Resonance Raman Spectra of the Reaction Intermediates of Horseradish Peroxidase Catalysis

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Horseradish peroxidase (HRP) catalyzes the oxidation of a variety of substrates by a specific oxidizer of H_2O_2. The catalysis proceeds via sequential intermediates of the enzyme called here compound I and compound II, which are known to stay in the higher oxidation states by 2 and 1 equiv, respectively, than the resting ferric state.[1,2] Currently the localization of the extra oxidation equivalents and identities of the heme axial ligands of the intermediates have been a matter of various spectroscopic concern. We report here new pieces of Raman information about compound II and the other intermediate, probably compound I, and point out involvement of the fifth ligand in stabilization of the intermediates having the higher oxidation state.

Mössbauer studies on compound I and compound II[3] and the corresponding intermediates of Japanese radish peroxidase[4] revealed little difference between the iron oxidation states of compound I and compound II. However, the visible spectrum of the two intermediates are quite variant. The similarity of the visible spectrum of compound I to that of a synthetic porphyrin π cation radical[5] suggested that the porphyrin ring itself could serve as a site of the extra oxidizing equivalent of compound I. Subsequent studies of compound I with ESR,[6] NMR,[7] and ENDOR,[8] and theoretical studies[9,10] have supported that suggestion. Resonance Raman scattering from heme proteins provides information about strength of chemical bonds of the heme through the observations of the vibrational frequencies and indeed has revealed structural details of the heme and its vicinity for compound II[11,12] as well as the resting and reduced states of HRP.[13-15] However, so far there has been no report of the resonance Raman spectrum of compound I, presumably due to its photolability toward laser irradiation. To overcome the difficulty, we adopted cryogenic measurements in this study.

Isozyme C of HRP (Toyobo, Grade I-C) with the R_z value ($=A_{408\,nm}/A_{280\,nm}$) of 3.2 was dissolved in a 0.05 M phosphate (pH 7) or 0.05 M carbonate buffer (pH 9.6) for the formation of compound I or compound II, respectively, so that the most stable intermediates solution with the concentration of 0.5 mM HRP could be obtained. Compound I was generated in a Raman cell by addition of a little excess of H_2O_2 at 0 °C, and immediately the cell was immersed into liquid nitrogen. The Raman cell, containing the frozen sample at −190 °C, was quickly attached to our homemade cryostat, which was promptly filled with liquid nitrogen after pumping off the inside air. Compound II was obtained in the same way as compound I except for the addition of equimolar $K_4[Fe(CN)_6]$ as an electron donor to the sample solution prior to freezing. Raman scattering was excited at 441.6 nm with a He/Cd laser (Kinmon Electrics, CDR80SG) and recorded on a JEOL-400D Raman spectrometer. Temperature of the sample was measured with a copper–constantan thermocouple touched to the Raman cell. The frequency calibration of the Raman spectrometer was performed with indene and CCl_4 as a standard.

