OXYGEN BINDING TO HEMOCYANIN: A RESONANCE RAMAN SPECTROSCOPIC STUDY

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SUMMARY: Oxygenation of hemocyanin gives rise to resonance Raman peaks at 742 and 282 cm^{-1}. The 742 cm^{-1} peak which is in resonance with the 575 nm charge transfer band shifts to 704 cm^{-1} when $^{18}O_2$ is substituted for $^{16}O_2$. Our results establish that the bound oxygen is in the form of peroxide (O_2^{2-}). The 282 cm^{-1} peak which is in resonance with the 340 nm optical transition is insensitive to isotopic substitution, suggesting that the 282 cm^{-1} peak corresponds to a vibration involving the magnetically-coupled Cu(II)··Cu(II) centers.

INTRODUCTION: The chemistry of the reversible binding of oxygen to copper in hemocyanin has been the subject of much conjecture (1). Although there is considerable circumstantial evidence for the oxidation of copper upon binding oxygen, direct evidence for the existence of Cu(I) or Cu(II) has been difficult to obtain by magnetic measurements because both colorless deoxyhemocyanin and blue oxyhemocyanin are diamagnetic (2). Resonance Raman spectroscopy is currently being applied to the study of metalloproteins in order to determine the mode of binding of metal ions to the proteins (3,4) and their substrates (5,6). Using this technique we demonstrate that hemocyanin-bound oxygen is a peroxide and that copper is, thus, present as Cu(II) in the oxygenated protein.

MATERIALS AND METHODS: Hemocyanin and apohemocyanin were prepared from the hemolymph of the Pacific crab, Cancer magister (2,7). The purified protein was dialyzed against 0.01 M Tris-Cl, 0.01 M $MgCl_2$ and 0.05 M $NaClO_4$ (pH 8.5). Perchlorate ion was included as an internal standard for measuring Raman intensities. Addition of $NaClO_4$ had no effect on oxygen binding as judged by optical absorbance at 340 nm. The protein concentration was 60 mg/ml (1.5 mM in Cu).

Deoxygenation and equilibration with $^{18}O_2$ were carried out in an evacuable Pyrex cell consisting of a chamber (5 ml in volume) with a side arm (10 mm x 5 mm o.d.). The hemocyanin (0.2 ml) was deoxygenated by repeated, careful evacuation of the chamber followed by equilibration with N_2 (Airco, prepurified) saturated with water vapor. The resulting, colorless deoxyhemocyanin was then equilibrated with $^{18}O_2$ (Miles Laboratories, 91.07 atom% ^{18}O) at ~250 torr. Raman spectra were recorded with hemocyanin solutions in the side arm of the cell or in 1.7 mm capillary tubes.

Spectra were obtained on a Jarrell-Ash 25-300 Raman spectrophotometer using a Coherent Radiation Laboratories (CRL) model 52 argon ion laser (5145, 4880, 4765 and 4579 Å excitations) or a Spex Raman spectrophotometer with a CRL model 52DG Ar^+/Kr^+ laser (5682 Å excitation). No Raman spectra could be obtained with a 6328 Å excitation (Spectra-Physics model 125 helium-neon laser). Spike filters were used to eliminate laser plasma lines. Neutral density filters were employed to maintain the laser power at the sample between 50 and 120 mw. There was no observable change in either visible-uv or Raman spectra after

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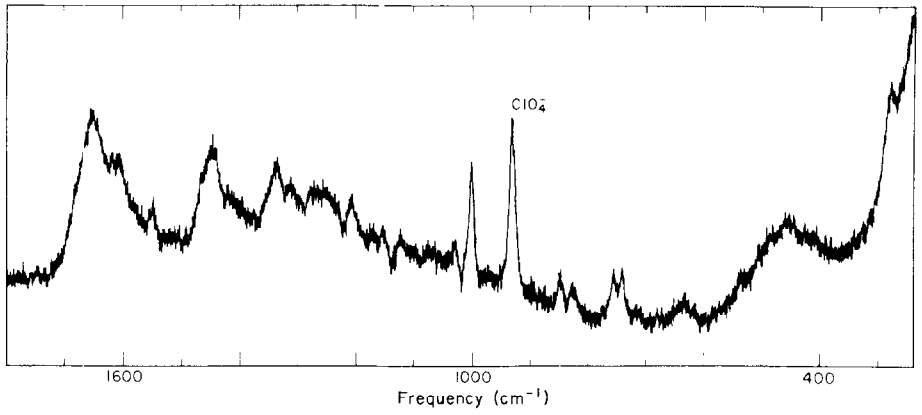

FIGURE 1: Raman spectrum of oxyhemocyanin using 4880 Å excitation, 20 cm^{-1}/min scanning rate, 2 second time constant and 8 cm^{-1} spectral slit width.

