

Simultaneous observation of the O—O and Fe—O₂ stretching modes in oxyhemoglobins

Tapan Kanti Das[†], Manon Couture[†], Yannick Ouellet[‡], Michel Guertin[‡], and Denis L. Rousseau^{†§}

[†]Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461; and [‡]Department of Biochemistry, Faculty of Sciences and Engineering, Laval University, Quebec, QC, Canada G1K 7P4

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Understanding of the chemical nature of the dioxygen moiety of oxyhemoglobin is crucial for elucidation of its physiological function. In the present work, direct Raman spectroscopic observation of both the Fe—O₂ and O—O stretching modes unambiguously establishes the vibrational characteristics of the oxygen-bound heme moiety in the hemoglobins of *Chlamydomonas eugametos* and *Synechocystis* PCC6803. In addition to providing the resonance Raman assignment of the O—O stretching mode (1136 cm⁻¹ for *Chlamydomonas*, 1133 cm⁻¹ for *Synechocystis*) in an oxyhemoglobin with an iron-porphyrin, this study also reports unusually low frequencies for the Fe—O₂ stretching modes (554 cm⁻¹). The effect of strong hydrogen bonding to the bound oxygen is confirmed by changes in the frequency of the Fe—O₂ stretching mode on mutation of distal residues. These findings suggest an enzymatic function rather than an oxygen transport role for these hemoglobins.

There has been a renewed interest in understanding the function of hemoglobins in recent years. Vertebrate hemoglobins, which are established as oxygen carrier proteins, have been proposed to have additional cellular activities (See for example, refs. 1 and 2). In the nonvertebrates, hemoglobins are expressed in a wide range of phyla (3–5). Although the nonvertebrate hemoglobins bind oxygen and other ligands just as the vertebrate hemoglobins do, the kinetic and structural properties of the oxygen complexes differ significantly, suggesting functional diversity. However, identification of the physiological functions remains elusive. One of the most vital clues to discerning hemoglobin function lies in the structure and stability of its oxygen complex. Despite the fact that hemoglobin is one of the most studied biological molecules, the energetics and structure of the oxy complexes of the many different hemoglobins that have been recently discovered remain poorly understood. For example, whereas the interactions of the distal residues with the bound oxygen play a central role in determining the stability and reactivity of the oxy complex, thus critically influencing the function of the protein, it is not established how the distal factors affect the orientation and strength of the Fe—O₂ bond.

Resonance Raman spectroscopy is a powerful tool for studying heme proteins. Assignments of several useful heme marker bands that are sensitive to oxidation, coordination, and spin states are known (6). Furthermore, assignments of the iron-ligand vibrational modes have been established for several intrinsic as well as exogenous ligands (see ref. 7). Determination of the frequency of the iron-ligand stretching mode not only gives a direct estimate of the bond strength, but also allows assessment of how the surrounding environment may affect the stability of such a bond. In heme proteins, such information is very crucial to understand their function because the heme prosthetic group can display a variety of biological activities depending on the nature of its surroundings.

In the present work, we have studied the oxy complex of two novel hemoglobins by resonance Raman spectroscopy with the aim of assigning the Fe—O₂ as well as the O—O stretching modes and determining their sensitivity to the environment in the heme pocket. There is no consensus on the assignment of the O—O stretching mode in oxyhemoglobins largely because the mode displays substantial vibrational coupling with other modes, resulting

in very complex spectra. In addition, this mode has not been reported in the resonance Raman spectra of globins with an iron-containing heme coordinated by a proximal histidine, so indirect methods, such as replacing the heme iron by cobalt, were used (8) to measure the O—O stretching frequency. In independent studies, the intrinsic O—O stretching mode in cobalt-porphyrin-substituted hemoglobin was assigned by one group at 1122 and 1153 cm⁻¹, representing two different species (8), by another group of investigators as a single species at 1134 cm⁻¹ (9), and by a third group as a single conformation at 1139 cm⁻¹ (10). The O—O stretching mode of iron-containing heme in hemoglobins is present in their infrared spectra, but the spectrum is also complicated by vibrational coupling. Caughey and coworkers (11) carried out a detailed and careful study of the multiple oxygen-sensitive lines in various hemoglobins and myoglobin and interpreted the infrared spectra as arising from two conformers of FeO₂ with O—O stretching frequencies at 1155 and 1125 cm⁻¹ (for HbA). However, Bruha and Kincaid (12), on the basis of elaborate studies on model complexes, provided an alternative interpretation, arguing that the multiple frequencies arose from vibrational coupling of internal modes of the proximal histidine, with a single O—O stretching mode having an inherent frequency of ≈1135 cm⁻¹.

We report here the resonance Raman scattering spectrum of the O—O stretching mode in the oxygen complex of hemoglobins with an iron-containing heme. The mode was detected in the hemoglobins from both *Chlamydomonas* (13) and *Synechocystis* (14). We also report an unusually low frequency of the Fe—O₂ stretching mode in these oxyhemoglobins and discuss the role of the heme pocket residues in modulating the vibrational frequencies to identify the hydrogen bond donors to the heme-bound oxygen. Confirmation of hydrogen bonding to the iron-bound oxygen is presented, judged by the shift of the frequency of the Fe—O₂ stretching mode on single point mutations of the distal residues.

