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Resonance Raman Studies of Hemerythrin–Ligand Complexes†

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ABSTRACT: Resonance Raman spectroscopy has been used as a probe of the structure of ligands at the active site of hemerythrin. Molecularly revealing insights have been obtained with oxyhemerythrin and with metazidohemerythrin. This spectroscopic technique has also facilitated a comparison of oxygen carrier within erythrocytes with that in solution. The electronic state of the bound O_2 is the same in the natural environment as in the artificial one.

In hemerythrin, the non-heme oxygen-carrying pigment of several invertebrate phyla, two Fe atoms bind one molecule of O_2 (Klotz and Klotz, 1955; Klotz et al., 1957; Boeri and Ghiretti-Magaldi, 1957). In view of this stoichiometry it has been assumed that O_2 bridges the two Fe atoms. On the basis of chemical and spectroscopic evidence originally (Klotz, 1955) and Mössbauer, optical and magnetic studies more recently (Okamura et al., 1969; Garbett et al., 1969, 1971; Dawson et al., 1972), the state of the active site has been represented as

$$Fe^{III}\text{-}O_2^{2-}\text{-}Fe^{III} \quad (1)$$

The techniques mentioned examine the state of the Fe atoms in hemerythrin, and the oxidation state of the oxygen, O_2^{2-}, has been assigned largely indirectly. Recently we have demonstrated that resonance Raman spectroscopy can scrutinize the vibrational modes of the oxygen at the active site of hemerythrin (Dunn et al., 1973). This probe has now been extended to hemerythrin within erythrocytes and studies have been expanded to ligands other than molecular oxygen.

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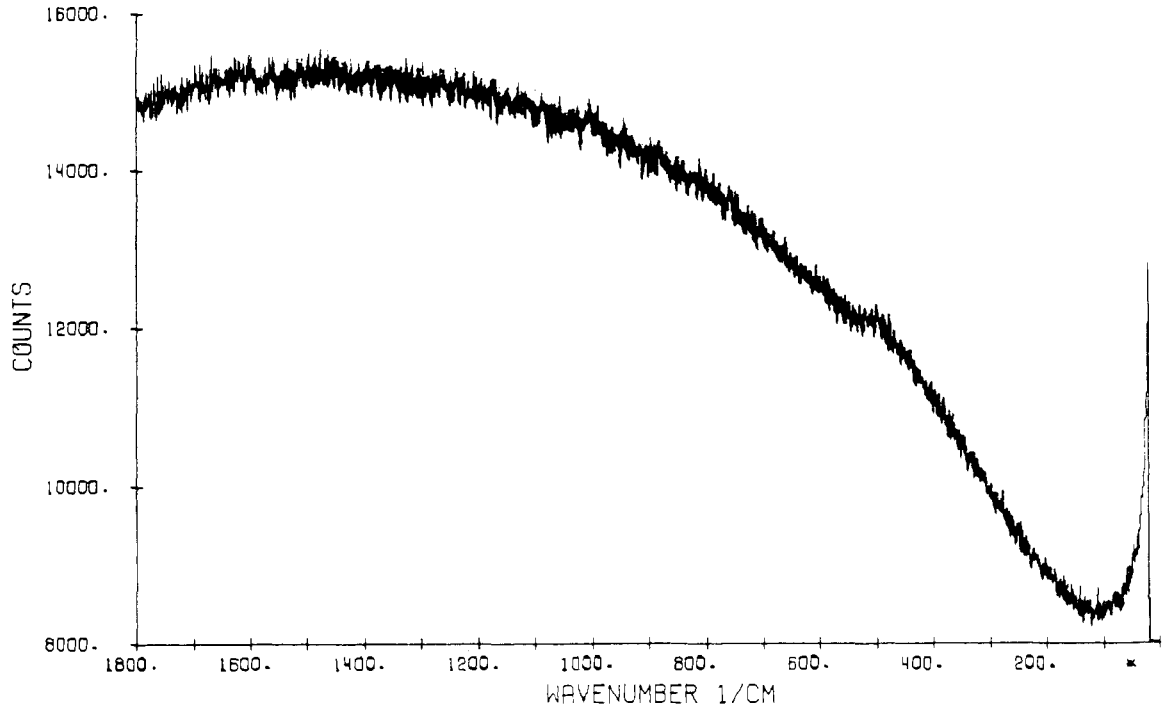

FIGURE 1: Resonance Raman spectrum of deoxyhemerythrin in solution.

