Resonance Raman Spectra of Horseradish Peroxidase and Bovine Liver Catalase Compound I Species

EVIDENCE FOR PREDOMINANT $^1\Lambda_{\text{a}}$, $\pi$-cation Radical Ground State Configurations

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The nature of the porphyrin $\pi$-cation radicals in the horseradish peroxidase and bovine liver catalase (BLC) compound I species has been investigated by studying their resonance Raman spectra. A variety of laser excitation and sample interrogation procedures have been employed in order to minimize previously documented problems arising from photoinduced conversions. With Soret band excitation, the spectra obtained for both species resemble that of a compound II-like photoproduction unless the samples are excited with residence times in the microsecond regime with very low (~1 milliwatt) powers. When these precautions are taken, spectra attributable to the compound I species themselves are obtained. The spectrum for horseradish peroxidase compound I is similar to that reported by Paeng and Kincaid (Paeng, K.-J., and Kincaid, J. R. (1988) Am. Chem. Soc. 110, 7913–7915) using a similar approach. Both horseradish peroxidase and BLC compound I exhibit frequency shifts relative to their compound II species that are in the direction observed for model $\pi$-cation radicals with predominant $^2\Lambda_{\text{a}}$ character. The magnitudes of these shifts are smaller than those observed for heme models that lack aromatic axial ligands, but agree well with these observed on formation of the compound I analog of N$_2$-acetyl microperoxidase-8 that has His as a proximal ligand. This observation is consistent with partial delocalization of the radical density onto the proximal His-170 and Tyr-357 ligands in horseradish peroxidase and BLC, respectively. The strong ligand field provided by these ligands on the proximal side and oxo ligand on the distal side of the heme group is apparently sufficient to reverse the $^2\Lambda_{\text{a}}$, radical ground state preference observed for heme-like porphyrin species (e.g., octaethylporphyrins) with weak axial fields. Enhancement of several bands assigned to the Tyr-357 ligand has also been observed for BLC compound I with 406.7-nm excitation. This is attributed either to resonance with a tyrosinate $\rightarrow$ Fe(IV) charge transfer band or to the coupling provided by radical spin delocalization onto the tyrosinate ligand.

Horseradish peroxidase (EC 1.11.1.7) and bovine liver catalase (BLC) (EC 1.11.1.6) are hydroperoxidases that catalyze the oxidation of their substrates by hydrogen peroxide (1, 2). The resting form of both enzymes contains a five-coordinate high spin, ferric heme cofactor, where the proximal ligand is His-170 (3) in horseradish peroxidase and Tyr-357 (4–6) in BLC. An initial step in the mechanism of both enzymes in their two-electron oxidation by hydrogen peroxide to form a species referred to as compound I. There is now convincing evidence that this oxidation occurs by removal of one electron from the iron atom with stabilization of the Fe(IV) state by an oxo atom derived from the peroxide to form an oxyferryl center, Fe(IV)=O (7, 8), while the second electron is removed from a porphyrin orbital to form a $\pi$-cation radical (1, 2).

Horseradish peroxidase compound I oxidizes a wide variety of small molecules, while BLC and other catalases exhibit a pronounced catalatic activity in which compound I selectively oxidizes a second molecule of hydrogen peroxide to molecular oxygen in each round of catalysis. A high valent species similar to compound I is believed to be an active intermediate in the catalytic cycle of other heme enzymes such as cytochrome P450, and the detailed elucidation of the structure and reactivity of such species has been the subject of intensive study.

A question of long standing with regard to the electronic structure of horseradish peroxidase and BLC compound I species has been the nature of the porphyrin radical and the distribution of radical spin density. The radical can be formed formally by removal of an electron from either of the nearly degenerate $a_{1u}$ or $a_{2u}$ orbitals of the porphyrin to give the $^1\Lambda_{\text{a}}$, or $^1\Lambda_{\text{a}}$, ground state configurations, respectively. The relative energies of these two orbitals are sensitive to the axial ligands and peripheral substitution of the porphyrin (9–11). EPR data show that Mg(II) or Zn(II) tetraphenylporphyrin (TPP) $\pi$-cation radicals with no axial ligands have $^1\Lambda_{\text{a}}$, ground states, while the corresponding complexes with octaethylporphyrin (OEP) give $^1\Lambda_{\text{a}}$, ground states (9, 12). The $^1\Lambda_{\text{a}}$, complexes have a distinct band in their optical spectra near 670 nm that was originally thought to be characteristic of this radical ground state. Since the $\pi$-cation radical of Co(III)(OEP)(Br)$_2$ exhibits a band in its optical spectrum at 670 nm, but that of Co(III)(OEP)(ClO$_4$)$_2$ does not, these species were originally thought to be $^1\Lambda_{\text{a}}$, and $^1\Lambda_{\text{a}}$, radicals, respectively. This suggested that the radical ground state configuration could be modulated by the axial ligands (13). Based on the similarity of the optical spectra of the BLC and horseradish peroxidase compound I species to those of the $\pi$-cation radicals of Co(III)(OEP)(Br)$_2$ and Co(III)(OEP)(ClO$_4$)$_2$, they were also assigned as $^1\Lambda_{\text{a}}$, and $^1\Lambda_{\text{a}}$, $\pi$-cation radicals, respectively, where

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The abbreviations used are: BLC, bovine liver catalase; rR, resonance Raman; OEP, octaethylporphyrin; TPP, tetraphenylporphyrin; mW, milliwatt(s).

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the difference in ground state configuration was attributed to the different proximal ligands in these two enzymes (13–18).

More detailed spectroscopic studies of porphyrin π-cation radicals, however, have shown that their optical spectra are not a reliable indicator of their ground state configuration (19–23). In addition, evidence now exists for the mixing of the 3A_1g and 3A_2g ground states in certain π-cation radical species (19, 22–24), indicating the appropriateness of referring to the “predominant character” of these species. Important to the subject of this investigation is the report that the π-cation radical of Co(III)(OEP)(ClO_4)_2 is now believed to be predominantly 3A_1g in character (23). Thus, the predominant radical ground state assignments for the horseradish peroxidase and BLC compound I species have remained unsettled.