TABLE I: Variation in Raman Band Intensities [relative to $\nu_1(ClO_4^-)$ at 932 cm^{-1}] with Laser Excitation Wavelength.

	Wavelength, Å			
$^{16}O_2$-Hemocyanin	4579	4765	4880	5145
282 cm^{-1}	.72	.45	.29	.14
742	.19	.24	.28	.61
758	.26	.23	.28	.28
1003	.63	.70	.65	.75
$^{18}O_2$-Hemocyanin				
282 cm^{-1}	.73	.36	.31	.12
704	∿.06	.12	.27	.55
758	.26	.19	.21	.25
1003	.73	.84	.80	.68

long laser exposure. Intensities were measured as peak heights relative to the 932 cm^{-1}, $\nu_1(ClO_4^-)$ line. Frequencies are accurate to ±2 cm^{-1}.

RESULTS AND DISCUSSION: The Raman spectrum of oxyhemocyanin is shown in Figure 1. Most of the spectral lines are typical in position and intensity of those observed in proteins (8) and do not change significantly in intensity (relative to the internal standard) as the excitation frequency is changed. As an example, the relative intensity behavior of the tryptophan and phenylalanine vibrations at 758 and 1003 cm^{-1}, respectively, (8) is shown in Table I. In contrast, the peaks at 742 cm^{-1} and 282 cm^{-1} are not characteristic protein bands. The intensities of these two peaks do depend on the wavelength of laser excitation (Fig.2 and Table I), indicating that these are vibrational modes which are in

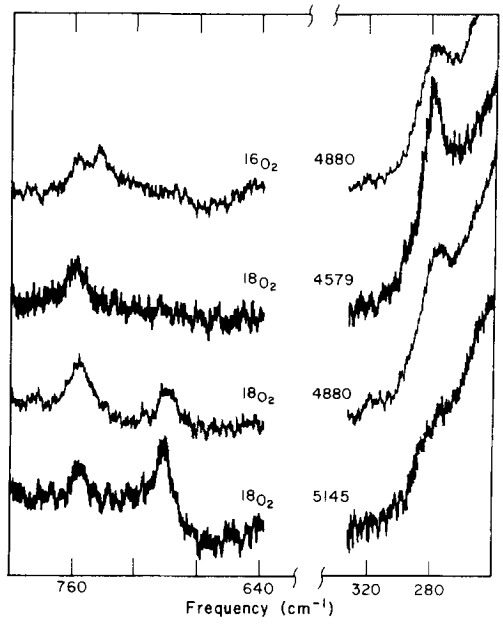

FIGURE 2: Raman spectra of oxyhemocyanin containing $^{18}O_2$ or $^{16}O_2$. Spectra were obtained with 4579, 4880, or 5145 Å excitation, 20 cm^{-1}/min scanning rate, 2 second time constant and 10 cm^{-1} slit width.

resonance with electronic states. The appearance of both resonance-enhanced and normal Raman modes in the same spectrum is the result of the high protein concentration used to compensate for the weakness of hemocyanin absorption in the visible region. The assignment of the 742 and 282 cm^{-1} peaks to chromophore-associated vibrational modes is supported by the selective disappearance of these two lines upon removal of oxygen or copper from the protein. The relative intensity of the 742 cm^{-1} peak increases with increasing wavelength of excitation while the intensity of the 282 cm^{-1} peak increases with decreasing wavelength of excitation (Fig.2 and Table I). Even greater intensity-enhancement was observed for the 742 cm^{-1} peak using the Kr^+ 5682 Å excitation line. Both the 742 and 282 cm^{-1} peaks are polarized.

The principal electronic absorption bands for C. magister oxyhemocyanin are at 340 nm (ε_{Cu} = 10,000 $M^{-1}cm^{-1}$) (2) and at 575 nm (ε_{Cu} = 600 $M^{-1}cm^{-1}$) (9). At 77°K the absorption bands in the visible region are observed at 420, 566 and 652 nm, all having similar absorptivities (10). From the wavelength dependence of intensity enhancement, it appears that the 742 cm^{-1} Raman line is in resonance with either the 575 or 652 nm absorption band while the 282 cm^{-1} line could be in resonance with electronic states at either 420 or 340 nm. These possibilities are resolved by calculating the expected enhancement of Raman in-

TABLE II: Resonance Enhancement (relative to intensity of 4880 Å excitation) of Hemocyanin Raman Bands.