Materials and Methods

Erythrocytes and hemerythrin were obtained from *Golfingia gouldii* by procedures described previously (Klotz et al., 1957; Okamura et al., (1969). Blood from the coelomic cavity, after filtration through glass wool, was centrifuged to separate nonpigmented material from erythrocytes. The erythrocytes were washed with sea water and centrifuged in several cycles. The nonpigmented layer was separated manually. Nonpigmented material was also treated by washing and centrifugation. A portion of the erythrocytes was set aside for direct spectroscopic examination. The remainder was processed to yield crystalline oxyhemerythrin. Some of this was converted to deoxyhemerythrin by titration with sodium dithionite under a blanket of nitrogen. $^{18}O_2$–oxyhemerythrin was obtained from deoxyhemerythrin contained within a closed sample tube by injection of ^{18}O-enriched oxygen gas (93.6% ^{18}O, 5.75% ^{16}O, 0.65% ^{17}O) obtained from Bio-Rad Laboratories. Enriched oxygen-18 oxyhemerythrin was prepared directly within a Raman sample tube to minimize possible exposure to external $^{16}O_2$. Solutions of metaquo- and methydroxyhemerythrin were obtained by titration of oxyhemerythrin with potassium ferricyanide, followed by dialysis to remove excess reagent and reaction products. Conversion to metazidohemerythrin was achieved by addition of potassium azide. Nitrogen-15 enriched metazidohemerythrin was prepared with ^{15}N triply labeled KN_3 obtained from the Wilmad Glass Company.

Laser Raman spectra were recorded with a Spex 1401, 0.85-m double monochromator with a cooled RCA C31034 GaAs photomultiplier and photon-counting electronics. A Spectra Physics 164 Ar^+ laser with light stabilization was used for sample illumination. A backscattering geometry was employed with the angle between the incident beam and the spectrometer axis being approximately 170°. A strict 180° back-scattering geometry was used during measurements of depolarization ratios. A 90-mm focal length cylindrical lens served to focus the laser beam at the sample. Samples were contained in Pyrex tubes of 10-mm i.d. sealed with a tight-fitting rubber septum. A spinner rotated the tubes at 1800 rpm to reduce thermal decomposition and the thermal lens effect. With pigmented erythrocytes viscosity hindered mixing and laser irradiation produced some bleaching, manifested as a thin light band around the tube at the position of the incident beam. Therefore these spectra were recorded as quickly as possible. In some experiments the sample was cooled to a temperature near 0° by passage of evaporated gas from liquid nitrogen around the tube. Spectra were recorded with a monochromator band pass of 3–7 cm^{-1} and a power level for the illuminating source of 300 mW at the sample. Operating parameters were varied to produce a maximum signal near 25,000 counts per second to maintain a good signal-to-noise ratio.

Visible absorption spectra were obtained with a Cary 14 recording spectrophotometer.

Results

Deoxyhemerythrin showed a high fluorescent background but no Raman bands (Figure 1) under illumination conditions that excited the characteristic peaks in oxyhemerythrin.

Excitation of concentrated samples of oxyhemerythrin with all of the blue and green laser lines of the argon laser generated two Raman bands of moderate intensity at 844 and at 500 cm^{-1} (Figure 2). The 844-cm^{-1} band was also observed as a weak feature on the anti-Stokes side. A very weak band was also discovered at 180 cm^{-1}.

Labeled oxyhemerythrin, prepared from deoxyhemerythrin and oxygen-18 gas, revealed the mass dependence of the two Raman bands (Figure 2). When the bound $^{16}O_2$ is replaced by $^{18}O_2$, the 844-cm^{-1} peak is shifted to 798 cm^{-1}, and the 500 cm^{-1} feature appears as two peaks, one at 500 and the other at 478 cm^{-1}. Reduction and subsequent reoxygenation with air produced a spectrum identical with that of the original oxyhemerythrin. No definite features above background were observed with the 647 nm K^+ laser excit-

