New Insights into Horseradish Peroxidase Function in Benzene from Resonance Raman Spectroscopy

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Abstract: Resonance Raman spectroscopic study of ferric and ferrous carbon monoxide forms of polyethylene glycolated horseradish peroxidase (HRPPEG) in benzene solution is reported. Ferric HRPPEG in benzene solution assumes a 6-coordinate high spin (6-c HS) heme system, binding water, rather than the usual 5-c structure. Low-frequency vibrations associated with porphyrin deformations and propionate bending (ν_8 and $\delta(C_\beta C_c C_d)$) are upshifted by 5 cm^{-1}, indicating that a heme propionate group is perturbed. The ferrous carbon monoxide complex of HRPPEG (Fe(II)HRPPEG-CO) exists in benzene as a single 6-c species characterized by a weak Fe(II)−H170 bond and weak distal and proximal H-bonding heme pocket interactions. The structural changes are similar to those observed when an aromatic substrate analogue, benzohydroxamic acid (BHA) binds to HRP in aqueous solution. The structural changes are attributed to the formation of an enzyme−substrate complex between HRPPEG and benzene, a known substrate for HRP. Taken together the resonance Raman spectral data for Fe(III)HRPPEG and Fe(II)HRPPEG-CO in benzene suggest that the heme active site in HRPPEG is significantly affected by substrate binding. Heme active site structural alterations may be attributed to dehydration which disrupts critical H-bonding interactions in both the distal and proximal heme pockets, allows substrate access to the heme iron, and promotes salt bridge formation between the propionate and the distal R38 side chain. These structural changes can explain the surprising observation that HRPPEG can hydroxylate benzene, with the incorporation of peroxide oxygen.

Introduction

Few spectroscopic studies have been reported to date that examined the structure of enzymes in nonaqueous media.[1−7] The majority of these reports have focused on the study of heterogeneous systems, specifically, enzyme powders and cross-linked enzyme crystals (CLCs) suspended in organic solvents. Overall, results to date have suggested that enzymes remain largely structurally intact in organic media and that their structure is not significantly perturbed by the solvent medium. This finding is somewhat surprising given the remarkable changes in reactivity and reaction chemistry that characterize nonaqueous enzymology.[8−14]

While the majority of studies in the field of nonaqueous enzymology have focused on the use of enzyme powders or CLCs, another approach involves the preparation and study of polyethylene glycolated (PEG) enzymes, which are readily soluble and catalytically active in a number of organic solvents such as chloroform[15] and benzene.[16] Since nonaqueous solutions of PEG enzymes are homogeneous and optically transparent, these systems are readily amenable to rigorous characterization and interpretation by optical spectroscopic probes such as UV−visible, circular dichroism, and resonance Raman spectroscopies.

The heme prosthetic group is the active site in a number of metalloproteins and enzymes. This moiety has demonstrated itself to be capable of a wide range of different functions in these heme proteins including electron transfer, oxygen transport, and catalysis.[17,18] The unique function achieved in each case appears to be imposed by the protein surrounding the heme. The specific mechanism for achieving this diversity and specificity of function continues to be a subject of intense scrutiny in mechanistic bioinorganic chemistry.

One of these heme enzymes is horseradish peroxidase. Due to its ability to catalyze the oxidation of a wide variety of organic and inorganic compounds including amines and phenols using

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(1) Burke, P. A.; Smith, S. O.; Bachovchin, W. W.; Klibanov, A. M. *J. Am. Chem. Soc*. **1989**, *111*, 1.
(2) Ryu, K.; Dordick, J. S. *Biochemistry* **1992**, *31*, 2588−2598.
(3) Burke, P. A.; Griffin, R. G.; Klibanov, A. M. *J. Biol. Chem*. **1992**, *267*, 20057−20064.
(4) Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8653−8657.
(5) Fitzpatrick, P. A.; Ringe, D.; Klibanov, A. M. *Biochem. Biophys. Res. Commun*. **1994**, *198*, 675−681.
(6) Mabrouk, P. A. *J. Am. Chem. Soc*. **1995**, *117*, 2141−2146.
(7) Wangikar, P. P.; Michels, P. C.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc*. **1997**, *119*, 70−76.
(8) Fitzpatrick, P. A.; Klibanov, A. M. *J. Am. Chem. Soc*. **1991**, *113*, 3166−3171.
(9) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192−3196.
(10) Zaks, A.; Klibanov, A. M. *Science* **1984**, *224*, 1249−1251.
(11) Sakurai, T.; Margolin, A. L.; Russell, A. J.; Klibanov, A. M. *J. Am. Chem. Soc*. **1988**, *110*, 7236−7237.
(12) Zaks, A.; Klbanov, A. M. *J. Am. Chem. Soc*. **1986**, *108*, 2767−2768.
(13) Kazandjian, R. Z.; Klibanov, A. M. *J. Am. Chem. Soc*. **1985**, *107*, 5448−5450.
(14) Rubio, E.; Fernandez-Mayorales, A.; Klibanov, A. M. *J. Am. Chem. Soc*. **1991**, *113*, 695−696.
(15) Souppe, J.; Urrutigoity, M. *New J. Chem*. **1989**, *13*, 503−506.
(16) Mabrouk, P. A. In *The Use of PEG-Enzymes in Non-Aqueous Enzymology*; Mabrouk, P. A., Ed.; American Chemical Society: Washington D. C., 1997; Vol. 680, pp 118−133.
(17) Ortiz de Montellano, P. R. *Acc. Chem. Res*. **1987**, *20*, 289−294.
(18) Dawson, J. H. *Science* **1988**, *240*, 433−439.

