Resonance Raman Study on the Active-Site Structure of a Cooperative Hemerythrin†

Shoji Kaminaka,‡ Hideo Takizawa,§ Takashi Handa,§ Hiroshi Kihara,‖ and Teizo Kitagawa*,‡

Institute for Molecular Science, Okazaki National Research Institutes, Myodaiji, Okazaki, 444 Japan, Tokyo Science College, Kagurazaka, Shinjuku, Tokyo, 278 Japan, and Jichi Medical College, Yakushiji, Minamikochi-cho, Kouchigun, Tochigi, 329-04 Japan

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ABSTRACT: Resonance Raman spectra were observed for the oxy and azidomet forms of a cooperative hemerythrin (Hr) isolated from *Lingula unguis* and a noncooperative Hr from *Siphonosoma cumanense*. The O–O stretching frequency of the oxy derivative of the *L. unguis* Hr was lower in the high-affinity form generated at pH 7.6 than in the low-affinity form generated at pH 6.2, while that of the *S. cumanense* Hr did not change at those two pH values. The Fe–O–Fe symmetric stretching mode of *L. unguis* azidomet-Hr exhibited a frequency shift between pH 7.6 and 6.2, while that of *S. cumanense* was not shifted. However, the corresponding band of the oxy form did not show a pH-dependent frequency change. Therefore, it is noted that the azidomet form is not a suitable model for studying a mechanism of cooperativity, contrary to the structural similarity between the oxy and azidomet forms. The Fe–O_2 as well as Fe–N_3 stretching frequencies were found to have no relation with the oxygen affinity. Upon exchange of solvent from H_2O to D_2O, the O–O and Fe–O_2 stretching modes of *L. unguis* Hr were shifted to higher and lower frequencies, respectively, and their magnitudes were the same for the high- and low-affinity forms. The same frequency shifts were observed for *S. cumanense* Hr. These observations suggested that the binding of oxygen to an arbitrary subunit is communicated to another subunit through hydrogen bonding between bound oxygen and a surrounding amino acid residue and that this hydrogen bond stabilizes more the oxy structure of the high-affinity form than that of the low-affinity form.

Hemerythrin (Hr) is an oxygen transport protein found in some marine invertebrates. The protein is typically an octamer with $M_r = \sim 108\,000$ (Klippenstein, 1980; Klotz et al., 1976; Ward et al., 1975), and each monomer contains a nonheme iron binuclear center which reversibly binds one molecule of oxygen (Boeri & Ghiretti-Magaldi, 1957). The met form of Hr is known to have two high-spin Fe^{3+} ions with a large antiferromagnetic exchange coupling (Schugar et al., 1972) mediated by an endogenous μ-oxo bridge, the presence of which was demonstrated by X-ray crystallographic (Stenkamp et al., 1985) and EXAFS studies (Hendrickson et al., 1982). Recently, the structures of the deoxy and oxy forms were solved at the level of 2.0-Å resolution, and the coordination environments around two irons were revealed in detail (Holmes et al., 1991); one iron (Fe1) is always hexacoordinated, but the other (Fe2) is penta- or hexacoordinated depending on the two states. Fe1 and Fe2 are coordinated by three and two histidine residues, respectively, and are joined by the carboxyl side chains of glutamic and aspartic acid residues besides the μ-oxo bridge. As suggested by earlier studies (Reem & Solomon, 1984; Stenkamp et al., 1985; Zhang et al., 1988), the μ-oxo bridge of the oxy form is converted to a μ-hydroxo bridge in the deoxy form and the antiferromagnetic exchange coupling is greatly reduced (Reem & Solomon, 1987).

Resonance Raman (RR) studies explored that the oxygen binding is of an end-on type (Kurtz et al., 1976) and the bound oxygen assumes a peroxide (Dunn et al., 1975, 1977; Dawson et al., 1972; McLendon & Martell, 1976; Sanders-Loehr et al., 1980) to which a proton is attached (Shiemke et al., 1984). This proton is deduced to be hydrogen bonded to the μ-oxo bridging oxygen (Shiemke et al., 1986). This presumption was certified by the recent X-ray crystallographic analysis (Holmes et al., 1991). Single-crystal polarized absorption spectra (Gay & Solomon, 1978) indicated structural similarity in the ligand binding sites between the oxy and azidomet forms. These structural and spectroscopic properties of Hrs are reviewed by Klotz and Kurtz (1984), Sanders-Loehr (1989), Vincent et al. (1990), and Kurtz (1990).