Materials and Methods

Preparations of the recombinant hemoglobins of *Chlamydomonas eugametos* (13) and *Synechocystis* PCC6803 (14) have been described elsewhere. The resonance Raman measurements were done as described previously (13). The oxy complex of *Chlamydomonas* and *Synechocystis* hemoglobins was prepared either by using a ferredoxin-based enzymatic reduction method or by exposing the desalted dithionite-reduced protein to air (13). OxyHb samples of either ¹⁶O₂ or ¹⁸O₂ (ICON, Mt. Marion, NY) isotopic composition were prepared in 100 mM sodium phosphate buffer, pH 7.4. All measurements reported here were carried out by using a laser excitation wavelength of 413.1 nm and a power of ≈2–5 mW at the sample. The sample cell was spun

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Abbreviations: Hb, hemoglobin; H/D, H₂O/D₂O.

[§]To whom reprint requests should be addressed. E-mail: rousseau@acom.yu.edu.

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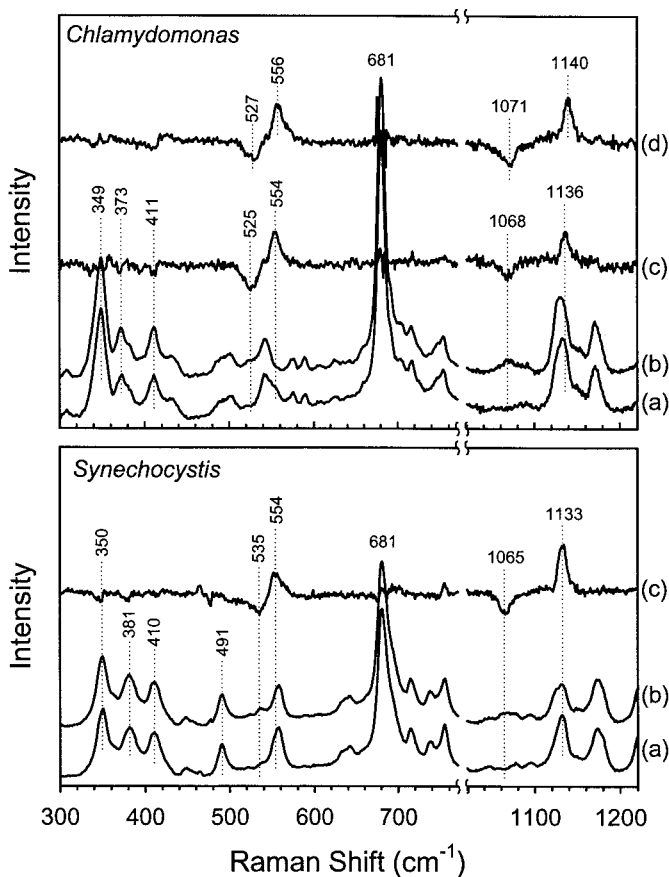


Fig. 1. Assignment of the frequencies of the O—O and Fe—O₂ stretching modes in the resonance Raman spectra of *Chlamydomonas* and *Synechocystis* oxyhemoglobins. Spectra shown are (a) ¹⁶O₂ in H₂O, (b) ¹⁸O₂ in H₂O, and (c) the ¹⁶O₂ – ¹⁸O₂ difference spectrum in H₂O. The ¹⁶O₂ – ¹⁸O₂ difference spectrum for the oxy complex of K(E10)A mutant of *Chlamydomonas* is shown in (d). The values of $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ are, respectively, 1136 (1068 with ¹⁸O₂) and 554 (525) cm⁻¹, for wild-type *Chlamydomonas* Hb; 1140 (1071) and 556 (527) cm⁻¹ for the K(E10)A mutant of *Chlamydomonas* Hb; and 1133 (1065) and 554 (535) cm⁻¹ for wild-type *Synechocystis* Hb.

at 6000 rpm to avoid laser heating. Under these conditions, there was no evidence of any photodissociation of the Fe—O₂ moiety.

Results

To assign the Fe—O₂ ($\nu_{\text{Fe}-\text{O}_2}$) and O—O ($\nu_{\text{O}-\text{O}}$) stretching modes, the spectrum of the ¹⁶O₂ adduct was compared with that of the ¹⁸O₂ adduct as shown in Fig. 1. In *Chlamydomonas* hemoglobin (Fig. 1 Upper), the lines at 1136 and 554 cm⁻¹ are assigned to the frequencies of the $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ modes, respectively, determined from the difference spectrum (¹⁶O₂ – ¹⁸O₂, spectrum c). Similarly, in *Synechocystis* hemoglobin (Fig. 1 Lower), the lines at 1133 and 554 cm⁻¹ are assigned to the $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ modes, respectively, from the difference spectrum (¹⁶O₂ – ¹⁸O₂, spectrum c). The observation of isotope shifts of 68 cm⁻¹ for the lines at 1136 cm⁻¹ and 1133 cm⁻¹ in *Chlamydomonas* and *Synechocystis* hemoglobins, respectively, agrees well with the calculated value (65 cm⁻¹) for the stretching mode of an isolated O—O group. On the other hand, the isotope shift of 29 cm⁻¹ for the Fe—O₂ stretching mode in *Chlamydomonas* hemoglobin (Hb) is higher than the predicted value of 20 cm⁻¹. Although there is no clear origin for this difference, it should be noted that, in heme oxygenase, a similarly large isotope shift has been attributed to a highly bent Fe—O—O conformation (15). In *Synechocystis*, the shift of 19 cm⁻¹ is in agreement with the predicted value.