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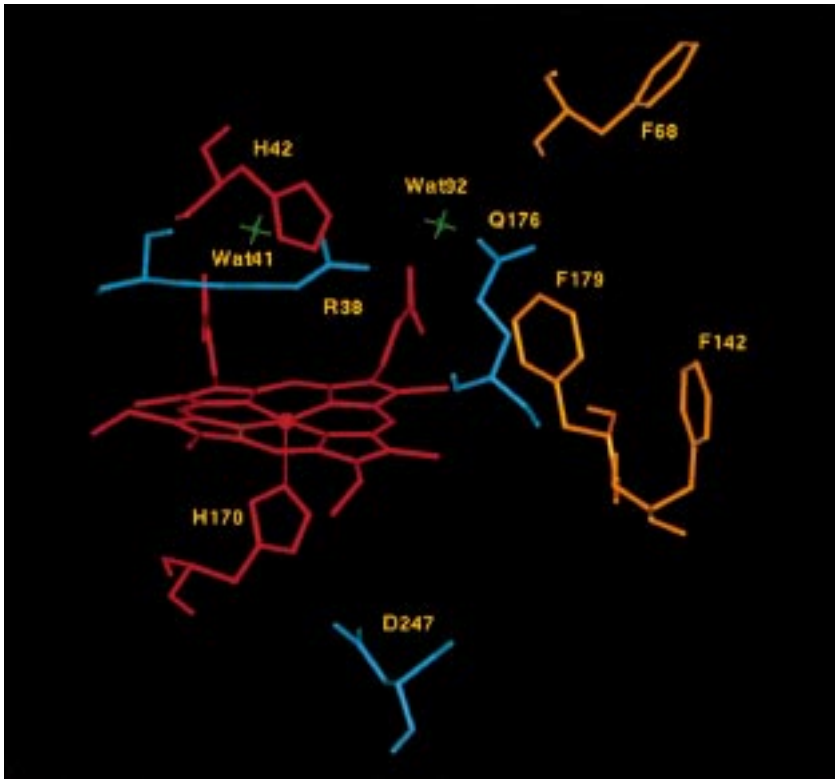

Figure 1. Model of the key amino acid and bound water residues in the distal and proximal heme pockets of HRP-C based on the recent X-ray crystal structure of recombinant HRP-C.[27]

hydrogen peroxide, horseradish peroxidase (HRP) has been the subject of much recent interest.[2,18−24] Recently, HRP was shown to efficiently catalyze the hydroxylation of benzene in the presence of peroxide when benzene was used as the reaction medium. Intriguingly, HRP displayed a catalytic activity comparable to that of cytochrome P450.[25] In the same study, the authors showed that 95% of the oxygen atoms incorporated into the phenol product was transferred from the peroxide oxidant rather than from water or molecular oxygen. Since benzene is not a normal substrate for HRP, since peroxide is not the usual source for the oxygen atom in HRP catalysis, and since cytochrome P450 is the only heme enzyme known to catalyze the hydroxylation of aromatic hydrocarbons,[26] these findings are intriguing and suggest a change of chemistry for HRP in benzene solution.

X-ray and spectroscopic studies of recombinant ferric horseradish peroxidase C (HRP-C)[27] have led to the identification of several key structural characteristics of the heme active site environment of plant peroxidases (Figure 1). H170 is the proximal ligand in the 5-coordinate high spin (5-c HS) heme active site. Hydrogen bonding interactions appear to be extremely important in the active site structure and reactivity of HRP. There is an extensive hydrogen-bonding network on both the proximal and distal sides of the heme active site. These distal and proximal hydrogen bond networks appear to be connected at Q176.[27] On the proximal side, a direct hydrogen bond exists between the proximal H170 $N_{\delta 1}$ and the carboxylate oxygen from D247 that is believed to increase the basicity of H170 helping to stabilize the high oxidation states of the heme iron. On the distal side, the X-ray crystal structure of HRP-C, shows a water molecule, Wat92, close to the heme iron (3.2 Å).[27] The relatively large distance between the water molecule and the heme iron suggests that at best the water molecule is weakly coordinated to the heme iron. Indeed, resonance Raman studies of horseradish peroxidase have shown that the distal coordination site is only partially occupied by water.[28] This water is also hydrogen-bonded to key residues in the distal binding pocket, specifically, the N_{ϵ} of R38 and the $N_{\epsilon 2}$ of H42 and also to another water molecule which also H-bonds to $N_{\eta 2}$ of R38.[27] On the distal side, R38, F41, and H42 form the distal binding pocket. Hydrogen bonding between $N_{\delta 1}$ of H42 and the side chain oxygen atom from N70 is believed to control the hydrogen-bonding at the distal histidine.[29] The distal histidine is considered to play a central role as an acid−base catalyst facilitating the formation of the initial Fe−OOH complex by deprotonating hydrogen peroxidase and promoting heterolytic O−O bond cleavage by protonating the distal oxygen atom. ^{1}H NMR[30] and suicide inhibitor studies[31] have shown that aromatic

(19) Penel, C.; Gaspar, T.; Greppin, H. In *Plant Peroxidases 1980−1990. Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects*; Penel, C.; Gaspar, T.; Greppin, H., Ed.; University of Geneva: Geneva, 1992.

