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Articles

Identification and Properties of an Oxoferryl Structure in Myeloperoxidase Compound II†

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Received February 9, 1988

ABSTRACT: Myeloperoxidase compound II has been characterized by using optical absorption and resonance Raman spectroscopies. Compared to compounds II in other peroxidases, the electronic and vibrational properties of this intermediate are strongly perturbed due to the unusual active-site iron chromophore that occurs in myeloperoxidase. Despite this difference in prosthetic group, however, other properties of myeloperoxidase compound II are similar to those observed for this intermediate in the more common peroxidases (horseradish peroxidase in particular). Two forms of the myeloperoxidase intermediate species, each with distinct absorption spectra, are recognized as a function of pH. We present evidence consistent with interconversion of these two forms via a heme-linked ionization of a distal amino acid residue with a $pK_a \simeq 9$. From resonance Raman studies of isotopically labeled species at pH 10.7, we identify an iron–oxygen stretching frequency at 782 cm^{-1}, indicating the presence of an oxoferryl ($O{=}Fe^{IV}$) group in myeloperoxidase compound II. We further conclude that the oxo ligand is not hydrogen bonded above the pK_a but possibly exhibits oxygen exchange with the medium at pH values below the pK_a due to hydrogen bonding of the oxo ligand to the distal protein group.

Peroxidases are utilized by the immune response systems of mammals (Klebanoff & Clark, 1978). Most peroxidase enzymes contain protoheme IX; however, the prosthetic group of myeloperoxidase (from granulocytes) is uncertain. Optical spectra of the pyridine hemochrome of myeloperoxidase suggest a heme *a* structure (Schultz & Shmukler, 1964; Newton et al., 1965), but resonance Raman measurements do not detect the expected formyl substituent (Babcock et al., 1985). Both Raman and magnetic circular dichroism studies were taken to suggest the presence of a saturated pyrrole ring, thus indicating an iron chlorin chromophore (Eglington et al., 1982; Sibbett & Hurst, 1984; Babcock et al., 1985; Ikeda-Saito et al., 1985; Stump et al., 1987). Still neither of these suggestions is sufficient to explain the distinctly red-shifted absorption spectra of several forms of the enzyme (Wever & Plat, 1981).

During turnover, myeloperoxidase (MPO) reacts with H_2O_2 to form a compound I intermediate which is capable of cat-

† This study was supported by grants from the Netherlands Organization for the Advancement of Pure Research (ZWO) under the auspices of the Netherlands Foundation for Chemical Research (SON) to R.W. and from the National Institutes of Health (GM 25480) to G.T.B. R.W. acknowledges receipt of a travel grant from the Netherlands Organization for the Advancement of Pure Research. This collaboration was made possible by Nato Research Grant 86/734.
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0006-2960/88/0427-5395$01.50/0 © 1988 American Chemical Society

alyzing the two-electron oxidation of Cl^- to HOCl, with concomitant re-formation of the native enzyme (Agner, 1970; Stelmaszyńska & Zgliczyński, 1974; Harrison & Schultz, 1976). In the presence of excess H_2O_2 and particularly at high pH, MPO compound I may be reduced by one electron to yield compound II (Harrison, 1976). Compound II is inactive in the formation of HOCl and slowly decays to the native state; thus, accumulation of this species leads to progressive inhibition of MPO catalysis (Bakkenist et al., 1978).

Like those of various ferrous and ferric forms of the enzyme, the optical absorption spectra of the MPO intermediates, compounds I and II, are distinctly red-shifted compared to those of other peroxidases (Manthey & Hager, 1985; Renganathon & Gold, 1986). Although the absorption spectra of MPO catalytic transients were reported earlier [see Odajima and Yamazaki (1970) and Harrison et al. (1980) and references cited therein], only recently was the MPO compound II spectrum found to be dependent on pH (Hoogland et al., 1987). From this work, two forms of this intermediate species, each with distinct absorption spectra, are recognized. The spectrum at pH 7 is characteristic of one form, while the spectrum obtained at pH 11 characterizes the other.