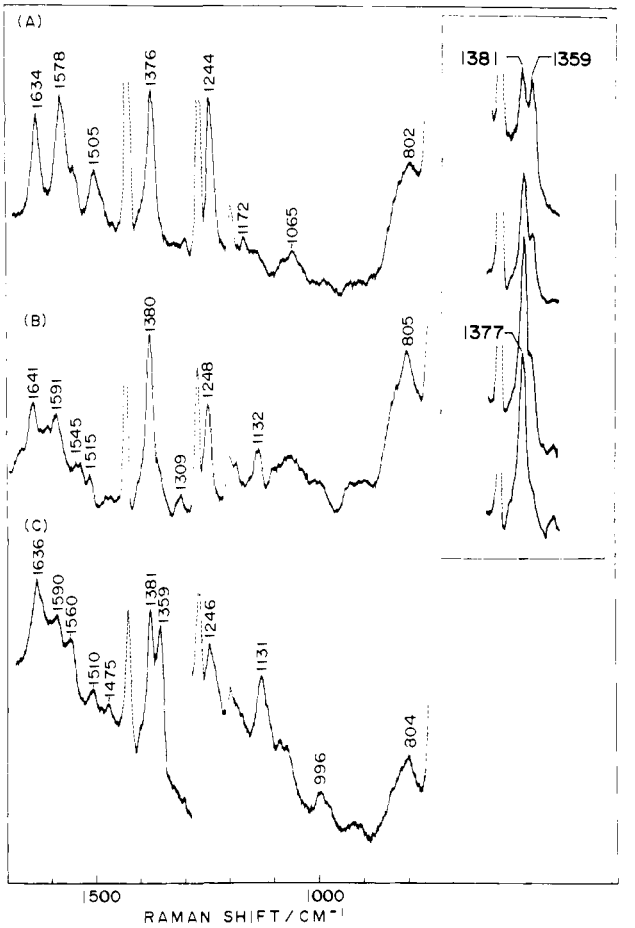

Figure 1. Resonance Raman spectra of ferriHRP at pH 7.4 (A), compound II at pH 9.6 (B), and compound I at pH 7.0 (C) excited at 441.6 nm. Samples were kept at −120 °C during the measurements (see text). The dotted parts of the spectra indicate the plasma lines of a He/Cd laser. The formation of compound I and compound II were confirmed by the characteristic visible absorption spectra at room temperatures. It was also confirmed that both samples that displayed spectra B and C gave the identical spectrum with spectrum A when the temperature of the samples was raised to room temperature and lowered again. The inset figure represents a change of the relative intensity of the two ν_4 lines of spectrum C with rising temperature. Because of instability of compound II at this pH, the bottom of the inset stands for a spectrum of a mixture of compound II and the restored native HRP.

Figure 1 displays the resonance Raman spectra of native HRP (A), compound II (B), and a mixture of compound I and compound II (C), all of which were measured at −120 °C. The frequencies of Raman lines of native HRP and compound II were slightly higher (2–3 cm^{-1}) than those at room temperatures. Distinct Raman features of compound II, which were previously pointed out for the ν_{10} (1641 cm^{-1}) and ν_4 lines (1381 cm^{-1}) by Spiro and co-workers[11] and Felton et al.,[12] are well reproduced in spectrum B, although the relative intensities of Raman lines were varied due to different excitation wavelength. Upon excitation at 441.6 nm, polarized lines are generally intense. In accord, the polarized line of compound II at 1591 cm^{-1} is assigned to ν_2[16,17]

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rather than to ν_{19}, which was previously noted as the marker line of compound II for the green excitation.[12]

In spectrum C there are two ν_4 lines at 1381 and 1359 cm^{-1}. Their relative intensity was variable with every preparation,[18] but when a temperature was raised to 0 °C, the 1359-cm^{-1} line always diminished and at the same time the 1381-cm^{-1} counterpart was shifted to lower frequency by 4 cm^{-1}. The spectral change from C to B at pH 7.0 is illustrated in the inset of Figure 1, where the top is the ν_4 part of spectrum C and the bottom denotes the spectrum after the temperature was raised. Spectrum A was finally restored from the sample that had once given spectrum C. Therefore, it is reasonable to assume that spectrum C arose from a mixture of reaction intermediates. Since compound II exhibits ν_4 at 1380 cm^{-1} (spectrum B) and the line would be apparently shifted to a lower frequency if the 1376-cm^{-1} line of the restored native enzyme were overlapped to it and unresolved, the 1381-cm^{-1} line is likely to arise from compound II contaminating the compound I preparation. The 1359-cm^{-1} line is inferred to be brought about by compound I. One may argue that the irradiation of laser light caused a photodecomposition of compound I,[19] which yielded the 1359-cm^{-1} species. With regard to this possibility, we recall that the visible absorption spectrum of the photoreacted compound I (λ_{max}^{Soret} 416 nm) is much closer to that of compound II (λ_{max}^{Soret} 419 nm) than that of compound I (λ_{max}^{Soret} 400 nm),[19] and therefore it is less likely that the photoreacted compound I exhibits the ν_4 line at distinctly different frequency from the ν_4 frequency of compound II. However, we cannot rule out a possibility for the presence of compound I′ which is slightly modified from compound I due to light illumination. We note that the Raman intensity of compound I is generally much less resonance-enhanced than those of compound II due to the much smaller absorbance and shorter wavelength of the Soret maximum. Therefore, the amount of compound II contaminating the compound I preparation is considered to be much smaller than that inferred from the equal intensity of the 1381- and 1359-cm^{-1} lines in spectrum B.

