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Mössbauer Spectroscopic Studies of Hemerythrin from *Phascolosoma lurco* (syn. *Phascolosoma arcuatum*)†

Paul E. Clark and John Webb*

ABSTRACT: Hemerythrin from coelomic cells of *Phascolosoma lurco* (syn. *P. arcuatum*) was isolated by gel filtration as two components, hemerythrin-I (25%) and hemerythrin-II (75%). The Mössbauer spectrum of oxyhemerythrin-II consisted of two pairs of lines of the same isomer shift (0.5 mm s^{-1}) corresponding to Fe(III) but different quadrupole splitting (1.01 and 2.02 mm s^{-1}). Application of a 2.5-T magnetic field at 4.2 K caused no significant spectral broadening. The $2Fe{\cdot}O_2$ binding site thus contains two nonequivalent high-spin Fe(III) ions that are antiferromagnetically coupled. The Mössbauer spectra of the minor component, hemerythrin-I, indicated an identical binding site. On deoxygenation, the spectrum was dominated by a simple quadrupole split doublet corresponding to Fe(II), indicating that the binding site in this derivative contains two identical Fe(II) ions that interact only weakly, if at all. The Mössbauer spectra of azidohemerythrin-II indicated that this derivative also contains a pair of antiferromagnetically coupled Fe(III) ions with the same isomer shift (0.5 mm s^{-1}) but quadrupole splittings (1.40 and 1.96 mm s^{-1}) that are not identical with those in oxyhemerythrin.

Hemerythrin, the oxygen-transport protein found in several phyla of marine invertebrates, binds dioxygen in the stoichiometry of $2Fe:O_2$. Many investigations using a wide range of biochemical, spectroscopic, and magnetic studies have been designed to elucidate the nature of the dioxygen binding site. Notwithstanding this extensive series of reports, the structure of the dioxygen binding site is still uncertain and controversial, as recounted in recent reviews (Okamura & Klotz, 1973; Klotz et al., 1976; Kurtz et al., 1977; Stenkamp & Jensen, 1979; Loehr & Loehr, 1979; Klippenstein, 1980).

Detailed structures for the binding site have been proposed on the basis of X-ray studies of metazidohemerythrin from *Themiste* (syn. *Dendrostomum*) *zostericola*, formerly known as *T. pyroides* (Hendrickson et al., 1975; Hendrickson & Ward, 1977), metazidohemerythrin from *Phascolopsis* (syn. *Golfingia*) *gouldii* (Ward et al., 1975), and metaquohemerythrin from *T. dyscritum* (Stenkamp et al., 1976a,b, 1978a,b). The differences among these structures have been reviewed in detail recently (Stenkamp & Jensen, 1979). In summary, two structures have been proposed: first, the metaquohemerythrin structure, which involves a (face-sharing) pair of octahedrally coordinated Fe(III) centers that share a face via oxygens from a water molecule and an asparate and glutamate side chain, and second, the metazidomohemerythrin structure, which also contains two octahedrally coordinated Fe(III) centers but now joined by a single μ-oxo bridge. Recent refinements of the crystallographic data have reduced the differences between the two models, with the confacial bioctahedron now containing a Fe–O–Fe bridging unit (J. Sanders-Loehr, personal communication).

In this paper we report results of Mössbauer studies of hemerythrin isolated from coelomic cells of a different species from those mentioned above, namely, *Phascolosoma lurco* (syn. *P. arcuatum*) (Stephen & Edmonds, 1972).

Mössbauer spectroscopy has been used extensively in studies of the hemerythrin dioxygen binding site for it is a particularly appropriate technique for detecting the presence of magnetic interaction between iron atoms (Trautwein, 1974; Johnson, 1975; Webb, 1975). Antiferromagnetic coupling between iron atoms in derivatives of several hemerythrins has been demonstrated clearly in previous studies (Okamura et al., 1969; York & Bearden, 1970; Trautwein, 1974; Garbett et al., 1974). The present study compares the electronic environment of the two iron atoms in two components of oxyhemerythrin isolated from *P. lurco* as well as the deoxy and azido derivatives of the major component.