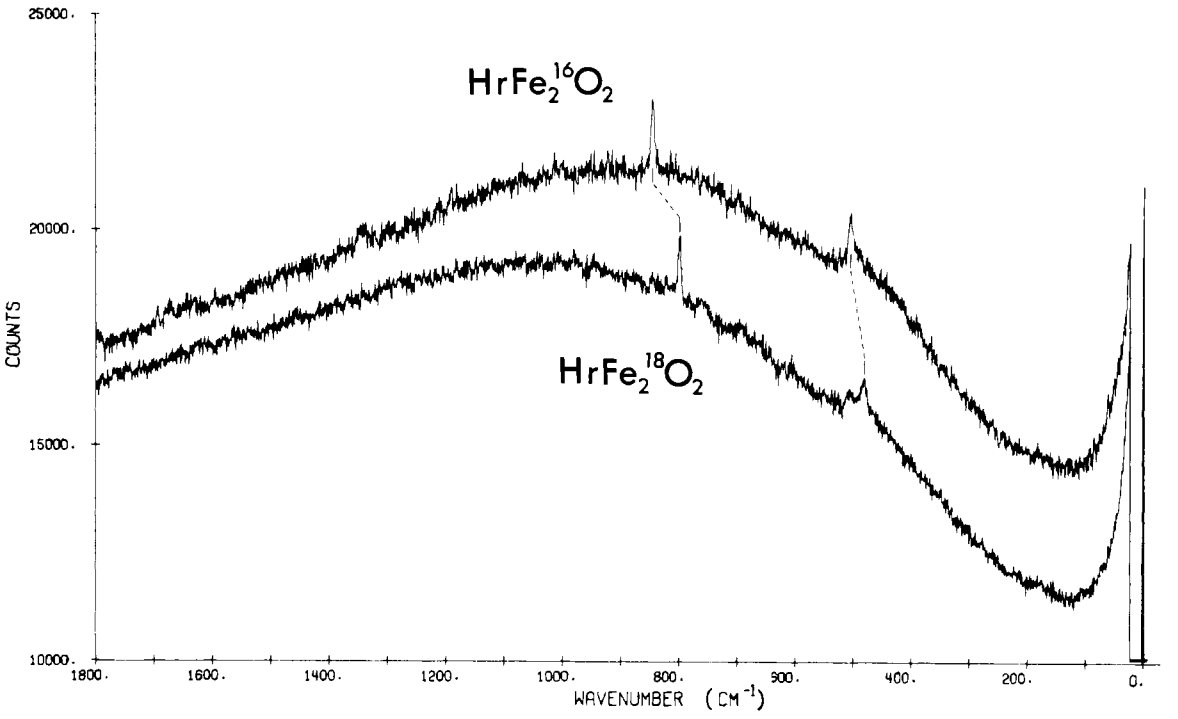

FIGURE 2: Resonance Raman spectra of oxyhemerythrin in solution.