A technique with the potential to contribute meaningfully to this issue is resonance Raman (rR) spectroscopy, since it can monitor the strengths of porphyrin bonds that are predicted to be differently strengthened or weakened in 3A_2g versus 3A_1g π-cation radicals. Indeed, a number of rR studies on model porphyrin π-cation radicals have been carried out (21, 22, 25–30) and definitive trends established. Unfortunately, however, the study of the horseradish peroxidase and BLC compound I species by the rR technique has been greatly hampered by the considerable photolability (31–33) of these species compared to model porphyrin π-cation radicals. Many different attempts to record the rR spectrum of horseradish peroxidase compound I have been made (34–43), and there is currently no agreement as to the correct spectrum of this species. Conversely, no studies of BLC compound I have been reported. The acquisition of the rR spectrum of compound I species is critically influenced by the sample handling and laser excitation procedures. Accordingly, a number of different techniques including continuous versus pulsed laser irradiation, visible versus ultraviolet excitation, and interrogation of the sample in a spinning cell, continuous flowing stream or microdroplet stream have been employed to study horseradish peroxidase compound I. In an effort to clarify conflicting results in the literature, we have systematically investigated the rR spectrum of horseradish peroxidase compound I using a variety of these methods while extending them to the study of BLC compound I.

EXPERIMENTAL PROCEDURES

Materials—BLC (type C-100) and horseradish peroxidase (type IV) were obtained from Sigma, and both were purified further by chromatography over Sephacryl S-200. Their concentrations were determined spectrophotometrically using ε_{max} = 1.0 × 10^3 and ε_{max} = 9.5 × 10^3 M^{-1} cm^{-1}, respectively. D_2O (99%) and peracetic acid (32% w/v) were purchased from Aldrich and reagent grade H_2O_2 (30% v/v) from J. T. Baker Chemical Co. H_2O (99% g) and D_2O were obtained from ICON Services, Inc. H_2O_2 was prepared by oxidation of 2-ethylanthraquinol in xylene/acetophenone/octanol (13:43:34 weight %) with excess H_2O_2 (44) followed by extraction with degassed H_2O under nitrogen in a glove box. The 2-ethylanthraquinol solution was prepared in situ by hydrogenation of 2-ethylanthraquinone in the presence of 2% Pd-AI_2O_3. The product H_2O_2 was confirmed from its Raman spectrum (45) and its concentration determined by iodometry. BLC compound I was prepared by addition of a 25-fold excess of peracetic acid to ferri-BLC, while horseradish peroxidase compound I was produced by addition of a 1.5-fold excess of hydrogen peroxide to ferri-horseradish peroxidase.

Pumping-New methods—The optical spectra of all samples were recorded with a Varian model 210 or 219 spectrophotometer in 0.1- or 1-cm path length quartz cuvettes. The optical spectra of all species were examined both before and after the rR spectra were recorded. All rR spectra were recorded with a Spex model 1877 Triplemate equipped with 600 grooves/mm gratings in the filter stage and 1200, 1800, or 2400 grooves/mm holographic gratings in the spectrograph stage. The detection system consisted of a model 1421 intensified diode array (EG&G Princeton Applied Research Corp.) used with a model 1461 detector interface. Most spectra are the sum of ten 1-min scans and were excited with either the 406.7-nm line of a Spectra Physics model 2020 krypton ion laser or the 514.5-nm line of a Coherent Radiation Innova model 90 argon ion laser. For some experiments, pulsed excitation (404 nm, 10 ns pulses, 0.1 mJ/pulse, 10 Hz) was employed by pumping Exciton DCM laser dye in a Quanta Ray Pulsed Dye Laser with the third harmonic output (355 nm) from a Quanta Ray DCR 2A Nd:YAG laser. In all cases, the samples were illuminated in a 90° scattering geometry. The spectra were recorded with powers of 1–20 mW at an instrumental resolution of 6 cm^{-1}.

Three different procedures were used to illuminate the samples. In the first arrangement, samples were held in a cell stirred from above and cooled by contact with a Peltier cooler. Second, some cooled samples were forced through a quartz flow cell with a syringe pump and examined as a continuous stream whose linear velocity was controlled by the pump. Third, a microdroplet generator similar to that described by Kincaid and associates (46, 47) has been constructed and used to examine the sample as a microdroplet stream. In this apparatus, the compound I species are prepared by using compressed nitrogen to force the enzyme and peroxide solutions held in separate chambers of a Plexiglas sample holding cell through a Durrum mixing jet, a 10-μm membrane filter, a 22-gauge stainless steel syringe needle, and a 100-μm-diameter, 12-cm-long, fused-silica capillary tube (diameter of the tip, 50–75 μm). The capillary tube is inserted through and glued into a hole in a rectangular piezoelectric strip that is connected to a variable frequency oscillator employing a circuit described by Seymour and Boss (48). The application of a square wave to the bimorph results in a regular axial perturbation along the jet stream and formation of a uniform droplet stream. The sample is delivered to the droplet generator at various linear velocities and delay times determined by the pressure and length of tubing connecting the sample holding cell and steel syringe, respectively. The residence time of the sample in the beam could be varied from approximately 1–100 μs.

RESULTS

Predicted Effects of 3A_1g Versus 3A_2g π-Cation Radical Formation on Porphyrin Skeletal Stretching Frequencies—The goal of this study is to record the rR spectra of the horseradish peroxidase and BLC compound I species and to use these spectra to establish whether the π-cation radicals in these intermediates have predominantly the 3A_1g or 3A_2g ground state configurations. The skeletal stretching frequencies of the heme group are sensitive to the coordination number and spin state (36) of the iron atom (50). Accordingly, the determination of the radical ground state from rR data is made by comparing the frequencies of the porphyrin skeletal stretching bands for species with the same coordination sphere and spin state before and after oxidation of the porphyrin ring. Thus, the relevant parameter is the rR frequency shift on oxidation, Δ_rR, and is obtained for these enzymes by comparing the compound I frequencies with those of the analogous compound II species. Since the heme group in both compounds I and II are six coordinate low spin, they differ primarily with respect to the porphyrin oxidation state. In order to provide a basis for interpreting the Δ_rR values determined below, it is informative to first review briefly the trends expected for formation of 3A_1g versus 3A_2g radicals.