Experimental Procedures

Preparation of Hemerythrin. Oxyhemerythrin was isolated from the coelomic cells of approximately 200 specimens of *P. lurco* collected less than 24 h previously from the mangrove swamps of the Brisbane River, Queensland, Australia. The

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Table I: Mössbauer Parameters

sample	temp (K)	isomer shift relative to α-Fe (mm s^{-1}) (±0.02 mm s^{-1})	quadrupole splitting (mm s^{-1}) (±0.02 mm s^{-1})	line widths (FWHM)[a] (mm s^{-1}) (±0.02 mm s^{-1}) negative velocity	positive velocity	fractional area of total spectrum (±0.05)
oxyhemerythrin-II	77	+0.50	2.02	0.26	0.28	0.49
		+0.51	1.01	0.37	0.28	0.51
	4.2	+0.51	1.96	0.30	0.29	0.50
		+0.52	0.95	0.36	0.30	0.50
deoxyhemerythrin-II	77	+1.15	2.70	0.30 =[b]		0.77
		+0.54	1.80	0.33 =		0.23
	4.2	+1.14	2.76	0.29 =		0.79
		+0.50	1.86	0.32 =		0.21
azidohemerythrin-II	77	+0.49	1.96	0.29	0.28	0.50
		+0.50	1.40	0.56	0.45	0.50
	4.2	+0.51	1.95	0.26	0.28	0.45
		+0.51	1.47	0.47	0.41	0.55
oxyhemerythrin-I	4.2	+0.52	1.93	0.26 =		0.47
		+0.51	0.91	0.34 =		0.53

[a] FWHM is full width at half-maximum height. [b] The symbol = indicates that the two lines of the doublet were constrained to be equal in line width and intensity.

isolation procedure was related to that commonly used in studies of hemerythrin (Klotz et al., 1976, and references therein). As the final step in the preparation, oxyhemerythrin was fractionated on a Sephadex G-150 gel column into two components (numbered in the order of their elution from the column), hemerythrin-I and hemerythrin-II, which were used for subsequent Mössbauer studies. The major fraction, corresponding to 75% of the protein eluted, was the lower molecular weight fraction hemerythrin-II, while hemerythrin-I was the minor (25%) fraction. This composition of oxyhemerythrin was observed in several different preparations.

Deoxyhemerythrin was prepared by the addition of an aliquot of sodium dithionite, and the resultant yellow solution was transferred under nitrogen to the Mössbauer sample holder (a low-iron aluminum cylinder, of 0.25-mL capacity, with Mylar ends). Conversion of oxyhemerythrin to metazidohemerythrin was achieved by adding sodium azide solution in a 2:1 stoichiometric excess (N_3^-/Fe). Samples were rapidly frozen in liquid nitrogen and maintained at or below 77 K throughout the experiment.

Mössbauer Spectroscopy. Mössbauer spectra were obtained by using a 25 mCi ^{57}Co in Rh Mössbauer source mounted on a constant acceleration spectrometer. The velocity scale was calibrated by using a foil of natural α-Fe at room temperature, and all isomer shift measurements are referred to the center of the α-Fe spectrum.

Spectra were measured with both the Mössbauer source and the absorber sample at either 77 K or 4.2 K. A superconducting solenoid was used at 4.2 K to apply an external magnetic field of 2.5 T parallel to the direction of γ-ray propagation. Compensation coils on the solenoid were used to minimize the fringing magnetic field experienced by the Mössbauer source.