In the present work, we present resonance Raman (RR) spectra which establish the occurrence of an oxoferryl ($O{=}Fe^{IV}$) group in MPO compound II. We find the iron–oxygen stretching frequency of this species at high pH to be similar to that of other oxoferryl peroxidase intermediates (Terner et al., 1985; Hashimoto et al., 1984, 1986b; Oertling & Babcock, 1988). Using both optical absorption and RR measurements, we report a heme-linked ionization in MPO compound II which suggests the presence of a distal amino acid group capable of hydrogen bonding to the oxo ligand.

Materials and Methods

Myeloperoxidase was purified from human leukocytes as described by Bakkenist et al. (1978). Its concentration was determined by using an absorbance coefficient of 89 mM^{-1} cm^{-1} per heme at 428 nm (Agner, 1958). The ratio A_{428nm}/A_{280nm} for the preparation was 0.8 or higher. H_2O_2 solutions were prepared by dilution of 30% stock (Mallinckrodt) and quantified by using an absorption coefficient at 240 nm of 43.6 M^{-1} cm^{-1} (Beers & Sizer, 1952). $H_2{}^{17}O_2$ and $H_2{}^{18}O_2$ were prepared from $^{17}O_2$ (72.1%; MSD Isotopes, Montreal, Canada) and $^{18}O_2$ (98.5%; Amersham International PLC, Amersham, England), respectively, with the glucose/glucose oxidase (Sigma, type V) system (Asada & Badger, 1984). The resulting peroxide solutions (40–50 mM) were assayed by comparison of the rate of chlorination catalyzed by MPO to those obtained in the presence of known H_2O_2 concentrations according to Bakkenist et al. (1980).

Samples for the Raman measurements were prepared in a cylindrical quartz cell maintained at 0–5 °C and spun during laser irradiation. At neutral pH, MPO compound II (30 μM) was generated by two to five repetitive additions of 0.33 mM H_2O_2 to a solution of MPO in sodium phosphate buffer. Compound II (30 μM) at high pH was prepared by addition of 0.60 mM H_2O_2 to the native enzyme in sodium carbonate buffer at pH 10.7. The pD of solutions prepared in D_2O buffer was measured by using a pH meter and assuming pD = pH-(observed) + 0.4.

Using optical absorption measurements (Perkin-Elmer Lambda 5 or Perkin-Elmer Lambda Array), we estimated the $t_{1/2}$ for the decay of the compound II samples. Subsequently, Raman experiments were performed on a time scale that ensured that compound II was present in excess of 80–90%. This was accomplished by using a diode array based detection

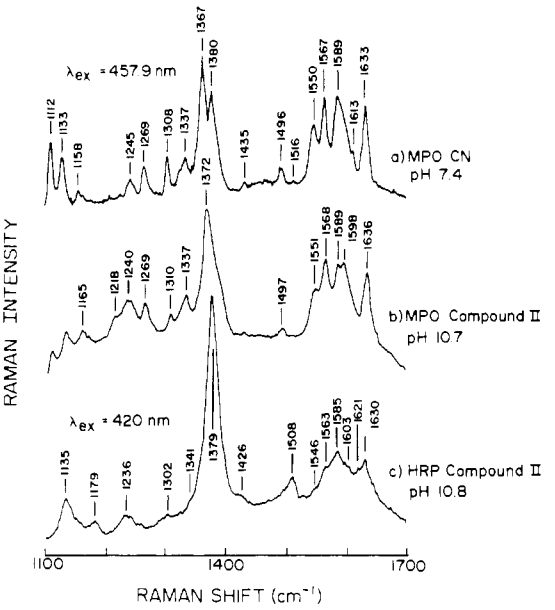

FIGURE 1: High-frequency RR spectra of MPO and HRP species (30 μM). Laser power of (a) 45 mW, (b) 30 mW, and (c) 20 mW.

