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Resonance Raman Studies of the Electronic State of Oxygen in Hemerythrin

(oxygen carriers/iron pigments/peroxide/oxygen-18/laser)

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ABSTRACT **Excitation of oxyhemerythrin with radiation within the envelope of its strong oxygen → iron charge-transfer band generates two Raman frequencies, at 844 cm^{-1} and 500 cm^{-1}. These are assigned to O–O and Fe–O stretching modes. Confirmation of these assignments is provided by the observed shifts in frequency when $^{16}O_2$ is substituted by $^{18}O_2$ as ligand. Comparison of the position of the 844-cm^{-1} band with band positions of small molecules of known oxidation state clearly establishes that the bound oxygen is in an O_2^{2-}, peroxide-type, electronic state.**

The electronic states of atoms at the active sites of oxygen-carrying pigments have been the subject of continuing experimental investigations and theoretical speculation. Among invertebrates, one of the major chemical types of oxygen carrier is hemerythrin (1). This nonheme pigment has Fe attached directly to side chains of residues of the polypeptide chain. Each subunit of hemerythrin contains 2 Fe atoms and binds 1 mol of O_2.

It was suggested some years ago (2), on the basis of meager evidence, that conversion of deoxy- to oxyhemerythrin should be described by the following transition in electronic states:

$$\underset{\text{deoxyhemerythrin}}{Fe^{II}\ Fe^{II}} + O_2 \rightleftarrows \underset{\text{oxyhemerythrin}}{Fe^{III}\text{–}O_2^{2-}\text{–}Fe^{III}} \qquad [1]$$

The oxidation state of Fe^{II} in deoxyhemerythrin was easily established by simple chemical experiments (2). However, an unequivocal test of the representation of the oxygenated state proved much more elusive. Ultimately Mössbauer, optical, and magnetic studies (3–6) showed clearly that the oxidation state of iron in oxyhemerythrin could be described as Fe^{III}, with antiferromagnetic coupling between the two iron atoms.

These spectroscopic and magnetic techniques scan the Fe atoms in hemerythrin. The oxidation state assigned to the oxygen, O_2^{2-}, emerges by implication. The two electrons given up by the 2 Fe^{II} of deoxyhemerythrin, to produce 2 Fe^{III} in oxyhemerythrin, have been assumed to reside on the oxygen. It would clearly be desirable to have more direct evidence on the electronic state of the oxygen.

Access to such evidence has been opened recently by development of techniques, based on resonance Raman spectroscopy, for establishing vibration frequencies of solutes in dilute aqueous solution. A resonance condition may be achieved by illumination of the sample with light of a frequency that lies within an electronic absorption band. Under these circumstances one can engender enormous intensification of Raman scattering by vibrational modes associated with the chromophore (7, 8), other modes being essentially unaffected. As a result, the resonance Raman bands often manifest themselves to the exclusion of all others.

Since the active site of oxyhemerythrin has a strong oxygen → iron charge-transfer band (Fig. 1) centered at 500 nm (1, 4), resonance Raman spectroscopy becomes attractive as a probe of the electronic state of the atoms, particularly the liganded O_2, at this site.

MATERIALS AND METHODS

Oxyhemerythrin from *Golfingia gouldii* was isolated by procedures described (3). Some samples were crystallized from a solution of 20% ethanol–0.4% NaCl and redissolved just before spectroscopic study. Other samples examined directly without crystallization showed the same Raman peaks.

Deoxyhemerythrin was prepared from fresh oxyhemerythrin, under a blanket of nitrogen, by titration with sodium dithionite to a colorless or light-green solution. Re-exposure of this material to air or oxygen regenerated oxyhemerythrin, with a resonance Raman spectrum identical to that of the original nonreduced protein. Oxyhemerythrin binding $^{18}O_2$ was prepared from deoxyhemerythrin, contained within a closed sample tube by injection of oxygen gas of composition of 93.6% oxygen-18, 0.65% oxygen-17, and 5.75% oxygen-16. This composition leads to the following diatom percentages: $^{18}O_2$ 87.6%, ^{18}O–^{16}O 10.8%, ^{18}O–^{17}O 1.22%, and $^{16}O_2$ 0.33%. All other combinations of O atoms are present in negligible amounts. To minimize contamination with extraneous $^{16}O_2$, $^{18}O_2$ was added with a gas-tight syringe within a nitrogen-filled glove-bag.

