Resonance Raman Spectra of Native and Mesoheme-reconstituted Horseradish Peroxidase and Their Catalytic Intermediates*

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Resonance Raman studies of native and mesoheme-reconstituted horseradish peroxidase and their catalytic intermediates, known as Compounds I and II, have been conducted using both near UV (~350 nm) and visible (406.7 nm) excitation. Careful power studies indicate that the authentic Compound I spectra are obtainable using near UV excitation, but that use of visible excitation results in contamination of the Compound I spectrum with the spectrum of a Compound II-like photoproduct. Using H$_2$O$_2$, the Fe(IV) stretching modes for both systems are unambiguously identified, for the ferryl porphyrin on the heme moiety, which is then properly formulated as a 2A$_{1u}$ cation radical. Early NMR studies (14), led to the conclusion that HRP-I is best formulated as an 2A$_{2u}$ cation radical. Early NMR studies (15) of HRP-I were also interpreted in terms of an 2A$_{2u}$ formulation, but more recent studies by this method favor an 2A$_{1u}$ formulation (16, 17).

The issue of the precise electronic structure of these primary, ferryl π-cation, intermediates (i.e. 2A$_{1u}$ versus 2A$_{2u}$ formulation) obviously may carry important biological implications. Thus, the observed differences in the optical spectra of the Compound I derivatives of catalases and peroxidases was initially suggested to be ascribable to a difference in the ground state formulations (i.e. 2A$_{1u}$ for catalase and 1A$_{2u}$ for HRP-I) and, by implication, for the difference in their reactivities (1–5). Also, it has been pointed out that, in the case of HRP, an 2A$_{2u}$ ferryl π-cation intermediate would place the orbital hole in closer proximity to the substrate binding site than would an 2A$_{1u}$ ferryl π-cation (17). Clearly, it is possible that variations in the reactivities of this ubiquitous intermediate, among different classes of oxidative heme enzymes, may arise as a consequence of specific active site architectures which stabilize one or the other of the two formulations. Thus, it is important to document the electronic ground state configuration for each system.

In principle, resonance Raman (RR) spectroscopy is well-suited to investigate this issue. Not only is it potentially able to provide direct documentation for the ferryl fragment, via observation of the v(Fe=O) stretching mode as in the case of the Compound II analogues (18, 19), but the precise position of certain high frequency macrocycle stretching modes may be effective in characterizing the nature of the electronic structure of the cation radical as either 2A$_{1u}$ or 2A$_{2u}$ (9–11, 20). Thus, in systematic RR studies of well-behaved model compounds (10, 11), it was shown that for 2A$_{2u}$-like metalloporphyrin π cation radicals (MP$^{2+}$) certain key modes (so-called “marker modes”) shift to higher frequencies, while these same modes shift to lower frequency for 2A$_{1u}$-like radicals.

Given this potentially informative probe, many attempts have been made to acquire the RR spectrum of HRP-I. Collectively, these studies have generated much controversy and methods have been applied to various enzymatic species in attempts to reveal this information. Perhaps the most thoroughly studied example is that of the readily available horseradish peroxidase (HRP). While optical absorption spectroscopy provides support for the existence of a ferryl porphyrin π-cation radical in the case of the Compound I intermediate (HRP-I) (6), this technique does not permit a clear distinction between the 2A$_{1u}$ and 2A$_{2u}$ formulations (7–12). The results of various magnetic resonance measurements are also conflicting. Thus, elegant ENDOR studies by Hoffman and co-workers (13), as well as magnetic circular dichroism studies by Stillman and co-workers (14), led to the conclusion that HRP-I is best formulated as an 2A$_{2u}$ cation radical. Early NMR studies (15) of HRP-I were also interpreted in terms of an 2A$_{2u}$ formulation, but more recent studies by this method favor an 2A$_{1u}$ formulation (16, 17).