(20) *Plant Peroxidases. Biochemistry and Physiology*; University of Copenhagen and University of Geneva: Elsinore, Denmark, 1993.

(21) Dordick, J. S.; Marletta, M. A.; Klibanov, A. M. *Biotechnol. Bioeng.* **1987**, *30*, 31−36.

(22) Dordick, J. S.; Marletta, M. A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6255−6257.

(23) Kazandjian, R. Z.; Dordick, J. S.; Klibanov, A. M. *Biotechnol. Bioeng.* **1986**, *28*, 417−421.

(24) Gorman, L. A. S.; Dordick, J. S. *Biotechnol. Bioeng.* **1992**, *39*, 392−397.

(25) Akasaka, R.; Mashino, T.; Hirobe, M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1861−1864.

(26) Ortiz de Montellano, P. R. *Cytochrome P-450*; Plenum Press: New York, 1986.

(27) Gajhede, M.; Schuller, D. J.; Henriksne, A.; Smith, A. T.; Poulos, T. L. *Nature Struct. Biol.* **1997**, *4*, 1032−1038.

(28) Evangelista-Kirkup, R.; Crisanti, M.; Poulos, T. L.; Spiro, T. G. *FEBS Lett.* **1985**, *190*, 221−226.

(29) Nagano, S.; Tanaka, M.; Watanabe, Y.; Morishima, I. *Biochem. Biophys. Res. Commun.* **1995**, *207*, 417−423.

(30) Veitch, N. C.; Williams, R. J. P. *Eur. J. Biochem.* **1990**, *189*, 351−362.

substrates bind perpendicularly to the heme plane[32] in a pocket near the heme edge bearing the 8-CH_3 group and close to a phenylalanine residue about 8−11 Å from the heme iron. The X-ray crystal structure of ferric HRP-C has revealed that there is a unique ring of three peripheral phenylalanine residues, 142, 68, and 179, which appear to guard the entrance to the exposed heme edge. On the basis of the crystal structure and NMR work, F179 has been proposed to be the critical site at which a pre-electron transfer complex is formed between aromatic substrates and the catalytically active high oxidation state intermediates.[27]

Resonance Raman spectroscopy has proven to be an extremely sensitive and powerful probe of the heme active site structure in hemoproteins and heme enzymes including horseradish peroxidase.[33] With Soret band excitation, the intensity of porphyrin skeletal modes in the spectral region above 1300 cm^{-1}, often called the heme marker bands, become significantly enhanced and can be selectively and sensitively probed. The frequencies of the marker bands are extremely useful diagnostics of heme structure and provide information on heme iron oxidation state, spin state, and porphyrin core size. For example, one of these marker bands, ν_3, which falls in the spectral region 1460−1500 cm^{-1}, is a particularly useful diagnostic for the coordination number of the heme system.[34] A 5-c HS heme system is characterized by ν_3 at 1495 cm^{-1}. The frequency of ν_3 downshifts to 1480 cm^{-1} in a 6-c heme. On the basis of spectral changes most notably in ν_3, HRP has been shown to exist as a predominantly 5-c HS heme system in aqueous phosphate buffer.[28] Information on the proximal and distal heme environment can be obtained through the resonance Raman study of different redox forms and small molecule complexes such as the carbon monoxide complex of the reduced protein. For example, the vibrational frequencies for the Fe(II)CO and the C−O stretching modes for the Fe(II)CO hemoprotein complexes are inversely correlated and have been demonstrated to be very sensitive probes of the distal heme environment.[35,36]

Recently, we prepared and examined HRPPEG in benzene solution using UV−vis, CD, and resonance Raman spectroscopy and demonstrated that the enzyme remains structurally intact and is catalytically active in benzene.[6] In the same study, we showed that the catalytically active and usually thermally and photochemically reactive intermediate Compound II can be generated and stabilized toward spectroscopic study in benzene solution.

In view of the continued interest in peroxidases, the utility of resonance Raman spectroscopy in the study of heme systems, and the unusual reaction chemistry of HRP in benzene, we wish to report the characterization of ferric HRPPEG and the ferrous carbon monoxide complex of HRPPEG in benzene solution by resonance Raman spectroscopy.

Experimental Section

Horseradish peroxidase (Sigma, type VI) was used as received without further purification. Sigma type VI peroxidase, the highest specific activity formulation of this peroxidase, is a mixture of basic isozymes, principally isozyme C (>75%) based on SDS PAGE assay.

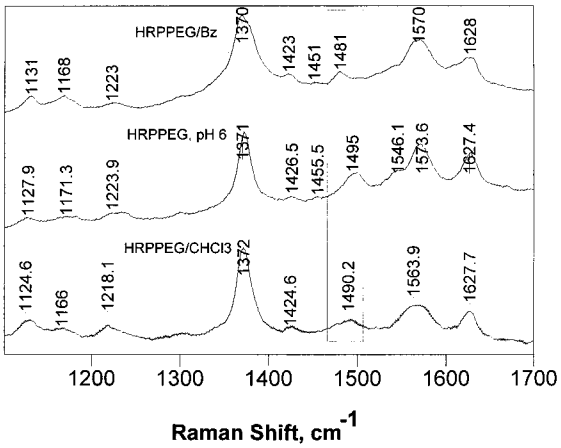

Figure 2. RR spectra of (top) ferric HRPPEG (1000−1700 cm^{-1}) dissolved in benzene solution; (middle) in aqueous 0.1 M potassium phosphate, pH 6.0; and (bottom) in chloroform. Experimental conditions: [HRPPEG] (1 mM, 406.7 nm excitation wavelength, 6 cm^{-1} resolution, 30 mW laser power at the sample.