system (OMA-II, PAR EG&G) with a triple monochromator (Spex 1877). In all cases, the Raman spectra presented represent the accumulated results from multiple samples, each freshly prepared. Absorption spectra obtained following the Raman measurement confirmed sample integrity. Raman scattering from compound II was selectively enhanced by using laser irradiation at 457.9 nm (Coherent Innova 100 argon ion), in resonance with the Soret absorption of the intermediate. In this way, spectral contributions from the native enzyme were minimized. The resonance Raman spectrum of cyanoferric MPO was obtained with a Spex 1401 scanning monochromator and PMT detection. RR spectra of horseradish peroxidase compound II were obtained as described elsewhere (Oertling & Babcock, 1988).

Results

Figure 1a,b shows high-frequency (1200–1700 cm^{-1}) resonance Raman scattering from cyanoferric MPO at pH 7.5 and MPO compound II at pH 10.7. The analogous spectrum of compound II at neutral pH (not shown) is similar to Figure 1b. We discussed the normal mode composition of several vibrations of the cyanoferric derivative earlier (Babcock et al., 1985). In particular, the bands at 1496 and 1633 cm^{-1} in Figure 1a and at 1497 and 1636 cm^{-1} in Figure 1b are thought to arise from C_aC_m stretching vibrations of the macrocycle, $\nu(C_aC_m)$ (Kitagawa et al., 1975; Abe et al., 1978). These frequencies are known to be inverse linear functions of the core size (macrocycle center to pyrrole nitrogen distance) of both iron porphyrin (Spiro et al., 1979) and iron chlorin (Ozaki et al., 1986a) complexes. Ozaki et al. (1986a) have demonstrated that low-spin ferric complexes of octaethylporphyrin and octaethylchlorin display similar $\nu(C_aC_m)$ frequencies and that the core size dependencies of ν_3 and ν_{10} displayed by iron octaethylporphyrin derivatives are duplicated by the analogous vibrations in iron octaethylchlorin compounds. Although the wavenumber values for these $\nu(C_aC_m)$ vibrations of the MPO species are indicative of low-spin iron complexes, they are significantly smaller than those of the analogous species of both horseradish peroxidase (Rakhit & Spiro, 1976) and lactoperoxidase (Manthey et al., 1986). This suggests that the macrocycle core of these MPO species is larger by ~0.01 Å than those of the other peroxidase derivatives mentioned. Table I collects the relevant vibrational frequencies and core

Table I: Vibrational Frequencies and Core Size Estimates for Various Low-Spin Ferric and Ferryl Peroxidase Species

	$\nu_3(C_aC_m)$ (cm^{-1})	$\nu_{10}(C_aC_m)$ (cm^{-1})	core size, d^a (Å)
HRP·CN	1507	1642[b]	1.99
HRP–II	1508	1641[b]	1.99
LPO·CN	1503[c]	1638[c]	1.99
LPO–II	1507[c]	1640[c]	1.99
MPO·CN	1496	1633	2.00
MPO–II	1497	1636	2.00

[a] Core size, d, was estimated by using the relationship $\nu = K(A - d)$; e.g., see Spiro et al. (1979). [b] Rakhit & Spiro (1976). [c] Manthey et al. (1986).