The catalytic cycles of a large number of oxidative heme enzymes involve intermediates which contain high-valent (ferryl) hemes (1, 2). Hydroperoxidases, whose essential function is to eliminate or utilize hydrogen peroxide, react with this oxidant to generate a primary intermediate (so-called Compound I) which is two oxidizing equivalents higher than the resting state (ferric) enzyme. In many cases, such as mammalian catalases and plant peroxidases, both oxidizing equivalents reside on the heme moiety, which is then properly formulated as a ferryl porphyrin π-cation radical; i.e. Of(IV)(P$^+$). The primary intermediate for peroxidases undergoes a one-electron reduction by substrate to generate the so-called Compound II, a ferryl porphyrin; i.e. Of(IVP).

While it is generally accepted that the primary intermediate in the cases of typical plant peroxidases (e.g. horseradish peroxidase) and mammalian catalases is indeed ferryl π-cation radicals, there is continuing uncertainty regarding the electronic and molecular structure of such species. Thus, the two highest occupied molecular orbitals (HOMOs) of metalloporphyrins, designated a$_{1u}$ and a$_{2u}$, are nearly degenerate, their relative energies being dictated by many factors, including: the nature and position of porphyrin peripheral substituents, the identity and disposition of the axial ligands to the iron, as well as more subtle factors such as environment and temperature (3–5).

Given this susceptibility to structural and environmental control of relative HOMO energies, it becomes difficult to predict the exact formulation (as either 2A$_{1u}$ or 2A$_{2u}$) of the primary intermediate for a given enzyme, and many spectroscopic

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confusion, the essential problem being the well-documented (14, 21, 22) photolability of HRP-I; i.e., the RR (laser) excitation source can, itself, efficiently generate photoproducts (23). Thus, the first reported RR spectrum of HRP-I (24) was later (23) shown to be ascribable to a photogenerated mixture of other species. Later studies, using conventional flow methods (25) or short laser pulses (26, 27), yielded spectra which were significantly different from that of HRP-II and were interpreted as being either that of HRP-I (25) or that of an HRP-II-like photoproduct (27). We (28) and others (29) attempted to overcome this photolability problem by using a novel microdroplet sampling device, first employed in our laboratory (28, 30, 31), which effectively reduces sample residence time in the beam (to less than 5 μs). The results of these latter two studies were in essential agreement, giving a new spectrum at low laser powers which, at higher powers, reverted to the spectrum of a (presumably photoreduced) product which resembled that of authentic HRP-II. Furthermore, the position of the key marker modes observed in these studies were indicative of an $^2A_{2u}$ formulation for HRP-I.

Independently, and apparently simultaneously, Palaniappan and Terner (32) employed a different strategy to acquire the RR spectrum of HRP-I, which involved using ~350 nm excitation; the reasoning being that HRP-I ($\lambda_{\text{max}} = 402$ nm) would be selectively enhanced relative to an HRP-II-like photoproduct ($\lambda_{\text{max}} = 419$ nm). In this way, these workers were able to acquire a unique spectrum (i.e., different from that of HRP-II), although no power studies were reported to demonstrate that the reported spectrum reverted to an HRP-II-like spectrum at high excitation powers. Most importantly, the RR spectrum reported by Palaniappan and Terner (32) exhibited marker mode frequencies which are most consistent with an $^2A_{2u}$ formulation for HRP-I.

EXPERIMENTAL PROCEDURES

Horseradish peroxidase (Type II, Sigma) was purified by the method of Shannon et al. (33). The fractions containing pure isoenzymes B and C, eluted from the CM52 (Whatman) column, were collected and pooled.

