Resonance Raman Spectroscopy of Horseradish Peroxidase Derivatives and Intermediates with Excitation in the Near Ultraviolet*

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Resonance Raman enhancement of derivatives and intermediates of horseradish peroxidase in the near ultraviolet (N-band excitation) results in intensity and enhancement patterns that are different from those normally observed within the porphyrin Soret (B-band) and α-β (Q-band) absorptions. In particular it allows the resolution of resonance Raman spectra of horseradish peroxidase compound I. The bands above 1300 cm⁻¹ can be assigned to porphyrin vibrational modes that are characteristically shifted in frequency due to removal of an electron from the porphyrin ring. The resonance Raman frequency shifts follow normal mode compositions. Relative to resonance Raman spectra of compound II, the v₂ frequency (primarily C₅-N) exhibits a 20 cm⁻¹ downshift. The v₂, v₁₁, and v₃₇ vibrational frequencies whose mode compositions are primarily porphyrin C₅-C₅, exhibit 10–20 cm⁻¹ upshifts. The v₁₀, v₁₀, and v₃₈ frequencies, whose mode compositions are primarily C₅-C₅, exhibit downshifts. The downshifts for v₂ and v₁₀ are small, 3–5 cm⁻¹; however, the downshift for v₃₈ is 14 cm⁻¹. These frequency shifts are consistent with those of previously published resonance Raman studies of model compounds. In contrast to reports from other laboratories, the data presented here for horseradish peroxidase compound I can be attributed unambiguously to resonance Raman scattering from a porphyrin π-cation radical.

Horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is a heme-containing glycoprotein that catalyzes reactions of hydrogen peroxide and organic hydroperoxides (1, 2). The physiological intermediates of horseradish peroxidase are known as compounds I and II (3–5). Compound I is formed by a two-electron oxidation of the resting enzyme by peroxide and contains an Fe(IV) porphyrin π-radical cation heme (6, 7). A one-electron reduction of compound I results in the compound II intermediate which contains an Fe(IV) (ferryl) heme (8–10). In addition to interest in horseradish peroxidase compounds I and II as examples of activated heme oxidants which occur ubiquitously in biological systems (11), these intermediates are considered as models for the postulated intermediates of heme enzymes, such as cytochrome P-450, which have been difficult to observe spectroscopically (12).

Resonance Raman spectroscopy is currently being used to great advantage for the elucidation of structural aspects of the active site of horseradish peroxidase compound II (13–18) and ferryl-type activated hemes (19–25). However, recent reports of resonance Raman spectra of horseradish peroxidase compound I (26–30) have been inconsistent and at variance with resonance Raman spectra of metalloporphyrin π-radical cations (29, 31–35). Some of the recent reports (27–29) have been suggestive of a compound II-type ferryl heme, such that it has been thought that the radical of compound I might be extensively delocalized to the axial ligands (29). By contrast, in this report we present resonance Raman spectra of compound I that are, in fact, characteristic of model metalloporphyrin π-cation radicals. We attribute previous difficulties in resolving the resonance Raman spectra of compound I to photolability, the use of laser pulses with high photon flux and unfavorable excitation wavelengths which result in preferential enhancement of ferryl-type species and/or the resting enzyme.

The electronic absorptions of porphyrins are typified by three prominent bands. The most intense is the Soret absorbance (or B-band), centered near 400 nm. The visible absorbance near 550 nm is known as the α-β or Q-band. A third band, the N-band, occurs in the near ultraviolet. The origins of these bands have been discussed extensively (36–39). Soret and α-β excitation have been used for resonance Raman spectroscopy of heme proteins almost exclusively because of the strong resonance enhancements traditionally obtained in these wavelength regions (40, 41). Near ultraviolet excitation has been used only infrequently below 390 nm for resonance Raman studies of heme proteins (42–46). We have found it advantageous to make use of the decreased near ultraviolet enhancements of the horseradish peroxidase resting enzyme and compound II relative to the enhancement of compound I. It is our experience that strong resonance enhancements of the resting enzyme, compound II, or compound II-like species (even in small amounts) produce strong signals which overwhelm weak resonance Raman scattering from compound I under Soret excitation.