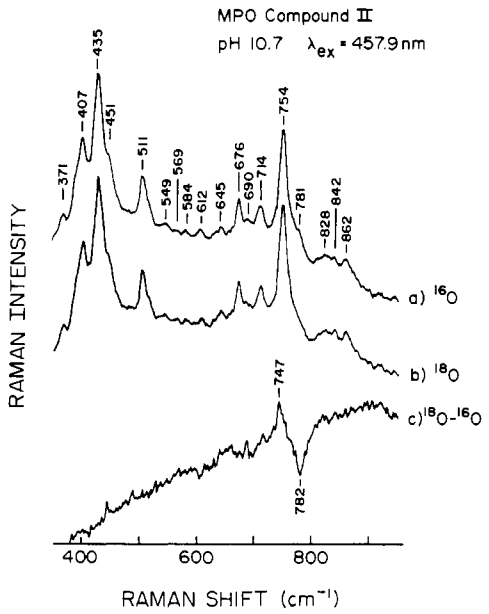

FIGURE 2: Low-frequency RR spectra of MPO compound II (30 μM) at pH 10.7. Laser power 30 mW.

size estimates of these low-spin ferric and ferryl enzyme species.

In Figure 1, the most intense feature, occurring at 1367 and 1372 cm^{-1} in the MPO cyanoferric and compound II spectra, respectively, derives from a predominantly C_aN stretching vibration of the macrocycle (Abe et al., 1978; Babcock et al., 1985) and is sensitive to the iron oxidation state. The 5 cm^{-1} increase in frequency of this feature in the compound II spectrum relative to that of the cyanoferric species is considered consistent with an Fe^{IV} assignment of the former compound (Rakhit & Spiro, 1976).

Comparison of Soret excited RR spectra of myeloperoxidase compound II presented here with those of horseradish peroxidase compound II [cf. Figure 1c; also see Hashimoto et al. (1984, 1986a–c), Terner et al. (1985), and Sitter et al. (1985)] illustrates the significantly different vibrational properties of the two chromophores. As these vibrations arise primarily from the macrocycle, these differences indicate contrasting structures for both the core and peripheral substituents of the heme ring systems of the MPO and HRP active sites. Thus, the strong similarity of the iron–axial ligand vibrations of the two compound II species, which we describe below, is noteworthy.

Indisputable characterization of the oxoferryl group by RR spectroscopy can be made only by the identification of an iron–oxygen stretching frequency in the ~750–900 cm^{-1} region (Adams, 1968; Bajdor & Nakamoto, 1984). Figure 2 shows RR spectra of MPO compound II at pH 10.7 prepared with both $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$. The shoulder located at ~781 cm^{-1}

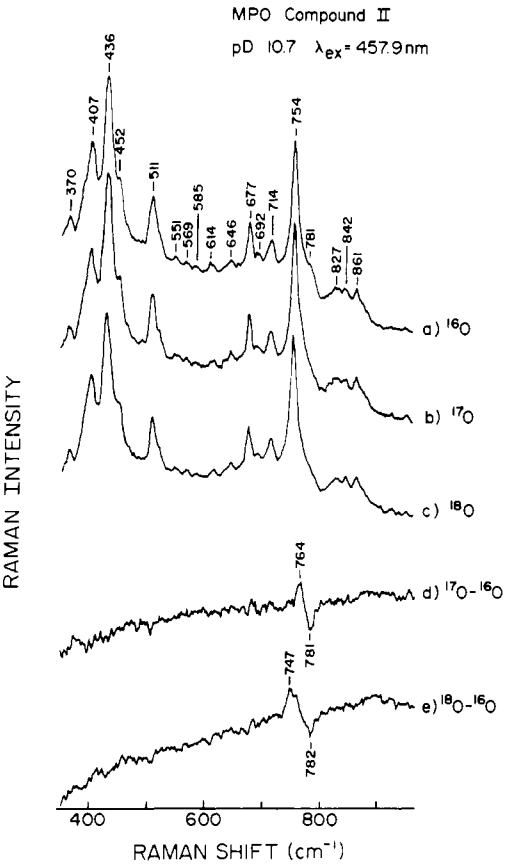

FIGURE 3: Low-frequency RR spectra of MPO compound II (30 μM) in D_2O buffer, pD 10.7. Laser power 30 mW.