While ambiguity persists regarding the electronic configuration of HRP-I, it is generally agreed that $^2H$ NMR evidence (17) conclusively demonstrates that the corresponding derivative of mesoheme-reconstituted HRP (i.e., mesoheme-HRP-I) is properly formulated as an $^2A_{2u}$-like radical. Thus, in an attempt to clarify these issues, the present work is undertaken to acquire the RR spectrum of mesoheme-HRP-I. In the process, the RR spectra of resting state mesoheme-HRP and mesoheme-HRP-II as well as their deuterium-labeled analogues have also been acquired. Spectra were acquired with the microdroplet sampling approach (28–31), using both 406.7 and 356.4 nm excitation. In addition, the RR spectrum of native HRP-I has been acquired using the same procedures (i.e., the microdroplet device and both 406.7 and 356.4 nm excitation). The entire series of experiments are thoroughly discussed with reference to previous work, and the results for mesoheme-HRP-I are interpreted as being supportive of an $^2A_{1u}$ formulation, as expected (16, 17). While considerable spectral congestion in the high frequency region prevents an unambiguous assignment of certain key marker modes, the general appearance of the spectrum for native HRP-I is quite similar to that originally reported by Palaniappan and Terner (32).
(Aldrich) (enzyme:H2O2 = 1:2.75), held in separate reservoirs, into a mixing chamber and subsequently through the microdroplet generator (30, 31). A low temperature stirred cell (36) was employed for the measurement of Compound II species, while a conventional spinning cell was used for the resting state samples. Methyl group deuteriation to give [2H12]mesohemin was carried out by the procedure described by Goff and co-workers (37). A sample of H218O2 was prepared by a previously described procedure (38, 39).

The RR spectra were acquired with a Spex 1269 spectrometer equipped with a Princeton Instruments ICCD-576 UV-enhanced detector, and the 406.7 nm and 356.4 nm excitation lines were from a Coherent Innova model 100-K3 Kr+ ion laser.

The absorptionspectra of Compound I and Compound II, shown in Fig. 1 for meso-heme-HRP, were monitored both before and after the RR spectra were acquired to ensure sample purity (in the case of native HRP via reference to reported spectra (35)). The UV-visible absorption spectra were recorded with a Hewlett-Packard 8452A spectrophotometer.

RESULTS

Mesoheme-reconstituted HRP

Resting State and Compound II—The RR spectra of resting state meso-heme-HRP and its 2H12-deuterated analogue, acquired with both 406.7 and 356.4 nm excitation, are given in Fig. 2. The high frequency spectra of the Compound II derivative and its deuterated analogue, acquired with 406.7 nm excitation, are given in Fig. 3. The low frequency spectra of meso-heme-HRP-II and its derivative prepared with H218O2 hydrogen peroxide are given in Fig. 4. The assignment of the feature located at 785 cm⁻¹ in the spectrum of meso-heme-HRP-II to the ν(Fe=O) stretching mode is confirmed by its shift to 754 cm⁻¹ upon using H218O2 in place of H216O2 (the difference spectrum is given in trace C of Fig. 4). Assignment of the higher frequency marker modes are made with reference to well-documented previous assignments for metalloporphyrins (40–42) and other heme proteins (20, 38, 39). These assignments, along with the observed shifts upon methyl group deuteriation, are summarized in Table I.

The frequencies observed for the meso-heme-HRP-II derivative, relative to the resting state species, are entirely consistent with expectations and the behavior observed for other ferryl heme-containing enzymes (20). Thus, ν2, ν3, ν4, ν10, and ν11 shift to higher frequencies by 9–20 cm⁻¹, compared to their positions for the ferric derivative.

Compound I—In Fig. 5 are shown the RR spectra of meso-heme-HRP-I at pH 7.5, acquired with 406.7 nm excitation, using both low and high excitation powers. The high-power spectrum (trace B) is virtually identical with the corresponding spectrum of meso-heme-HRP-II acquired with 406.7 nm excitation (trace C) and is indicative of an efficient photoreduction process as has been previously observed (26–29, 32) in the case of HRP-I (see below). At this wavelength, even the low power excitation (trace A) apparently produces substantial amounts of reduced photoproduct, as is evidenced by the similarity of all the spectra shown in Fig. 5.