MATERIALS AND METHODS

Horseradish peroxidase (Type II, Sigma) was purified by DEAE- and CM-Sepharose (Pharmacia LKB Biotechnology Inc.) ion exchange chromatography following the procedures of Shannon et al. (47). Isoenzymes B and C were used predominantly since these have the largest abundance; however, identical results were obtained with isoenzymes A-1 and A-2. Compound I was formed by mixing (at 20 °C) equal volumes of a 2:1 (H₂O₂:heme) mole ratio of H₂O₂ (Fisher) in aqueous buffer with buffered (0.01 M sodium phosphate, pH 6.8)
horseradish peroxidase (800 μM) in a modified Ballou 4-jet mixer (49) fed by syringes driven from a Harvard Bioscience (South Natick, MA) model 975 syringe pump. The exit port of the mixer was a 1-cm length of 26-gauge hypodermic tubing which produced a horizontal jet stream of sample through the open air. The flow rate was 0.2 ml/s with a time delay of 30 ms between mixing and observation. The sample stream was collected by a small (10 ml) glass catch bottle. The sample jet was excited transversely relative to the resting (ferric) enzyme. The sample jet was excited transversely relative to a reservoir containing an excess of ascorbic acid, regenerating the sample (Sigma) solution. Resonance Raman spectra of the resting enzyme were obtained by continuously recirculating the enzyme through the mixer preliminary to mixing the enzyme with the H₂O₂ solution. The resonance Raman spectra shown in the figures are the sum of two or three 5-min vidicon exposures.

Resonance Raman spectra of compound II are only weakly enhanced relative to the resting enzyme under the near ultraviolet excitation wavelengths used for this report. Small contributions of the resting enzyme to the near ultraviolet resonance Raman spectra of compound II were removed by computer subtraction. The compound II resonance Raman spectra (Figs. 1c and 2c) were the only resonance Raman spectra shown in this report for which spectral subtraction procedures were employed.

The following horseradish peroxidase derivatives (200 μM, at pH 6.8, 0.01 M sodium phosphate, unless otherwise noted) were contained in a spinning quartz Raman cell (Spex Industries, Edison, NJ). Horseradish peroxidase ferricyanide was formed by adding crystals of potassium cyanide until the enzyme turned from brown to red. The ferric enzyme was formed by deoxyanion with N₂ followed by reduction with sodium dithione under N₂. The ferrocytochrome derivative was formed by the addition of potassium cyanide to the dithionite-reduced enzyme under N₂. The ferric fluoride derivative was formed by adding an excess of sodium fluoride to the resting enzyme. The ferrous-CO derivative was formed by stirring the dithionite-reduced ferrous enzyme under CO and by stoppering under a slight positive pressure of CO. Resonance enhancement of the five-coordinate ferrous enzyme, produced by photolysis of the ferrous-CO derivative, is relatively strong in the near ultraviolet, and has a tendency to overwhelm weaker scattering from the CO-ferrous derivative. It was necessary to use very low (1 milli Watt or less) laser power and high sample concentration (1.4 mM) to obtain the resonance Raman spectrum of the photolabile ferrous-CO derivative in the near ultraviolet.

The ferric-NO derivative was formed by deoxyanion of the enzyme with N₂ followed by gently stirring under NO causing the sample to change from brown to red (49). The ferric-NO derivative was formed either by dithionite reduction of the ferric-NO form or by gentle stirring of the ferrous enzyme under NO.

Compound III (oxygenferrous form) was formed by adding a 100-fold excess of H₂O₂ (50). The alkaline form of isoenzyme A-1 (hydroxycyanide) was formed by buffering at pH 10.5 in 0.02 M sodium carbonate (13). Complexes with benzohydroxamic acid (Fairfield Chemical Co., Blythewood, SC), flavone, and trimethylamino (Aldrich) (51, 52) were prepared by adding to the buffered ferric or ferrous enzyme, a small excess of these substrates dissolved in a minimum of 30% ethanol, or by incubation of the aqueous enzyme with a few crystals of the substrate.

Raman scattered light was collected by a 55-mm f/1.4 Rolleinol MC lens and imaged by a quartz 180-mm f/4.5 lens (Melles Griot, Irvine, CA) onto the slit of a 0.5-mm spectrophotograph (Spex model 1870) containing interchangeable 1800 and 2400 groove/mm holographic gratings (John-Yvon/Instruments SA, Edison, NJ) or a 1200 groove/mm ruled grating (Spex). The excitation sources were a Spectra-Physics (Mountain View, CA) model 171 krypton ion laser equipped with a model 210 argon-ion single-frequency laser (Spectra-Physics model 2025) with enhanced ultraviolet output. The detection system was an optical multichannel analyzer (EG&G Princeton Applied Research Corp., Princeton, NJ, model 1460V/1216) with a model 1254 silicon-intensified vidicon detector head.