In comparison of spectra B and C, the ν_4, ν_{10}, and ν_2 lines of compound I are shifted by $\Delta\nu_4 = -22$ cm^{-1}, $\Delta\nu_{10} = -5$ cm^{-1}, and $\Delta\nu_2 = 0$ cm^{-1} with regard to those of compound II. The potential-energy distribution calculated for these modes indicates that ν_4, ν_{10}, and ν_2 involve primarily the $C_\alpha N$ stretching (53%), $C_\alpha C_m$ stretching (49%), and $C_\beta C_\beta$ stretching term (60%), respectively.[17] The frequency shifts of these modes are deduced to reflect a different π distribution in the heme of compound I and compound II. Accordingly, the data of the frequency shifts would suggest a significant reduction of the $C_\alpha N$ bond strength, an appreciable reduction of the $C_\alpha C_m$ bond strength, and little change of the $C_\beta C_\beta$ bond strength upon removal of 1 oxidizing equiv from the highest filled π orbital of the Fe(IV) porphyrin. This is surprisingly consistent with the recent suggestions from the NMR,[7] ENDOR,[8a] and theoretical studies[10] that the spin density of the $^2A_{2u}$ cation radical is evidently higher at pyrrole nitrogens or meso carbons (C_m) compared with that at C_β.

Spectra B and C exhibit a new Raman line at 1131–1132 cm^{-1}. This line gave no frequency shift upon preparation with ^2H_2O and $^2H_2O_2$. Although the Raman line is relatively intensified in spectrum C, it is conceivable that both compound I and compound II involve the 1131-cm^{-1} mode. Hence it would not be associated with porphyrin vibrations characteristic of a porphyrin π cation radical, one of which was recently observed at about 1270–1290 cm^{-1} in the IR spectra.[20] We speculate that the Raman line may possibly be associated with the Fe(IV)=O stretching mode, and if this were the case, the size of its frequency and no frequency shift in ^2H_2O would be unfavorable to the Fe(IV)–OH structure of the intermediate.