In contrast, as is shown in Fig. 6, the spectra acquired with 356.4 nm excitation clearly demonstrate a dependence on the power of the excitation beam. Thus, the spectrum acquired with 1 milliwatt of power (trace A) is distinctly different from the corresponding spectrum of meso-heme-HRP-II shown in Fig. 3. Furthermore, as the power of the excitation beam is increased to 5 and 25 milliwatts (Fig. 6, traces B and C), new features (e.g. 1379 cm⁻¹) begin to appear whose intensities increase with increased excitation power. This behavior is most reasonably interpreted as follows. The authentic meso-heme-HRP-I spectrum is obtained with low power (1 milliwatt) exci-
tation at 356.4 nm, but is contaminated at higher powers by contributions (e.g. 1379 cm$^{-1}$) from a photogenerated reduction product whose spectrum resembles that of mesoheme-HRP-II. Finally, as is shown in Fig. 7, the difference spectrum generated by the subtraction of the spectra acquired at 1 milliwatt with H$_2$O$_2$ and H$_2$O$_2$ reveals the $\nu$(Fe$^{IV}$-O) stretching mode at 793 cm$^{-1}$ (H$_2$O$_2$). The observed isotopic shift (34 cm$^{-1}$) is in close agreement with the theoretical value (33 cm$^{-1}$) and that observed for other ferryl hemes (20).

Native HRP

The existing controversy regarding the authentic RR spectrum of the Compound I derivative of native HRP (23–29, 32), taken together with the present results for mesoheme-HRP-I, prompted us to reinvestigate this issue. Shown in Fig. 8 are the RR spectra of HRP-I, acquired with 356.4 nm excitation at various power levels. As was the case for mesoheme-HRP-I, the spectrum acquired with 1 milliwatt of power (trace A) becomes contaminated by additional features (i.e. 1379 and 1509 cm$^{-1}$ bands) as the power is increased to 10 and 25 milliwatts (traces B and C). It is noted that the frequencies observed in trace A (i.e. apparently the authentic spectrum of HRP-I) are in close agreement with those originally reported by Palaniappan and Terner (32). In the present work, this spectrum is shown to become contaminated at higher excitation powers, producing a set of features whose frequencies are similar to those of HRP-II; a fact which is consistent with production of an “HRP-II-like” photoproduct, as expected (21–23, 26–29, 32).

Table I: Resonance Raman frequencies for native and mesoheme-reconstituted horseradish peroxidase and its intermediates

| Mode | Resonance Raman frequencies for native and mesoheme-reconstituted horseradish peroxidase and its intermediates |
|------|---------------------------------------------------------------------------------------------------------------|
|      | Mesoheme HRP                                                                                                    |
|      | Native HRP                                                                                                      |
| $v_1$ | 1634(−5) 1645(−2) 1633                                                                                           |
| $v_{10}$ | 1631 1639 1633                                                                                                   |
| $v_{13}$ | 1582(−5) 1602(−5) 1610                                                                                           |
| $v_{14}$ | 1550 1560 1572                                                                                                   |
| $v_{15}$ | 1499(−6) 1514(−2) 1498                                                                                           |
| $v_{16}$ | 1374(0) 1382(0) 1358                                                                                             |
| $v_{17}$ | 783(31)$^e$ 790(35)$^e$                                                                                          |

* Frequency differences between the species and their deuterated analogues are given in parentheses.
* Species are obtained at pH 10.8 in this work.
* Species are prepared at pH 7.5. Values obtained using 3564 Å excitation from this work.
* From Refs. 18 and 19.
* Isotopic shifts upon substitution of H$_2$O$_2$ for H$_2$O$_2$ are given in parentheses.

**Fig. 5.** Resonance Raman spectra obtained with 406.7 nm excitation (1 and 10 milliwatts) of mesoheme horseradish peroxidase Compound I generated by mixing 0.6 mM meso-HRP with 2.75 eq of H$_2$O$_2$ at pH 7.5. Trace C, resonance Raman spectrum of mesoheme-HRP Compound II acquired with 406.7 nm excitation.