To a first approximation, the effects of removal of an electron from an a_1g versus a_2g orbital of a metalloporphyrin on a particular skeletal stretching mode can be understood by considering the molecular orbital phasing diagrams for these two orbitals (51) in conjunction with the internal coordinates (52, 53) that are the major contributors to each mode (21, 22, 25, 26). Modes π_2 and π_11 involve mainly C=C bond stretching. The a_1g orbital is antibonding with respect to the C=C bond, and removal of an electron from this orbital is predicted to increase the C=C bond length and raise the frequencies of modes π_2 and π_11. In contrast, the a_2g orbital is bonding with respect to the C=C bond, and removal of an electron should weaken these bonds and decrease the frequencies of these
modes. Modes $v_{20}$, $v_{29}$, and $v_{1}$ are characterized by predominant C=C=C stretching character. The $a_{2u}$ orbital is bonding with respect to the C=C=C bond, and removal of an electron is predicted to decrease the frequencies of these four modes. The $a_{3u}$ orbital is formally nonbonding with respect to the C=C=C bond, but bonding with respect to the C=C=C bonds. Thus, a downshift of $v_{1}$ is expected on formation of an $^{1}A_{2u}$ radical. For the $a_{20}$ orbital, both the C=C=C and C=C=C interactions are antibonding and the shift in $v_{1}$ should be positive. These predicted signs for $\Delta v_{a}$ on formation of $^{1}A_{2u}$ and $^{3}A_{2u}$ cation radicals are summarized in Table I where they are compared with the values actually observed for a variety of four- and five-coordinate metalloporphyrins (21, 22, 26). With the exception of mode $v_{12}$ for $^{1}A_{2u}$ radicals, there is generally good agreement between the predicted and observed signs for $\Delta v_{a}$. Although the magnitudes of these $\Delta v_{a}$ values depend somewhat on the identity of the metal and the axial ligand(s), the directions of the shifts should be diagnostic toward distinguishing between the formation of a predominantly $^{1}A_{2u}$ versus $^{3}A_{2u}$ radical. In particular, mode $v_{1}$ shows large opposite shifts for these two species.

High Frequency rR Spectrum of HRP Compound I—The rR spectrum of horseradish peroxidase compound I has been obtained using several different sample handling and excitation techniques. The spectra of ferri-horseradish peroxidase (trace A), horseradish peroxidase compound I obtained by different procedures (traces B-E), and horseradish peroxidase compound II (trace F) acquired with Soret band excitation are shown in Fig. 1. The spectra of ferri-horseradish peroxidase and horseradish peroxidase compound II were obtained with continuous wave 406.7-nm excitation (10 mW) and are essentially identical with published spectra of these species (54). The same spectra were obtained whether the samples were examined in a spinning cell, continuous flowing stream, or microdroplet stream. The first rR spectrum of horseradish peroxidase compound I (trace B) was obtained using a pulsed laser (404 nm, 10 ns pulse, 0.1 mJ/pulse, 10 Hz repetition rate) with a continuously flowing sample at a flow rate that delivers fresh sample into the beam between laser pulses (residence time, 3 ms). If the rate of photoreduction of the sample were slower than 10 ns, then the correct rR spectrum of horseradish peroxidase I should be obtained. However, the spectrum represented by trace B is very similar to that reported by Oertling and Babcock (38, 41) using conditions similar to those employed here and appears to be a mixture of ferri-horseradish peroxidase and either compound II or a compound II-like photoprotein. Thus, the photoreduction process that depletes horseradish peroxidase compound I from the scattering element occurs faster than 10 ns.

In view of this, attempts have been made to overcome the photolability of compound I by using continuous wave excitation and minimizing the total number of photons absorbed by the sample in the volume element illuminated by the laser. To accomplish this, the sample has been directed through the beam (406.7-nm excitation, 10 mW) in the form of either a continuous (trace C) or microdroplet (traces D and E) stream to reduce its residence time. The linear velocities used for traces C-E give residence times of 3 ms, 130 ms, and 4.5 $\mu$s, respectively. Unfortunately, all of these spectra correspond most closely to previously published rR spectra of horseradish peroxidase compound II or a compound II-like photoprotein (37, 41, 54). There are reproducible changes in the intensities of the bands in the 1560–1610 cm$^{-1}$ region; however, that suggest that the composition of the photoinduced mixture is sensitive to the number of photons absorbed. Although spectrum E was obtained under conditions similar to those reported by Paeng and Kincaid (3.5-$\mu$s residence time, 10–50 mW) (42), the spectrum observed by us does not resemble theirs. With regard to this, it should be noted that there can be considerable difficulty in illuminating the droplet stream with a tightly focused beam, making the true photon field experienced by the sample difficult to assess. Thus, differ-

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**Table I**

Predicted and observed shifts in porphyrin skeletal stretching frequencies on oxidation of porphyrin ring to $^{1}A_{2u}$ and $^{3}A_{2u}$ cation radicals

<table>
<thead>
<tr>
<th>Mode</th>
<th>Assignment</th>
<th>Predicted</th>
<th>Observed$^\text{a}$</th>
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<tr>
<td>$v_{11}$</td>
<td>$\epsilon$(C=C)</td>
<td>Zero or negative</td>
<td>Negative</td>
</tr>
<tr>
<td>$v_{12}$</td>
<td>$\epsilon$(C=C)</td>
<td>Zero or negative</td>
<td>Negative</td>
</tr>
<tr>
<td>$v_{13}$</td>
<td>$\epsilon$(C=C)</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>$v_{14}$</td>
<td>$\epsilon$(C=C)</td>
<td>Zero or negative</td>
<td>Negative</td>
</tr>
<tr>
<td>$v_{15}$</td>
<td>$\epsilon$(C,N)</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

$^\text{a}$ Taken from Refs. 21, 22, and 26.