Near ultraviolet laser wavelengths used were 3638, 3564, and 3507 Å. Frequency and assignments for intermediates and derivatives were verified with 5309 (α-β band), and 4131 and 4067 Å (Soret band) excitation, except for compound I, which we were only able to observe under ultraviolet excitation. Vibrational frequencies were calibrated with the known frequencies of indene (55) and cyclohexene. The resolution was 1 cm⁻¹. Band frequencies are accurate to ±1 cm⁻¹ for strong isolated bands. The band assignments and numbering system follow those given for the modes of nickel octahedralporphyrin and protoporphyrin hemes (54-57), and are listed in Table 1. The resolution of the Raman bands are located by frequency and enhancement patterns and band depolarization ratios (41). Aₘ modes are polarized, A₂ₚ modes are anomalously polarized, and B₁ₚ and B₂ₚ modes are depolarized. Infrared active Eₘ modes appear in the resonance Raman spectra due to the rotation of the porphyrin symmetry from strict D₄h symmetry by the vinyl groups and inequivalence of the axial ligands (58). Eₘ modes were measured to be polarized or depolarized, evidently due to decomposition to Raman active Aₘ or Bₘ-type symmetry components under the reduced symmetry environment.

Stopping base lines, due to fluorescent backgrounds in many of the ultraviolet excited resonance Raman spectra, were removed by computer subtraction of a straight diagonal line. No smoothing procedures were employed for any of the resonance Raman spectra shown in this paper.

RESULTS

Resonance Raman spectra of horseradish peroxidase derivatives with near ultraviolet excitation (3507, 3564, and 3638 Å), given in this report, show a modified intensity pattern relative to that observed under Soret excitation in the violet region of the visible spectrum (4067 and 4131 Å) (13). The near ultraviolet (N-band) excited resonance Raman spectra in the region from 1300 to 1700 cm⁻¹ are dominated by modes other than νₚ. The heme oxidation state marker, νₚ (Aₚ), is normally the most intense of the porphyrin modes under Soret excitation (41). We have noticed that certain modes which have substantial intensity under Soret excitation, such as A₁ₕ modes ν₁ (679 cm⁻¹ (14, 15)), ν₆, and ν₈ are usually very weak under ultraviolet excitation. However another A₂ₕ mode, ν₅, can have substantial intensity. ν₁₀ (Bₘ₈) is also noted to have high intensity relative to the other bands. ν₁, ν₁₈, and other modes above 1550 cm⁻¹ involve expansion and contraction motions of the outer periphery of the porphyrin ring (ν₇₋₇ and ν₈₋₈) to a greater extent than ν₅ or ν₁, which are ring breathing modes involving motions of atoms (ν₇₋₇) in the inner core (54). The enhanced intensities of ν₁₀ and ν₁₈ in the near ultraviolet might be therefore reflective of displacement of equilibrium nuclear positions, in the excited state relative to the ground state, of the heme periphery.

A band at 1630 cm⁻¹, which has been proposed to be an infrared active vinyl mode (13), appears almost invariably in Soret excited spectra, obscuring ν₁₀ (a heme spin-state marker frequency). The 1630 cm⁻¹ band is weaker and sometimes absent in the near ultraviolet excited spectra, frequently allowing a clear delineation of ν₁₀. Infrared active Eₘ modes appear prominently in Soret excited resonance Raman spectra as well as with near ultraviolet excitation. The intensity patterns of the near ultraviolet excited resonance Raman spectra of horseradish peroxidase are similar to those obtained with α-β (Q-band) excitation (13), with prominent B₁ₕ and B₂ₖ modes, and the appearance of A₁ₕ modes; although the A₂ₖ modes do not dominate at the excitation wavelengths used in this report. Like the Soret and α-β bands, the electronic transition symmetry of the N-band is Eₘ (59). The B₁ₕ, B₂ₖ, and A₁ₕ modes would therefore be expected to be enhanced in the near ultraviolet through vibronic interaction of the N-band with the intense Soret (B-band) absorption in a manner similar to that observed for α-β (Q-band) enhancement (41).