Current interest in the Hr studies is focused on appearance of allostery (Reem et al., 1989), which seems to be crucial in its physiological function. The cooperative oxygen binding is observed for two brachiopods, *Lingula reevii* (Richardson et al., 1983) and *Lingula unguis* (Manwell, 1960). It was found recently that these two cooperative Hrs adopt $\alpha_4\beta_4$ octamer (Satake et al., 1990; Zhang & Kurtz, 1991) and are distinct from others in this regard. Although X-ray structures are not available for these two Hrs, detailed spectroscopic analysis was carried out for *L. reevii* Hr (Richardson et al., 1987). According to it, the O–O stretching frequency is lower by 1.5 cm^{-1} in the high-affinity state (high-pH form) than in the low-affinity state (low-pH form). However, there has been no such detailed study on *L. unguis* Hr, while there was a preliminary report (Joshi & Sullivan, 1973). Since the analysis of oxygen binding properties of *L. unguis* Hr was completed (Imai et al., 1991b), we investigated, in this study, resonance Raman spectra of oxy and azidomet forms of *L. unguis* Hr under the same solution conditions as used for the analysis of the oxygen binding equilibrium. For comparison, we also treated Hr isolated from *Siphonosoma cumanense*, which

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* Author to whom correspondence should be addressed.
‡ Okazaki National Research Institutes.
§ Tokyo Science College.
‖ Jichi Medical College.

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exhibits no cooperative oxygen binding at any pH and is proved to be a trimer (Satake et al., 1989; Fuseya et al., 1989).

MATERIALS AND METHODS

L. unguis collected in the Ariake sea of Japan were deshelled, and the inner substance was washed with 0.1 M phosphate buffer, pH 8.0, containing 0.25 M Na_2SO_4. The solution containing insoluble fractions was filtered through a glass wool column and centrifuged. The precipitates (erythrocyte-like cells) were washed with the same buffer twice and then hemolyzed. Hr was purified from the supernatant of the hemolyzed solution using CM-Sephadex C-50 chromatography according to Joshi and Sullivan (1973). The *S. cumanense* Hr was purified from the coelomic fluid of the marine worm (*S. cumanense*) collected in the Seto Inland Sea of Japan according to Addison and Dougherty (1982). The purified Hrs were rapidly frozen in liquid N_2 and stored at −80 °C until Raman experiments.

The protein concentration of Hr, which was represented in terms of the subunit mole concentration, was determined spectrophotometrically by using the molar absorption coefficients of 1800 M^{-1} cm^{-1} at 500 nm and 29 400 M^{-1} cm^{-1} at 280 nm for the oxy form (Zimmer et al., 1986). Prior to the experiments, the absorption ratio, A_{280}/A_{500}, was checked to avoid large contamination of metHr. For the measurements of Raman spectra, the concentrated Hrs were diluted to the concentration of 1.0 mM/subunit by 50 mM phosphate or Tris–acetate buffer at pH 7.6 or 6.2. A D_2O solution of Hr was obtained by dialyzing the H_2O solution against 250-fold excess of D_2O buffer for 8 h at 7 °C, and it was repeated twice. AzidometHr was obtained by adding a 5-fold molar excess amount of ferricyanide and a 50-fold molar excess amount of NaN_3 to the oxyHr.

Raman spectra of the oxy and azidomet forms were excited by the 514.5- or 363.8-nm line. For the 363.8-nm excitation, the sample solution was flowed by a peristaltic pump through a capillary cell made of synthetic quartz (diameter = 1.5 mm, flow speed = 30 mL/min), which was kept at 7 °C by flushing with cold N_2 gas against the sample reservoir. Raman scattering was excited with an Ar^+ ion laser (Spectra Physics 2045) and recorded on a triple monochromator (Spex 1877) equipped with an 1800 grooves/mm nonblazed holographic grating and an optical multichannel analyzer (PAR 1420 UV/OMA II or 1421 UV/OMA III). The laser power at the sample point was 10 mW, and the spectral slit width was 5 cm^{-1}.