in Figure 2a is assigned to the iron–oxygen stretching frequency, ν(Fe=O), of MPO compound II. This weak feature of the RR spectrum in Figure 2a disappears in Figure 2b and is replaced by a shoulder on the high-energy side of the intense macrocycle vibration at 754 cm^{-1}. Figure 2c represents the difference of the spectra in Figure 1b and Figure 1a. Thus, the maximum at 747 cm^{-1} derives from the iron–oxygen stretch of the sample prepared with $H_2{}^{18}O_2$, and the minimum at 782 cm^{-1} corresponds to the analogous vibration of the ^{16}O species. The 35 cm^{-1} shift between these frequencies is predicted by a two-body harmonic oscillator approximation. Coupling of this vibration to other vibrational modes of the iron–macrocycle complex would lessen the observed isotope shift. This result is consistent with incorporation of a single oxygen atom from the peroxide which becomes bonded to the heme iron. The value of ν(Fe=O) of 782 cm^{-1} is within the region expected for an O=Fe^{IV} group and can be used to predict an Fe–O bond length of ~1.6 Å (Proniewicz et al., 1986).

Figure 3 shows results from analogous samples prepared in D_2O. Spectra from MPO compound II prepared with $H_2{}^{16}O_2$, $H_2{}^{17}O_2$, and $H_2{}^{18}O_2$ appear in parts a, b, and c, respectively, of Figure 3. Figure 3d shows the difference between the spectra of the ^{17}O vs ^{16}O sample. This identifies a band at 781 cm^{-1} shifting to 764 cm^{-1} upon substitution of ^{17}O for ^{16}O. Again, the 17 cm^{-1} shift is in accord with a two-body harmonic oscillator and provides additional confirmation of an O=Fe^{IV} group. Comparison of Figure 3a,c,e with Figure 2 shows that identical results are obtained in H_2O and D_2O buffers for ν(Fe=^{16}O) and ν(Fe=^{18}O). This implies that there is no hydrogen bonding of the oxo ligand at pH 10.7.

Resonance Raman spectra of MPO compound II at neutral pH appear in Figure 4. In the first approximation, these spectra are very similar to those obtained at pH 10.7 (Figure 2). However, at pH 7, no difference could be detected in

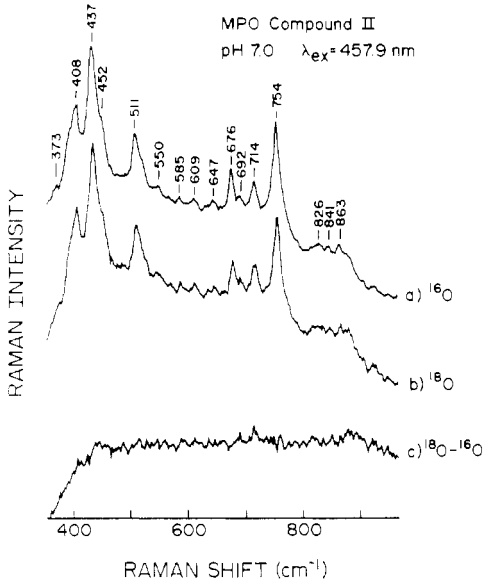

FIGURE 4: Low-frequency RR spectra of MPO compound II (30 μM) at neutral pH.

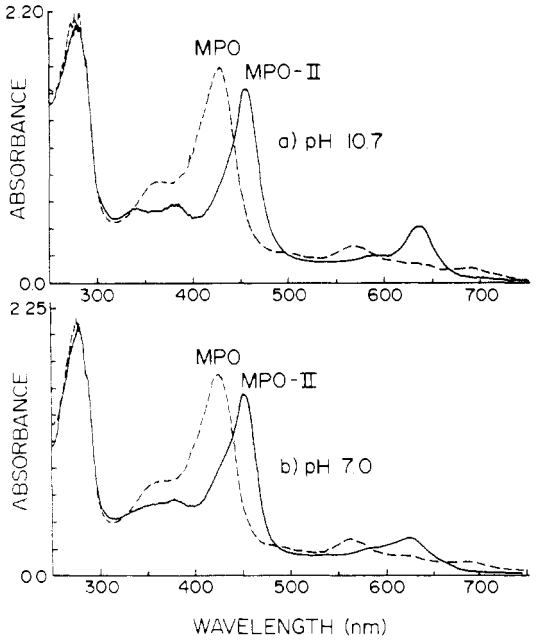

FIGURE 5: Optical absorption spectra of (a) native MPO and compound II at pH 10.7 and (b) native MPO and compound II at pH 7.0.