Near ultraviolet excited resonance Raman spectra of resting horseradish peroxidase, isoenzymes B and C, for the region from 1300 to 1700 cm⁻¹, are shown in Figs. 1d and 2d with mode assignments listed in Table 1 and are in agreement.
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FIG. 1. Resonance Raman spectra (3 milliwatts, 3564 Å excitation) of horseradish peroxidase (0.01 M sodium phosphate, pH 6.8) as (a) the ferrous form (300 μM) produced by dithionite reduction of the resting enzyme; (b) compound I (CPD I) (400 μM) produced by mixing the resting enzyme with a 2:1 ratio of H₂O₂ to heme; (c) compound II (CPD II) (200 μM), formed by mixing equimolar sodium ascorbate with compound I; and (d) the resting enzyme (ferric state), 800 μM.

with previously reported near ultraviolet excited data (42, 44-46). The vibrational frequencies directly correspond with those previously reported for Soret and α-β excitation (13). Similarly, frequencies and assignments are listed in Table I for compound II (13) (Figs. 1c and 2c), the dithionite-reduced (ferrous) enzyme (Figs. 1a and 2a) (42, 59, 60), the alkaline form of isoenzyme A-1 (21) (Fig. 3b), compound III (61, 62) (Fig. 3c), and the ferric fluoride (63) (Fig. 3d), ferrous-NO (Fig. 4b), ferric-NO (49) (Fig. 5a), ferrous-CO (Fig. 4a) (60), ferric cyanide (Fig. 3d), and ferrous cyanide (Fig. 3b) (63-65) derivatives.

The resonance Raman spectra of the CN- and CO-ferrous enzymes obtained with 3638 Å excitation are dominated by ν₄ (1362 cm⁻¹ in Fig. 5b and 1375 cm⁻¹ in Fig. 4a) and are thus more typical of heme Soret excited resonance Raman spectra. However, data obtained for the CN- and CO-ferrous forms with 3564 Å excitation were complicated by strong enhancement of the 1547, 1567, 1585, 1605, and 1630 cm⁻¹ bands of the five-coordinate ferrous enzyme which is a small contaminant in these samples. The resonance enhancements of the CO- and CN-ferrous enzymes under 3638 Å excitation appear to be influenced by the B(1,0) absorption (the high energy shoulder on the Soret band) (36), accounting for the large relative intensities of the ν₄ (A₁g) vibrations in Figs. 4a and 5b.

Resonance Raman spectra of compound I in the 1300–1700 cm⁻¹ region are shown in Figs. 1b and 2b. Band assignments are made from the frequency pattern and band polarizations. The following bands were observed to be polarized: ν₁ (E₁g) at 1614 cm⁻¹, ν₂ (A₁g) at 1606 cm⁻¹, ν₃ (A₂g) at 1504 cm⁻¹ and ν₄ (A₁g) at 1359 cm⁻¹. The following bands were depolarized: ρ₁₀ (B₁g) at 1636 cm⁻¹, ρ₃₁ (B₁g) at 1570 cm⁻¹, and ρ₃₅ (B₂g) at 1458 cm⁻¹.

The resonance Raman spectra of compound I (Figs. 1b and 2b) can be compared with resonance Raman spectra of compound II (Figs. 1c and 2c). Both compounds I and II contain a six-coordinate low-spin (S = 1) Fe(IV) (66, 67), although compound I has an electron removed from the porphyrin ring (68). As for compound II, the iron atom of compound I is bound to a single peroxide-derived oxygen atom, most likely as an oxoferryl, Fe(IV)=O (69).

It is also useful to compare the resonance Raman spectra of compound I with six-coordinate low-spin ferric hemes, since resonance Raman spectra of compound II have been shown to be similar to those of ferric six-coordinate low-spin horseradish peroxidase derivatives (13). Resonance Raman fre-
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TABLE I

Resonance Raman band frequencies (in cm⁻¹) and assignments (following Refs. 54–57) for the horseradish peroxidase (HRP) derivatives and intermediates shown in the figures (with the exception of the flavone, benzhydroxamic acid, and trimethylaniline derivatives)

Frequencies quoted are obtained from 3564 or 3638 Å excitation unless otherwise noted.