The following reagents were obtained commercially as analytical grade or better and were used as received: potassium phosphate monobasic (Baker), methoxypoly(ethylene glycol) activated with cyanuric chloride (Sigma), sodium tetraborate decahydrate (Aldrich), L-ascorbic acid (Aldrich), dimethyl sulfoxide (Fisher), chloroform (Fisher, spectranalyzed), and benzene (Fisher, spectranalyzed). The sodium hydrosulfite (Aldrich) was technical grade (~85%).

HRPPEG was prepared and characterized as described by Mabrouk.[6] The protein was reduced by the anaerobic addition of 5 mL of a saturated solution of either sodium dithionite or ascorbic acid dissolved in nitrogen-purged dimethyl sulfoxide to a previously prepared and nitrogen-purged solution of HRPPEG dissolved in benzene. The carbon monoxide complex was prepared by purging a freshly prepared solution of ferrous HRPPEG in benzene with ^{13}CO (99%; Isotec Matheson) or ^{12}CO (Matheson), as desired, for five minutes.

Resonance Raman spectra were obtained at room temperature by excitation with 406.7 or 413.1 nm from a Kr^+ laser (Coherent). A Spex Triplemate spectrometer (2400 grooves/nm grating) was used as the spectrograph, and an optical multichannel analyzer (Princeton Instruments) was used as the detector. Raman scattering was sampled for solutions contained in spinning 5-mm NMR tubes using a backscattering configuration. HRPPEG solution concentrations were ~1 mM. The laser power at the sample was typically 30 mW. Spectral acquisition times were between 2 and 10 min. Absorption spectra (190−820 nm) were measured on a Hewlett-Packard 8452A diode array spectrometer immediately prior to and following each Raman experiment. No degradation was detected under the conditions used in this study. All Raman spectra were calibrated using benzene (300−1700 cm^{-1}), toluene (300−1700 cm^{-1}), or d_6-dimethyl sulfoxide (1700−2200 cm^{-1}) as an external frequency standard. The spectral features of benzene and chloroform have been subtracted from the resonance Raman spectra obtained in these solvents. All spectral data were processed with Grams-32 Spectral Notebase software (Galactic Industries, Co.). None of the spectra were smoothed.

Results

***Ferric* HRPPEG.** Resonance Raman spectra in the high-frequency marker band region (1000−1700 cm^{-1}) (Figure 2) reveal a change in coordination number when ferric HRPPEG is dissolved in benzene. The coordination number of ferric heme can be determined by examining the frequency of the ν_3 skeletal porphyrin ring mode which appears in a clear region of the spectrum between 1480 and 1500 cm^{-1}.[34] The frequency of ν_3 is 1495 cm^{-1} in a 5-c HS heme and downshifts to 1480 cm^{-1} in a 6-c HS heme. The boxed section of Figure 2 shows the key finding that the resonance Raman spectra for HRPPEG

(31) Ator, M. A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1987**, *262*, 1542−1551.
(32) Sakurada, J.; Takahashi, S.; Hosoya, T. *J. Biol. Chem.* **1986**, *261*, 9657.
(33) Spiro, T. G. In *Resonance Raman Spectroscopy of Metalloporphyrins*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1988; Vol. 3, pp 1−37.
(34) Spiro, T. G. *Adv. Protein Chem.* **1985**, *37*, 111−159.
(35) Uno, T.; Nishimura, Y.; Tsuboi, M.; Makino, R.; Iizuka, T.; Ishimura, Y. *J. Biol. Chem.* **1987**, *262*, 4549−4556.
(36) Ray, G. B.; Li, X.-Y.; Ibers, J. A.; Sessler, J. L.; Spiro, T. G. *J. Am. Chem. Soc.* **1994**, *116*, 162−176.

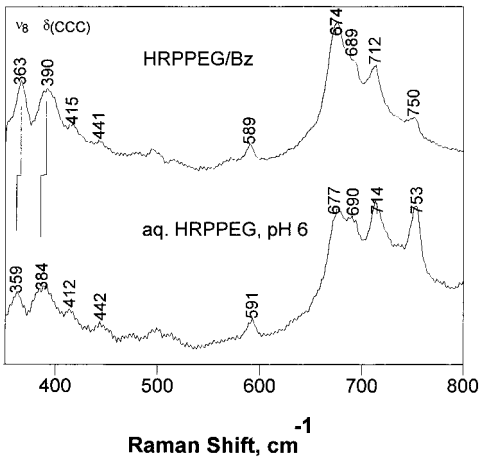

Figure 3. RR spectra of ferric HRPPEG (350−800 cm^{-1}) in benzene solution (top) and in aqueous 0.1 M potassium phosphate, pH 6.0, (bottom). Experimental conditions: [HRPPEG] (1 mM, 406.7 nm excitation wavelength, 6 cm^{-1} resolution, 30 mW laser power at the sample.