samples prepared with $H_2{}^{16}O_2$ vs $H_2{}^{18}O_2$.

Optical absorption spectra of native MPO and compound II at pH 10.7 and pH 7.0 appear in Figure 5. Hoogland et al. (1987) have established that the most apparent spectral change upon an increase in the pH of compound II samples from 7 to 11 is manifest by the red-shift and increase in absorption coefficient of the visible absorption band from 628 nm (18 mM^{-1} cm^{-1}) to 635 nm (25 mM^{-1} cm^{-1}). The transition from the low-pH to high-pH form of MPO compound II exhibits an isosbestic point at 617 nm. As is the case with other peroxidases, in the presence of high concentrations of H_2O_2, MPO may form a ferrous oxy species called compound III. Preparations of compound II may be contaminated with compound III, and the ratio A_{625nm}/A_{456nm} can be used to quantify the relative amounts of the low-pH form of compound II and compound III in mixtures of the two species (Hoogland et al., 1987); similarly, under conditions that assure that no compound III is present, the ratio A_{635nm}/A_{456nm} can be used

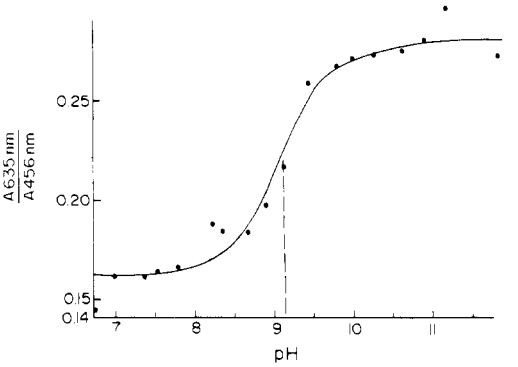

FIGURE 6: Absorbance ratio A_{635nm}/A_{456nm} vs pH illustrating the heme-linked ionization of MPO compound II.

to monitor the relative amounts of the low- and high-pH forms of compound II as a function of pH. Figure 6 plots this dependency and can be used to estimate a p$K_a \cong 9$ for the transition of the low- to high-pH form of MPO compound II.

Discussion

Of all peroxidase enzymes, horseradish peroxidase (HRP) is the most extensively studied. In particular, the compound II catalytic intermediate has been well characterized. It is of interest to discuss vibrational characteristics of the oxoferryl group of HRP compound II in order to compare them to those we find for the analogous MPO species.

Recently, independent RR studies by Terner and co-workers (Sitter et al., 1985) as well as by Kitagawa and co-workers (Hashimoto et al., 1986a,c) have identified two forms of HRP compound II that are interconverted via a heme-linked ionization of a distal protein residue with a pK_a of ~8.8. At pH 11, HRP compound II exhibits a ν(Fe=O) at 787 cm^{-1} shifting to 753 cm^{-1} upon substitution of ^{18}O for ^{16}O (Hashimoto et al., 1984; Sitter et al., 1985; Oertling & Babcock, 1988). No change in this vibration occurs if D_2O rather than H_2O buffer is used. This establishes the existence of an O=Fe^{IV} group that does not exhibit hydrogen bonding at pH 11.