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<th>NO-ferrous</th>
<th>NO-ferric</th>
<th>Ferric cyanide</th>
<th>Alkaline HRP</th>
<th>Compound I</th>
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* Frequency differences (Δ cm⁻¹) for compound I relative to compound II.
* From Ref. 56.
* The abbreviations used are: 6 c., l.s., six-coordinate low-spin heme; 5 c., h.s., five-coordinate high-spin heme;
   6 c., h.s., six-coordinate high-spin heme; dp, depolarized; p, polarized; ap, anomalously polarized.
* Value obtained using 4867 Å excitation.
* vₒ or vₓ value listed is typical for ferric heme (63−65, 71).
* Value obtained using 5300 Å excitation.
* May include contribution from vₓ (A₄).
* Polarization anomaly (vₒ ≥ 1/9) observed for the 1372 cm⁻¹ band of resting horseradish peroxidase (3564 Å excitation), as reported in related studies (42, 44).
* NO, O₂ (compound III), and CO ferrous derivatives exhibit vₓ frequencies typical of ferric derivatives due to π-acceptor properties of these ligands (41).}

Quencies above 1300 cm⁻¹, of ferryl peroxidases, which are sensitive to heme core size and oxidation state, are known to occur within the ranges observed for low-spin ferric hemes (13, 23, 70). Examples of ferric six-coordinate low-spin horseradish peroxidase derivatives are the ferric cyanide (Fig 3d), NO-ferric (Fig. 5a), and alkaline (hydroxylferryoxydride (21), Fig. 3b) forms. In addition, the oxyferrous (62) (also known as compound III, Fig. 3c), CO-ferrous (Fig. 4a) and NO-ferrous (Fig. 4b) derivatives exhibit the elevated vₓ frequencies typical of ferric hemes due to backbonding from the Fe(II) dₓ orbitals to the p* orbitals of O₂, NO, and CO which are strong π-acceptors (41).

Additional horseradish peroxidase heme-state variations are illustrated in the figures. Resonance Raman spectra of the ferric fluoride derivative (Fig. 3a), ferrous enzyme (Figs. 1a and 2a) and CN-ferrous (Fig. 5b) derivatives are typical of six-coordinate ferric high-spin, five-coordinate ferrous high-spin, and six-coordinate ferric low-spin hemes, respectively (41, 63, 71).

The most apparent features of the compound I resonance Raman spectra relative to compound II (Table I) are the substantial downshifts of vₓ (20 cm⁻¹), and vₒ (14 cm⁻¹), and upshifts of vₓ (10 cm⁻¹), vₓ (19 cm⁻¹), and vₒ (12 cm⁻¹). Smaller, but still significant, downshifts are seen for vₒ (3 cm⁻¹) and vₓ (5 cm⁻¹). The frequency differences between compound I and the six-coordinate low-spin ferric-type derivatives give similar values.

The 1359 cm⁻¹ vₓ frequency of compound I (Figs. 1b and 2b) is downshifted by 20 cm⁻¹ from the compound II vₓ frequency of 1379 cm⁻¹ (Figs. 1c and 2c). It is fortuitously coincident with the 1358 or 1359 cm⁻¹ vₓ frequency of the reduced (ferrous) enzyme (Figs. 1a and 2a). A previously reported 1359 cm⁻¹ vₓ band of compound I (26) has been reinterpreted as the vₓ band of a ferrous photoproduct (72).

We can demonstrate, however, that the compound I resonance Raman spectra of Figs. 1b and 2b of the present report are not due to the ferrous enzyme. Resonance Raman spectra of the five-coordinate ferrous enzyme (formed by dithionite reduction of the resting enzyme) are shown in Figs. 1a and 2a. While the five-coordinate ferrous enzyme shows a vₓ frequency at 1359 cm⁻¹, there are additional strong features at 1427, 1475, 1548, 1567, 1558, 1605, and 1630 cm⁻¹ (assignments are listed in Table I) which form a pattern that is distinctive from the compound I frequency pattern seen in Figs. 1b and 2b. The ferrous flavone (Fig. 5c) and trimethylaniline (Fig. 5d) derivatives show frequencies similar to the five-coordinate
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Figure 3. Resonance Raman spectra of 300 μM horseradish peroxidase (a) Fe(III) fluoride, formed by an excess of NaF; (b) alkaline (hydroxyferric) form of isoenzyme A-1, 0.02 M Na₂CO₃-NaHCO₃, pH 10.5; (c) compound III (CPD III) (oxyferrous derivative), formed by a 100-fold excess of H₂O₂; and (d) the ferric cyanide derivative, formed by an excess of KCN. 10 milliwatts, 3564 Å excitation.