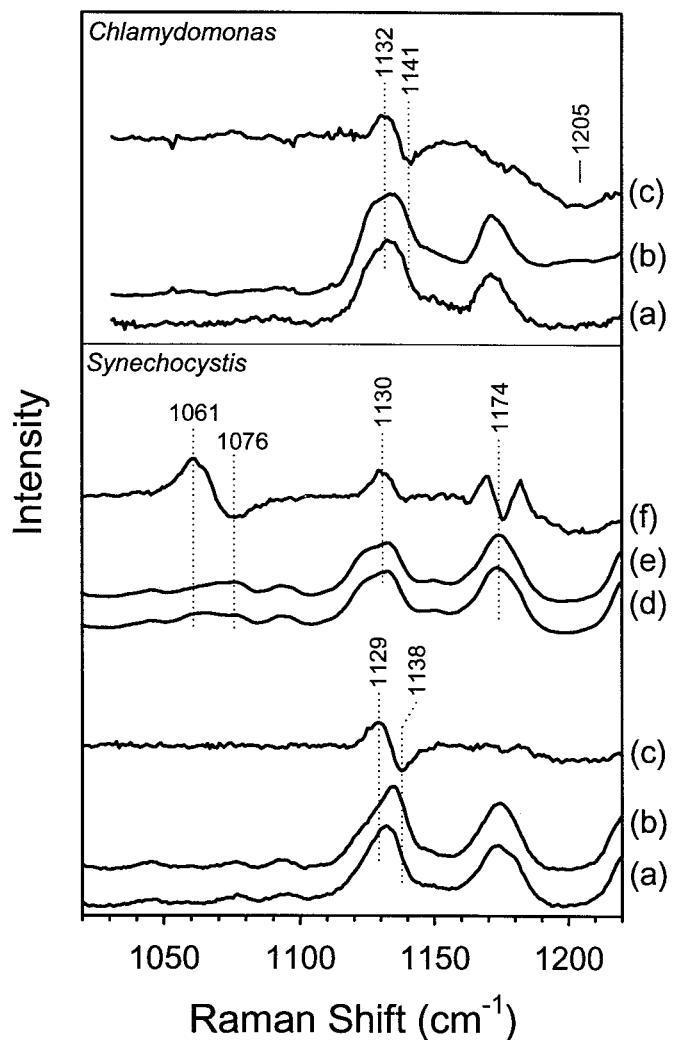


Fig. 2. The H/D sensitivity of the frequency of the O—O stretching mode in the resonance Raman spectra of *Chlamydomonas* and *Synechocystis* oxyhemoglobins. Spectra shown are (a) ¹⁶O₂ in H₂O, (b) ¹⁶O₂ in D₂O, and (c) the H₂O – D₂O difference spectrum for ¹⁶O₂. The sensitivity of the $\nu_{\text{O}-\text{O}}$ mode to H₂O/D₂O is seen in the difference line at 1132/1141 cm⁻¹ for *Chlamydomonas* HbO₂, and at 1129/1138 cm⁻¹ for *Synechocystis* HbO₂. The broad line at \approx 1205 cm⁻¹ results from the D—O—D bending vibration of D₂O. Also shown, for *Synechocystis* oxyHb, are (d) ¹⁸O₂ in H₂O, (e) ¹⁸O₂ in D₂O, and (f) the H₂O – D₂O difference spectrum for ¹⁸O₂, which shows a difference feature at 1061/1076 cm⁻¹.

The H/D sensitivity of the $\nu_{\text{O}-\text{O}}$ region spectra of *Chlamydomonas* and *Synechocystis* oxyhemoglobins is shown in Fig. 2. The H₂O – D₂O difference spectra (spectra c) are obtained by subtracting spectra b (¹⁶O₂ in D₂O) from spectra a (¹⁶O₂ in H₂O). Difference features are observed in the $\nu_{\text{O}-\text{O}}$ region spectra of both hemoglobins (1132/1141 cm⁻¹ in *Chlamydomonas* and 1129/1138 cm⁻¹ in *Synechocystis*). Similarly, difference bands are observed at 1061/1076 cm⁻¹ for the ¹⁸O₂ derivative of *Synechocystis* Hb (spectra d–f). Thus, all of the observed $\nu_{\text{O}-\text{O}}$ mode isotope shifts are to higher frequency in D₂O, as shown by the 8–15 cm⁻¹ apparent upshift in the difference spectra. The absolute value of the shifts is substantially smaller than the maximum and minimum in the difference spectra for these H/D shifts and are estimated to be a few cm⁻¹ by using mathematical methods described elsewhere (16). The $\nu_{\text{Fe}-\text{O}_2}$ mode does not show any appreciable H/D sensitivity in either hemoglobin.

To identify the amino acid residues involved in hydrogen bonding to the iron-bound oxygen, three distal pocket mutants of *Chlamy-*