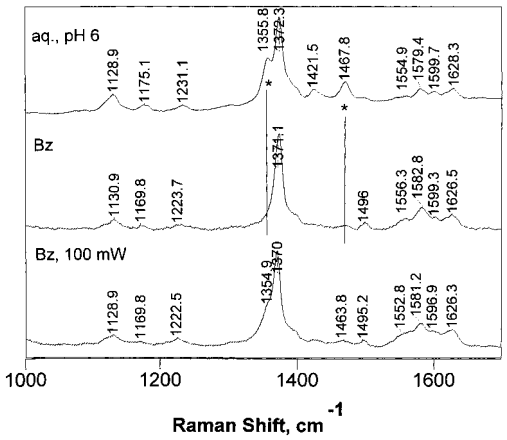

Figure 4. RR spectra of (top) Fe(II)HRP-CO (1000−1700 cm^{-1}) in aqueous 0.1 M potassium phosphate, pH 6.0, (middle and bottom) Fe(II)HRPPEG-CO in benzene solution reduced using ascorbic acid. Experimental conditions: (top and middle) [HRPPEG] (1 mM, 413.1 nm excitation wavelength, 6 cm^{-1} resolution, 30 mW defocused laser power at spinning sample; (bottom) [HRPPEG] (1 mM, 413.1 nm excitation wavelength, 6 cm^{-1} resolution, 100 mW focused laser power at stationary sample.

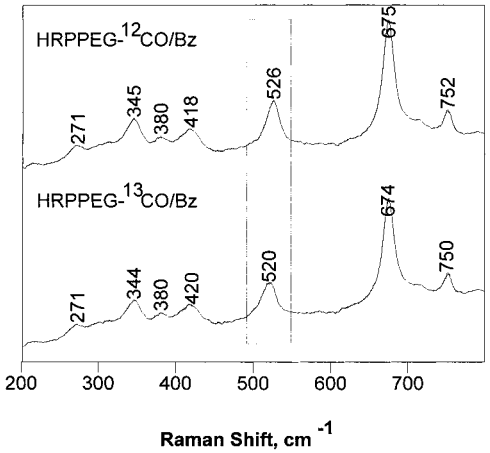

Figure 5. RR spectra of (top) Fe(II)HRPPEG−^{12}CO; (bottom) Fe(II)HRPPEG-^{13}CO (350−800 cm^{-1}) in benzene solution reduced using ascorbic acid. Experimental conditions: [HRPPEG] (1 mM, 413.1 nm excitation wavelength, 6 cm^{-1} resolution, 30 mW defocused laser power at the sample. Each data set at the right represents average of 600 accumulations (7 s/scan).

dissolved in chloroform or aqueous phosphate buffer, pH 6.0, show features consistent with the presence of a predominantly 5-c HS heme, as previously reported,[28] while the spectrum for HRPPEG dissolved in benzene shows features consistent with a 6-c HS heme. However, in chloroform, the heme remains mostly 5-c. Thus, Fe(III)HRPPEG changes from 5-c to 6-c when dissolved in benzene but not in $CHCl_3$ or water.

The low-frequency resonance Raman spectra (Figure 3) indicate an altered conformation for one or both propionate substituents because the band assigned to $\delta(C_\beta C_c C_d)$[33] at 384 cm^{-1} is 5 cm^{-1} higher in benzene than in water. Likewise, ν_8, a porphyrin deformation mode with peripheral substituent involvement[33] shifts up 5 cm^{-1} in benzene. The likeliest explanation is reorientation of a propionate group. An intensity loss for the ν_7 porphyrin skeletal mode at 753 cm^{-1} (Figure 3) is also suggestive of a conformational change.

Aqueous pH has been identified as an important factor in determining the catalytic activity of enzymes in nonaqueous media.[9] In an earlier study, Mabrouk[6] observed that the intensity of the Soret band in the UV−vis spectrum of HRPPEG in benzene was sensitive to the aqueous pH. The intensity of the Soret band increased by a factor of 2 when benzene extracted with aqueous phosphate buffer was used to prepare HRPPEG in benzene solutions. For this reason we also investigated the structural significance of aqueous pH for Pegylated HRP. However, extraction of benzene with aqueous buffer was found to produce no significant spectral changes in the marker bands (1000−1700 cm^{-1}). The only differences observed were subtle changes in ν_8, attributed to the porphyrin deformation and ν(Fe−N) and $\delta(C_\beta C_c C_d)$, the propionate bending mode, in the low-frequency resonance Raman spectrum of HRPPEG in benzene (not shown).

Fe(II)HRPPEG and Fe(II)HRPPEG−CO. The reduced form produced by ascorbic acid or sodium dithionite reduction of HRPPEG in nitrogen-purged benzene solution was extremely unstable. However, addition of CO to these solutions produced a stable carbon monoxide adduct. The specific reducing agent (ascorbic acid or dithionite) used was not found to produce any detectable change in the resonance Raman spectrum of the CO adduct in benzene (data not shown).