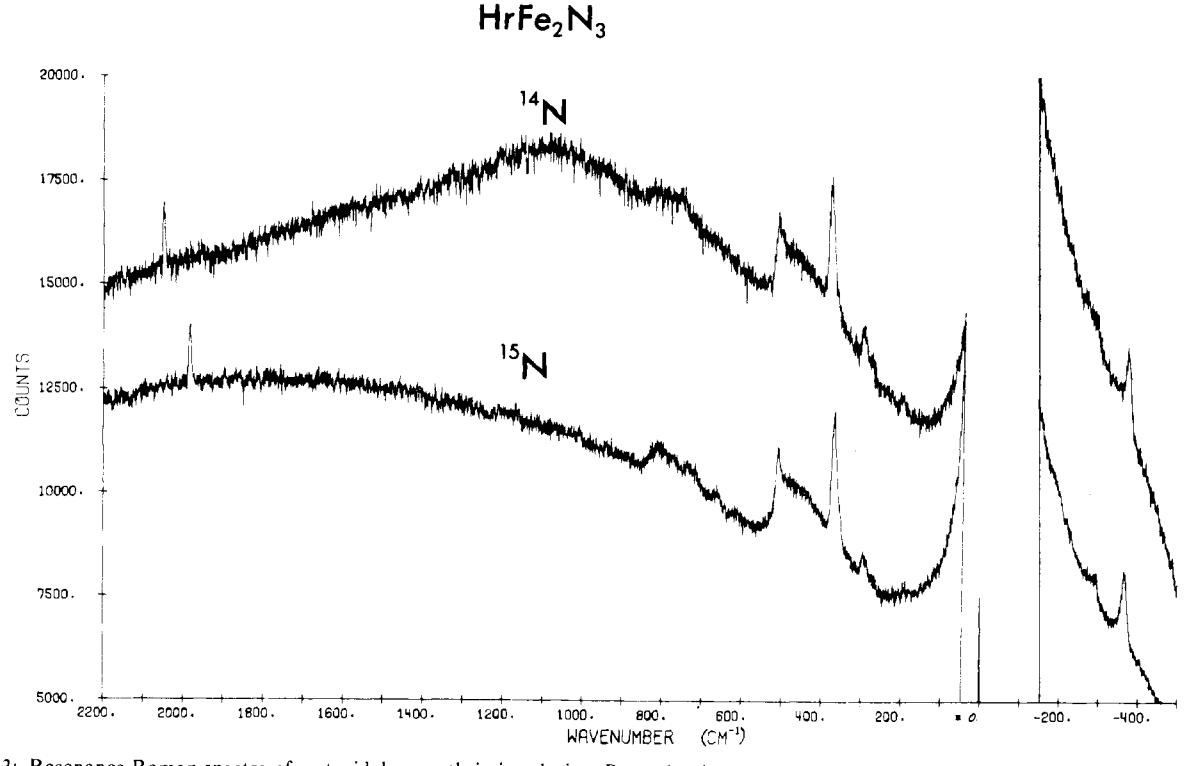

FIGURE 3: Resonance Raman spectra of metazidohemerythrin in solution. Power levels were 600 mW on the anti-Stokes side and 250 mW on the Stokes side.

ing line under comparable experimental conditions. Some minor decomposition of oxyhemerythrin was observed over the time course of the experiment. If the loss of the primary species was greater than 25%, the sample was replaced. The stability of different samples of oxyhemerythrin varied, but most survived over 10 hr of exposure to the laser.

The resonance Raman spectrum of metazidohemerythrin, obtained with blue and green laser lines, consists of two major peaks at 2049 and 376 cm^{-1} and three minor peaks, at 500, 292, and 188 cm^{-1} (Figures 3 and 4). The three lowest energy bands were also observed as weak anti-Stokes bands. The resonance Raman spectrum of metazidohemerythrin prepared with nitrogen-15 azide manifested the expected mass dependence of position of the two major peaks (Figure 3). The 2049-cm^{-1} peak was shifted to 1983 cm^{-1}, the 376 cm^{-1} to 368 cm^{-1}. Shifts were not observed in the 500-, 292-, and 188-cm^{-1} bands. The latter two bands are very weak and broad so a shift of less than 5 cm^{-1} might

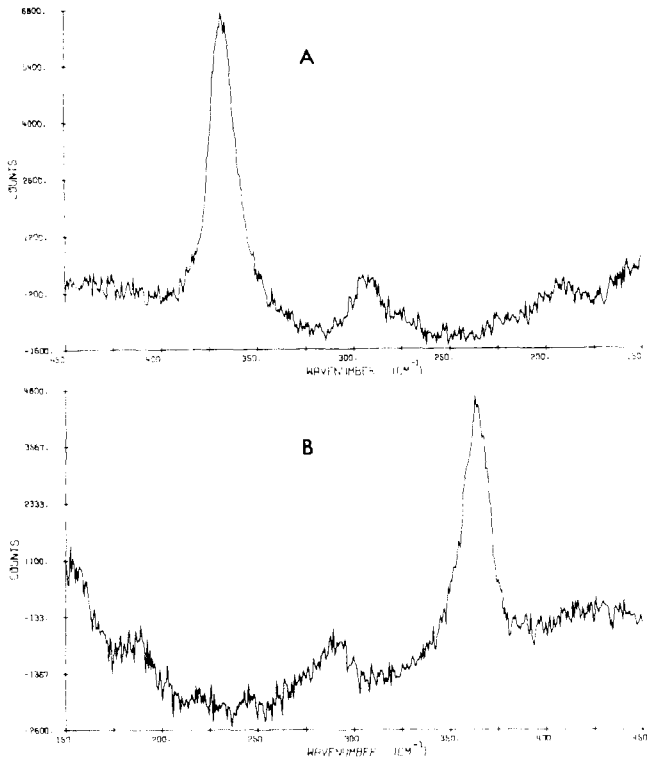

FIGURE 4: Low energy peaks of resonance Raman spectrum of $HrFe_2{}^{15}N_3$. Stokes scattering is shown in A, anti-Stokes peaks in B.

not be detected. Even though the feature at 500 cm^{-1} is sharp it occurs on the high frequency edge of the broad glass band, so a small shift might go undetected. Metazidohemerythrin was exceptionally stable in the laser Raman experiments. Samples showed no detectable decomposition after several hours of exposure to the laser.