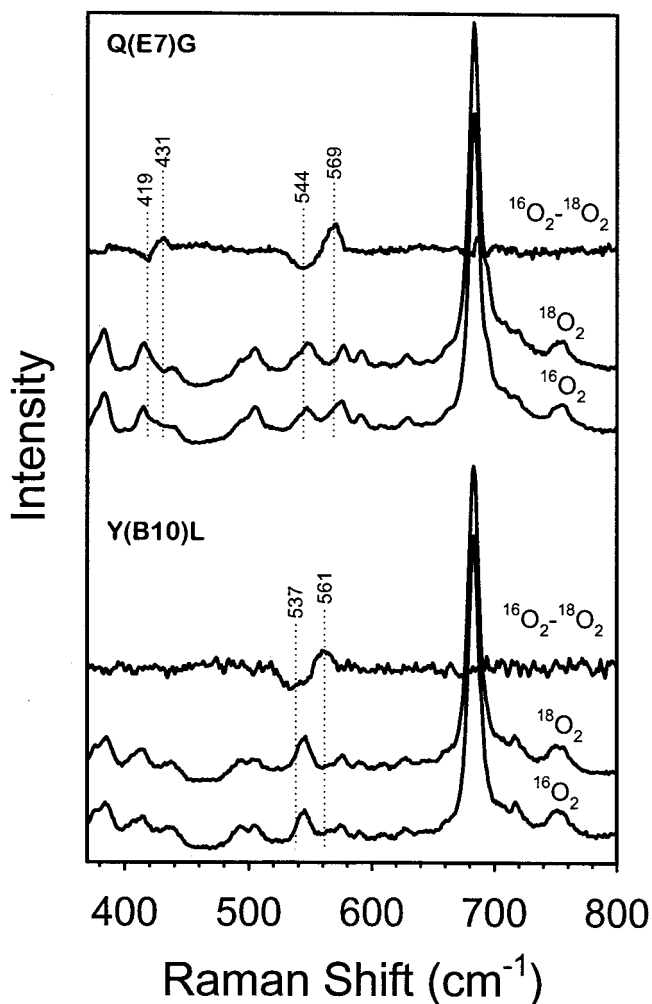


Fig. 3. Resonance Raman spectra of the oxy complex of Q(E7)G and the Y(B10)L mutants of *Chlamydomonas* oxyHb. The spectra with $^{16}\text{O}_2$ and $^{18}\text{O}_2$, as well as the difference spectra ($^{16}\text{O}_2 - ^{18}\text{O}_2$), are shown for both the mutants. The frequencies of the $\nu_{\text{Fe}-\text{O}_2}$ mode are assigned at 569 (544 with $^{18}\text{O}_2$) and 561 (537) cm^{-1} for Q(E7)G and Y(B10)L, respectively. The difference feature at 431/419 cm^{-1} in Q(E7)G may arise from the Fe—O—O bending mode (see refs. 23 and 35), which is not detected in Y(B10)L. The $\nu_{\text{O}-\text{O}}$ modes are not detected in these two mutants.

domonas Hb were studied. Single point mutations were made at the B10 (Y63), E7 (Q84), and E10 (K87) helical positions. The resonance Raman $^{16}\text{O}_2 - ^{18}\text{O}_2$ difference spectra of the Y(B10)L and Q(E7)G oxyHb mutants of *Chlamydomonas* are presented in Fig. 3. The effects of the mutations are clearly seen in the frequency of the $\nu_{\text{Fe}-\text{O}_2}$ mode, which increases from 554 cm^{-1} in the wild-type protein (Fig. 1) to 561 cm^{-1} in Y(B10)L and 569 cm^{-1} in Q(E7)G. However, in the oxy complex of K(E10)A (Fig. 1 *Top*, spectrum d), the $\nu_{\text{Fe}-\text{O}_2}$ mode (556 cm^{-1}) has a frequency similar to that found in the wild-type protein. The $\nu_{\text{O}-\text{O}}$ mode of K(E10)A is assigned at 1140 cm^{-1} , close to that in the wild-type protein (1136 cm^{-1}). In contrast, $\nu_{\text{O}-\text{O}}$ modes were not detected in the Y(B10)L and Q(E7)G mutants.

Discussion

The data reported here contain three major findings. (i) The frequency of the $\nu_{\text{O}-\text{O}}$ mode in oxyhemoglobin is identified in the resonance Raman spectra of iron-heme-containing globins. (ii) The frequencies of the $\nu_{\text{Fe}-\text{O}_2}$ mode are lower than that reported in any other globin. (iii) Large effects of the distal E7

and B10 mutations on the $\nu_{\text{Fe}-\text{O}_2}$ frequency suggest that these two residues are strong hydrogen bond donors to the bound oxygen in *Chlamydomonas* Hb.

The Frequency of the $\nu_{\text{O}-\text{O}}$ Mode. The $\nu_{\text{O}-\text{O}}$ frequencies in heme proteins and model heme complexes all appear in the 1120–1200 cm^{-1} range, consistent with a ferric-iron-superoxide configuration (17) ($\nu_{\text{O}-\text{O}} \approx 1100\text{--}1150 \text{ cm}^{-1}$ in O_2^- , metal superoxides) and not a ferrous-iron-neutral oxygen complex ($\nu_{\text{O}-\text{O}} \approx 1556 \text{ cm}^{-1}$ in O_2 , molecular oxygen) (see Table 1). Location of the $\nu_{\text{O}-\text{O}}$ frequency at 1136 cm^{-1} in *Chlamydomonas* Hb and at 1133 cm^{-1} in *Synechocystis* Hb is consistent with this typical oxy-heme configuration. A low-spin ferric superoxide structure ($\text{Fe}^{+3}-\text{O}-\text{O}^-$) in these oxy-Hbs is also consistent with frequencies of the modes of the heme macrocycle oxidation state marker (ν_4) and the spin-state marker (ν_3) bands in *Chlamydomonas* oxyHb that appear at 1376 and 1502 cm^{-1} , respectively (ν_4 and ν_3 for *Synechocystis* oxyHb are at 1374 and 1498 cm^{-1} , respectively) (spectrum not shown), which are typical for low-spin ferric hemes. Similar frequencies of these two lines are observed in mammalian oxyHbs and oxymyoglobins. In ferrous 6-coordinate low-spin heme complexes (in which the axial ligand is not a π -acid, i.e., not O_2 , CO, or NO), ν_4 and ν_3 appear at ≈ 1360 and $\approx 1490 \text{ cm}^{-1}$, respectively (13).