Experimental spectra were fitted to a number of Lorentzian lines by using a least-squares computer program. In the case of oxyhemerythrin-II and azidohemerythrin-II, the spectra were fitted with four lines which were allowed to vary independently. The lines were then matched into doublets to obtain values for the isomer shift and quadrupole splitting of each doublet. For the deoxyhemerythrin-II in which two of the lines were unresolved and the oxyhemerythrin-I which has poorer statistics, doublets were assigned, and the two lines of each doublet were constrained by the program to be equal in line width and in intensity. The fractional contribution of each doublet to the final spectrum was determined from the total area of both lines in the doublet. The resolution afforded by the applied field of 2.5 T used was insufficient to allow the sign of the electric field gradient to be unambiguously determined.

Results and Discussion

Chemical studies of hemerythrin from the nominated species of sipunculid *P. lurco* have been reported previously (Addison & Bruce, 1977; Manwell, 1977). The study by Addison & Bruce (1977) was apparently based on a misidentification of the organism used, which has now been identified as *Siphonosoma sp.* [A. W. Addison, quoted in Klippenstein (1980)]. Manwell (1977), using specimens from the source used in the present study, has reported that on electrophoresis *P. lurco* hemerythrin consists of four components. Most of the protein is accounted for by two components of M_r 28 000 and 60 700, with two trace components at M_r 29 000 and 65 800. Using a calibrated Sephadex column, Appleby [quoted by Manwell (1977)] has determined the molecular weight of one component to be 56 000.

Our preparations behave on gel filtration in a manner consistent with these data. In our nomenclature, hemerythrin-I is the component of molecular weight of about 56 000, and hemerythrin-II is the second component at about half this molecular weight, i.e., 28 000. Appleby suggests further that the M_r 56 000 hemerythrin (our hemerythrin-I) is tetrameric, not trimeric as reported for hemerythrin from *P. agassizii* (Liberatore et al., 1974) and by Addison & Bruce (1979) for their *Siphonosoma sp.* It is likely then that hemerythrin-II, the component on which most of the following Mössbauer spectra were recorded, is a dimeric protein.

Mössbauer studies have yielded isomer shift, quadrupole splitting, line-width, and fractional area data. These are listed for oxy, deoxy, and metazido derivatives of hemerythrin-II and for oxyhemerythrin-I in Table I. Spectra are shown in Figures 1–4.

The spectrum of oxyhemerythrin-II at 77 and 4.2 K (Figure 1) consists of two quadrupole split pairs of lines with the same isomer shift value but quite different quadrupole splittings (see Table I). The computer-fitted curves are shown as the full lines in Figure 1. The isomer shift and quadrupole splitting for each site show little variation between the two temperatures. Each doublet accounts for about 50% of the total spectrum. The values of the isomer shift and quadrupole splitting are consistent with the formulation of the iron atoms

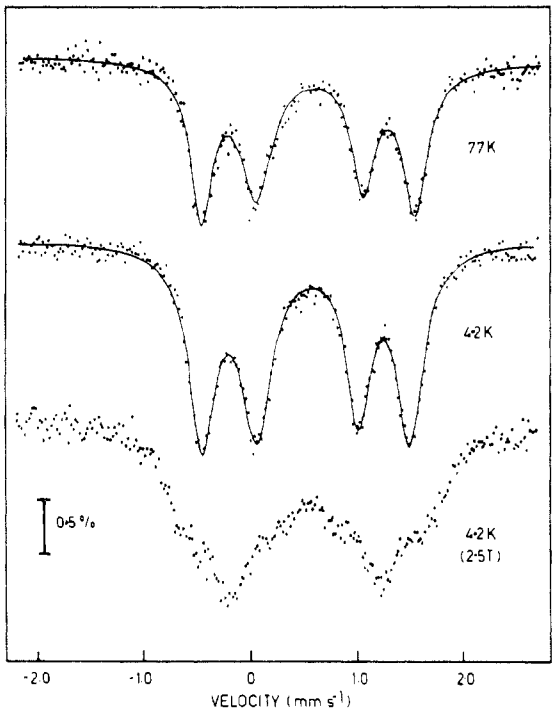

FIGURE 1: Mössbauer spectra of oxyhemerythrin-II at temperatures of 77 and 4.2 K and in an applied magnetic field of 2.5 T at 4.2 K.