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**Fig. 1.** Comparison of the high frequency region of the rR spectra of ferri-horseradish peroxidase (A), horseradish peroxidase compound I (B–E), and horseradish peroxidase compound II (F). Samples were dissolved in 10 mM potassium phosphate, pH 7.5, at a concentration of 150 $\mu$M. All spectra were obtained at an instrumental resolution of 6 cm$^{-1}$. Spectra A and F were obtained with 406.7-nm excitation at a power of 10 mW. The same spectra were obtained whether the sample was examined in a spinning cell, continuous, or microdroplet stream. Spectra B and C were obtained with a flowing sample with a residence time of 3 ms, while spectra D and E were obtained on samples in a microdroplet stream with residence times of 130 and 4.5 $\mu$s, respectively. Spectrum B was obtained with pulsed 404-nm excitation (100 ns pulse, 0.1 mJ/pulse, 10 Hz) and C–E with continuous 406.7-nm excitation (10 mW).
ences between studies carried out under the same nominal conditions are not surprising.

To reduce the absorption by the sample further, rR spectra with 406.7-nm excitation have been acquired with a 1.5-μs residence time at lower powers. The rR spectra of horseradish peroxidase compound I (traces A–C) obtained with powers of 1, 3, and 20 mW, respectively, and horseradish peroxidase compound II (trace D) obtained with 10 mW are shown in Fig. 2. Again, the spectrum obtained for compound I excited with 20 mW of power (trace C) is very similar to that of horseradish peroxidase compound II (trace D). However, as the power is reduced from 20 to 3 to 1 mW, the spectrum obtained for compound I gradually changes. There is a monotonic shift in the 1378 band to 1376 cm⁻¹, in the 1509 band to 1504 cm⁻¹, in the 1632 band to 1634 cm⁻¹, and a collapse in the doublet near 1563/1584 to a band at 1574 cm⁻¹. The best rR spectrum of horseradish peroxidase compound I that we have been able to acquire with 406.7-nm excitation was with a power of 1 mW and a residence time of 1.0 μs. This spectrum (trace B) is compared with that of ferri-horseradish peroxidase (trace A), and horseradish peroxidase compound II (trace C) in Fig. 3.

The bands observed for horseradish peroxidase compound I at 1376, 1504, 1574, and 1634 cm⁻¹ are assigned to the symmetric porphyrin modes $v_4$, $v_3$, $v_2$, and $v(C=C)$, respectively, where the nomenclature is that of Abe and co-workers (52). Relative to compound II, modes $v_4$, $v_3$, and $v_2$ for compound I are shifted by −3, −6, and −13 cm⁻¹, respectively. The directions of these shifts indicate that they are due to formation of an $^1A_{2u}$ π-cation porphyrin radical (Table I). The spectrum of compound I shown in Fig. 3 is now similar to that reported by Paeng and Kincaid (42) who report $\Delta v(C=C)$ values of −10, −7, and −15 cm⁻¹, respectively. Interestingly, the spectrum of compound I is similar to that of ferri-horse-
as radish peroxidase. This is because the downshifts predicted relative to compound II due to $^1A_{2u}$ π-cation radical formation are all in the same direction as those that accompany the core expansion associated with conversion to the five-coordinate high spin ferri-horseradish peroxidase (49).

![Fig. 2. Comparison of the high frequency region of the rR spectra of horseradish peroxidase compound I (A–C) and horseradish peroxidase compound II (D) obtained with excitation at 406.7 nm. Samples were dissolved in 10 mM potassium phosphate, pH 7.5, at a concentration of 150 μM. Spectra were obtained at an instrumental resolution of 6 cm⁻¹ at powers of 1 (A), 3 (B), 20 (C), and 10 (D) mW with a residence time of 1.5 μs.](image-url)

It is known from earlier studies of horseradish peroxidase compound I that irradiation of a frozen solution with white light changes the optical spectrum to a horseradish peroxidase compound II-like photoproduct (32). This is most likely the same photoconversion observed here (Fig. 1). The most effective wavelengths for this photoconversion were reported to be in the 320–450-nm region (32), suggesting that one might be able to overcome the photolability with Q-band excitation. This was indeed the case and the rR spectra of ferri-horse-
as radish peroxidase and horseradish peroxidase compounds I and II (traces A–C, respectively) obtained with 514.5-nm excitation (10 mW) as continuous streams (residence time, 450 μs) are shown in Fig. 4. The spectrum of compound I is noisy, but can be seen to exhibit bands at 1373, 1552, 1572, and 1636 cm⁻¹ that are assigned to porphyrin modes $v_4$, $v_3$, $v_1$, and $v_{10}$, respectively. These bands are shifted by −6, −8, −14, and −5 cm⁻¹, respectively, with respect to compound II. Again, with the exception of $v_{10}$, the downshifts observed for these modes reduce them almost to the values observed for the five-coordinate high spin ferric enzyme. However, the differences in the spectra of the more intense bands of compound I from those of the ferric enzyme are reproducible. We are not aware of any reports of the direct photoinduced conversion of horseradish peroxidase compound I to the five-coordinate high spin ferric enzyme. In an earlier study, we showed that the photoreduction appears to proceed in a series of one-electron steps in the sequence horseradish peroxidase compound I → compound II-like product → ferri-horseradish peroxidase → ferro-horseradish peroxidase (37). In contrast, the data in Figs. 1–3 show that a reduction in absorption by the sample results in the compound II-like spectrum switching to the ferri-horseradish peroxidase-like spectrum that we attribute to compound I. There has been a report that photolysis of horseradish peroxidase compound I yields a six-coordinate low spin ferric form of horseradish peroxidase (33). However, the rR spectrum that we attribute to horseradish peroxidase compound I is not consistent with such a species. Thus, the resemblance of the rR spectrum of horseradish

![Fig. 3. Comparison of the high frequency region of the rR spectra of ferri-horseradish peroxidase (A), horseradish peroxidase compound I (1 mW with a 1.0-μs residence time) (B), and horseradish peroxidase compound II obtained with excitation at 406.7 nm (C). Samples were dissolved in 10 mM potassium phosphate, pH 7.5, at a concentration of 150 μM. Spectra were obtained at an instrumental resolution of 6 cm⁻¹.](image-url)
peroxidase compound I to resting horseradish peroxidase is fortuitous.