Unusually Low Frequency for the $\nu_{\text{Fe}-\text{O}_2}$ Mode. The $\nu_{\text{Fe}-\text{O}_2}$ mode in *Chlamydomonas* and *Synechocystis* oxyHbs appears at an unusually low frequency (554 cm^{-1}) compared with those in other heme proteins containing an axial histidine ligand (see Table 1). This frequency is also the lowest among the axial imidazole-containing heme model compounds in solution (in the range of 560–576 cm^{-1}) (17–21) and the peroxide complex of horseradish peroxidase ($\approx 562 \text{ cm}^{-1}$) (ref. 22; Table 1). However, for thiolate axial ligand-containing heme proteins, such as cytochrome P450 in which the strength of the trans axial ligand (thiolate) is substantially higher in comparison with that in the histidine-ligated heme proteins, the $\nu_{\text{Fe}-\text{O}_2}$ band appears in a different frequency domain ($\approx 540 \text{ cm}^{-1}$) (refs. 23 and 24; Table 1). We attribute the significant lowering ($\approx 14 \text{ cm}^{-1}$) of the $\nu_{\text{Fe}-\text{O}_2}$ frequency in both *Chlamydomonas* and *Synechocystis* oxyHbs relative to mammalian oxyHb ($\approx 568 \text{ cm}^{-1}$) to arise predominantly from relatively strong hydrogen bonding of the bound O—O moiety on the distal side of the heme. Both *Chlamydomonas* and *Synechocystis* hemoglobins contain a tyrosine at helical position B10 and a glutamine at E7, in the distal heme pocket (13, 14, 25). We postulate that these two residues serve as hydrogen bond donors (13, 14) to both the oxygen atoms in oxyHb (Fig. 4), which is also evident from mutagenesis data discussed below.

Factors That Modulate the Frequency of the $\nu_{\text{Fe}-\text{O}_2}$ Mode. The strength of the Fe—O₂ bond in heme proteins may be modulated by (i) direct distal interactions, (ii) proximal effects, and (iii) heme effects. We show below that the distal interactions play the major role in lowering the frequency of the $\nu_{\text{Fe}-\text{O}_2}$ mode in *Chlamydomonas* and *Synechocystis* oxyHbs as compared with mammalian hemoglobins.

In the distal pocket, direct hydrogen bonding interactions between the bound oxygen and neighboring residues play a dominant role in influencing the frequency of the $\nu_{\text{Fe}-\text{O}_2}$ mode. Lowering of the $\nu_{\text{Fe}-\text{O}_2}$ frequency (to $\approx 560 \text{ cm}^{-1}$) indeed has been observed in oxy complexes of some amide-basket-handle hemes and attributed to intramolecular hydrogen bonding of the secondary amide group with the oxygen (21). In the oxy complexes of *Paramecium* (26) and *Mycobacterium* (HbN) (27) Hbs (Table 1), observations of relatively lower frequencies have been attributed to distal hydrogen bonding with the bound oxygen. We postulate that the greater reduction in the frequency of the $\nu_{\text{Fe}-\text{O}_2}$ mode in *Chlamydomonas* oxyHb ($\approx 14 \text{ cm}^{-1}$ in comparison with mammalian oxyHb) is caused by relatively stronger distal side hydrogen bonding. The involvement of the distal

Table 1. The Fe—O₂ ($\nu_{\text{Fe—O}_2}$)^a and O—O ($\nu_{\text{O—O}}$) stretching mode assignments of the oxy complexes of globins, other hemeproteins, and some selective model complexes

Type	Protein/porphyrin	$\nu_{\text{Fe—O}_2}$	$\nu_{\text{O—O}}$	Ref
Globin	<i>Synechocystis</i> Hb ^b	554	1133	This work
	<i>Chlamydomonas</i> WT Hb ^c	554	1136	This work
	<i>Chlamydomonas</i> K(E10)A Hb ^c	556	1140	This work
	<i>Chlamydomonas</i> Y(B10)L Hb ^c	561	—	This work
	<i>Chlamydomonas</i> Q(E7)G Hb ^c	569	—	This work
	Human adult HbA	568	1155, 1125	35, 11
	Human adult HbA	—	≈1135	12
	<i>Paramecium</i> Hb ^d	563	—	26
	<i>Mycobacterium</i> HbN ^e	564	—	27
	<i>Ascaris</i> Hb ^f	570	—	31
	<i>Scapharca</i> Hbl ^g	570	—	36
	Horse Mb	571	—	35
	Elephant Mb	572	—	37
	Soybean legHb ^h	576	—	38
	Cobalt Hb (CoO ₂) ⁱ	—	1122, 1153	8
Cobalt Hb (CoO ₂) ⁱ	—	1134	9	
Cobalt Hb (CoO ₂) ⁱ	—	≈1139	10	
Peroxidase	HRP ⁱ	≈562	—	22
Oxygenase	Heme oxygenase	565	—	15
Heme-Cu oxidase	Bovine aa ₃ ^k	568–572	—	35,39,40
	<i>E. coli</i> bo ₃ ^l	568	—	41
Model heme-6C	TPP(Pip) ^m	575	1157	18
	TpivP(1,2-Me ₂ Im) ⁿ	564	1159	17
	TpivP(1-MeIm) ⁿ	568	1159	17,19,20
	[(Piv) ₂ C ₁₂](1-MeIm) ^o	563	—	21
	[(Piv) ₂ C ₉](1-MeIm) ^o	560	—	21
	[(Piv) ₂ C ₈](1-MeIm) ^o	563	—	21
Model heme-5C	TPP ^m	508	1195	42
	TMP ^p	522	1171	34
Model heme-6C (—S ⁻) ^q	TpivP(C ₆ HF ₄ S ⁻) ^q	527	1140	24
	TpivP(C ₆ F ₅ S ⁻) ^q	536	1147	24
P450 (—Cys ⁻) ^r	Cytochrome P450	540–541	1139–1140	23,24
	NOS ^s	—	1135	43
O ₂ gas			1556	44
O ₂ (metal-O ₂ ⁻)			1100–1150	44