Tris-acetate buffer, 50 mM (pH 8.5), or phosphate buffer, 0.1 M (pH 8.0), served as solvent for the hemerythrins. Except for the occasional appearance of small bands due to the buffer, the resonance Raman spectra of the hemerythrins were the same in both solvents.

Laser Raman spectra were obtained with a Spex 1401, 0.85-m double monochromator, a cooled RCA C31034 Ga–As photomultiplier, and photon-counting electronics. A light-stabilized Spectra Physics 164 Ar^+ laser operating at 488 nm and delivering about 220 mW at the sample was used for most of the work. Other Ar^+ and Kr^+ laser lines were used at comparable power levels to test for the resonance condition. The samples were contained in sealed 10-mm (inner diameter) Pyrex tubes, which were rotated at 1800 rpm to minimize sample decomposition and the thermal lens effect (9). In several experiments the sample was cooled to near 0°C. At the lower temperature there was some reduction in sample decomposition but no change in peak positions or shapes as

compared to those found in scans at ambient temperature. A back-scattering geometry was used for sample illumination, with the angle between incident laser beam and spectrometer axis usually being 173°. Depolarization ratios were determined with 180° back-scattering and a polaroid analyzer in the scattered beam. In all experiments the laser was focused into the sample with a 90-mm focal-length cylindrical lens. In general, an interference filter was not used so that the plasma lines could provide internal wavenumber markers.

RESULTS

Excitation of oxyhemerythrin with the 488.0-nm Ar^+ laser line generated two Raman frequencies of moderate intensity at 844 cm^{-1} and 500 cm^{-1} (Fig. 2). Illumination with 514.5-nm radiation at the same power level produced both Raman lines with slightly greater intensities. As expected, no Raman features above background were observed with illuminating radiation of 647 nm.

The peak at 844 cm^{-1} is polarized, with a ρ value of 0.33; that at 500 cm^{-1} shows a depolarization ratio of about 0.4. The latter value is more uncertain because of overlap from a broad polarized glass band around 480 cm^{-1}.

The frequency of each Raman peak depends on the mass of the O_2 bound by hemerythrin. If $^{16}O_2$ is replaced by $^{18}O_2$, the 844-cm^{-1} peak is shifted to 798 cm^{-1}, and the 500-cm^{-1} peak is displaced to 478 cm^{-1} (Fig. 2). Under conditions that produced peaks with oxyhemerythrin, no Raman features were observed for deoxyhemerythrin.

DISCUSSION

Since Raman peaks at 844 and 500 cm^{-1} appear with oxyhemerythrin but not with the deoxygenated protein, it is likely that these bands are associated with O_2 vibrations. Such an assignment is confirmed by the frequency shifts accompanying the replacement of $^{16}O_2$ by $^{18}O_2$ (Fig. 2). Furthermore, it seems reasonable to assign the 844-cm^{-1} line to an O–O stretch, with relatively little mixing with other vibrational coordinates, since the calculated frequency, 796 cm^{-1}, for the ^{18}O isotopic species agrees almost exactly with

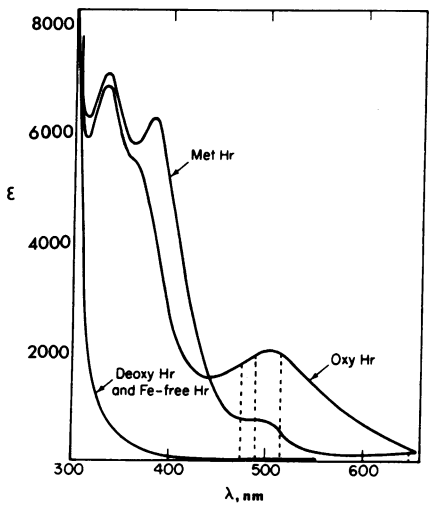

FIG. 1. Absorption spectra of hemerythrin (*Hr:* oxy, deoxy, and met). The positions of various laser-exciting frequencies are indicated by *broken vertical lines.*