A comparison of the high frequency rR bands of horseradish peroxidase compound I and II detected with both Soret and Q-band excitation is shown in Table II. The directions of the shifts observed for modes \(v_2\) and \(v_4\) are substantially smaller than those observed for most of the model porphyrins that have been reported in the literature (Table I). This is consistent with NMR data for horseradish peroxidase compound I that indicate that some of the radical density is delocalized onto the proximal His-170 residue (55). This reduction in unpaired spin density on the porphyrin should reduce the values of \(\Delta \nu_{ox}\) expected for this species and explain why the \(\Delta \nu_{ox}\) values observed here are lower than for the models cited in Table I. It is noteworthy that none of the models for which \(\Delta \nu_{ox}\) values are cited have an aromatic axial ligand and, thus, are not good models for horseradish peroxidase. In contrast, however, we have recently reported the rR spectrum of the compound I analog of \(N_\alpha\)-acetyl microperoxidase-8 which has a His residue as a proximal ligand (56). Relative to its compound I analog, modes \(v_4, v_5, v_6, \) and \(v_{10}\) were shifted by -5, -17, -16, and -3 cm\(^{-1}\), respectively, establishing that it is a predominantly \(1^2\)A\(_{2u}\) radical. With the exception of the \(v_1\) band (that is broad in \(N_\alpha\)-acetyl microperoxidase-8 and hard to assign a frequency), the magnitude of these shifts are much closer to those observed here for horseradish peroxidase compound I than those for the models cited in Table I. This implies that a His axial ligand may function as a site of radical delocalization.

**Low Frequency rR Spectrum of Horseradish Peroxidase Compound I**—The oxyferryl stretching region of the rR spectra of ferri-horseradish peroxidase (trace A) and horseradish peroxidase compound I (traces B–D) obtained with 406.7-nm excitation in a microdroplet stream with a residence time 1.0 \(\mu s\) are shown in Fig. 5. The spectrum of ferri-horseradish peroxidase is essentially identical with published spectra (57–59). The spectra of horseradish peroxidase compound I prepared with either \(H_2^{18}O_2\) (trace B) or \(H_2^{16}O_2\) (trace C) obtained with a power of 20 mW at pH 7.5 are similar to those published for horseradish peroxidase compound II (57, 59). A weak \(\nu(Fe=O)\) band is observed at 776 cm\(^{-1}\) in the spectrum of the sample prepared with \(H_2^{16}O_2\) that is replaced by a band at 745 cm\(^{-1}\) in the sample prepared with \(H_2^{18}O_2\). Once again, this shows that horseradish peroxidase compound I converts to a horseradish peroxidase compound II-like species at high laser power. The spectra obtained with a power of 1 mW, however, show different features (traces D and E). There appears to be a shoulder on the 717 cm\(^{-1}\) band in trace D at \(-721\) cm\(^{-1}\) that could be due to a \(\nu(Fe=^{18}O)\) band. This band is replaced by a \(\nu(Fe=^{16}O)\) band at \(-690\) cm\(^{-1}\) in trace E. This observation is similar, but not in good quantitative agreement with, that of Paeng and Kincaid (42) who report better defined bands with frequencies of 737 and 698 cm\(^{-1}\), respectively. If the 721 cm\(^{-1}\) band is truly a \(\nu(Fe=O)\) band, its 55-cm\(^{-1}\) downshift upon \(\pi\)-cation radical formation implies a substantial weakening of the Fe=O bond in horseradish peroxidase compound I.

**rR Spectra of BLC Compound I**—A similar approach has been used to study BLC compound I. The rR spectra of ferri-BLC (trace A), BLC compound I (traces B and C), and BLC compound II (trace D) obtained with 406.7-nm excitation (2 mW) with samples in continuous (trace B) and microdroplet (traces A, C, D) streams are shown in Fig. 6. The spectra of ferri-BLC and BLC compound II are essentially identical with published spectra (60, 61). As with horseradish peroxidase compound I, the rR spectra of BLC compound I show a residence-time dependence. The spectrum in trace B (3-ms residence time) most closely resembles ferri-BLC, while trace C (130-\(\mu s\) residence time) appears to be almost completely a compound II-like photoproduct.

When the residence time is decreased to 1.0 \(\mu s\), however, the true rR spectrum of BLC compound I is obtained (Fig. 7, trace B). The bands at 1371, 1506, \(-1500, 1571,\) and 1634 cm\(^{-1}\) are assigned to porphyrin modes \(v_4, v_5, v_{11}, v_2,\) and \(v_{10}\) [overlapped with \(\nu(C=C)\)], respectively (Table III). These frequencies indicate that BLC compound I has its bands shifted by \(-4, -7, -12, -12,\) and \(-6\) cm\(^{-1}\), respectively.