^aThe $\nu_{\text{Fe—O}_2}$ frequency is from the ¹⁶O₂—¹⁸O₂ difference spectrum where available; Ref, reference; Hb, hemoglobin; Mb, myoglobin. ^b*Synechocystis* PCC6803 Hb. ^c*Chlamydomonas eugametos* Hb; WT, wild-type protein. ^d*Paramecium caudatum* Hb. ^e*Mycobacterium tuberculosis* HbN. ^f*Ascaris suum* Hb. ^gHbl of *Scapharca inaequivalvis*. ^hSoybean leghemoglobin. ⁱCobalt-porphyrin substituted hemoglobin; $\nu_{\text{Co—O}_2}$ appears at 537 cm⁻¹. ^jHorseradish peroxidase. ^kBovine aa₃ cytochrome c oxidase. ^l*Escherichia coli* bo₃-type quinol oxidase. ^mTPP, tetraphenylporphyrin; Pip, piperidine. ⁿTpivP, meso-tetra(α,α,α,α-o-pivalamidophenyl)porphyrin (picket fence porphyrin); MeIm, methylimidazole. ^o[(Piv)₂C_n, α,α-5,15-[2,2'-(X)diphenyl]-α,α-10,20-bis(o-pivalamidophenyl)porphyrin (n = 8, X = octanediamido; n = 9, X = nonanediamido; n = 12; X = dodecanediamido). ^pTMP, 5,10,15,20-tetramesitylporphyrin. ^qS⁻-ligation in the proximal side. ^rP450 type heme proteins that contain proximal Cys⁻ ligand. ^sOxygenase domain of rat brain neuronal nitric oxide synthase. 5C, five-coordinate; 6C, six-coordinate.

groups in providing hydrogen bonds is confirmed by the mutagenesis data. The $\nu_{\text{Fe—O}_2}$ frequency increases from 554 cm⁻¹ in the wild-type protein to 561 cm⁻¹ in Y(B10)L. In Q(E7)G, the $\nu_{\text{Fe—O}_2}$ frequency goes all the way to 569 cm⁻¹, similar to that of HbA (Table 1). Thus, relative to the wild-type protein, the hydrogen bonding to the oxygen is reduced in both mutant proteins. This conclusion based on the spectroscopic results is consistent with the oxygen kinetics data, which show a large increase in oxygen dissociation rate in these mutants (0.948 and 0.444 s⁻¹, respectively, in Y(B10)L and Q(E7)G) in comparison to a very low rate (0.014 s⁻¹) in the wild-type protein (13). Interestingly, mutation of the E7

glutamine causes the $\nu_{\text{Fe—O}_2}$ frequency to move to the 569 cm⁻¹ region that is typical for $\nu_{\text{Fe—O}_2}$ in mammalian oxyhemoglobin (and oxymyoglobin), whereas mutation of the B10 tyrosine shifts the $\nu_{\text{Fe—O}_2}$ frequency only halfway. Thus, it is very likely that E7Gln interacts primarily with the proximal oxygen atom and that B10Tyr interacts primarily with the terminal oxygen. With such a hydrogen-bonding pattern, the distal hydrogen bonding structure of *Chlamydomonas* and *Synechocystis* oxyHbs (Fig. 4) resembles that of *Ascaris* oxyHb, which also has an E7 glutamine and a B10 tyrosine (28); and from the crystal structure of *Ascaris* oxyHb (28), it has been shown that the glutamine forms a hydrogen bond to the

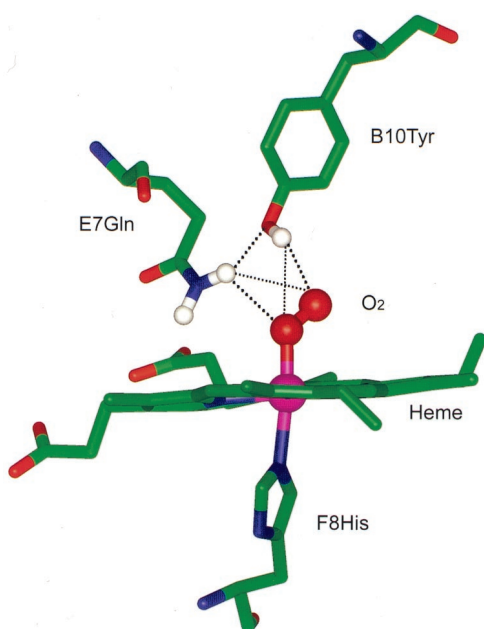


Fig. 4. Proposed structural model of the active site in *Chlamydomonas* and *Synechocystis* HbO₂. Only selective residues in the vicinity of the heme are shown. The model was built by using the structural coordinates of HbO₂ of *Ascaris suum* (1ash.pdb, Brookhaven Protein Data Bank). The dotted lines represent hydrogen bonds (thick line, strong hydrogen bond; thin line, weak hydrogen bond). The E10 helical positions (not shown here) are occupied by a Lys and a His, respectively, in *Chlamydomonas* and *Synechocystis* Hb.

proximal oxygen atom. It may be noted that, in the Hbs with this type of distal pocket, E7Gln also is in hydrogen bonding contact with B10Tyr (25, 28), thus providing extra stability to the hydrogen bond network.