Exchange of bound O_2 from $^{16}O_2$ to $^{18}O_2$ was carried out by deoxygenating the oxyHr under N_2 gas in the flow cell and filling it with $^{18}O_2$. Since no optical rearrangements were involved between the measurements of $^{16}O_2$ and $^{18}O_2$ derivatives, the $^{16}O_2$ minus $^{18}O_2$ difference spectrum is sufficiently precise. The 514.5-nm excited spectra were measured with an Ar^+ ion laser (NEC GLG3200) and a JEOL-400D Raman spectrometer equipped with a cooled RCA-31034a photomultiplier. The measurements were carried out with a spinning cell (diameter = 20 mm, 1800 rpm) which was kept below 7 °C by flushing with cold N_2 gas. The laser power used was 60 mW at the sample point, and the spectral slit width was 7 cm^{-1}.

RESULTS

Figure 1 shows the absorption spectra of *L. unguis* Hr in the oxy and azidomet forms. The spectral features are similar

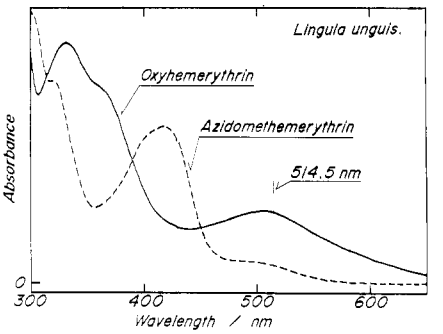

FIGURE 1: Visible–near-UV absorption spectra of oxy- (solid curve) and azidomet forms (dashed curve) of Hr isolated from *L. unguis*.

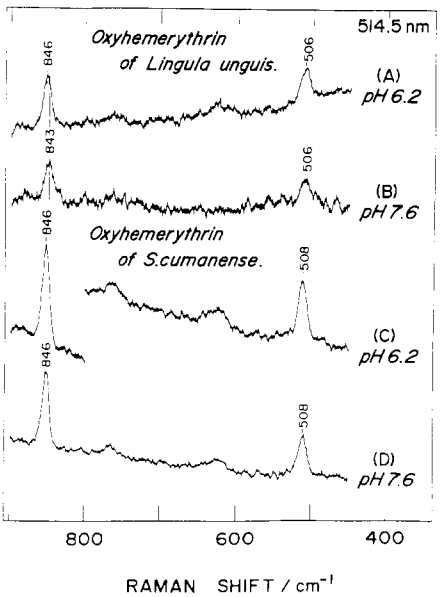

FIGURE 2: Resonance Raman spectra of oxyHr of *L. unguis* in phosphate buffer (A and B) and of *S. cumanense* in Tris–acetate buffer (C and D). (A) pH 6.2; (B) pH 7.6; (C) pH 6.2; (D) pH 7.6. Excitation, 514.5 nm.

to those of other Hrs. The bands of oxyHr around 510 nm and of azidometHr at 420 nm are assignable to charge-transfer (CT) bands from exogenous ligand to Fe^{3+}, while the band of oxyHr around 360 nm is assignable to a CT band from the μ-oxo-bridged oxygen to Fe^{3+}. Accordingly, we chose two excitation lines at 514.5 and 363.8 nm to resonance-enhance the ligand–metal stretching Raman bands selectively.