![Fig. 4. Comparison of the high frequency region of the rR spectra of ferri-horseradish peroxidase (A), horseradish peroxidase compound I (B), and horseradish peroxidase compound II (C) obtained with excitation at 514.5 nm examined as a continuous stream with a residence time of 450 \(\mu s\).](image)

**Table II**

<table>
<thead>
<tr>
<th>Mode</th>
<th>Assignment</th>
<th>Ferrhorseradish peroxidase</th>
<th>Horseradish peroxidase-I</th>
<th>Horseradish peroxidase-II</th>
<th>(\Delta \nu_{ox})</th>
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<tr>
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<td>(\nu(C,C_m))</td>
<td>1629</td>
<td>1641</td>
<td>1636</td>
<td>-5</td>
</tr>
<tr>
<td>(v_2)</td>
<td>(\nu(C,C_m))</td>
<td>1571</td>
<td>1586</td>
<td>1572</td>
<td>-14</td>
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<tr>
<td>(v_4)</td>
<td>(\nu(C,C_m))</td>
<td>1574</td>
<td>1587</td>
<td>1575</td>
<td>-12</td>
</tr>
<tr>
<td>(v_{11})</td>
<td>(\nu(C,C_m))</td>
<td>1549</td>
<td>1560</td>
<td>1552</td>
<td>-8</td>
</tr>
<tr>
<td>(v_1)</td>
<td>(\nu(C,C_m))</td>
<td>1499</td>
<td>1510</td>
<td>1505</td>
<td>-5</td>
</tr>
<tr>
<td>(v_0)</td>
<td>(\nu(C,N) + \nu(C,C_m))</td>
<td>1374</td>
<td>1379</td>
<td>1373-1376</td>
<td>-3 to -6</td>
</tr>
<tr>
<td>(\nu(Fe=O))</td>
<td></td>
<td></td>
<td></td>
<td>776</td>
<td>721</td>
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</table>
Resonance Raman Spectra of Compound I Species

FIG. 5. Comparison of the oxyferryl stretching region of the rR spectra ferri-horseradish peroxidase (A) and horseradish peroxidase compound I (B-E) obtained with excitation at 406.7 nm in a microdroplet stream (1.0-μs residence time). Samples were dissolved in 10 mM potassium phosphate, pH 7.5, at a concentration of 150 μM. Spectra were obtained at an instrumental resolution of 6 cm⁻¹ at powers of 10 (A), 20 (B and C), and 1 mW (D and E).

FIG. 6. Comparison of the high frequency region of the rR spectra of ferri-BLC (A), compound I (B and C), and BLC compound II (D) obtained with excitation at 406.7 nm (10 mW). Samples were dissolved in 10 mM potassium phosphate, pH 7.5, at a concentration of 150 μM. Spectra were obtained at an instrumental resolution of 6 cm⁻¹ with a residence time of 1 μs (A), 3 ms (B), 130 μs (C), and 2 ms (D).

relative to BLC compound II (trace C). The shifts are also in the direction that makes the spectrum of compound I more like that of the ferric enzyme (trace A). Unlike horseradish peroxidase, however, the spectrum of BLC compound I is quite distinct from that of ferri-BLC, particularly with respect to the p₁ band and the proximal Tyr-357 mode at 1618 cm⁻¹ (see below). The directions of the Δνmax shifts for BLC compound I (Table III) are the same as those observed for horseradish peroxidase compound I (Table II), indicating that it is also a predominantly A₂ ground-state cation porphyrin radical (Table I). More interesting, however, is the observation that the magnitudes of the shifts for the horseradish peroxidase and BLC compound I species are very similar and that both are smaller than those observed for model porphyrins that do not have aromatic axial ligands. This implies that the porphyrin radical in BLC compound I may also be partially delocalized, most likely onto the proximal Tyr-357 residue. No new bands could be reproducibly detected for BLC compound I in the oxyferryl stretching region, while severe problems with fluorescence prevented spectra with Q-band excitation from being acquired.

In addition to the porphyrin bands discussed above, bands are reproducibly observed for BLC compound I with 406.7-nm excitation at 1618 and 1450 cm⁻¹ (Fig. 7, trace B). Moreover, the rR spectrum of BLC compound I in the 500-1300 cm⁻¹ region (Fig. 8, trace A) shows that there is an intense band at 598 cm⁻¹ and a shoulder near 1245 cm⁻¹ that are not present in BLC compound I. Three of these four bands are very close in frequency to those observed in Hb M Boston and Iwate (62), native ferric catalases (60, 63), and other Fe(III)-tyrosinate species (64). These bands have been attributed to the tyrosinate ligand in these proteins. Using the nomenclature for benzene normal mode vibrations (6.5), the tyrosinate bands at 1618, 1245, and 598 cm⁻¹ are assigned to modes v₁, v₂, and v₃, respectively. The band at 1450 cm⁻¹ is considerably lower in frequency than the value of approximately 1500 cm⁻¹ observed for tyrosinate mode v₄ in other ferric tyrosinate proteins (64). Thus, it is not clear whether this is due to a tyrosinate mode or possibly a porphyrin mode.

![Graph of Raman Shift vs Intensity](image1)

![Graph of Raman Shift vs Intensity](image2)

**TABLE III**

Comparison of rR spectra of BLC species at pH 7.5

<table>
<thead>
<tr>
<th>Mode</th>
<th>Assignment</th>
<th>Ferri-BLC</th>
<th>BLC-II</th>
<th>BLC-I</th>
<th>Δνmax</th>
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<tbody>
<tr>
<td>p₁₀</td>
<td>r(C,C)</td>
<td>1626</td>
<td>1640</td>
<td>~1634</td>
<td>~6</td>
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<tr>
<td>p₂</td>
<td>r(C,C)</td>
<td>1570</td>
<td>1563</td>
<td>1571</td>
<td>-12</td>
</tr>
<tr>
<td>p₁₁</td>
<td>r(C,C)</td>
<td>1552</td>
<td>1562</td>
<td>~1550</td>
<td>-12</td>
</tr>
<tr>
<td>p₃</td>
<td>r(C,C)</td>
<td>1487</td>
<td>1513</td>
<td>1506</td>
<td>-7</td>
</tr>
<tr>
<td>p₄</td>
<td>r(C,C) + r(C,C)</td>
<td>1373</td>
<td>1375</td>
<td>1371</td>
<td>-4</td>
</tr>
</tbody>
</table>

[Diagram 1: Graph showing Raman Shift vs Intensity]

[Diagram 2: Graph showing Raman Shift vs Intensity]
such as $\nu_{329}$. In the ferric proteins, these tyrosinate bands are resonance enhanced by excitation within an Fe(III)-tyrosinate charge transfer band. In ferri-BLC enhancement only occurs with wavelengths in the 470-500-nm range (60). The unexpected enhancement of these modes with 406.7-nm excitation in BLC compound I suggests either that there is an Fe(IV)-tyrosinate charge transfer transition under the Soret band or that the delocalization of the $\pi$-cation radical onto the proximal Tyr-357 ligand is responsible for the enhancement.