The resonance Raman spectra of the cyanato, cyano, chloro, and aquo derivatives of methemerythrin revealed no Raman peaks. The fluorescence varied slightly, but was comparable to that of Figure 1 for deoxyhemerythrin. These derivatives of methemerythrin were fairly unstable in the laer beam.

The resonance Raman spectrum of oxygenated erythrocytes is composed of five major peaks at 1525, 1159, 1008, 844, and 500 cm^{-1} (Figure 5). Deoxygenated erythrocytes and the nonpigmented material revealed Raman bands at 1525, 1159, and 1008 cm^{-1}, but no peaks at 844 or 500 cm^{-1}.

The observed Raman bands for all the samples studied are assembled in Table I for purposes of comparison. The depolarization ratios of some of the bands have been determined and are also listed in this table.

Discussion

The appearance of two moderately intense Raman peaks at 844 and 500 cm^{-1} for oxyhemerythrin, excited near its 500-nm electronic absorption maximum, suggests that these peaks are resonance-enhanced vibrational transitions of the active site. These peaks are not observed with excitation outside of the absorption envelope, nor are they present in the Raman spectra of deoxyhemerythrin and methemerythrins. Thus it is evident that the chromophoric active site is involved in the expression of these vibrational transitions.

To test this presumption that bound oxygen is involved in these resonance enhanced vibrational modes, we prepared oxyhemerythrin with oxygen-18 gas. The spectrum showed shifted bands, the 844-cm^{-1} peak going to 798 cm^{-1} and a portion of the 500-cm^{-1} peak going to 478 cm^{-1}. These isotopic frequency shifts confirm the assignment of these bands to active site modes, specifically those involving O_2 vibrations.

Since these two bands are polarized, they must be associated with totally symmetric modes (A_1). It seems reasonable, therefore, to assign the 844-cm^{-1} peak in oxyhemerythrin to an O-O symmetric stretch. The observed new

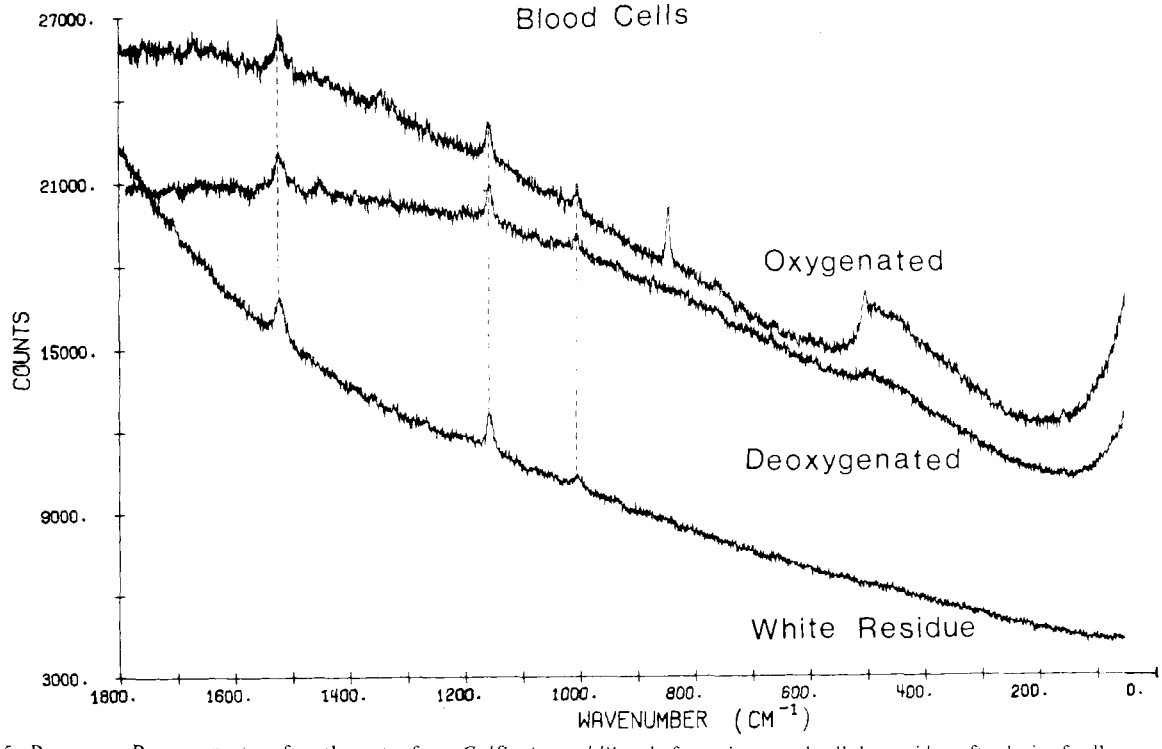

FIGURE 5: Resonance Raman spectra of erythrocytes from *Golfingia gouldii* and of nonpigmented cellular residue after lysis of cells.