Figure 4. Resonance Raman spectra of 200 μM horseradish peroxidase (0.01 M sodium phosphate, pH 6.8) in the (a) CO-ferrous form formed by binding CO to the dithionite-reduced enzyme, 0.1 milliwatt, 3638 Å excitation; the following were obtained with 10 milliwatts, 3564 Å excitation: (b) the NO-ferrous derivative formed by stirring the dithionite-reduced enzyme under NO; complexes of the ferric enzyme with (c) benzhydroxamic acid (BHA); and (d) trimethylamine.

The 20 cm⁻¹ downshift of ν₄ that we observe from compound II to compound I is consistent with ν₄ downshifts reported for octaethylporphyrin π-radical cations which range from 12 to 34 cm⁻¹ (25, 31-35). Additionally, the resonance Raman frequency shifts for compound I, relative to compound II and the six-coordinate low-spin ferric derivatives, follow normal mode compositions. These shifts conform well with the characteristic shifts recently described for octaethylporphyrin π-cation radical model compounds (25, 35). The resonance Raman frequency shifts for compound I relative to compound II are listed in Table I. As stated above, the ν₄ frequency (primarily C₅-N) exhibits a 20 cm⁻¹ downshift. The ν₁, ν₁₁, and ν₄₁ vibrational frequencies whose mode compositions are primarily C₅-C₇, C₅-C₇, and C₅-C₇, exhibit 10-20 cm⁻¹ upshifts. Downshifts are observed for the ν₃, ν₁₀, and ν₂₈ frequencies, whose mode compositions are primarily C₅-C₇. The downshifts for ν₃ and ν₁₀ are small, 3-5 cm⁻¹; however, the downshift for ν₂₈ is 14 cm⁻¹.

Of the organic substrates known to form complexes with the heme group of horseradish peroxidase (51, 52, 73-77) we found several that displayed distinctive resonance Raman spectra under near ultraviolet excitation. In addition to the ferrous complexes mentioned previously, ferric enzyme complexes with benzhydroxamic acid and trimethylamine are shown in Fig. 4, c and d. These complexes exhibit enhancement patterns typical of near ultraviolet excitation. The near ultraviolet resonance Raman spectra of the ferric benzhydroxamic acid complex (Fig. 4c) contain the same frequencies as previously reported for Soret excitation (52), but with the expected variations in intensities. Benzhydroxamic acid does not bind to the iron atom (78, 79), but causes formation of a high-spin heme (80).

DISCUSSION

Resonance Raman scattering from porphyrin π-radical cations under Soret and α-β excitation is known to be weak...
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Fig. 5. Resonance Raman spectra of the (200 μM) horseradish peroxidase (a) ferric NO derivative (20 milliwatts, 3564 Å excitation) in 0.005 M sodium acetate, pH 4.4; (b) cyanoferric form (20 milliwatts, 3638 Å excitation) formed by addition of KCN to the dithionite-reduced enzyme, 0.01 M sodium phosphate, pH 6.8; and complexes of the ferrous enzyme (0.01 M sodium phosphate, pH 6.8) with (c) flavone (20 milliwatts, 3564 Å excitation); and (d) trimethylammonium (20 milliwatts, 3564 Å excitation).