**DISCUSSION**

The structures of horseradish peroxidase and BLC compound I species, which are deficient by two electrons from the resting ferric enzymes, have been the subject of numerous studies. Mosbach's spectroscopy first revealed the presence of low spin Fe(IV) in horseradish peroxidase compound I (7), establishing that the first oxidizing equivalent is stored on the iron atom. Dolphin and co-workers (9, 12, 13, 15-18) subsequently suggested that the second oxidizing equivalent in these species resides on the porphyrin macrocycle. EPR data provided support for the facile formation of $\pi$-cation radicals on oxidation of a wide range of metallo-TPP and -OEP derivatives (9, 10, 12). Since the relative energies of the top-filled $a_{2u}$ and $a_{g}$ orbitals depend upon the structure of the porphyrin and on its axial ligands (9-11), two distinct ground state configurations are possible for the resultant $\pi$-cation radicals. Early conclusions regarding the $\pi$-cation radical ground state based on optical spectra are now recognized to be unreliable, emphasizing the need to address this question by other techniques.

A variety of spectroscopic studies including EPR (20), electron-nuclear double resonance (66), NMR (67), and magnetic circular dichroism (24) investigations, as well as theoretical studies (10, 11, 68), all favor a predominantly $2A_{2u}$ $\pi$-cation radical formulation for horseradish peroxidase compound I. In principle, $rR$ spectroscopy can also distinguish between $2A_{2u}$ and $2A_{2g}$ $\pi$-cation radicals, since the porphyrin vibrational frequencies observed for these species reflect the differences in the bond strengths that arise from differences in electron distribution. Predictions based on these principles generally agree well with the shifts in frequency actually observed on oxidation of model porphyrins to $2A_{2u}$ and $2A_{2g}$ $\pi$-cation radicals (Table I). Accordingly, several laboratories have attempted to use these trends to study the $\pi$-cation radical character of horseradish peroxidase compound I using $rR$ spectroscopy (38, 40-43). However, the spectra reported for compound I and the conclusions of these studies are in substantial disagreement.

A comparison of the different $rR$ spectra reported for horseradish peroxidase compound I is shown in Table IV. Studies employing either pulsed (41) or continuous (40) Soret band excitation of flowing samples with residence times in the millisecond range have reported compound II-like $rR$ spectra for horseradish peroxidase compound I. Ogura and Kitagawa (40) have attributed this to the true spectrum of horseradish peroxidase compound I and hypothesized that it resembles compound II because of extensive delocalization of the radical onto the axial ligands. Oertling and Babcock (41) offered this as one possible explanation, but also suggested that the radical delocalization might be photoinduced and, thus, the spectrum obtained was actually that of a photoprotein. Since both groups acquired their spectra under conditions shown here to yield that of a compound II-like photoprotein, the latter explanation seems more likely. In contrast, Paeng and Kincaid have reported a $rR$ spectrum for horseradish peroxidase compound I examined as a microdroplet stream with a residence time in the microsecond range (42). This technique minimizes problems due to photochemistry and gives $\Delta v_{329}$ shifts for three porphyrin skeletal stretching bands that are indicative of $2A_{2g}$ $\pi$-cation radical formation. The data reported here are most similar to those of Paeng and Kincaid (42) and indicate substantial $2A_{2g}$ character for horseradish peroxidase compound I, in agreement with other experimental (20, 24, 66, 67) and theoretical (10, 11, 68) studies.

Horseradish peroxidase compound I appears to exhibit a weak band near 721 cm$^{-1}$ whose shift on $^{18}$O substitution (31 cm$^{-1}$) is appropriate for a $\nu(\text{Fe=O})$ mode. In our spectra, this band is weaker and occurs at a slightly lower frequency than that observed by Paeng and Kincaid (42). If this band is truly a $\nu(\text{Fe=O})$ band, then the downshift of 39-55 cm$^{-1}$ observed for horseradish peroxidase compound I relative to compound II by us and Paeng and Kincaid (42) indicates that there is a substantial weakening of the Fe=O bond upon $2A_{2u}$-like radical formation. This contrasts with the upshift of ~15 cm$^{-1}$ observed by Oertling and Babcock for the compound II-like photoprotein (41). Since compound I-like species can oxygenate substrates, but compound II-like species cannot, this reduction in Fe=O bond strength in the former could signal their greater oxygen-transfer potential. Unfortunately, there is not yet agreement as to the effect of $2A_{2u}$ $\pi$-cation radical formation on the frequency of the $\nu(\text{Fe=O})$ mode of model metalloporphyrins (69-71). Thus, it is not presently possible to compare the large downshift in $\nu(\text{Fe=O})$ observed for horseradish peroxidase compound I with that for other species.