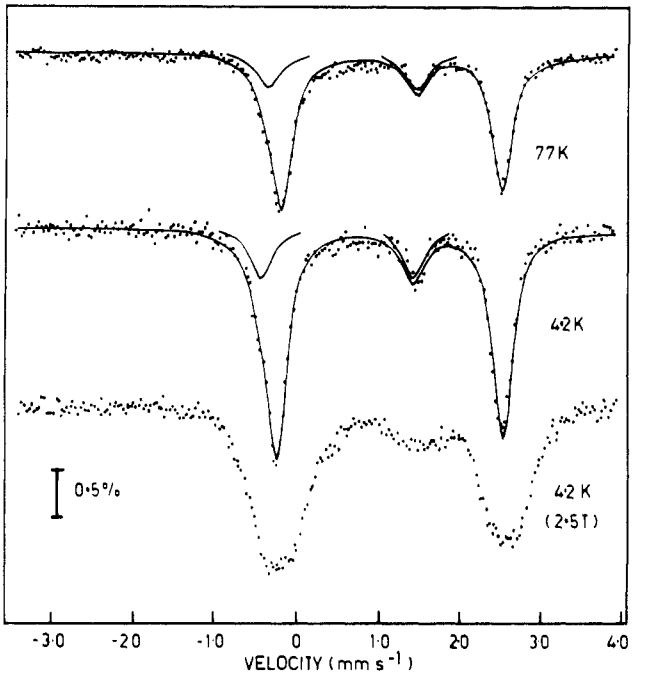

FIGURE 2: Mössbauer spectra of deoxyhemerythrin-II at temperatures of 77 and 4.2 K and in an applied magnetic field of 2.5 T at 4.2 K.

as high-spin iron(III). These results correspond closely with those reported for hemerythrins from other species, viz., an isomer shift of 0.5 mm s^{-1} and quadrupole splittings of 1.01 and 2.02 mm s^{-1} [Loehr & Loehr (1979) and references therein]. The presence of two distinct pairs of quadrupole-split absorption lines indicates that the two high-spin iron(III) sites in oxyhemerythrin differ in their electric field gradient and, to this extent, are not chemically equivalent.

Application of an external field (2.5 T) at 4.2 K (Figure 1) causes no broadening apart from that corresponding to the 2.5-T field itself. At this temperature and external field, any paramagnetic component would give contributions to the Mössbauer spectrum at velocities much higher than any observed here. A spectrum run at higher velocities exhibited no Mössbauer absorption other than that shown in Figure 1. This indicates that at this low temperature the iron atoms occupy a diamagnetic ground state. Antiferromagnetic spin coupling between the iron atoms in the active site would readily generate such a diamagnetic ground state. As with other hemerythrins, these Mössbauer results indicate that in the oxy derivative, the $2Fe{\cdot}O_2$ binding site contains two nonequivalent high-spin iron(III) ions whose electronic configurations are antiferromagnetically coupled sufficiently to render the protein diamagnetic at helium temperature.

Removal of the bound dioxygen by treatment with dithionite results in a spectrum for deoxyhemerythrin-II that differs greatly from that of oxyhemerythrin-II. As shown in Figure 2, the spectrum apparently consists of a simple quadrupole split doublet and a single line. This single line would have an isomer shift uncharacteristic of either Fe(II) or Fe(III), and it is most logical to asume that it is one of the lines of a doublet, the other line lying under the major peak at negative velocity. The fitted curves in Figure 2 were obtained on this basis. The major doublet accounts for about 77% of the spectrum, while the remaining 23% consists of a doublet whose parameters, viz., isomer shift and quadrupole splitting, are similar to those observed for one of the doublets in oxyhemerythrin-II (see Table I). A similar iron(III) component was observed in the spectrum of deoxyhemerythrin from *P. gouldii* and attributed to methemerythrin (Okamura et al., 1969). The relatively large isomer shift (1.15 mm s^{-1}) and quadrupole splitting (2.70 mm s^{-1}) of the major doublet indicate it is due to high-spin iron(II). These values correspond to those previously reported in several studies of other deoxyhemerythrins (Loehr & Loehr, 1979). The presence of only one iron(II) doublet indicates that on losing the bound O_2 the formerly nonequivalent iron(III) sites become indistinguishable on Mössbauer criteria.