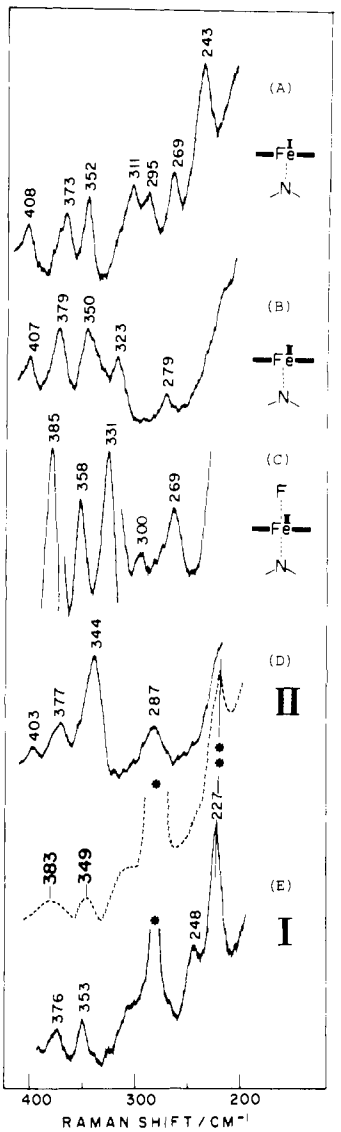

Figure 2. Low-frequency resonance Raman spectra of ferroHRP (A), native ferriHRP (B), ferriHRP fluoride (C), compound II (D), and compound I (E) excited at 441.6 nm. Spectra A–C and the solid line of D were observed at room temperature, but the broken line of D and spectrum E were observed at −120 °C. The Raman line of the frozen samples at 227 cm^{-1} arises from the lattice vibration of ice, which was confirmed with the pure frozen buffer. The scattering peaks marked by an asterisk indicate the plasma line. For compound I and compound II, the measurements of the lower frequency region were always preceded and followed by the measurements of the ν_4 region, and thus its identity was confirmed, although the relative intensity, I_{1381}/I_{1359} was somewhat variant with every preparation. It was confirmed that the frozen sample of compound II gives the spectrum identical with the spectrum represented by the solid line when it was remelted after the measurement at −120 °C.

Figure 2 compares the resonance Raman spectra in the Fe–His stretching region of ferroHRP (A), ferriHRP (B), ferriHRP fluoride (C), compound II (D), and compound I (E). Spectra A–D were measured in the solution state to avoid the overlapping of the plasma line, but spectrum E was obtained at −120 °C to stabilize compound I. For comparison, the spectrum of compound II was also observed in the frozen state and is depicted by a broken line. It is unknown why the spectral pattern is altered by freezing, but such freezing effect is also seen for hemoglobin[21] and possibly

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due to a change in van der Waals contact between heme and protein, because the low-frequency modes involve significant contribution from the peripheral groups.[22]

The Raman line of ferroHRP at 243 cm^{-1} and that of ferriHRP fluoride at 269 cm^{-1} were previously assigned to the Fe–His stretching vibration on the basis of the observed ^{54}Fe isotopic frequency shift.[15] Due to the analogy of their similar frequencies, the Raman lines of ferriHRP at 279 cm^{-1}, compound II at 287 cm^{-1}, and compound I at 248 cm^{-1} might be assignable to the Fe–His stretching mode. It was recently pointed out that the Fe–His stretching frequency of ferroHRP (243 cm^{-1}) serves as a distinct property in iron coordination environments of plant tissue peroxidases[23] and is caused by a considerably strong hydrogen bond of the proximal histidine.[15b] The frequency shifts of the Fe–His stretching mode from 279 to 248 cm^{-1} upon formation of compound I[24] and to 287 cm^{-1} upon formation of compound II are significantly large, suggesting a concomitant change of the charge distribution of the Fe–His bond and thus involvement of the proximal histidine in activation of the ligated oxygen atom at its trans position.

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Photoacoustic Measurement of Photophysical Properties. Lowest Triplet-State Energy of a Free Base Porphyrin

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Via a calorimetric method[2-4] based on the photoacoustic effect,[5,6] we have measured certain photophysical properties of free base *meso*-tetra-*p*-tolylporphyrin. In particular, we determine the product of the quantum yield of intersystem crossing and the triplet-energy level to be 0.93 eV. By contrast, current literature values give a range for this product from 1.07 to 1.26 eV. To demonstrate that our calorimetric method is viable, we have also investigated two other compounds with well-known photophysical properties, zinc tetraphenylporphyrin and 1-chloroanthracene. In these cases, the experimental values we obtain are in agreement with literature values.