relative to porphyrins containing an oxidized ring. The weak scattering is presumed to correlate with the diminished intensity of the Soret absorption relative to intact porphyrins (81, 82). Early attempts to obtain resonance Raman spectra of compound I (82, 83), resulted in reports of elevated values of the v4 (oxidation state marker) frequency, which were almost certainly due to interference from compound II or a compound II-like ferryl species. However, removal of an electron from tetraphenylporphyrins has been characterized by a lowering of resonance Raman frequencies, including v4 (84, 85). An apparent v4 frequency of 1359 cm⁻¹ for compound I, relative to a v4 frequency of 1380 cm⁻¹ for compound II, was reported by Kitagawa's laboratory in a low temperature resonance Raman study (26). However, these results were demonstrated by Van Wart and Zimmer (72) to be due to the ferrous enzyme, generated by photoreduction of compound I. An ensuing account from Kitagawa's laboratory (27) showed an elevated (1380 cm⁻¹) compound I v4 frequency relative to compound II (1379 cm⁻²) consistent with model compound studies that showed upshifts for resonance Raman v4 frequencies for model 3Aum porphyrin π-radical cations (31). It was subsequently shown that the upshifted 3Aum model compound v4 frequencies were due to discid impurities (33) and that both 3Aum and 4Aum porphyrin π-radical cation v4 frequencies appeared to exhibit downshifts relative to the nonradicals (33). The recent attempts of Ogura and Kitagawa (27), Oertling and Babcock (28, 29), and Paeng and Kincaid (30) to obtain resonance Raman spectra of horseradish peroxidase compound I have given rise to conflicting results, showing substantial similarity to published resonance Raman spectra of either horseradish peroxidase compound II or the resting enzyme (13), or both, and poor correspondence to known porphyrin π-radical cation species (31–35).

Our results do not agree with recent reports of high frequency (1300–1700 cm⁻¹) resonance Raman spectra obtained for horseradish peroxidase compound I (29, 30),² which were stated to contain no vibrational frequencies characteristic of a metalloporphyrin π-radical cation (29), or showed relatively small downshifts of v4, v3, and v2 from the compound II frequencies (30). By contrast, we observe a downshift for v4 of twice the magnitude, a large upshift of v3, plus additional significant frequency shifts which have been shown to be typical of porphyrin π-radical cation model compounds (31–35).

We have been able to reproduce the data of Refs. 27–29 that had been previously stated to represent resonance Raman spectra of compound I. Using low powered (less than 1 milliwatt) Soret excitation (4067 Å) for our mixing experiments, we obtained bands at 1378, 1507, 1563, 1620, and 1632 cm⁻¹, as reported (28, 29). In certain cases we also observed the reported value for v4 that is slightly elevated (1380 or 1381 cm⁻¹) (27). These data (27–29) correspond closely to previously published resonance Raman spectra of compound II (13), although with some intensity variations. The data presented in this paper, resonance Raman spectra which are interpretable in terms of a strict porphyrin π-cation radical (Figs. 1b and 2b), are strong evidence that the data of Refs. 27–30 are not resonance Raman spectra of compound I; but rather, they represent preferential Soret enhancement of ferryl (compound II-like) or ferric components that are not porphyrin π-radical cation hemes, but reduction products of the compound I porphyrin radical. The photoabsorbance of compound I has been described (72, 86–88). It is likely that formation of the ferryl component occurs by photo-assisted reduction of the radical by an endogenous electron donor, or movement of the radical from the porphyrin to an amino acid residue, analogous to compound ES of cytochrome c peroxidase (67).

We have obtained resonance Raman spectra of compound I (shown in Figs. 1b and 2b) by tuning the excitation into the ultraviolet, away from the strong Soret enhancements of the ferryl state (compound II) and the resting enzyme. Electronic absorption spectra of resting horseradish peroxidase and compounds I and II have been well documented (2–5, 81, 87–90). The Soret maximum of compound II occurs near 420 nm. The Soret maxima of the resting enzyme and compound I both occur near 400 nm (403 nm for the resting enzyme and 400 nm for compound I), although the molar absorption intensity at λmax of compound I is considerably diminished from that of the resting enzyme. However, the electronic absorption of compound I has a component that extends into the ultraviolet such that at wavelengths shorter than 400 nm; the compound I absorption is significantly stronger than compound II (87).

²These reports (29, 30) also discussed resonance Raman data from the oxoferryl region (600–950 cm⁻¹) which we do not address in the present account.
As with Soret excitation, compound I is photolable under near-ultraviolet excitation. To avoid contributions from ferryl and ferric photoproducts, we use minimum laser power, high protein concentrations, and relatively rapid sample flow rates to circumvent the inherent photolability. Under 3564 and 3507 Å excitation, we find that higher laser power and/or lower sample concentration induces contributions to the resonance Raman spectra by the resting (ferric) enzyme and, to a lesser extent, by compound II (the ferryl state). Under 3638 Å excitation, which is closer to the Soret absorption of compound II, the ferryl contaminant becomes more prominent.