**Fig. 6.** Resonance Raman spectra acquired with 356.4 nm excitation of mesoheme HRP Compound I with various excitation powers: 1 milliwatt (trace A), 5 milliwatts (trace B), and 25 milliwatts (trace C).
for a sample prepared with H$_2$O$_{18}$, and trace C shows the difference spectrum which reveals a positive component at ~790 cm$^{-1}$ (H$_2$O$_{16}$) and a negative component at 755 cm$^{-1}$. These frequencies agree very well with those observed for mesoheme-reconstituted HRP-I.

**DISCUSSION**

Native Horseradish Peroxidase Compound I (HRP-I)—In the present work, an advantageous sampling technique (28–31), which permits very short sample residence times in the laser beam, was employed with two different excitation lines (i.e., near 400 and 350 nm). In contrast to earlier reports by us (28) and others (29), a unique spectrum for HRP-I could not be obtained using 406.7 nm excitation. Instead, the spectrum observed at this excitation wavelength resembled that of authentic HRP-II and is most reasonably attributed to the spectrum of a photogenerated (reduced) product (24–28) which contains a (nonradical) ferryl heme, i.e. the equivalent to that of the heme moiety of HRP-II.

On the other hand, as is shown in Fig. 8, the spectrum of HRP-I acquired with 356.4 nm excitation is distinctly different from that of authentic HRP-II acquired with the same excitation line (32). In fact, the spectrum given in trace A of Fig. 8 is quite similar to that originally reported by Palaniappan and Terner (32) using ~350 nm excitation. The essential deficiency of the original report (32), which in fact made it susceptible to later criticism (20, 29), was that the expected power dependence of the HRP-I spectrum was not demonstrated, i.e. the unique HRP-I spectrum was not shown to revert to that of an HRP-II-like photoproduct upon increasing the power of the excitation beam.

The present work clearly demonstrates this expected power dependence. Thus, as is shown in Fig. 8, the spectrum acquired with 1 milliwatt exhibits characteristic features at 1358 ($\nu_3$) and 1503 ($\nu_2$). Upon increasing the power of the excitation beam to 10 milliwatts, new features begin to appear at 1379 ($\nu_4$) and ~1510 ($\nu_3$). Furthermore, the relative intensities of these new features increase further upon increasing the laser power to 25 milliwatts. The positions of these new features are virtually identical to those observed for authentic HRP-II (18–20) and for HRP-I acquired with 406.7 nm excitation (24–29); i.e. the photoproduct of HRP-I.

Given the authenticity of the HRP-I spectrum acquired with 356.4 nm excitation reported here and (originally) by Palaniappan and Terner (32), it seems appropriate to address the contrary results previously reported by us (28) and others (29). While several of the earlier studies (24–27) obtained spectra similar to HRP-II spectra, which are correctly attributable to an HRP-I photoproduct (27), two studies (28, 29) using 406.7 nm excitation reported unique spectra for HRP-I. In both cases, as was pointed out in the most recent report (29), this “unique” HRP-I RR spectrum resembled that of resting state (i.e. ferric) HRP, and convincing arguments were made there to suggest that this similarity is not altogether unexpected.

However, in view of the results obtained using 356.4 nm excitation, in retrospect, it now seems likely that the similar spectra (obtained with 406.7 nm excitation) reported by the two independent laboratories (28, 29) were actually contaminated by the spectrum of the resting state (HRP) enzyme. Thus, given the weak enhancement of the actual HRP-I sample, relative to that of a resting state (HRP) contaminant, at low power (i.e. in the absence of an HRP-II-like photoproduct), the observed spectrum would likely be dominated by (even small amounts) of
resting state HRP. Upon increasing the laser power, the HRP-I component is effectively photoreduced to produce an HRP-II-like photoproduct. As the power of the excitation beam is increased, the relative concentration of the photoproduct increases and then begins to dominate the spectrum. Thus, the most reasonable explanation for the documented (28, 29) power dependence of the previously reported (406.7 nm excited) spectra is that the observed spectral power dependence arose as a result of contamination of the sample with small amounts of resting state HRP whose spectrum was masked at higher excitation powers, are clearly useful in providing such information.