When a system of absorbing molecules is exposed to intensity-modulated exciting light, the populations of the excited electronic states produced will also be modulated. Deexcitation of these excited states through nonradiative routes constitutes the heat source for the photoacoustic effect. Since the relaxation of the triplet state is slow, the modulation of the triplet population will be delayed and lag in phase the modulation of other rapidly relaxing states. Quenching the triplet state to a sufficiently short lifetime eliminates this phase lag. We measure the phase-angle difference, θ, between the photoacoustic signals of an unquenched sample and of the same sample but with the triplets quenched. This angle is given by the theoretical expression[7]

$$\tan\theta = \omega\tau/[1 + R(1 + \omega^2\tau^2)]$$

$$R = (h\nu - \phi_{fl}E_s - \phi_{isc}E_T)/\phi_{isc}E_T$$

where $h\nu$ and ω are the energy and angular modulation frequency of the exciting light, E_s and E_T are the energies of the O–O transitions of fluorescence and phosphorescence, and τ is the triplet lifetime. ϕ_{fl} and ϕ_{isc} are quantum yields of fluorescence and intersystem crossing. We have assumed $\phi_{fl} + \phi_{isc} + \phi_{ic} = 1$, where ϕ_{ic} is the quantum yield of internal conversion.

Neither fluorescence excitation nor emission spectra of the samples described below indicate any fluorescence other than the transition from the lowest vibrational state of S_1 to the ground-state vibrational manifold, as is consistent with Kasha's Rule.[8] These same spectra also indicate a phosphorescence quantum yield less than 1/100th that of fluorescence; we have accordingly neglected phosphorescence.

The molecules were dissolved into a rigid matrix consisting of a polymer[9-11] of either polystyrene (Aldrich 18,242-7) or cellulose triacetate (Aldrich 18,100-5) (both used without further purification). This was necessary to lengthen the triplet lifetime τ of unquenched samples to the order of milliseconds, so that $\omega\tau \sim 1$ within the normal frequency range of photoacoustic spectroscopy, and an appreciable phase shift will result. Samples were placed in a photoacoustic cell employing nitrogen as the coupling gas for

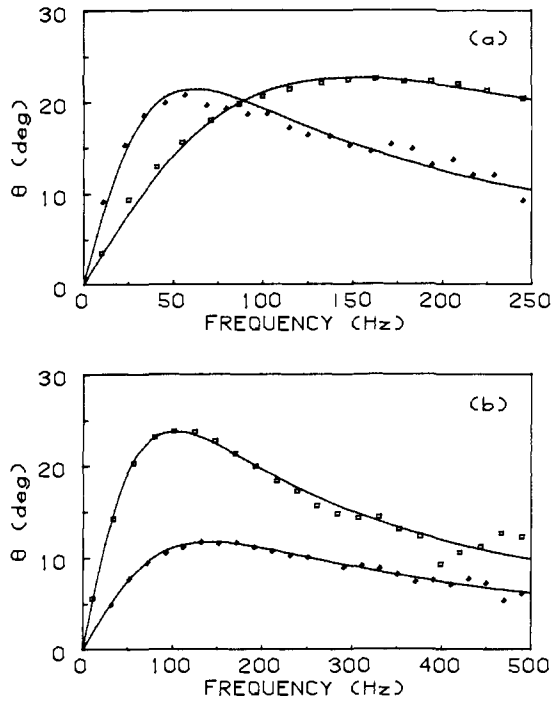

Figure 1. (a): (□) 1×10^{-4} M zinc tetraphenylporphyrin in polystyrene, 422-nm excitation, fitted curve parameters $R = 0.80$, $\tau = 1.6$ ms; (◇) 1×10^{-2} M 1-chloroanthracene in cellulose triacetate, 365-nm excitation, curve parameters $R = 0.87$, $\tau = 3.8$ ms. (b): *meso*-tetra-*p*-tolylporphyrin (◇) 4×10^{-3} M in cellulose triacetate, 420-nm excitation, fitted parameters $R = 1.96$, $\tau = 1.4$ ms; (□) 8×10^{-3} M in polystyrene, 650-nm excitation, parameters $R = 0.74$, $\tau = 2.4$ ms.

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