RESONANCE RAMAN EVIDENCE FOR Fe(IV) IN COMPOUND II OF HORSERADISH PEROXIDASE

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SUMMARY

Resonance Raman spectra have been obtained for Compound II of horseradish peroxidase. Its prophyrin vibrational frequencies are consistent with a planar low-spin heme containing Fe(IV). The oxidation-state marker band is found at the unprecedentedly high value of 1382 cm^{-1}. This band was also observed in solutions of myoglobin and cytochrome c peroxidase to which H_2O_2 had been added. No evidence was found for an actual Fe=O double bond in Compound II.

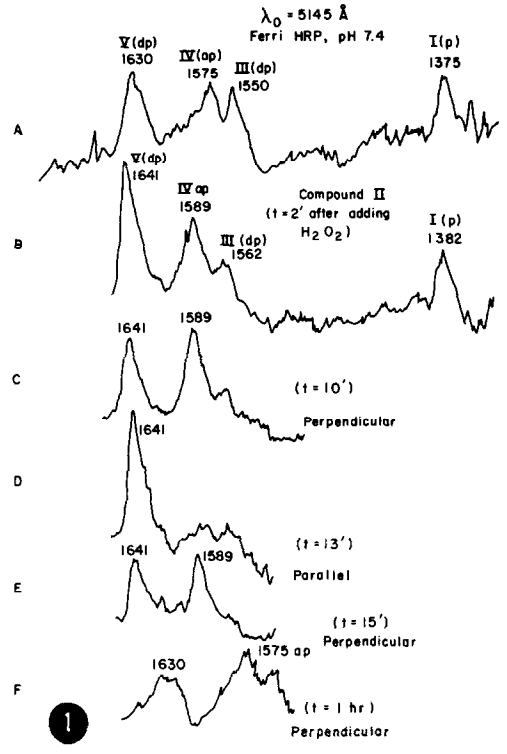
The horseradish peroxidase (HRP) catalyzed oxidation of substrates by hydrogen peroxide is known to proceed via sequential enzyme intermediates, Compound I and Compound II, which are respectively two and one oxidation equivalents above the resting enzyme (1,2). In the absence of substrate, the rate of disappearance of these transient species, following addition of H_2O_2 to HRP, can be slowed sufficiently to allow their characterization by physical techniques, and they have been subjected to intensive study (1,2). Since the resting enzyme is known to contain Fe(III) protoporphyrin, it was early proposed that Compounds I and II contain Fe(V) and Fe(IV) respectively (3,4). Magnetic susceptibility measurement (4) on Compound II showed it to be paramagnetic, with a moment of 3.6 Bohr magneton, the approximate spin-only value for two unpaired spins, consistent with low-spin Fe(IV). Mossbauer measurements gave the same iron isomer shift for Compounds I and II, and the value suggested oxidation state(IV)(5). The absorption spectrum of (red) Compound II is a normal prophyrin spectrum, but that of (green) Compound

I resembles the spectrum of a porphyrin cation radical, leading to the suggestion that Compound I in fact contains an Fe(IV) porphyrin cation radical (6).

Resonance Raman spectra have been obtained for a number of heme proteins (7), including various derivatives of HRP (8). The strongly enhanced porphyrin vibrational bands have been shown (9,10) to be sensitive to the iron oxidation and spin state. It is logical to extend these studies to the HRP intermediates. Unfortunately, Compound I is highly photoactive, and is rapidly converted to Compound II upon illumination with visible light, even at low temperatures (11). We have been unable to obtain a Raman spectrum for Compound I, even with a rapid flow technique (12).

Compound II, however, does give rise to good quality resonance Raman spectra. In our present Raman study, HRP (RZ 3.0-3.2) was used as purchased from Worthington Biochemical. Compound II was generated by adding a two-fold molar excess of H_2O_2 to 0.1-0.2 mM HRP soln, in 0.05M phosphate buffer pH 7.4. Spectra were obtained in a spinning cell, maintained at about 4°, by blowing cold nitrogen, to minimize laser heating. 4579Å and 5145Å Ar^+ laser excitations were employed in transverse scattering geometry. The instrumental techniques have been described elsewhere (8).