It is known that *L. unguis* Hr exhibits cooperative oxygen binding with n_{max} = 1.78 at pH 7.6 but no cooperativity at pH 6.2 (Manwell, 1960; Imai et al., 1991b) while *S. cumanense* Hr is noncooperative at both pHs. Accordingly, *L. unguis* Hr at pH 7.6 and pH 6.2 should stand for the high- and low-affinity quaternary states, respectively. Figure 2 shows the 514.5-nm excited RR spectra of the oxy forms of *L. unguis* and *S. cumanense* Hrs at pH 6.2 and pH 7.6. The Raman bands around 506–508 and 845 cm^{-1} are assigned to the Fe–O_2 stretching (ν_{Fe-O_2}) and the O–O stretching (ν_{OO}) modes, respectively (Kurtz et al., 1976, 1977; Dunn et al., 1973). The general features of RR spectra are similar between the cooperative and noncooperative Hrs, and also the structure of the binuclear center scarcely depends on the oligomeric size. The observed ν_{OO} frequencies imply the peroxide structure as pointed out previously (Dawson et al., 1972; McLendon & Martell, 1976; Sanders-Loehr et al., 1980). It was confirmed in repeated measurements that the ν_{OO} frequency of *L. unguis* Hr is lower by 3 cm^{-1} in the high-affinity form (B) than in the low-affinity form (A), while the

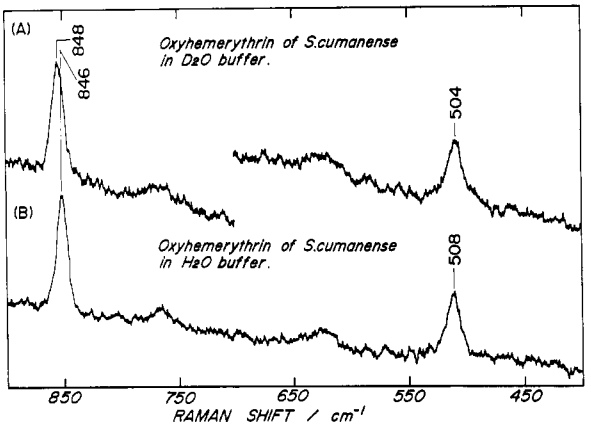

FIGURE 3: Resonance Raman spectra of *S. cumanense* oxyHr in D_2O, pD 7.6 (A), and in H_2O, pH 7.6 (B). Excitation, 514.5 nm.

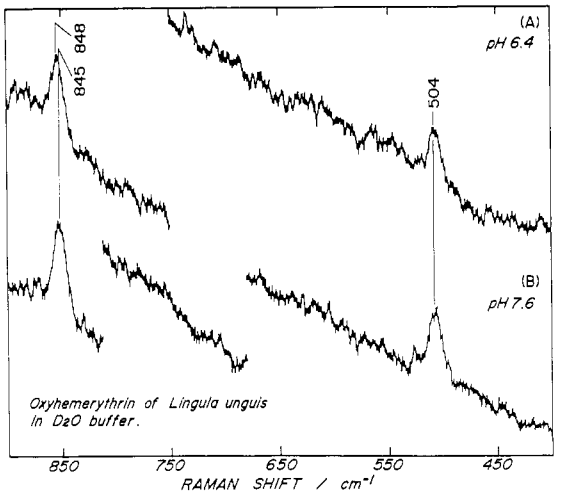

FIGURE 4: Resonance Raman spectra of *L. unguis* oxyHr in D_2O. (A) pD 6.4; (B) pD 7.6. Excitation, 514.5 nm.

ν_{Fe-O_2} frequency remains unshifted. This trend is in agreement with the other cooperative Hr isolated from *L. reevii* which gave the ν_{OO} bands at 844 and 845.5 cm^{-1} at pH 7.7 (high-affinity form) and pH 6.3 (low-affinity form), respectively, and the ν_{Fe-O_2} band at 505 cm^{-1} at both pHs (Richardson et al., 1987). On the other hand, *S. cumanense* Hr did not exhibit the pH-dependent frequency shift as shown by spectra C and D. Therefore, it is highly likely that the energy of cooperativity is partially attributed to interactions between the bound oxygen and surroundings.