Table I: Frequencies and Depolarization Ratios of Resonance-Enhanced Raman Peaks of Hemerythrin.

Sample	Raman Frequencies (cm)	Depolarization Ratio
$HrFe_2{}^{16}O_2$	844	0.3
	500	0.4
	180	
$HrFe_2{}^{18}O_2$	798	0.3
	478	0.4
	(180)	
Oxyerythrocytes	1525	
	1159	
	1008	
	844	
	500	
Deoxyerythrocytes	1525	
	1159	
	1008	
Nonpigmented Cells	1525	
	1159	
	1008	
$HrFe_2{}^{14}N_3$	2049	0.6
	376	0.3
	292	
	188	
$HrFe_2{}^{15}N_3$	1983	0.6
	368	0.3
	290	
	188	

band frequency (798 cm^{-1}) on isotopic substitution of oxygen-18 in oxyhemerythrin agrees almost exactly with the frequency calculated (796 cm^{-1}) for a diatomic model. These results are consistent with a symmetric stretch of the bound oxygen with little mixing of the other vibrational coordinates.

The frequency of the oxygen–oxygen symmetric stretch can be compared to that of diatomic oxygen in other molecular species. Vibrational and structural parameters of model compounds have been assembled in Table II. From these data for small molecules it is evident that O–O stretching frequency reflects the oxidation state of diatomic oxygen. The observed frequency in oxyhemerythrin, 844 cm^{-1}, clearly establishes that the bound oxygen is in a peroxide-type electronic state. Thus the Raman spectrum provides a direct confirmation of the description (Klotz and Klotz, 1955) of the oxygenation reaction as involving the transfer of $2e^-$ from the two Fe^{II} atoms to O_2 to produce a bound peroxide.

The peak at 500 cm^{-1} is in the general region of an Fe–O stretching mode, and for $^{16}O_2$ oxyhemerythrin it is assigned to a composite of the Fe–O_2 stretch and an Fe–O stretch associated with an unidentified ligand. The composite nature of this band is revealed by the observation of two features in this region for $^{18}O_2$ oxyhemerythrin, and it is further confirmed by the observation of a 500-cm^{-1} band in metazidohemerythrin. A tyrosine residue in the protein, coordinated through its phenolic oxygen, is a possible candidate as the ligand associated with this 500-cm^{-1} feature.

The observation of a weak band at 188 cm^{-1} in metazidohemerythrin spurred an intensive search for low energy bands in oxyhemerythrin. One very weak peak was observed at 181 cm^{-1}, and it may be due to the same transition as the 188-cm^{-1} band in metazidohemerythrin, the small difference in frequency being a response to the ligand change. Since this peak is not shifted on isotopic substitution of $^{16}O_2$ to $^{18}O_2$ it is not likely to be associated with the ligand but rather with another portion of the chromophoric site. It is also possible that these features near 180 cm^{-1} arise from the buffer, or some other, unidentified source.

The vibrational frequencies at 844 and 500 cm^{-1} in oxygenated erythrocytes correspond within experimental error with those of isolated, purified oxyhemerythrin. The identity in positions demonstrates that the electronic state of the active site is the same in the natural environment of oxyhemerythrin, the erythrocyte, as in the artificial one in buffered aqueous solution.

The three additional peaks observed with oxygenated erythrocytes, at 1525, 1159, and 1008 cm^{-1}, also appeared

Table II: Oxygen Stretching Frequencies.