When the Mössbauer spectrum is recorded in the presence of a strong external magnetic field of 2.5 T, little spectral broadening is observed. Further, the spectrum contains no components at higher velocities. This absence of any hyperfine interaction has been reported by Garbett et al. (1974) also for deoxyhemerythrin from *P. gouldii*. For this latter protein, magnetic susceptibility data show little, if any, antiferromagnetic coupling between the two iron(II) ions (Okamura et al, 1969; York & Bearden, 1970). It has been suggested (Garbett et al., 1974) that such results are consistent with a large negative field splitting parameter, *D*, resulting in a doubly degenerate ground state. For *P. lurco* deoxyhemerythrin, magnetic susceptibility data are not yet available. However, the probable structure of the active site is one which contains two identical iron(II) ions that interact only weakly, if at all.

A comparison of the spectra for the oxy derivatives of both components of hemerythrin from *P. lurco*, viz., hemerythrin-I and hemerythrin-II, is shown in Figure 3. These spectra indicate that the electronic environments of the iron atoms in oxyhemerythrin-I and oxyhemerythrin-II are essentially the same. Spectral parameters are closely comparable (see Table I). The lower intensity of the oxyhemerythrin-I spectrum resulted from using a sample of lower concentration than that used for oxyhemerythrin-II. Thus, although the two hemerythrins are of different molecular weight and subunit complexity, they employ the same dioxygen binding site.

For the azido derivative of hemerythrin-II, the Mössbauer spectrum (Figure 4) consists of two doublets, with isomer shifts similar to those observed in oxyhemerythrin-II (see Table I), but with more closely comparable quadrupole splittings. The iron ions are thus identified as high-spin iron(III). The inner doublet, which appears as distinct shoulders on the peaks of

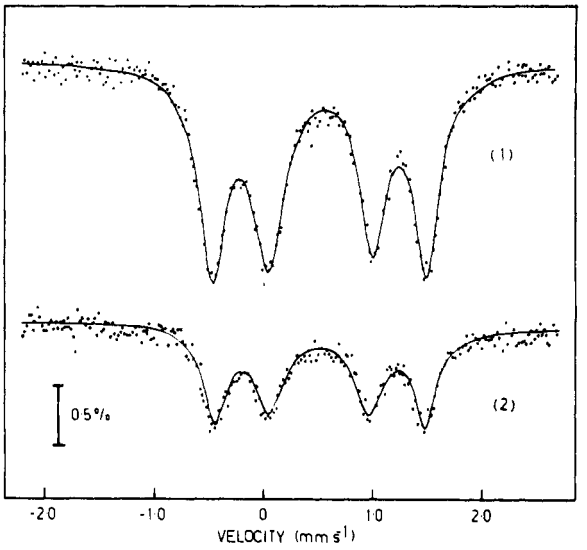

FIGURE 3: Mössbauer spectra at 4.2 K of (1) oxyhemerythrin-II and (2) oxyhemerythrin-I.