	Observed Intensity Enhancement[a] Wavelength, Å			
$^{16}O_2$-Hemocyanin	4579	4765	4880	5145
282 cm^{-1}	2.5	1.5	1.0	0.49
742[b]	-	-	1.0	2.2
$^{18}O_2$-Hemocyanin				
282	2.4	1.2	1.0	0.41
704	0.21	0.46	1.0	2.1

		Calculated Relative Enhancement Factors(F_A^2)[c] Wavelength, Å			
$\Delta\nu$	ν_e	4579	4765	4880	5145
280 cm^{-1}	340 nm	2.4	1.4	1.0	0.52
280	420	10.3	2.1	1.0	0.27
700	575	0.31	0.61	1.0	4.2
700	650	0.52	0.76	1.0	2.0

a) relative intensities in Table I divided by intensity with 4880 Å.

b) overlapped with 758 cm^{-1} peak.

c) $F_A = \nu^2(\nu_e^2 + \nu_o^2)/(\nu_e^2 - \nu_o^2)^2$, where ν_o = excitation frequency; ν_e = virtual electronic state responsible for resonance; $\nu = \nu_o - \Delta\nu$; $\Delta\nu$ = Raman shift (Ref. 11). Data relative to 4880 Å enhancement.

tensities as a function of excitation wavelength assuming enhancement from a single electronic state (11) (Table II). The correlation between the observed and predicted enhancements is excellent for the 282 cm^{-1} vibration in resonance with the 340 nm electronic level. In view of the less accurate experimental data for the weaker 742 and 704 cm^{-1} peaks, the neglect of damping in the calculation of F_A when near an absorption maximum, the somewhat larger absorptivity of the 575 nm band as compared to the 650 nm band, and the absence of a Raman spectrum at 6328 Å, resonance enhancement of the 742 cm^{-1} vibration by the 575 nm electronic level is more probable.

In order to determine the involvement of bound oxygen in the resonance-enhanced vibrational modes, hemocyanin was equilibrated with $^{18}O_2$. This results in a shift of the 742 cm^{-1} peak to 704 cm^{-1}, but no observable change in the position of the 282 cm^{-1} peak (Fig.2 and Table I). The isotope shift to 704 cm^{-1} agrees well with the calculated value of 700 cm^{-1} for diatomic O-O stretching. The frequency of the O-O vibration is most characteristic of a peroxide-contain-

ing molecule in a hydrophobic environment, e.g. 738 cm^{-1} for Na_2O_2 in the anhydrous crystalline solid (12). The oxygen bound to the nonheme-iron protein, hemerythrin, has also been identified as peroxide by resonance Raman spectroscopy (6). Since oxygen is apparently reduced to peroxide in oxyhemocyanin, the copper atoms to which it binds must be oxidized to the Cu(II) state.

Since hemocyanin contains two copper atoms at the oxygen-binding site (13), it is possible for the oxygen to be bridging the two coppers (Cu-O-O-Cu) or for it to be attached to only one of them (Cu-O-O). It is unlikely that the oxygen is in a π-bonded complex ($Cu\langle^{O}_{O}$) since these species exhibit O-O stretching frequencies 100 - 150 cm^{-1} higher (14) than that observed for oxyhemocyanin. Recent infrared evidence indicates end-on (Fe-O-O) binding of oxygen in hemoglobin (15).

Identification of species participating in resonance enhancement can also provide insight into the nature of the electronic states responsible for the resonance effect. The fact that the oxygen stretching mode observed at 742 cm^{-1} is in resonance with the 575 nm absorption band suggests that this electronic transition involves $O_2^{2-} \rightarrow$ Cu(II) charge transfer, rather than a copper-localized d-d transition. The failure to observe an isotope effect on the 282 cm^{-1} peak suggests that the bound oxygen molecule is not involved in this vibration or in the electronic state(s) responsible for resonance-enhancement at 282 cm^{-1}. Thus, the 340 nm absorption band is probably related to electronic transitions involving the antiferromagnetically-coupled Cu(II) centers in oxyhemocyanin (2), rather than to $O_2^{2-} \rightarrow$ Cu(II) charge transfer. Similar absorption bands are observed in other proteins containing diamagnetic Cu(II) pairs (16) and in dimeric Cu(II) complexes with subnormal magnetic moments (17). The electronic transitions have been ascribed to charge transfer from groups bridging the copper atoms and/or copper ligands.

A survey of the literature reveals the following possibilities for the vibrational mode responsible for the 282 cm^{-1} peak: (i) Cu-S stretching vibration. This is unlikely as no free sulfhydryl groups are released upon removal of copper (7). (ii) Cu-Cu stretching vibration. Metal-metal bond vibrations generally occur at frequencies below 250 cm^{-1} and this type of bonding is not well substantiated for copper (18). However, an Fe-Fe double bond has been observed at 284 cm^{-1} (19). (iii) Cu-O stretch of a copper coordinated to the carboxyl group of an amino acid (20). (iv) Cu-O-Cu symmetric stretch for linear or nearly linear M-O-M system (21), although such bonding is not common for metals with more than six d-electrons (22). Copper coupling as in (iv) would explain the greater diamagnetism and lower absorption wavelengths (340 nm vs. 380 nm) observed in diamagnetic copper proteins as compared to cupric acetate dimers (17)

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