Species	ν_{O-O} (cm^{-1})[a]	Ref
Molecular oxygen		
O_2	1555	Herzberg (1950)
Superoxides		
HOO	1101	Milligan and Jacox (1963)
$Co(acac)_2en(py)O_2$	1123	Crumbliss and Basolo (1970)
$Co(acac)_2en(CH_3\text{-}py)O_2$	1195	Crumbliss and Basolo (1970)
$Co(acac)_2en(NH_2\text{-}py)O_2$	1132	Crumbliss and Basolo (1970)
$Co(acac)_2en(CN\text{-}py)O_2$	1140	Crumbliss and Basolo (1970)
$Co(CN)_5O_2(NEt_4)_3$	1138	White et al. (1972)
$[(His)_2Co(O_2)Co(His)_2][NO_3^-]_3\cdot 2H_2O$	1120	Freedman et al. (1975)
$[(NH_3)_5Co(O_2)Co(NH_3)_5]Cl_5\cdot 4H_2O$	1122	Shibahara (1973)
Peroxides[b]		
H_2O_2 (Solid)	878	Bain and Giguère (1955)
$IrCl(CO)(PPH_3)_2O_2$	860	Vaska (1963)
$Pt(PPh_3)_2O_2$	828 (782)	Nakamura et al. (1971)
$Pd[(CH_3)_3CNC]_2O_2$	893 (837)	Nakamura et al. (1971)
$Ni[(CH_3)_3CNC]_2O_2$	898 (848)	Nakamura et al. (1971)
$RhCl(PPh_3)_2[(CH_3)_3CNC]O_2$	892 (842)	Nakamura et al. (1971)
$(His)_2Co(O_2)Co(His)_2$	805	Freedman et al. (1974)
$(NH_3)_5Co(O_2)Co(NH_3)_5(SO_4)_2$	808	Freedman et al. (1974)
Biological oxygen carriers		
Oxyhemerythrin	844 (798)	Dunn et al. (1973)
Oxyhemoglobin	1107 (1062)	Barlow et al. (1973)
Oxyhemocyanin	742 (704)	Loehr et al. (1974)

[a] Values in parentheses are frequencies observed for $^{18}O_2$ isotopically substituted compounds. [b] See also Griffith and Wickins (1968).

with deoxyerythrocytes and with the residual material after lysis and removal of hemerythrin. Clearly these peaks arise from some component of the membrane or associated insoluble constituents. The positions of the peaks do not correspond with those of heme chromophores (Loehr and Loehr, 1971; Brunner et al., 1972; Spiro and Strekas, 1974) nor with those of other metalloproteins (Tomimatsu et al., 1973; Carey and Young, 1974; Siiman et al., 1974). On the other hand, there is a remarkable coincidence with the three major resonance-enhanced peaks of the carotenoids (Rimai et al., 1970; Gill, et al., 1970; Heyde et al., 1971) lycopene (1516, 1156, and 1004 cm^{-1}) and β-carotene (1527, 1158, and 1006 cm^{-1}). It seems very likely, therefore, that the corresponding peaks in erythrocytes arise from a carotenoid constituent embedded in or attached to the membranes (Lippert, 1974).

The resonance Raman spectrum of metazidohemerythrin contains two major peaks that are dependent on the mass of the nitrogens in the azide ligand—one at 2049 cm^{-1} and another at 376 cm^{-1}. The peak at 2049 cm^{-1}, which is only slightly polarized, correlates with the asymmetric stretch of free azide. The use of triply labeled ^{15}N azide in the preparation of metazidohemerythrin leads to a shift of this band to 1983 cm^{-1}. This observed frequency is reasonably close to 1980 cm^{-1}, the calculated value assuming no interaction between the $^{15}N_3^-$ asymmetric stretch and other coordinates in the molecule.

The spin state of iron has an effect on the azide stretching frequency (McCoy and Caughey, 1970). In metazidohemoglobin and metazidomyoglobin two frequencies are observed, one near 2045 cm^{-1} for the complex in the high spin state and one at 2025 cm^{-1} for the low spin state of the iron. In metazidohemerythrin it is known from Mössbauer and magnetic properties that the iron is in a high spin state. The azide asymmetric stretch at 2049 cm^{-1} thus is consistent with these data. In metazidohemoglobin and metazidomyoglobin the N_3^- is attached in a nonbridging manner to a single high spin Fe^{III}. The closeness of vibration frequency in metazidohemerythrin to that in the heme proteins suggests that the azide is not a bridging ligand in the former either but is bound to only one iron of the pair at the active site, as has been conjectured also for the O_2 ligand (Okamura, 1970; Dawson et al., 1972; Okamura and Klotz, 1973; Dunn, 1974). However, the shifts in frequency between bridging and nonbridging azides within and between different series of model compounds are not consistent enough to provide a basis for a definitive choice in metazidohemerythrin.