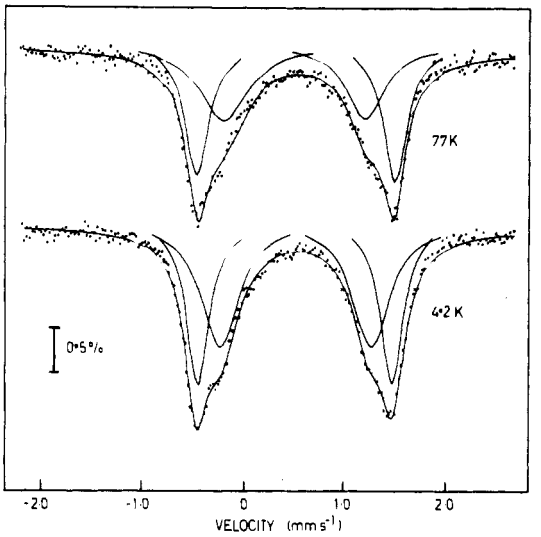

FIGURE 4: Mössbauer spectra of azidohemerythrin-II at temperatures of 77 and 4.2 K.

the outer doublet, nevertheless accounts for 50% of the total spectrum. The presence of a diamagnetic ground state in this derivative also was indicated by the very limited spectral broadening induced by an external magnetic field (not shown in Figure 4). The isomer shift value of 0.5 mm s^{-1} reported in other studies (Okamura et al, 1969; York & Bearden,1970) is similar to that observed in the present work. However, these other metazidohemerythrins show only one quadrupole-split doublet. The inequivalence of the two iron(III) ions in azidohemerythrin-II is quite marked, as shown by their quadrupole splittings of 1.96 and 1.40 mm s^{-1}. This difference is, however, less than that observed in oxyhemerythrin-II of 2.02 and 1.01 mm s^{-1}. Thus in this met derivative, the two iron(III) ions are antiferromagnetically coupled, as in oxyhemerythrin-II, and are distinguishable on the basis of their electric field gradient. Further, one of the iron(III) ions appears to be identical with one of the iron(III) ions in the oxy form, but the other has a quadrupole splitting that is appreciably different from those observed in oxyhemerythrin-II (see Table I). The line widths associated with this second quadrupole doublet are appreciably greater than any others measured in this study, suggesting that the local environment of this ion may not be well-defined.

In summary, the data reported in this paper indicate that the two iron atoms in the oxy and azido derivatives are nonidentical iron(III) ions that are proximate enough for antiferromagnetic coupling to occur, leading to a diamagnetic ground state. Unlike other hemerythrins, e.g., that from *P. gouldii* (Kurtz et al., 1976), dioxygen and azide derivatives of hemerythrin from *P. lurco* do not give identical Mössbauer spectra, suggesting some nonequivalence in the iron environments in the two derivatives. This comparison of the derivatives (Loehr & Loehr, 1979; Dunn et al., 1977) is crucial to X-ray crystallographic studies of the dioxygen binding site, for all such studies use methemerythrin as the stable crystal form (Stenkamp & Jensen, 1979). Differences between the two iron atoms in the O_2 binding site are now accessible by using the semi-met derivatives, i.e., containing [Fe(II·Fe(III)] centers (Babcock et al., 1980).

On deoxygenation, the electronic environment of both iron atoms changes to give iron(II) ions that are indistinguishable by Mössbauer spectroscopy. Further, the Mössbauer data on the two components of hemerythrin isolated from *P. lurco* indicate that the electronic environments of the two-iron site in both oxy derivatives are essentially identical.

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Unusual Low-Frequency Resonance Raman Spectra of Heme Observed for Hog Intestinal Peroxidase and Its Derivatives†