In contrast to the ferrous CO complex which had marked photoreactivity in aqueous solution,[35] HRPPEG-CO was observed to be stable to relatively intense laser irradiation (Figure 4). No spectral changes were observed to occur in HRPPEG-CO when the sample was irradiated with 30 mW of defocused 413.1-nm light, whereas in aqueous solution, photodissociation of CO was evident (Figure 4) in the appearance of the ν_3 and ν_4 bands at positions characteristic of the 5-c ferrous form, at 1469 and 1359 cm^{-1}, respectively.[35] Thus, geminate CO recombination appears to be significantly more efficient in benzene than in aqueous solution. Indeed, irradiation of a stationary sample with 100 mW of focused laser power was required to produce any significant change in the resonance Raman spectrum of the CO adduct in benzene (bottom trace of Figure 4). At this power level, there is a weak 1355 cm^{-1} shoulder on the ν_4 marker band and a small but significant change in the relative intensity of the ν_3 bands at 1464 and 1495 cm^{-1}.

A search for bound CO modes via ^{13}CO substitution revealed a single prominent ν(Fe−C) band at 526 cm^{-1} (Figure 5) and a single ν(CO) stretch at 1952 cm^{-1} (Figure 6). These shift down by 5 and 42 cm^{-1}, respectively, upon ^{13}CO substitution,

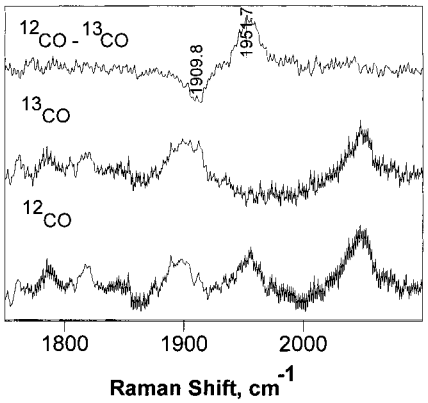

Figure 6. (top) Difference RR spectrum (1850−2100 cm^{-1}) obtained by subtracting (middle) Fe(II)HRPPEG-^{13}CO and (bottom) Fe(II)-HRPPEG-^{12}CO in benzene solution reduced using ascorbic acid. Experimental conditions: [HRPPEG] (1 mM, 413.1 nm excitation wavelength, 6 cm^{-1} resolution, 30 mW laser power at the sample. Each data set at right represents average of 600 accumulations (7 s/scan). The 1896 cm^{-1} feature is an overtone band.

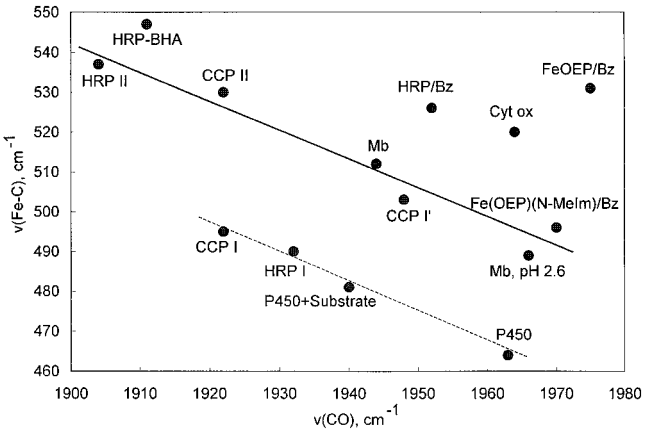

Figure 7. Plot of ν(Fe−C) vs ν(CO) frequencies in Fe(II)CO heme adducts for representative heme proteins and model complexes. Data are taken from refs 35,36 and 38. Abbreviations: Mb, myoglobin; CCP, cytochrome *c* peroxidase; P450, cytochrome P450; Bz, benzene; BHA, benzhydroxamic acid. I and II stand for CO conformers type I and II, respectively. The solid line represents the main correlation for neutral imidazole coordinated proximally. The dashed line represents the correlation for anionic imidazolates and thiolates.

as expected.[37] The frequencies differ substantially from those found in aqueous solution where multiple forms are encountered.[37] The δ_{FeCO} bending mode, seen weakly in aqueous solutions at 587 cm^{-1}, is absent from the spectrum of Fe(II)-HRPPEG-CO in benzene (Figure 5).

The ν(Fe−C) and ν(CO) frequencies are linearly correlated for a variety of heme protein−CO adducts. This negative correlation (Figure 7) is attributed to the effects of back-bonding in the FeCO unit.[38] The ferrous ion donates d-π electrons to the empty π^* orbitals of the CO ligand, decreasing the C−O bond order but increasing the Fe−C bond order. Variation in distal polar interactions such as H-bonding is primarily responsible for the distribution of the heme protein points which fall along the line. Positive polar interactions, which increase back-bonding, are characteristic of CO adducts that appear higher on the correlation, while negative polar interactions, which decrease back-bonding, are characteristic of CO adducts that appear lower on the correlation. For example, the upper left point of the line in Figure 7 is associated with the HRP type II conformer which has a strong H-bond between the bound CO and a positively charged arginine side chain in the distal pocket.[37] The hydrogen bond polarizes the CO and strengthens back-donation from the heme iron which produces the unusually high ν(Fe−C) and low ν(CO) frequencies characteristic of this CO conformer.

Large deviations from the central line are observed for adducts with different axial ligands.[36,38] Axial ligands such as imidazolate or thiolate that are stronger σ donors than imidazole compete more effectively with the CO ligand for the Fe(II) d_{z^2} orbital, and weaken the Fe−C σ bond. Consequently, CO adducts with these axial ligands produce a correlation that falls below the imidazole correlation.