In order to examine protonation of bound O_2, similar experiments were carried out for their D_2O solutions. Figure 3 compares the RR spectra of *S. cumanense* oxyHr in D_2O (A) with those in H_2O (B). It is clear that the ν_{OO} and ν_{Fe-O_2} RR bands are shifted to higher and lower frequencies, respectively, in D_2O. The frequency shifts to opposite directions were previously noted for *Phascolopsis gouldii* Hr (Shiemke et al., 1984), and from it, the protonation of bound oxygen and its hydrogen bonding to the μ-oxo-bridged oxygen were proposed (Shiemke et al., 1984, 1986). This proposal was justified by the high-resolution X-ray crystallographic analysis (Holmes et al., 1991).

Figure 4 displays the RR spectra of *L. unguis* oxyHr in D_2O at pD 6.4 (A) and 7.6 (B). The ν_{OO} frequency is lower by 3 cm^{-1} at pD 7.6 than at pD 6.4, while the ν_{Fe-O_2} frequency remains unchanged, in agreement with the results from the H_2O solution. In comparison with the spectra shown in Figure 2, we notice that the ν_{OO} band is upshifted by 2 cm^{-1} in D_2O whereas the ν_{Fe-O_2} band is downshifted by 2 cm^{-1}. The sizes

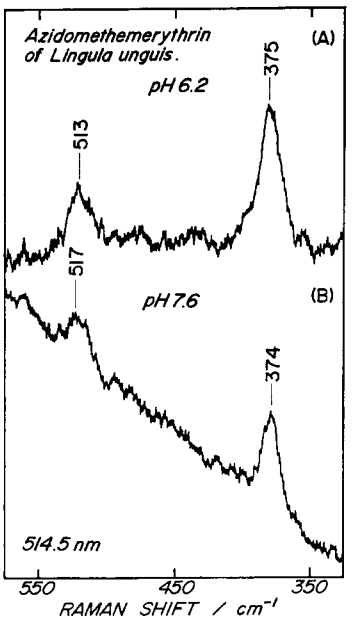

FIGURE 5: Resonance Raman spectra of *L. unguis* azidomet-Hr in phosphate buffer. (A) pH 6.2; (B) pH 7.6. Excitation, 514.5 nm.

of the deuteration shift of ν_{OO} and ν_{Fe-O_2} of *L. unguis* Hr are the same for pH 7.6 and 6.2 and are also close to those of the pH-insensitive *S. cumanense* Hr. Consequently, it is inferred that a large change in the hydrogen bonding of the proton attached to bound oxygen would not take place upon the transition between the high- and low-affinity quaternary states.

It was noted previously that O_2 and N_3^- were bound to the active site of Hr in analogous geometric disposition (Kurtz et al., 1976; Gay & Solomon, 1978). Therefore, information about the azidomet form might be useful to study the structure of the oxy form. Figure 5 shows the RR spectra of *L. unguis* azidometHr at pH 6.2 (A) and pH 7.6 (B) in the 300–600-cm^{-1} region. The Raman bands around 375 and 513 cm^{-1} are assigned to the Fe–N_3 stretching (ν_{Fe-N_3}) (Kurtz et al., 1976, 1977; Dunn et al., 1975) and the Fe–O–Fe symmetric stretching modes ($\nu^s_{Fe-O-Fe}$) (Freier et al., 1980; McCallum et al., 1984), respectively. It is stressed that the $\nu^s_{Fe-O-Fe}$ frequency is appreciably higher at pH 7.6, where oxygen binding is cooperative, than that at pH 6.2, while the ν_{Fe-N_3} band scarcely shifts. In contrast, the $\nu^s_{Fe-O-Fe}$ as well as ν_{Fe-N_3} bands of *S. cumanense* azidometHr exhibited no frequency shift upon the same pH change (not shown). Although the $\nu^s_{Fe-O-Fe}$ frequency is not known for the other allosteric Hr (Richardson et al., 1987), these results may suggest that the Fe–O–Fe linkage adopts a structure specific to the high- or low-affinity state.