Figure 1 shows spectra obtained with 5145Å excitation at various intervals after the addition of H_2O_2 to HRP. The initial spectrum is that of resting HRP, which has previously been reported (8). Within two minutes it is replaced by an entirely different spectrum that gradually decays back to the spectrum of resting HRP over a period of one hour. This time course is consistent with the development and decay of Compound II (13), which was independently monitored via the absorption spectrum of the solutions, taken before and after the Raman spectral runs. Figure 2 compares native enzyme and Compound II Raman spectra obtained with 4579Å excitation; at this wavelength the polarized bands are emphasized (9), particularly the band near 1375 cm^{-1}, which is the key frequency in the present analysis. Reproducible Raman frequencies (cm^{-1}) for Compound II were observed at 1641, dp, vs; 1592 p, w; 1589 ap, vs; 1587 p, w;1562 dp, w; 1508 p, m; 1382 p, vs; 1345 ap, w; 991 p, w; 758 dp, w; 725 p, vw; 684 p, w; 348 p, vw (14).



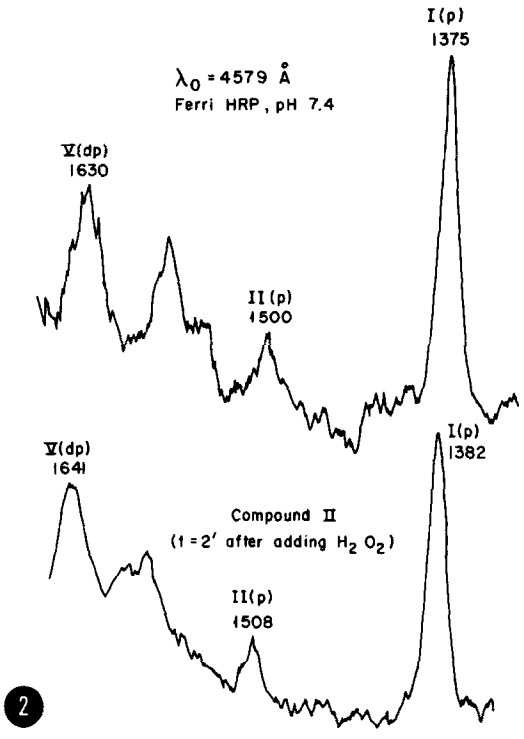


Figure 1. Comparison of resonance Raman spectra of native HRP and Compound II, recorded with 80 mw 5145Å excitation.

(A) Ferri HRP (~0.2 mM in heme) in 0.05 M phosphate buffer, pH 7.4. (B) Compound II. 2 minutes after addition of 0.4 mM H_2O_2 to above HRP solution. Bands I, III, IV and V refer to Table 1. (C), (D), (E) and (F) show portions of Raman spectra, recorded 10′, 13′, 15′ and 1 hour respectively after H_2O_2 addition. The scattered radiation is analyzed perpendicular (C, E and F) and parallel (D) to the incident polarization. The absence of native HRP bands in (B) - (E) indicate no detectable decay of Compound II, even after 15′ laser irradiation. At later times, however, overlapping bands appear due to resting HRP, and its regeneration is complete in about an hour, as indicated in (F). Instrumental Condition 10^3 x 2 cps, time constant 3 sec, spectral slit width 6 cm^{-1}, scan rate 60 cm^{-1}/min.

Figure 2. Comparison of resonance Raman spectra of native HRP and Compound II, recorded with 30 mw 4579Å excitation.

(A) Ferri HRP (~0.1 mM in heme) in 0.05M phosphate buffer, pH 7.4. (B) Compound II. 2 minutes after addition of 0.2 mM H_2O_2 to above native HRP solution. Instrumental Conditions, 10^3 x 2 cps, time constant 3 sec, spectral slit width 6 cm^{-1}, scan rate 60 cm^{-1}/min.