If the axial ligand is weak or is absent altogether, as in FeOEP-CO, the ν(Fe−C) and ν(CO) frequencies fall above the imidazole correlation. The CO adduct of cytochrome *c* oxidase exhibits a position above the imidazole correlation which has been attributed to an unusually weak bond between the proximal histidine and the heme iron.[39]

The point for HRPPEG-CO in benzene is quite close to that of cytochrome oxidase and similarly indicates a weakened Fe−ImH bond. Interestingly, the CO adduct of the Fe(II)HRP-benzhydroxamic acid (BHA) complex[35] likewise lies above the imidazole correlation (Figure 7) although the low ((CO) frequency implies significantly greater H-bonding than for HRPPEG-CO in benzene.

Discussion

Heme Active Site Structural Alterations. The structural changes we observe for ferric HRPPEG in benzene resemble those reported for HRP complexed to BHA, an aromatic substrate analogue.[35,40] In both cases, the Fe(III)heme is 6-c rather than 5-c (the disappearance of the 1501 cm^{-1} component of ν_3) and a propionate substituent is perturbed (frequency shifts for low-frequency heme modes including $\delta(C_\beta C_c C_d)$). Moreover, the Fe(II)CO adduct gives evidence for weakening of the bond to the proximal His (positive deviations of ν(Fe−C)/ν-(CO) from the imidazole correlation) in both cases. How can this common pattern be understood?

Gajhede et al.[27] identified F179 as the docking site for aromatic substrates, on the basis of the crystal structure and of NMR data from Veitch and Williams.[30] This residue is located next to Q176 (Figure 1). Q176 is H-bonded to one of the propionate groups. The same propionate group is H-bonded to a water molecule (Wat92) which in turn is H-bonded to the distal R38 residue. We propose that binding of an aromatic substrate at F179 perturbs Q176, disrupting its H-bond to the propionate and allowing the reorientation of the propionate (accounting for the low-frequency resonance Raman band shifts) which frees Wat92 to bind the heme Fe(III) (accounting for the switch to 6-coordination). These proposed changes in the heme pocket structure are illustrated in Figure 8.

On the proximal side of the heme, the Fe−His bond is affected by the substrate-induced perturbation because Q176 is connected by a 5-residue loop to the proximal H170. The H170 imidazole donates a H-bond to the carboxylate side chain of D247 and acquires substantial imidazolate character. This electronic effect strengthens the iron−histidine bond as long as the proximal loop is anchored by the heme propionate. When

(37) Evangelista-Kirkup, R.; Smulevich, G.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4420−4425.
(38) Li, X.-Y.; Spiro, T. G. *J. Am. Chem. Soc.* **1988**, *110*, 6024−6033.
(39) Argade, P. V.; Ching, Y. C.; Rousseau, D. L. *Science* **1984**, *225*, 329−331.
(40) Teraoka, J.; Kitagawa, T. *J. Biol. Chem.* **1981**, *256*, 3969−3977.

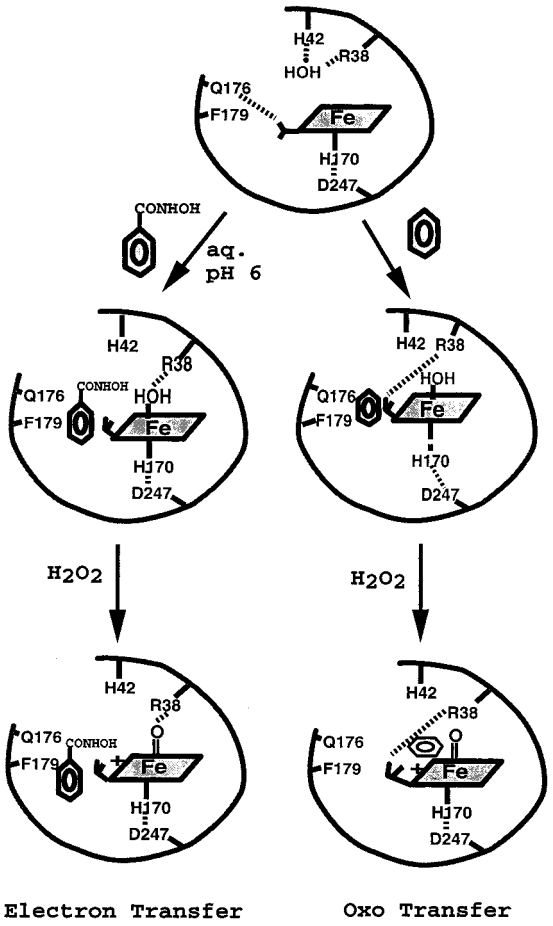

Figure 8. Schematic view of the proposed structural changes in the active site of HRP upon BHA or benzene binding at the aromatic pocket near F179 and the subsequent addition of hydrogen peroxide. Relevant H-bond interactions are indicated by a dashed line.

the anchor is dislodged, however, the H170 imidazole can approach D247 more closely, at the expense of the iron-histidine bond, at least in the Fe(II) form. This effect has been observed in cytochrome *c* peroxidase, which has an analogous proximal loop and His−Asp interaction. When this loop is dislodged by site-directed mutagenesis of the anchoring residue (H175 for cytochrome *c* peroxidase), the Fe(II)−His bond is weakened.[41] The positive deviations from the imidazole correlation for the CO adducts of HRPPEG in benzene, and for the HRP−BHA complex in water, are consistent with weakened Fe-His bonding.