To examine whether this idea can be applied to the oxy form, measurements of the $\nu^s_{Fe-O-Fe}$ band for the oxyHr are requisite, but the band was not observable with 514.5-nm excitation. However, upon excitation at 363.8 nm, two bands were observed around 500 cm^{-1} for *S. cumanense* oxyHr as depicted in Figure 6. In the previous report by Shiemke et al. (1986), a shoulder was recognizable at a higher frequency side of the strong 480-cm^{-1} band and was assigned to ν_{Fe-O_2}. The corresponding band is clearly resolved in Figure 6A for the $^{16}O_2$ derivative. When $^{18}O_2$ was used, the separation of these two bands became apparently worse as shown by Figure 6B. The difference spectrum between $^{16}O_2$Hr and $^{18}O_2$Hr, which is delineated by trace C in Figure 6, exhibited a derivative-like pattern with positive and negative peaks at 506 and 488 cm^{-1}, respectively. Therefore, we confirm that the 503-cm^{-1} band of oxyHr arises from the Fe–O_2 stretching

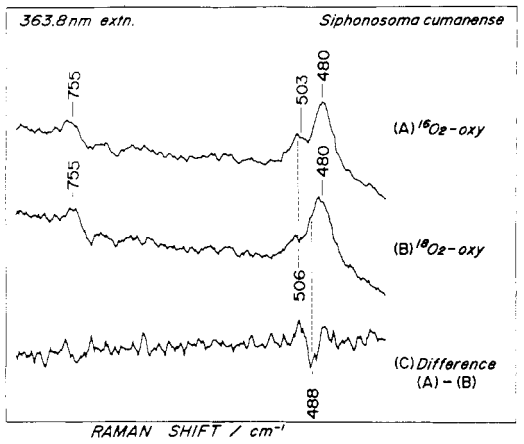

FIGURE 6: Resonance Raman spectra of *S. cumanense* oxyHr in phosphate buffer, pH 7.6. (A) $^{16}O_2$ derivative; (B) $^{18}O_2$ derivative; (C) difference spectrum (=spectrum A − spectrum B). Excitation, 363.8 nm.

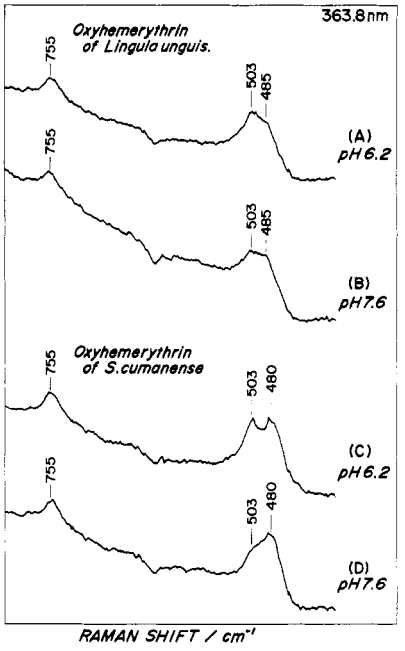

FIGURE 7: Resonance Raman spectra of *L. unguis* oxyHr in phosphate buffer (A and B) and *S. cumanense* oxyHr in Tris–acetate buffer (C and D). (A) pH 6.2; (B) pH 7.6; (C) pH 6.2; (D) pH 7.6. Excitation, 363.8 nm.

vibration and its apparent band position is shifted slightly to a lower frequency due to an overlapping band at a lower frequency. The remaining two bands of oxyHr at 480 and 755 cm^{-1} are assignable to $\nu^s_{Fe-O-Fe}$ and $\nu^a_{Fe-O-Fe}$, respectively (Shiemke et al., 1986).

Figure 7 shows the 363.8-nm excited RR spectra of oxyHr of *L. unguis* (A and B) and *S. cumanense* (C and D) at pH 6.2 and 7.6. The ν_{Fe-O_2} frequencies of *L. unguis* oxyHr at pH 6.2 and 7.6 are the same, indicating that the Fe–O_2 bond strength is not related with cooperativity. These frequencies are also identical with that of *S. cumanense* oxyHr, although all of them appear at lower frequencies than the true frequencies represented in Figure 2 due to overlapping with other bands ($\nu^s_{Fe-O-Fe}$ and a weak band around 500 cm^{-1}). The $\nu^s_{Fe-O-Fe}$ frequency of *L. unguis* oxyHr (485 cm^{-1}) is higher than that of *S. cumanense*. In both strains, the $\nu^s_{Fe-O-Fe}$ frequency is higher for the azidomet form than for the oxy form, which is also reported for other Hrs (Dunn et al., 1975; McCallum et al., 1984; Shiemke et al., 1984, 1986). In this regard, oxyHr is distinct from azidometHr. We note that both $\nu^s_{Fe-O-Fe}$ and $\nu^a_{Fe-O-Fe}$ frequencies of oxyHr do not depend on pH, contrary to the presumption derived with azidometHr.