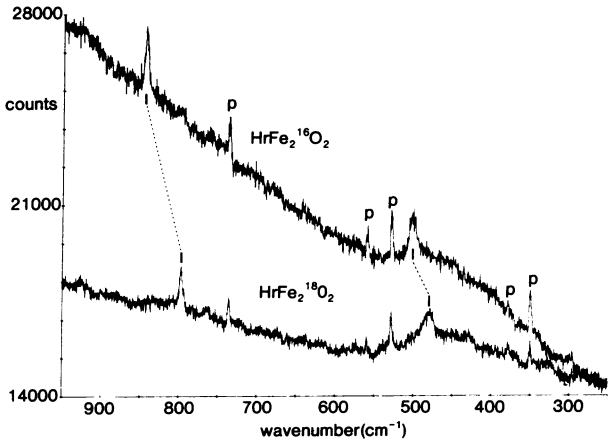

FIG. 2. Resonance Raman spectra of $HrFe_2 \cdot {}^{16}O_2$ (3.2 mM), and of $HrFe_2 \cdot {}^{18}O_2$ (3.2 mM). Laser plasma lines are indicated by *p*. The spectral band pass is 7 cm^{-1}. These tracings were produced directly from digital data. Absorbancy was 6. 9 at 488 nm.

that observed, 798 cm^{-1}. The observed depolarization ratio, ρ, of 0.33 is consistent with a symmetric stretch (A_1).

The frequency of the O–O stretch in oxyhemerythrin can now be compared with that of diatomic oxygen in other species. For such a comparison we note (10) that ν_{O-O} is 1556 cm^{-1} for gaseous O_2, 1145 cm^{-1} for the superoxide in KO_2, and 836 cm^{-1} for the peroxide in $(NH_4)(HO_2)$. The observed frequency in oxyhemerythrin, 844 cm^{-1}, clearly establishes that the bound oxygen is in a peroxide-type electronic state. Thus, Raman spectra provide a direct confirmation of the description of the oxygenation reaction, Eq. [1], as involving electron transfer from both Fe^{II} atoms to O_2 to produce O_2^{2-}.

The peak at 500 cm^{-1} can be assigned to an iron–peroxide stretch. The isotopic shift with ^{18}O confirms the involvement of oxygen in this vibration.

Although it does not seem profitable at this time to speculate on the implications of these observations with regard to the geometry of the dimeric iron-O_2 linkage, it is pertinent to note that the vibrational data provide evidence for the presence of only one type of O_2 species. It is also of interest that a sample containing both $^{16}O_2$ and $^{18}O_2$ showed only the characteristic Raman frequencies of the corresponding isotopic oxyhemerythrin species and gave no indication of isotopic scrambling on a time scale of hours.

Among oxygen-carrying pigments, hemoglobin has also been examined under resonance Raman conditions (11) with excitation by radiation in the range 470–550 nm. It reveals a complex set of bands, but none can be assigned to the liganded oxygen. It is possible that illumination at wavelengths within the envelope of other electronic absorption bands would be more successful. There have been arguments (12, 13) whether an Fe → O_2 electron transfer occurs in hemoglobin. A direct observation of the ligand vibration frequencies would permit a decision between the opposing viewpoints. Likewise in the third major category of oxygen-carriers, the hemocyanins, electron transfer may occur from Cu to O_2 at the active site (2). Here too some insight into the electronic state of the ligand may be obtained from resonance Raman spectroscopy, since hemocyanin shows an electronic absorption

band of moderate intensity near 600 nm and a strong band near 350 nm. It is clear from our results with hemerythrin* that, in favorable circumstances, resonance Raman spectroscopy may answer corresponding questions with regard to the electronic state of liganded atoms at the active sites of other oxygen carriers.

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* We have also observed ligand–metal and internal ligand vibrations in resonance Raman spectra of metazidohemerythrin.