In samples at neutral pH, both Terner et al. (1985) and Hashimoto et al. (1986a) report a ν(Fe=O) at 775 cm^{-1}, albeit with greatly reduced intensity compared to the high-pH result. This feature exhibits small but distinct increases in both wavenumber (+3 cm^{-1}) and intensity in samples prepared in D_2O. Although different explanations have been proposed for these spectral changes, both Sitter et al. (1985) and Hashimoto et al. (1986a) agree that these changes suggest hydrogen bonding of the oxo group at pH 7. Furthermore, Hashimoto et al. (1986a,c) have clearly demonstrated that the oxo ligand of HRP compound II will exchange with bulk water at pH 7. Thus, these studies indicate that the physiologically active form of HRP compound II [i.e., at pH < pK_a; see Dunford (1982)] most likely depends on the hydrogen bond to the oxo ligand for activity.

Our RR work presented here clearly indicates that at high pH, MPO compound II is similar to HRP compound II in terms of the O=Fe^{IV} center. The ν(Fe=O) we measure for the MPO intermediate is notably similar to that measured for HRP compound II (see Table II), and samples in D_2O indicate no hydrogen bonding of the oxo ligand at pH 10.7 (Figure 3). The optical absorption data we present for the pH titration of MPO compound II (Figure 6) indicate two forms of this species interconverting at ~pH 9. This is much like the heme-linked ionization in HRP compound II, which occurs at ~pH 8.8 (Dunford, 1982) and interconverts the hydrogen-bonded oxoferryl structure with the non-hydrogen-bonded one (Sitter et al., 1985; Hashimoto et al., 1986a). Thus, by

Table II: Compound II Oxoferryl Stretching Frequencies of HRP and MPO

	ν(Fe=O) (cm^{-1})			
	HRP		MPO	
	obsd	predicted[a]	obsd	predicted[a]
^{16}O	787		782	
^{17}O	766	769	764	764
^{18}O	753	752	747	747

[a] Based on the observed value for the ^{16}O derivative and the harmonic oscillator approximation.

analogy to the HRP compound II system, we may speculate concerning the significance of our MPO compound II result at pH 7 (Figure 4). The failure to detect a spectral change in $^{16}O{=}Fe^{IV}$ vs $^{18}O{=}Fe^{IV}$ samples may indicate hydrogen bonding of the oxoferryl and, in the latter case, exchange of ^{18}O for ^{16}O from the medium, as was shown for HRP compound II (Hashimoto et al., 1986a,c).

As discussed above, the stability of HRP compound II at high pH owes to the lack of reactivity of the oxoferryl structure in the absence of hydrogen bonding by the distal protein residue. Similarly, at high pH, MPO compound II is considerably more stable than at neutral pH. Although the chlorination reaction catalyzed by MPO proceeds via the compound I intermediate, the increased stability of MPO compound II at high pH implies that the heme-linked ionization reported here may have the same consequence for the MPO compound II activity involving organic electron donors as it does for HRP compound II.

The ν(Fe=O) values measured at pH 11 for HRP and MPO compound II are separated by only 5 cm^{-1} (Table I). This suggests that despite the significant optical and RR spectral differences between the two, the $O{=}Fe^{IV}$ moiety and its interaction with the protein are very similar. This is noteworthy in light of the large variability of ν(Fe=O) in both models and heme proteins [i.e., ~750–850 cm^{-1}; see Kean et al. (1987, 1988) for a discussion]. On the other hand, the large differences both in the remainder of the RR spectrum (Figure 1) and in the optical absorption spectrum obviously indicate large differences in the macrocycles of the two species. These macrocyclic differences do not seem to be translated to the iron–axial ligand system. This is in accord with comparative vibrational studies of iron porphyrins and iron chlorins (Ozaki et al., 1986b; Kean et al., 1988; Lopez-Garriga et al., 1988).