The contribution to the distal hydrogen-bonding network from another residue in *Chlamydomonas* Hb, E10Lys, located close to the distal pocket, was also evaluated in this work. Mutation of this residue has a large effect on the heme structure in the absence of any exogenous ligand (29). However, in the oxy complex of K(E10)A, only minor perturbations on the FeO₂ structure (increase in $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ by 2 and 4 cm⁻¹, respectively) relative to the wild-type proteins were observed. These small changes in the Fe—O—O modes are consistent with the observation of an oxygen dissociation rate (0.022 s⁻¹) in K(E10)A similar to that in the wild-type protein (0.014 s⁻¹) (13). In another exogenous ligand-bound form, the ferric cyanide derivative, mutation of E10Lys has no significant effect on the Fe—CN stretching frequency whereas the B10Tyr and E7Gln have significant effects on the Fe—CN mode (30). Thus, the binding of exogenous ligands (in both ferric and ferrous oxidation states) significantly modifies the structure of the heme pocket of *Chlamydomonas* (and also *Synechocystis*) Hb, demonstrating the adaptability of the heme pocket in these Hbs.

The H/D Sensitivity of the $\nu_{\text{O}-\text{O}}$ Mode. The observation of a frequency increase of the $\nu_{\text{O}-\text{O}}$ mode in D₂O relative to H₂O (Fig. 2) in the present study may suggest that the upshift is caused by hydrogen bonding to the bound oxygen, assuming that there are no other latent changes in the spectra caused by the H/D exchange. An increase in the frequency of the $\nu_{\text{O}-\text{O}}$ mode in D₂O in both the proteins could result from strong hydrogen bonding interactions that might cause an anomalous shift because of higher rigidity of the Fe—O—O...D— assembly compared with the Fe—O—O...H— assembly (31). Such an interpretation, however, could be flawed if some H/D-sensitive vibrational modes (either resonance enhanced or not) lie near the O—O stretching mode, which might couple with

$\nu_{\text{O}-\text{O}}$, causing anomalous shifts. Such frequency shifts may not reflect inherent sensitivity of $\nu_{\text{O}-\text{O}}$ to H/D. In a series of model cobalt-porphyrin oxy complexes, Kincaid and coworkers (10, 12, 32) have established that such vibrational couplings of the internal modes of trans axial ligands with $\nu_{\text{O}-\text{O}}$ are very abundant in the complexes and complicate the spectral pattern. They successfully extracted the inherent frequencies of the vibrational modes from the observed coupled frequencies, and provided an alternate interpretation of the H/D sensitivity of the $\nu_{\text{O}-\text{O}}$ mode in cobalt-porphyrin-substituted hemoglobins (9), concluding that the H/D-induced spectral changes arise solely from vibrational coupling and not from hydrogen bonding with the bound oxygen (10, 12). Thus, the H/D sensitivity observed here for the ¹⁶O₂ complex, as well as for the ¹⁸O₂ complex (Fig. 2), may be attributed either to vibrational coupling of $\nu_{\text{O}-\text{O}}$ with histidine internal modes or to hydrogen bonding of the distal residues with the bound oxygen. At this time, in the absence of internal mode assignments of proximal histidine in these hemoglobins, discrimination between these two possibilities is not straightforward. However, strong hydrogen bonds indeed exist between the distal residues and the heme-bound dioxygen in both of these hemoglobins, as indicated from the studies of the $\nu_{\text{Fe}-\text{O}_2}$ mode discussed in earlier sections.

Correlation Between the Frequencies of the $\nu_{\text{O}-\text{O}}$ and $\nu_{\text{Fe}-\text{O}_2}$ Modes.

The correlation between the frequencies of Fe—XO and X—O (where X = C, N, O) in heme-ligand complexes has been a topic of considerable interest. Although there are ample data on the FeCO derivative available in the literature, studies on FeO₂ and FeNO are limited. In the FeO₂ complex of the heme proteins containing an axial imidazole ligand, such a correlation could not be evaluated because of the non-availability of the $\nu_{\text{O}-\text{O}}$ frequency. However, for model heme complexes, some data are available, and it has been proposed that an inverse correlation between the $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ frequencies exists just as in FeCO, albeit with a different slope (24, 33, 34). It is also argued that the 5-coordinate (no proximal ligand) and 6-coordinate FeO₂ complexes lie in the same correlation line (34). The frequencies of $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ for hemoglobins obtained from the present work are plotted along with the 6-coordinate model heme complexes (Fig. 5) to evaluate the correlation. From the data, no meaningful correlation between $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ frequencies could be identified. In heme proteins, at least in the present examples of *Chlamydomonas* and *Synechocystis* Hbs, distal interactions with the bound oxygen modulate the two frequencies. However, unlike CO, because of the small d π — π^* back-bonding in the FeO₂ derivatives, modulation of the $\nu_{\text{Fe}-\text{O}_2}$ frequency is not inversely correlated with the $\nu_{\text{O}-\text{O}}$ frequency.