Table 1 lists Raman frequencies which are known to be sensitive to oxidation and/or spin state (9,10), for resting HRP, its cyanide complex, and Compound II. Binding cyanide to HRP changes the heme from high- to low-spin Fe(III). The accompanying shift to higher frequency of bands II, III, IV and V are believed to reflect

Table 1

Comparison of Oxidation and Structure Sensitive Marker Bands for Compound II with Ferri HRP and Ferri HRP Cyanide.[a]

HRP Derivative pH 7.4	Oxidation State	Spin State	I (p)	II (p)	III (dp)	IV (ap)	V dp
Ferri HRP	Fe(III)	high	1375	1500	1550	1575	1630
Ferri HRP-cn$^-$	Fe(III)	low	1375	1506	1562	1590	1642
HRP Compound II	Fe(IV)	low	1382	1508	1562	1589	1641

(a) p = polarized; dp = depolarized; ap = anomalously polarized

mainly the associated change in heme stereochemistry, from an out-of-plane (high-spin) to an in-plane (low-spin) structure (15). For Compound II these frequencies are the same as for HRP cyanide, implying a planar, low-spin heme.

Band I was early identified as a marker of oxidation state (16). It occurs at ~1360 cm^{-1} in Fe(II) porphyrins, and shifts to higher frequency upon coordination of π-acid ligands or upon oxidation (9,10). All known Fe(III) porphyrins have band I frequencies within 1-2 cm^{-1} of 1375 cm^{-1} (7,10). For Compound II, however, the frequency is significantly higher, 1382 cm^{-1}. The direction of the shift is consistent with further oxidation of Fe(III). Bands III and V are also known to shift up somewhat upon oxidation from Fe(II)to Fe(III) (10). For these bands the effect apparently saturates at Fe(III), since no further shift (with respect to the low-spin Fe(III) frequencies) is observed on Compound II formation. While the 1382 cm^{-1} frequency does not by itself establish the existence of Fe(IV), it does strongly reinforce other experimental observations cited above, which point in the same direction.

We have also observed the 1382 cm^{-1} band in solutions of "ferryl" myoglobin, the transient species obtained on adding H_2O_2 to metmyoglobin, which has an absorption spectrum like that of Compound II (17). Likewise we were able to detect the 1382 cm^{-1} band upon addition of H_2O_2 to cytochrome c peroxidase (18) (CCP). CCP forms an intermediate with two oxidizing equivalents above the resting enzyme, but with

an absorption spectrum similar to that of Compound II of HRP (13,19,20). Presumably the CCP intermediate also contains Fe(IV) porphyrin. The other oxidizing equivalent is apparently stored elsewhere in the protein as a free radical, whose epr spectrum has been observed (20).

The identities of the heme axial ligands of Compound II are unknown. Presumably the fifth ligand is imidazole, as inferred from the ^{15}N hyperfine pattern of the epr spectrum of the NO adduct of ferro-HRP (21), and ultraviolet difference spectra of Compound II (22). The sixth ligand is generally thought to involve an oxygen atom originating in H_2O_2 (23). The ferryl ion, Fe^{2+} formulation suggested by George (4) implies an oxo-metal bond. Such bonds have appreciable double bond character and are known to give rise to strong Raman bands near 900 cm^{-1} (24). An O^{2-} - F^{4+} unit would also be expected to produce appreciable charge transfer absorption in the visible or near-ultraviolet region, which could be expected to provide resonance enhancement of the Raman scattering. An Fe-O stretching mode has been detected by Brunner (25) in the resonance Raman spectrum of oxy-hemoglobin, for which Fe → O charge transfer absorption has been observed (26). We searched the low-frequency Raman spectra of Compound II carefully, but failed to observe any bands which have not already been identified as prophyrin modes (7).

Other possibilities for the sixth ligand are H_2O or OH^-. The former appears unlikely, since the pK_a of heme-bound H_2O in methemoglobin or myoglobin is ~8 (27), and it should shift to lower values for a Fe(IV) heme. If the ligand is OH^-, the pK_a would have to be below 4 to be consistent with the observation that Compound II does not undergo heme-linked protonation between pH 4 and 11 (28). An OH^- ligand should have an associated Fe-O vibration around 400-500 cm^{-1}, but it is expected to be weak; it has not been observed in hydroxy-methemoglobin (9) or hydroxy-HRP (8).

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