Compared to that of MPO compound II, the heme-linked ionization in HRP compound II affects the absorption spectrum little [an ~1-nm red-shift occurs in the Soret absorption when raising the pH from 7 to 11; cf. Critchlow and Dunford (1972)]. This implies that not only does the macrocycle contain different peripheral substituents but also the interaction of these substituents with the protein is likely to be more pronounced in MPO compound II than in HRP compound II and, hence, manifest in a greater spectral change upon interconversion at the pK_a. Thus, comparison of both the RR and optical absorption spectral properties of HRP and MPO compounds II suggests that the oxoferryl and distal protein residues in contact with the heme ligand are probably similar in both HRP and MPO compound II species. On the other hand, the macrocycle structure and the protein interactions with it may be very different in the two species. This suggests that catalytic reactions of compound II (which clearly involve the oxoferryl structure) may occur similarly in the two enzymes while reactions of compound I [which may involve the macrocycle edge; see Ator and Ortiz de Montelliano (1987)] may occur differently.

ACKNOWLEDGMENTS

W.A.O. thanks Jeff Carlo (Spex Industries) for technical assistance.

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A Nuclear Overhauser Effect Study of the Heme Crevice in the Resting State and Compound I of Horseradish Peroxidase: Evidence for Cation Radical Delocalization to the Proximal Histidine†

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ABSTRACT: The assignment of resolved hyperfine-shifted resonances in high-spin resting state horseradish peroxidase (HRP) and its double-oxidized reactive form, compound I (HRP-I), has been carried out by using the nuclear Overhauser effect (NOE) starting with the known heme methyl assignments in each species. In spite of the efficient spin–lattice relaxation and very broad resonances, significant NOEs were observed for all neighboring pyrrole substituents, which allowed the assignment of the elusive propionate α-methylene protons. In the resting state HRP, this leads directly to the identity of the proximal His-170 H_β peaks. The determination that one of the most strongly contact-shifted single proton resonances in HRP-I *does not* arise from the porphyrin dictates that the cation radical must be delocalized to some amino acid residue. The relaxation properties of the non-heme contact-shifted signal in HRP-I support the identity of this contributing residue as the proximal His-170. Detailed analysis of changes in both contact shift pattern and NOEs indicates that compound I formation is accompanied by a $\sim 5°$ rotation of the 6-propionate group. The implication of a porphyrin cation radical delocalized over the proximal histidine for the proposed location of the solely amino acid centered radical in compound I of related cytochrome *c* peroxidase is discussed.

Horseradish peroxidase, HRP,[1] is one of a number of functionally related heme enzymes for which the high-spin ferric resting state reacts with hydrogen peroxide to yield an initially reactive species two oxidizing equivalents above the resting state (Dunford, 1982; Dunford & Stillman, 1976; Morrison & Schonbaum, 1976). One of these oxidizing equivalents is invariably associated with oxidation of the iron center to Fe^{IV} (usually as the ferryl ion, Fe^{IV}=O) that is retained in compound II, with the second one residing on an organic moiety. For HRP compound I, HRP-I, optical spectra have been found characteristic for a porphyrin cation radical (Dolphin et al., 1971), while yeast cytochrome *c* peroxidase compound I, C*c*P-I, has been shown to possess a free radical on an amino acid side chain removed from the iron (Yonetani & Ray, 1965). While there is a paucity of hard structural information on HRP, the yeast cytochrome *c* peroxidase, C*c*P, has yielded to successful X-ray crystallographic structural analysis for both its resting-state C*c*P and compound I, C*c*P-I (Poulos & Kraut, 1980; Finzel et al., 1984; Edwards et al., 1987).

Solution NMR studies have been found particularly useful for delineating a number of structural features of the heme cavity of HRPCN, the cyanide complex of the resting state (Thanabal et al., 1987a,b, 1988), which could be interpreted

† This work was supported by a grant from the National Institutes of Health, GM 26226.
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[1] Abbreviations: HRP, horseradish peroxidase; HRP-I, compound I of horseradish peroxidase; C*c*P, cytochrome *c* peroxidase; C*c*P-I, compound I of cytochrome *c* peroxidase; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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