the globin structure and that CO is not dissociated before laser illumination in the N_2 atmosphere. In the previous study[34] the RR spectrum for $\Delta t_d = 200$ μs was the same as that of the CO-bound form, but in this experiment the RR spectrum for $\Delta t_d = 200$ μs is distinctly different; it is close to the spectrum of deoxyHb shown in Figure 3. This indicates that recombination of CO with the photodis-

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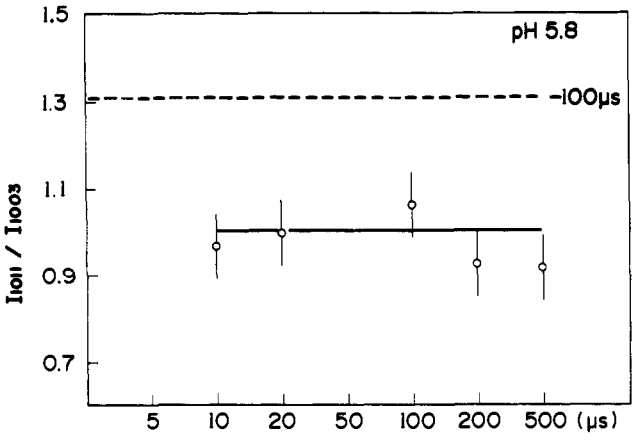

Figure 6. The quaternary structure change of COHb at pH 5.8 (50 mM bis-Tris/HCl) after photolysis. Other explanations are the same as those in Figure 5.

sociated Hb does not take place with the new circulation system.

Similar time-resolved measurements were carried out for various values of Δt_d between 5 and 500 μs. The RR spectrum for Δt_d = 5 μs (not shown) was similar to that for Δt_d = −100 μs. Accordingly, it confirmed the previous conclusion[34] that the Trp residue (presumably β37-Trp) responsible for the intensity change of the 1011 cm^{-1} band does not undergo any change at 5 μs after photolysis. The relative peak intensities (I_{1011}/I_{1003}) of the 1011 cm^{-1} band (Trp) to the 1003 cm^{-1} band (Phe) are plotted against Δt_d in Figure 5. The broken line indicates the value at Δt_d = −100 μs. It is apparent that I_{1011}/I_{1003} starts to change at Δt_d = 5 μs and finishes at Δt_d = 20 μs, in agreement with the results of Su et al.[33] and our previous work obtained from the relative intensity change of the 878 cm^{-1} band.[34] Note that the values after Δt_d = 50 μs are constant, indicating a two-state change. Since the I_{1011}/I_{1003} value for Δt_d = 10 μs is between the values for Δt_d = 5 and 20 μs, it is more reasonable to assume that there is no intermediate state between the CO-bound R and deoxy T structures so far as this Trp is concerned, although Su et al.[33] pointed out the presence of an intermediate state around Δt_d = 5 μs from the behavior of UVRR bands of Trp and Tyr residues obtained upon excitation at 229 nm.

The same series of measurements were carried out for the Hb solution at pH 5.8, where Bohr protons are attached and the oxygen affinity is lowered. Figure 6 shows the I_{1011}/I_{1003} versus Δt_d plots obtained for the sample at pH 5.8. Here again the broken line indicates the value at Δt_d = −100 μs; its magnitude is almost the same as that at pH 7.4. However, the value for Δt_d = 10 μs was different from that at pH 7.4. At pH 5.8, the I_{1011}/I_{1003} value remained constant, within experimental error, after Δt_d = 10 μs and the magnitude was very close to that at pH 7.4. This similarity suggests that the local structure of the Trp residue in question is not altered by binding of Bohr protons and that it exhibits a similar structural change upon photodissociation of CO, irrespective of the presence or absence of the Bohr protons. The only observable difference is the rate of structural change. The fact that the change to the T structure is much faster at acidic pH than at neutral pH is consistent with the fact that the quaternary equilibrium is shifted toward the T structure at acidic pH.