The $rR$ data presented here indicate that BLC compound I also has a predominantly $2A_{2u}$ $\pi$-cation radical character. This contradicts an earlier assignment based on its optical spec-

**TABLE IV**

<table>
<thead>
<tr>
<th>Mode</th>
<th>Refs.</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\nu_{329}$</td>
<td>1636</td>
<td>1632</td>
</tr>
<tr>
<td>$\nu_{329}$</td>
<td>1572</td>
<td></td>
</tr>
<tr>
<td>$\nu_{330}$</td>
<td>1569</td>
<td>1506</td>
</tr>
<tr>
<td>$\nu_{330}$</td>
<td>1545*</td>
<td>1570</td>
</tr>
<tr>
<td>$\nu_{330}$</td>
<td>1502</td>
<td>1504</td>
</tr>
<tr>
<td>$\nu_{330}$</td>
<td>1369</td>
<td>1359</td>
</tr>
<tr>
<td>$\nu(\text{Fe=O})$</td>
<td>737</td>
<td>791</td>
</tr>
</tbody>
</table>

* Band was reported but not assigned in Ref. 42.
The finding that both the horseradish peroxidase and BLC compound I species are predominantly $\Sigma_{\Delta a_{\pi}}$ $\pi$-cation radical species refocuses attention on the importance of the axial ligands in influencing the electron distribution in metalloporphyrins. The $\Delta_{\sigma}$ values observed for model metalloporphyrins that do not have strong field axial ligands indicate that all of the TPP radicals have predominantly $\Sigma_{\Delta a_{\pi}}$ character, while all of the OEP radicals have predominantly $\Sigma_{\Delta a_{\pi}}$ character (21, 22). The axial ligands, however, can reverse the relative orbital energies. Extended Hückel calculations indicate that the $\Delta_{\sigma}$ orbital is stabilized relative to the $\Delta a_{\pi}$ orbital when the donor strength of the axial ligands is increased (10, 11). The $\Delta a_{\pi}$ orbital concentrates electron density on the pyrrole N atoms, while the $\Delta a_{\sigma}$ orbital has nodes at these positions. Thus, although the electropositive metal ion stabilizes the $\Delta a_{\sigma}$ orbital more than the $\Delta a_{\pi}$ orbital, this tendency is countered by electron donation from the ligand. Increasingly strong donor ligands destabilize the $\Delta a_{\pi}$ orbital via this polarization effect and possibly also by direct interaction with filled ligand $\pi$ orbitals (e.g. imidazole, thiolate, tyrosinate). The porphyrin groups in BLC and horseradish peroxidase are both protoporphyrin IX which has a substituent pattern similar to OEP. Thus, in the absence of a strong axial field, the $\pi$-cation radicals in these species would be expected to have predominantly $\Sigma_{\Delta a_{\pi}}$ character. Apparently, the donor effects of the oxo ligand on one side, and the proximal histidine or tyrosinate ligand on the other side, are sufficient to reverse the $\Delta a_{\pi}$ and $\Delta a_{\sigma}$ orbital energies and stabilize the $\Sigma_{\Delta a_{\pi}}$ $\pi$-cation radical in horseradish peroxidase and BLC compound I species, respectively.

The influence of axial ligation in determining the $\pi$-cation radical ground state may explain the single report that horseradish peroxidase compound I is an $\Sigma_{\Delta a_{\pi}}$ radical (43). This report is based on a Raman spectrum of horseradish peroxidase compound I obtained using near ultraviolet excitation on a flowing sample with an unspecified residence time (43). The $\Delta_{\sigma}$ values reported in this study were very similar to those observed for four- and five-coordinate model metalloporphyrin $\Sigma_{\Delta a_{\pi}}$ $\pi$-radicals with weak axial fields (Table I). This finding would be explained if the ultraviolet excitation produced photodissociation of the proximal imidazole and/or oxo ligand(s). This would substantially weaken the axial field to give an $\Sigma_{\Delta a_{\pi}}$ $\pi$-radical and explain why the $\Delta_{\sigma}$ shifts agree with those observed for four- and five-coordinate OEP models. Although we have not attempted to investigate this possibility, we note that the photodissociation of endogenous ligands on laser excitation of heme proteins has been observed elsewhere (72, 73).

It is particularly interesting that the $\Delta_{\sigma}$ shifts observed on formation of both the horseradish peroxidase and BLC compound I species are considerably smaller than those observed for model metalloporphyrins that lack aromatic axial ligands. This is consistent with partial delocalization of the porphyrin radical onto the axial histidine and tyrosinate ligands in horseradish peroxidase and BLC, respectively. Indeed, NMR data have already provided evidence for this delocalization in horseradish peroxidase compound I (55). Extended Hückel calculations predict significant spin delocalization onto the axial ligand of $\Sigma_{\Delta a_{\pi}}$ radicals (11), and EPR data confirm that this delocalization occurs for model $\Sigma_{\Delta a_{\pi}}$ porphyrin $\pi$-cation radicals with an axial pyridine ligand (11). The $\Delta_{\sigma}$ shifts observed on formation of the compound I analog of N-acetyl microperoxidase-8 are very similar in magnitude to those observed here for horseradish peroxidase and BLC (66). This is the best known model for horseradish peroxidase compound I because it has a histidine residue and an oxo group as axial ligands. Collectively, these observations are consistent with the view that there is unpaired spin delocalization onto aromatic axial ligands of $\Sigma_{\Delta a_{\pi}}$ radicals in both model and enzymic species.

An observation of interest for BLC compound I is the resonance enhancement of modes of the proximal tyrosinate ligand with 406.1-nm excitation. These same modes are enhanced in Fe(III)-tyrosinate proteins via resonance with a tyrosinate $\rightarrow$ Fe(III) charge transfer band that lies near 490 nm for ferri-BLC (60). There may be a similar proximal tyrosinate $\rightarrow$ Fe(IV) charge transfer band in BLC compound I that is blue-shifted relative to that in ferri-BLC that is responsible for this resonance enhancement. Since these bands are not resonance enhanced with 406.1-nm excitation in BLC compound II which also contains the Fe(IV)-tyrosinate bond, an alternative explanation is that the delocalization of the $\pi$-cation radical density onto the proximal Tyr-357 ligand in compound I is responsible for this resonance enhancement. The tyrosinate band near 1618 cm$^{-1}$ is more intense and shifted slightly in frequency compared to ferri-BLC. Importantly, the observation of the resonance-enhanced tyrosinate bands for BLC compound I constitutes strong evidence that the proximal ligand remains bound and is not photodissociated during acquisition of the Raman spectra.

REFERENCES