DISCUSSION

Cooperative oxygen binding requires that oxygen binding to an arbitrary subunit is communicated to another subunit and thereby its oxygen affinity is altered. Therefore, discussion should be focused on the following two questions. (1) How is the oxygen affinity controlled by the molecular structure within each subunit? In other words, how is ΔG due to oxygen binding determined from a view of molecular structure? (2) What kind of structural changes upon binding of oxygen to an arbitrary binuclear center trigger a structural change at the intersubunit interface? Since all the cooperative Hrs have $\alpha_4\beta_4$ subunit compositions while noncooperative Hrs consist of identical subunits (Zhang & Kurtz, 1991), the α–β contact must be essential.

In the case of hemoglobin (Hb), which is a typical example of molecules with homotropic allostery, the ligand-bound form exhibits little difference between the low- and high-affinity states. Indeed, the Fe–O_2 stretching (Nagai et al., 1980), the Fe–CO stretching (Rousseau et al., 1984; Kaminaka et al., 1989), and the O=O stretching (Kaminaka et al., 1989) frequencies as well as porphyrin vibrations do not show any distinct shift between the two states. However, the Fe–histidine stretching frequency of the deoxy state exhibits a clear correlation with the oxygen affinity (Matsukawa et al., 1985). This implies that a key of Hb cooperativity is lowering of the oxygen affinity in the low-affinity deoxy form. In contrast, Hr cooperativity seems to arise from a change of the oxy state, since the oxygen binding equilibrium at low saturation range is insensitive to a pH change from 6.2 to 7.6, whereas that at high saturation is sensitive to the pH change (Reem et al., 1989; Imai et al., 1991b). Therefore, the oxy state is expected to show some structural difference between the low- and high-affinity states, and in this regard, Hr cooperativity has a feature different from that of tetrameric Hbs, but may have some similarity to giant Hbs (Chung & Ellerton, 1979; Yoshikawa et al., 1984).

The present study clarified that the Fe–N_3 and Fe–O_2 bonding interactions are almost the same between the high- and low-affinity states. A small but definite difference was observed for the ν_{OO} and $\nu^s_{Fe-O-Fe}$ frequencies of oxyHr and azidometHr, respectively. The X-ray crystallographic analysis (Stenkamp et al., 1984), EXAFS studies (Elam et al., 1982, 1983), and single-crystal absorption measurements (Gay & Solomon, 1978) suggested the structural similarity in the binuclear iron center between oxyHr and azidometHr. Since the $\nu^s_{Fe-O-Fe}$ frequency of azidometHr was altered by pH, the Fe–O–Fe moiety was anticipated to control the oxygen affinity. However, this presumption was denied by the finding that $\nu^s_{Fe-O-Fe}$ of oxyHr remained unaltered between the high- and low-affinity states.

According to X-ray crystallographic analysis of deoxyHr and oxyHr (Holmes et al., 1991), an iron ion (Fe1) of the binuclear center is bound to His73, His77, His101, Glu58, Asp106, and the μ-oxo-bridging oxygen, while the other iron (Fe2) is coordinated by His25, His54, Glu58, Asp106, and the μ-oxo oxygen as illustrated in Figure 8. Fe2 is the ligand binding site. The internal ligand trans to the external ligand (O_2 or N_3) is a carboxylate oxygen of Glu58, and the coordination geometry around this iron can be approximated by an octahedron in which the external ligand and an oxygen atom of Glu58 occupy the axial positions and the other four internal ligands constitute a square plane (Stenkamp et al.,