Discussion

Dynamics of Quaternary Structure. Previously, Sawicki et al.[11] measured the time dependent absorption changes (at 425 and 436 nm) following photolysis of COHb. They pointed out that the R to T transition is accelerated by lowering the pH; the time required was 200 μs at pH 9.4 and 50 μs at pH 6.0. Later Hofrichter et al.[13] determined the transition time to be 20 μs at pH 7.0 by using nanosecond transient absorption spectroscopy. The change of the Fe–His(F8) stretching frequency also occurs more rapidly at neutrality than under alkaline conditions.[19d] If the pH dependence observed by Sawicki et al.[11] is applied to Hofrichter et al.'s data[13] at neutral pH, the visible absorption spectral changes are expected to occur around 5 μs or sooner at pH 5.8. This is in agreement with the results shown in Figure 4.

In our previous time-resolved UVRR study,[34] the sample chamber was filled with CO, and therefore recombination of CO prevented detailed discussion on the kinetic behavior of the quaternary structure. The present flowing-sample system permits observation of the final deoxyHb at longer delay times. In the previous study the behavior of the relative intensity of two bands at 883 and 878 cm^{-1} suggested that the quaternary structural change occurs approximately 10–20 μs after photolysis.[34] The I_{1011}/I_{1003} versus Δt_d plots in Figure 5 indicate that the structural change takes place between 5 and 20 μs, in agreement with the previous results.[34]

The 883 cm^{-1} band is assigned to the W_{17} mode of Trp.[37c] Since this mode involves motion of the N–H group of the indole ring, its frequency is sensitive to hydrogen bonding at the indole ring and becomes lower when the N–H group is hydrogen bonded.[37] In the present study, however, the measurements of the W_{17} band of deoxyHb and COHb were repeated (20 scans) under identical experimental conditions, and finally we concluded that the W_{17} frequency was unaltered between deoxyHb and COHb, in agreement with Spiro and co-worker's observation.[38a] Therefore, one of Trp residues may undergo rearrangements of hydrogen bonding during the T – R transition, and only in the intermediate state does a part of the band exhibit a frequency shift. On the other hand, since the 1011 cm^{-1} mode (W_{16}) is associated with the in-plane C–H bending mode of the benzene ring, its frequency is insensitive to hydrogen bonding at N–H.[37b] Its intensity would likely be sensitive to π stacking at the benzene ring.

There are three Trp residues per αβ dimer: α14(A12), β15-(A12), and β37(C3). Two of them are contained in a helical region and their hydrogen bonding interactions are unaltered upon the quaternary structural change.[39] Accordingly, two-thirds of the intensity of Trp Raman bands would not be influenced by the quaternary transition. In contrast, β37-Trp is located at the α_1–β_2 interface where this residue is in contact with $\alpha_1$140-Tyr, $\alpha_1$94-Asp, and $\alpha_1$95-Pro in the deoxy state but is freed from the $\alpha_1$140-Tyr contact in the CO-bound state.[39] The UV absorption differences between the T and R structures of deoxy Hb[40a] and metHb[40b] have been attributed to environmental changes of β37-Trp and α42-Tyr. Furthermore, recently Spiro et al.[38b] observed a change in W3 band of Trp at Δt_d = 10 μs and attributed it to β37-Trp on the basis of the same measurements on a β37-mutant Hb (Hb Rothschild). Therefore, it is most reasonable to identify the Trp residue which exhibited the quaternary structure dependent UVRR spectral change with β37-Trp.

Structural Implications of Bohr Effects. There is general agreement that β146-His significantly contributes to the Bohr effect.[22,23] Perutz et al.[22] explained its structural aspect as follows. The protonated β146-His residue is hydrogen bonded with the CO_2^- group of β94-Asp in deoxyHb, whereas this hydrogen bond is cleaved in COHb. Recently its pK_a value was determined to be 8.1 for deoxyHb and 7.0 for COHb.[41] Therefore, this residue remains protonated in both the deoxy and CO bound forms at pH 5.8, but partial protonation occurs upon photodissociation of COHb at pH 7.4. This may cause the kinetic difference between the results shown in Figures 4 and 5. The second candidate for the Bohr residue is β143-His[24] which is involved in an electrostatic