**Mesoheme-reconstituted HRP (Mesoheme-HRP)—** In the present work, the power dependence of the mesoheme-HRP-I RR spectrum is directly analogous to that observed for native HRP-I. Thus, as is shown in Fig. 5, using 406.7 nm excitation, no unique spectrum is observed for the Compound I derivative; i.e. the “mesoheme-HRP-I” spectra (Fig. 5, traces A and B) are virtually identical with that of mesoheme-HRP-II (Fig. 5, trace C). However, a unique spectrum is again obtained using 356.4 nm excitation, as is shown in Fig. 6, trace A. As expected, as the power of the excitation beam is increased to 5 and 25 milliwatts, new features (e.g. 1379 cm\(^{-1}\)) grow in whose relative intensities increase with increasing laser power and whose frequencies match those observed in the RR spectrum of authentic mesoheme-HRP-II (Fig. 5, trace C).

In comparison with the RR spectra of native (i.e. protoheme-containing) HRP, the spectra of the meso-HRP derivative are considerably less complex. The presence of the vinyl groups in protoheme gives rise to several internal vinyl modes and also lowers the effective symmetry of the heme macrocycle so that several \(E\) modes are activated (42). Thus, the spectra of the meso-HRP derivatives exhibit only a few, relatively isolated, modes above 1300 cm\(^{-1}\). This relative simplicity in the spectra facilitates correlation of the various marker modes throughout the series (meso-HRP-RS, meso-HRP-II, and meso-HRP-I).

Thus, while both \(\nu_1\) and \(\nu_2\) occur between 1580 and 1600 in the spectrum of meso-HRP-II (Fig. 3 and Table I), no bands appear between 1550 and 1600 cm\(^{-1}\) in the spectrum of meso-HRP-I (Fig. 6A and Table I). In this case then, it becomes clear that \(\nu_2\) (and apparently \(\nu_{12}\)) is shifted to higher frequency (by at least 9 cm\(^{-1}\)) upon cation radical formation (i.e. \(\nu_2\) shifts from \(-1602\) to \(-1611\) cm\(^{-1}\)). Such shifts are consistent with an \(A_{1u}\) formulation for meso-HRP-I, a conclusion which is consistent with the recent \(^2\)H NMR results (16, 17).

Finally, it is interesting to consider the behavior of \(\nu(Fe=O)\) upon cation radical formation. The \(\nu(Fe=O)\) of meso-HRP-II is observed at 786 cm\(^{-1}\) (shifting to 754 cm\(^{-1}\) upon \(^18\)O substitution) as shown in Fig. 4. These spectra were acquired at pH 10.5, and it is well established that this mode is sensitive to pH, shifting by about 10 cm\(^{-1}\) to lower frequency at neutral pH (18–20). Thus, the expected \(\nu(Fe=O)\) frequency for meso-HRP-II at pH 7.5 is \(-776\) cm\(^{-1}\). The corresponding frequency for meso-HRP-I (at pH 7.5) is 793 cm\(^{-1}\) (shifting to 759 \(-1\) for native HRP-I) as shown in Fig. 9. This shift to higher frequency upon cation radical formation is consistent with model compound studies wherein the \(\nu(V=O)\) of vanadyl porphyrin \(\pi\)-cation radicals (which yield an \(2A_{1u}\) ground state) was shown to occur at a higher frequency than the corresponding (neutral) vanadyl porphyrin (43).

**Conclusions**—The use of near UV (\(-350\) nm) excitation facilitates acquisition of the RR spectra of Compound I derivatives of horseradish peroxidase and its analogues via enhancement of a Compound II-like photoproduct. The authentic RR spectra of the Compound I species of both the native and mesoheme-reconstituted proteins are indicative of an \(2A_{1u}\)-like ground state. Structural characterization of such species may be important from a biological perspective in that variations in the reactivities of such intermediates may depend on the electronic ground state configurations (\(2A_{1u}\) versus \(4A_{2u}\)) (1–5, 17). Carefully conducted RR studies, employing judiciously chosen excitation wavelengths over a range of incident powers, are clearly useful in providing such information.

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