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ABSTRACT: The resonance Raman spectra of hog intestinal peroxidase (IPO) and its cyanide and fluoride complexes were observed for the first time. The Raman spectra in the 100-500-cm^{-1} region were quite dissimilar to those of the A-, B-, and C-type hemes so far observed, although the spectra in the 1200-1700-cm^{-1} region were similar to those of the B-type heme. The Raman spectral characteristics, including the presence of two anomalously polarized lines at 1344 and 1306 cm^{-1} and the absence of any Raman lines between 1650 and 1700 cm^{-1}, indicated the presence of a vinyl group at position 2 or 4, at least, and the absence of a C_β—C(=O)—R (R = H, alkyl, or *O*-alkyl) linkage in the heme periphery. The ν_4 line (oxidation-state marker) was observed at normal frequencies for both the ferrous and ferric states, indicating that the axial ligand is not of an unusual type. The ν_{10} frequency of ferri-IPO (1622 cm^{-1}) suggested coordination of a water molecule to the sixth coordination position at the heme iron of native ferri-IPO, in contrast with ferri-horseradish peroxidase. The Raman spectral characteristics of native ferri-IPO remained unaltered between pH 5.6 and 9.3. The cyanide complex of ferri-IPO gave rise to ν_{10} at 1638 cm^{-1}, implying the coordination of cyanide anion to the axial position of the heme iron. The cyanide and the fluoride complexes of ferri-IPO retained the unusual feature of the low-frequency Raman spectrum. On the other hand, for ferro-IPO, two kinds of Raman spectra were observed by varying the pH. One ($4.5 < pH < 6.5$) was of the low-spin type and the other ($7 < pH < 10$) of the high-spin type. The spin change from high to low spin was irreversible, and its rate was very slow between pH 6 and 7. The Raman spectra of the cyanide complex of ferro-IPO displayed a distinctly different feature from those of the cyanide complex of ferro-horseradish peroxidase. Both the Raman and absorption spectra of the cyanide complex of ferro-IPO exhibited a drastic change on further addition of a large amount of KCN.

The presence of a heme-containing peroxidase is a likely origin of the high peroxidase activity in animal tissues (Neufeld et al., 1958). So far, hog mucosa intestinal peroxidase has been purified, and its visible spectral characteristics were shown to have a close resemblance to those of cow lactoperoxidase among animal peroxidases (Stelmaszyńska & Zgliczyński, 1971). The intestinal peroxidase (IPO)[1] is a true peroxidase which reacts with H_2O_2 as a substrate similarly to horseradish peroxidase (HRP), but its spectral as well as biochemical properties have somewhat different aspects from those of HRP (Kimura & Yamazaki, 1978, 1979). Such properties substantially depend upon the nature of the heme itself and the heme environment. Although much information has been accumulated about HRP [Yamazaki et al. (1978) and references cited therein], little is known about IPO.

Resonance Raman scattering from hemoproteins has provided detailed structural information on the heme proximity through selective observation of the molecular vibrations of the heme group (Spiro, 1975; Felton & Yu, 1978; Kitagawa et al., 1978). The structural implication of the heme-linked ionization of HRP was analyzed recently by resonance Raman spectroscopy, and the importance of the hydrogen bonding of proximal histidine to protein was pointed out as the difference in the heme proximity between HRP and myoglobin (Mb) (Teraoka & Kitagawa, 1981). Application of this technique to IPO may bring about crucial information on the structure-function relationship of the peroxidase and may distinguish the specific property of IPO from common properties of hemoproteins. Accordingly, we have investigated the resonance Raman spectra of hog mucosa intestinal peroxidase.

Materials and Methods

The intestinal peroxidase was isolated from the mucosa membrane of hog intestine tissue by the method of Stelmaszyńska & Zgliczyński (1971) with a slight modification. As a molar absorbance of IPO is not known, an approximate concentration was inferred from the comparative experiments on lactoperoxidase for which the concentration could be determined spectrophotometrically on the basis of $\epsilon_{mM} = 114$ at 412 nm (Morrison et al., 1957). The buffer system used for the Raman experiments was 0.1 M sodium

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[1] Abbreviations used: IPO, intestinal peroxidase; HRP, horseradish peroxidase; IPO-CN, cyanide complex of intestinal peroxidase; HRP-CN, cyanide complex of horseradish peroxidase.

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