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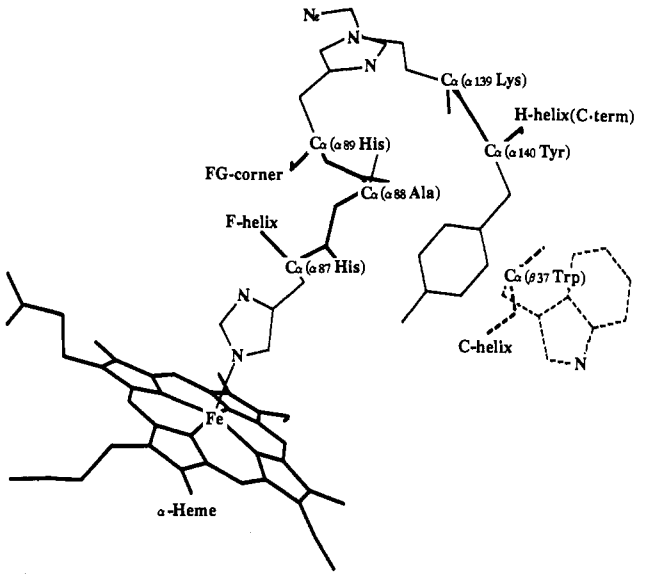

Figure 7. The structure of deoxyHb near α87-α89, α139-α140, and β37 residues taken from computer graphics (original, ref 43).

interaction with the ϵ-NH_3^+ group of β144-Lys, and therefore its protonated form is destabilized. Accordingly, the pK_a value of β143-His is low: 5.6 for deoxyHb and 6.1 for COHb.[41] At pH 7.4 this residue is deprotonated in both the deoxy- and CO-bound states, but at pH 5.8 partial deprotonation occurs upon photodissociation of COHb, which is opposite to the overall Bohr effect. Since the pK_a difference is not so large, this residue is considered to contribute little to the quaternary structure-dependent UVRR spectral change. The pK_a value of α20-His was determined to be 7.6 for deoxyHb and 7.0 for COHb.[41] This residue should be deprotonated in both the deoxy and CO-bound states at pH 5.8, but at pH 7.4 partial protonation occurs upon photodissociation of COHb, similar to β146-His.

The last candidate for the Bohr residue is α89-His which has the pK_a values of 7.2 for deoxyHb and 5.6 for COHb.[41] In this case, partial protonation takes place, both at pH 5.8 and 7.4, upon photodissociation of COHb, consistent with the fact that the I_{1011}/I_{1003} change is practically the same at pH 5.8 and 7.4. Since neutral α89-His can be hydrogen bonded with ϵ-NH_3^+ of α139-Lys and they repel each other upon protonation of α89-His, some movement of α139-Lys may take place upon protonation of α89-His. Consequently, the next residue, α140-Tyr, would also be expected to move. As a result, β37-Trp, which interacts with α140-Tyr,[39] would be affected. This view of the cooperativity mechanism is consistent with the recent observation of noncooperative oxygen binding and no Bohr effect for crystalline Hb[42] in which the intersubunit salt-bridges as well as intrasubunit tertiary structure are retained in the T quaternary structure by the lattice force.

α89-His is located at the end of the F-helix near the proximal His (α87) as illustrated in Figure 7.[43] The time-resolved visible RR spectra by Friedman et al.[19c-e] demonstrated that the Fe–His(F8) stretching frequency changes continuously in the time period from 10 ns to 10 μs after photolysis, but during this period the subunit interface exhibited no distinct change. It is noted that strain on the Fe–His(F8) bond is apparently correlated with the oxygen affinity,[26a,b] and also with the intersubunits hydrogen bonding. The latter was demonstrated recently for mutant Hb's obtained from site directed mutagenesis.[44] Consequently, it is likely (see Figure 7) that the dissociation of CO is first noticed by α87-His (proximal His), and then by α89-His (in the same F-helix as α87-His), which induces a change of α140-Tyr.

Finally, β37-Trp is altered by direct interaction with α140-Tyr. While the structural change itself is unaffected by pH, the rate of change is accelerated by protonation of Bohr residues. This was previously predicted by Perutz,[22a] and the increased rate can be ascribed to the shift of quaternary equilibrium toward the T structure. In conclusion, it is stressed that the allosteric effect of Hb is caused by mechanical strain originating from the heme moiety which is propagated by geometrical structure changes of the globins to other subunits.

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