The Raman band for metazidohemerythrin at 376 cm^{-1} is also mass dependent and its frequency is consistent with the metal azide stretches found in model compounds (Barrow, 1962). Bands found in model compounds suggest that the vibration at 294 cm^{-1} is due to a metal azide deformation. This band does not appear to be isotopically shifted in the nitrogen-15 metazidohemerythrin; however, it is weak and quite broad, and so a shift may not be detectable.

Thus with hemerythrin, resonance Raman spectroscopy has given insights into the structure of ligands at the active site that have not been attainable by any other probe. The technique is applicable to appropriate chromophoric systems in their natural environment as well as in isolated purified circumstances.

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The Binding of Azide to Human Methemoglobin A_0. Error Analysis for the Interpolative and Noninterpolative Methods†

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ABSTRACT: The binding of azide to human methemoglobin A_0 has been studied at 6°, pH 7, and $I = 0.2$ by three spectroscopic methods: (1) the conventional interpolative method, (2) an interpolative dialysis technique, and (3) a noninterpolative method. The interpolative methods assume that the fractional spectral change equals the fraction of heme sites bound by ligand, while the noninterpolative method measures the extent of binding directly, i.e., without the interpolative assumption. Both experiment and error analysis show that method 1 has low precision, and consequently, gives an inherently unreliable binding isotherm. Method 2 achieves high experimental and intrinsic precision. However, method 3, which also has high precision, clearly proves that the interpolative assumption of method 2 is incorrect. That is, the true fractional extent of binding becomes equal to the fractional spectral change only after about 97% of heme sites have been bound with ligands.

Recently, studies of the interactions of ferrihemoglobin A (Hb^+) with anionic ligands, none of which bind to ferrohemoglobin itself, have become especially germane, in that an understanding of these processes should increase our understanding of the in vivo function of ferrohemoglobin, especially in light of the reported crystallographic near-isomorphism of oxyferrohemoglobin and ferrihemoglobin (Perutz et al., 1974).

A review of the literature concerning the binding of azide to Hb^+ is particularly disconcerting. That is, several laboratories have reported substantially dissimilar estimates of the cooperativity of the binding process, although the experiments were conducted under essentially similar conditions. To wit: Banerjee et al. (1973) have reported a Hill coefficient ("n" value) of about 1.5 at pH 6, which indicates that the binding process may be somewhat cooperative (i.e., $n > 1$). In sharp contrast, Epstein and Stryer (1968) find $n = 1$ independent of temperature and pH (from 5.5 to 9.5). These results suggest that the binding of azide to Hb^+ might be noncooperative (four independent heme groups). All of these sets of Hill coefficients were obtained at moderate ionic strengths (0.05–0.1) and temperatures (10–25°) and should, therefore, have been more nearly in agreement, especially in the cases where the pH was the same.

In view of the discrepancies in Hill coefficients in the literature and in our own laboratory (vide infra) for the binding of azide to Hb^+, we have undertaken an investigation of the usual interpolative tube method for determining the Hb^+-N_3^- isotherm.

The interpolative tube method consists of this series of steps: a set of tubes containing a constant amount of Hb^+, buffer, and total azide concentrations of sufficient span to assure total azide/heme ratios of 0 to about 50 is incubated until equilibrium is reached. After incubation, the absorbance of these solutions is measured at an appropriate wavelength at the temperature of incubation. The ligand bound per heme is equated to the fractional spectral change, Y, which, in turn, is interpolated from the absorbance changes by the relationship

$$Y = (A_s - A)/(A_s - A_0) \quad (1)$$

where A_s, A_0, and A represent the absorbances at saturating, zero, and intermediate concentrations. The free azide concentration is subsequently calculated by

$$[L]_f = [L]_t - Y[Hb^+] \quad (2)$$

with $[L]_f$, $[L]_t$ = concentrations of free and total ligand, respectively, $[Hb^+]$ = concentration of protein in moles heme per liter, and with the product $Y[Hb^+]$ being taken as the concentration of bound ligand.

We have addressed ourselves to the following possible weaknesses in the interpolative tube method. First, does the increase in absorbance truly represent ligand binding alone, or can that increase also have contributions from other sources? That is, may the fractional spectral change, Y, be correctly equated to the fraction of heme ligated? Second, is the concentration of free ligand correctly and precisely described by eq 2?

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