Substantive spectroscopic (68, 89, 90) and theoretical (91–94) arguments have been made in favor of a $A_{2u}$ designation for the porphyrin $\pi$-radical cation of compound I of horseradish peroxidase (versus the $A_{1u}$ designation which is proposed for catalase compound I). However, recent theoretical (95, 96), magnetic circular dichroism (88, 97, 98), NMR (99, 100), EPR and Mossbauer (101, 102) studies have questioned the rigidity of the $A_{1u}$ vs $A_{2u}$ assignments for horseradish peroxidase and catalase compounds I. In particular, resonance Raman model studies have noted that resonance Raman frequency shifts of model octaethylporphyrin $\pi$-radical cations appear to be insensitive to $A_{1u}$ or $A_{2u}$ radical designation (34). This observation might be explained by recent reports that octaethylporphyrin $\pi$-radical cations are being proposed to be predominantly $A_{1u}$ rather than $A_{2u}$ (35, 103–105). The octaethylporphyrin radicals were originally assigned on the basis of characteristic optical absorption spectra, as either $A_{1u}$ or $A_{2u}$ with a reversible switch of symmetry states being attainable in certain cases by an appropriate choice of axial ligands (6, 81, 89).

The prevailing symmetry state assignment of the horseradish peroxidase compound I porphyrin $\pi$-radical cation is $A_{2u}$ (6, 89, 90). However, the characteristics of the resonance Raman frequency shifts we have observed are most similar to those reported for metallo-octaethyl porphyrin cation radicals which have recently been proposed to be predominantly $A_{1u}$ (35, 103). The $A_{2u}$ radical has large spin density on the bridging meso carbons (C$_6$) and pyrrole nitrogens, while the $A_{1u}$ radical has sizeable $\pi$-electron density in the pyrrole nitrogens (91, 93, 105–107). The C$_6$-C$_7$ interactions are bonding in the $A_{2u}$ orbital, and antibonding in the $A_{1u}$ orbital (106, 107). Removal of an electron from the $A_{2u}$ orbital is expected to result in decreases in frequency of the C$_6$-C$_7$ modes, $v_3$, $v_4$, and $v_{57}$ (31, 103). Large downshifts of these modes have, in fact, been reported for $A_{1u}$ type radical model compounds (103). Removal of an electron from the $A_{1u}$ orbital of model compounds has been reported to result in upshifts of C$_6$-C$_7$ modes (31, 35, 103). Our data show substantial upshifts (10–20 cm$^{-1}$) in $v_3$, $v_4$, and $v_{57}$. The C$_6$-N interaction is antibonding in the $A_{1u}$ orbital, however nodal planes pass through the nitrogen atoms in the $A_{2u}$ orbital. We observe a substantial (20 cm$^{-1}$) downshift in $v_3$, whose mode composition is primarily C$_6$. The C$_6$-C$_7$ modes $v_3$, $v_{10}$, and $v_{57}$ exhibit smaller but still significant downshifts (in the range from 3 to 14 cm$^{-1}$). We feel, nevertheless, that it is premature to make extensive comments on the $A_{1u}$ vs $A_{2u}$ assignment of horseradish peroxidase compound I at the present time, since assignments of the model metalloporphyrin cation radicals are reported to be still in progress (35, 103).

In summary, excitation of resonance Raman scattering from derivatives and intermediates of horseradish peroxidase, in the near ultraviolet, results in enhancement patterns that are modified from those commonly observed under Soret and $\alpha$-$\beta$ excitation. By tuning away from the strong interferences of the Soret enhanced ferryl and ferric states, we have been able to obtain resonance Raman spectra of horseradish peroxidase compound I, without interference from the signals of contaminating species that have been incorrectly attributed to compound I in recent reports from other laboratories (26–30). The resonance Raman spectra of compound I shown in this paper are characteristic of a low-spin six-coordinate $\pi$-cation radical heme with frequency shifts relative to the nonradicals that follow normal mode compositions.

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Note Added in Proof — The reader is referred to Ref. 108 which has recently appeared. It contains a full account of the work summarized in Ref. 103 and a further and extensive discussion of the $A_{1u}$ versus $A_{2u}$ symmetry state assignments for metalloporphyrin $\pi$-cation radicals.