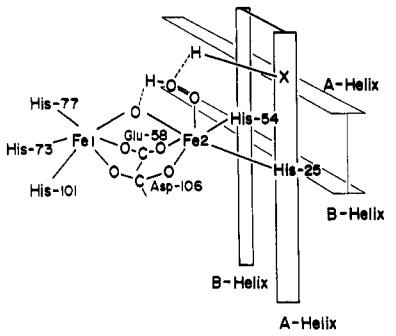

FIGURE 8: Schematic model for an active-site structure of the binuclear center of cooperative oxyHr. A- and B-helices of the α subunit are assumed to be in contact with the A- and B-helices of the β subunit. The X residue (unknown) is assumed to form a hydrogen bond to bound dioxygen which stabilizes more the high-affinity oxy structure than the low-affinity oxy structure. The active-site structure is transferred from that of *Themiste dyscritum* Hr reported by Stemkamp et al. (1976) and Holmes et al. (1991).

1981, 1983). The distance between the second oxygen atom of dioxygen and μ-oxo oxygen is determined to be 2.80 Å (Holmes et al., 1991). Therefore, the hydrogen-bonding interaction between bound oxygen and μ-oxo oxygen is present, but it seems to be a structural feature in common to the cooperative and noncooperative Hrs.

The X-ray study (Holmes et al., 1991) pointed out that binding of oxygen causes some changes in the Fe1–O–Fe2 bond angle (from 110.6° to 125°) and distances of the Fe2–O(μ) (from 1.88 to 1.79 Å) and Fe2–Glu58 bonds (from 2.14 to 2.20 Å) while other changes are little: Fe2–His54 (from 2.28 to 2.25 Å), Fe2–Asp106 (from 2.14 to 2.15 Å), and Fe2–His25 (from 2.15 to 2.14 Å). Although the structural change of the μ-oxo bridge is the largest, it does not seem to change with pH, because such changes should be sensitively reflected in the $\nu^{s}_{Fe-O-Fe}$ and $\nu^{a}_{Fe-O-Fe}$ frequencies (Sanders-Loehr et al., 1989). The next largest change is seen for the Fe2–Glu58 bond, which serves as a trans ligand of dioxygen. If this bond length controls the Fe2–O_2 interactions, the ν_{Fe-O_2} frequency should be altered with the affinity change. Since the Fe–O_2 (and also Fe–N_3) stretching frequencies do not exhibit a significant pH dependence, this model seems less likely.

Since only the ν_{OO} frequency exhibits an affinity-dependent change, it is more likely to assume the presence of a hydrogen bond between O_2 and the protein moiety as illustrated schematically in Figure 8. Generally, the ν_{OO} frequency becomes lower in the presence of hydrogen bonds. The hydrogen bond between the bound oxygen and μ-oxo oxygen present in all oxyHrs stabilizes the oxy form, but this would not be related with the cooperativity. If another hydrogen bond between the bound oxygen and protein (residue X in helix A in Figure 8) were present for the cooperative Hr and stronger at pH 7.6 than at pH 6.2, the oxy form would be more stabilized at higher pH than at lower pH, and simultaneously the binding of oxygen could be conveyed through the A-helix to the subunit interface. Since only the α–β contacts are effective to the cooperativity, a small Hill coefficient (1.78; Imai et al., 1991b) despite a large oligomeric structure (octamer) is understandable. Such a hydrogen bond would not be formed for an azidometHr on account of sterical mismatching. Therefore, it is reasonable that there is no cooperativity for binding of N_3^- to *L. unguis* Hr, even though the active-site structures of the oxy and azidomet forms were alike.

Involvement of direct interactions of the substrate with the surrounding amino acid residues in cooperativity was pointed out for phosphofructokinase, in which the interaction between fructose 6-phosphate and Arg162 was significantly altered between the low- and high-affinity states (Schirmer & Evans, 1990). For cooperativity of human HbA, it was recently demonstrated from site-directed mutagenesis experiments that a hydrogen bond between α40-Tyr and β99-Glu plays a pivotal role in its cooperativity (Imai et al., 1991a). Although the present interpretation for the Hr cooperativity is preliminary, this study has provided some essential information about the cooperativity mechanism of Hr.

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