Resonance Raman Enhancement of $\nu_{\text{O}-\text{O}}$ Mode. The reason the simultaneous enhancement of the $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ lines does not occur in the resonance Raman spectra of most oxyhemoglobins (and other histidine-ligated heme proteins) but occurs in cobalt porphyrin-substituted oxyhemoglobins was suggested to be due to a charge-transfer transition from the occupied π^* orbital to an empty antibonding σ^* orbital (8). Following this suggestion, it was argued that, in the oxy complex of P450, strong electron donation from the axial thiolate ligand to the metal π^* orbital makes the Fe(II) resemble Co(II), and thus both $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ bands are enhanced (24). Obviously, this argument does not explain the simultaneous enhancement of both the modes in *Chlamydomonas* oxyHb because its Fe—His bond (232 cm⁻¹) is much weaker than the Fe—Cys bond in P450 (351 cm⁻¹) (7). Whereas it remains to be clarified as to what extent the proximal ligand influences the enhancement of the $\nu_{\text{Fe}-\text{O}_2}$ and $\nu_{\text{O}-\text{O}}$ modes in oxyHb, we believe that distal interactions can contribute to the enhancement mechanism by altering the energy levels of the molecular orbitals of the FeO₂ group. This conclusion is consistent with the silence of the $\nu_{\text{O}-\text{O}}$ mode in the

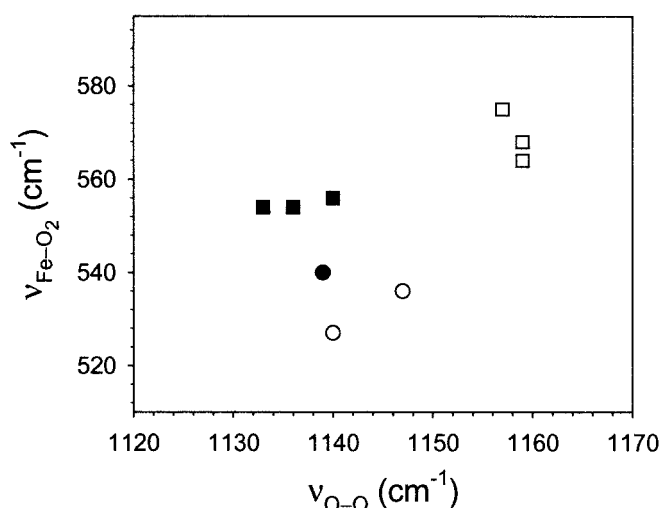


Fig. 5. A plot of the frequencies of the $\nu_{\text{Fe-O}_2}$ vs. $\nu_{\text{O-O}}$ modes for the oxy complexes of hemoproteins and heme model compounds containing histidine as the proximal ligand. The frequencies are listed in Table 1. *Chlamydomonas* and *Synechocystis* Hb (filled square), six-coordinate heme model complexes (open square), Cytochrome P450 (filled circle), and 6-coordinate model heme complexes containing 5⁻-ligation on the proximal side (open circle). No correlation between the frequencies of the $\nu_{\text{Fe-O}_2}$ and $\nu_{\text{O-O}}$ modes is apparent from this plot.

resonance Raman spectra of oxy complexes of the two distal pocket mutants [Y(B10)L and Q(E7)G] of *Chlamydomonas*, in which the stereochemistry of the Fe—O—O moiety could be significantly different from that in the wild-type protein. It will be interesting to find out precisely what factors render the intensity of the O—O stretching mode weak in some hemoproteins, while it is moderately or strongly enhanced in others.

Conclusions

The present work reports the simultaneous detection of the O—O and Fe—O₂ stretching modes in oxyhemoglobin, which until now

had not been detected by resonance Raman spectroscopy in any iron hemoprotein containing a proximal histidine. The detection of $\nu_{\text{O-O}}$ at 1133–1140 cm^{-1} in *Chlamydomonas* and *Synechocystis* Hbs with simple $^{16}\text{O}_2 - ^{18}\text{O}_2$ difference spectra, in contrast to those reported in cobalt-substituted globins, argues strongly for the assignment made by Kincaid and coworkers. Namely, the inherent O—O stretching mode is located in the 1133–1140 cm^{-1} region, and the extra lines seen in the infrared spectra and the resonance Raman spectra of the cobalt-substituted porphyrins are a consequence of mode coupling. It is also postulated that the bound oxygen is hydrogen-bonded not only to the terminal oxygen atom but also to the proximal atom, as has been shown by the crystal structure of *Ascaris* Hb. Deciphering the characteristics of the Fe—O and O—O bonds provides valuable insights not only for the mechanism of dioxygen stabilization and transport by oxyhemoglobin, but also for the dioxygen activation by histidine-ligated heme-enzymes such as cytochrome *c* oxidase and horseradish peroxidase. Dioxygen activation by cytochrome *c* oxidase is facilitated by the presence of redox-active groups such as Cu_B in close proximity to the heme-bound oxygen. In horseradish peroxidase, the O—O splitting after the peroxide reaction of the enzyme is aided by a trans effect from the proximal imidazolate as well as distal factors (hydrogen bonding of O—O with distal residues). The data reported here offer a clue for the understanding of the cellular function of these novel non-vertebrate hemoglobins. Strong hydrogen bonding of both oxygen atoms of FeO₂ in *Chlamydomonas* as well as in *Synechocystis* Hb make it unlikely that they could serve as oxygen transport proteins because, with such a low oxygen off-rate, the O₂ delivery would be very inefficient. Instead, the strong polar environment around the bound oxygen suggests that these proteins may activate dioxygen for an as yet undetermined enzymatic process. Experiments on the turnover rate of oxygen by the protein in a reducing environment will be a useful starting point to elucidate the mechanism of these novel proteins.

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