However, there are significant differences between the HRP−BHA complex in aqueous solution and HRPPEG in benzene solution. The low-frequency heme mode shifts are not as pronounced for BHA, and the Raman data for the Fe(II)HRP-BHA-CO complex on the ν(Fe−C)/ν(CO) plot (Figure 7) indicate greater back-bonding (higher ν(Fe−C), lower ν(CO)) for the BHA complex than for Fe(II)HRPPEG-CO in benzene. The difference is likely due to the dehydrating properties of benzene in the case of Fe(II)HRPPEG-CO in benzene solution, and to the presence of a hydroxamic acid group capable of H-bond interaction in BHA for the aqueous Fe(II)HRP-BHA-CO complex. This -OH group can interact with the nearby propionate group, or with the R38 side chain, replacing the dislodged Wat92 (Figure 8). Upon reduction and CO binding, the flexible R38 can readily rearrange to interact with the bound CO, thereby enhancing back-bonding and decreasing the ν(CO) frequency. In benzene, however, R38 is more likely to form a salt bridge with the heme propionate because of the lowered dielectric constant. This linkage would prevent polarization of the bound CO and produce a higher ν(CO) frequency.

A recent theoretical study of possible ligand binding modes in the ternary BHA/HRP-C/CN complex[42] found evidence supporting a possible role for the -OH group in BHA binding that supports our hypothesis. In the most favorable BHA binding mode, BHA binds in the distal heme pocket near F179 with the phenyl headgroup pointing outward while the polar hydroxamate tail group points in toward the heme iron. This BHA binding mode was found to be significantly stabilized by strong H-bond interactions between the hydroxamic acid group and R38 and the cyanide ligand.

Mechanistic Implications. Peroxidases are generally believed to operate via an electron-transfer mechanism,[43] in which substrates are oxidized in one-electron steps by the oxyferryl intermediates Compounds I and II. The redox potentials are +1.33 V for both the Fe(III)/Compound II and Compound I/Compound II redox couples,[44] requiring that the substrate redox potential be lower than this value. The common substrates are indeed readily oxidizable phenolic compounds.[45] The labeling studies of Ortiz de Montellano[31,46,47] establish that substrates do not approach the heme iron but remain at the heme periphery, from where electrons are transferred.

It is therefore quite surprising that HRP in benzene catalyzes hydroxylation of solvent molecules.[25] The redox potential for the oxidation of benzene is +2.16 V,[48] ruling out an electron-transfer mechanism. Further evidence for a different reaction mechanism in the case of HRPPEG in benzene is provided by Akasaka et al.[25] who found that 95% of the oxygen isotope incorporated into the phenol product was from peroxide, consistent with an oxo transfer mechanism. Indeed, the catalytic activity of HRP toward benzene was found to be comparable to that of cytochrome P450, the classic oxo transfer enzyme.

How is this change in mechanism accomplished by HRP? Oxo transfer requires that the substrate be adjacent to the ferryl oxygen of Compound I[26] when this highly reactive intermediate is generated. We propose that this becomes possible in benzene because of the disruption of the H-bond network and dehydration of the distal heme pocket. The decreased polarity of the pocket and the resulting conformation change may allow a benzene molecule to slip into the heme pocket. In addition, since the R38 side chain would not be available to stabilize the Compound I ferryl oxygen, as it normally is,[49,50] Compound I therefore retains the reactivity needed to attack the benzene.

Conclusions

The present study provides the first structural evidence for a change in the reaction mechanism for an enzyme, HRP, involved

(41) Smulevich, G.; Miller, M. A.; Kraut, J.; Spiro, T. G. *Biochemistry* **1991**, *30*, 9546−9558.
(42) Chang, Y.-T.; Veitch, N. C.; Loew, G. H. *J. Am. Chem. Soc.* **1998**, *120*, 5168−5178.
(43) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199−8206.
(44) Hayashi, Y.; Yamazaki, I. *J. Biol. Chem.* **1979**, *254*, 9101−9106.
(45) Campa, A. In *Biological Roles of Plant Peroxidases*: *Known and Potential Function*; Campa, A., Ed.; CRC Press: Boca Raton, 1991; pp 25−50.
(46) Ator, M. A.; David, S. K.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1987**, *262*, 14954−14960.
(47) Ortiz de Montellano, P. R.; Choe, Y. S.; DePillis, G.; Catalano, C. E. *J. Biol. Chem.* **1987**, *262*, 11641−11646.
(48) Osa, T.; Yilidiz, A.; Kuwana, T. *J. Am. Chem. Soc.* **1969**, *91*, 3994.
(49) Sitter, A. J.; Reczek, C. M.; Terner, J. *J. Biol. Chem.* **1985**, *260*, 7515−7522.
(50) Hashimoto, S.; Tatsuno, Y.; Kitagawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2417−2421.

in nonaqueous catalysis. In aqueous solution, HRP catalyzes peroxidation of readily oxidizable aromatic substrates via an electron-transfer mechanism. The efficiency of this process is enhanced by the docking of the substrate at an aromatic site adjacent to the heme and by H-bonding of substrate substituents to the heme periphery. In benzene, however, a conformation change in the heme pocket permits access of solvent molecules to the heme iron, permitting oxo transfer upon Compound I formation. This conformation change may result from salt bridge formation between R38 and the propionate heme substituent at the docking site. The salt bridge also prevents deactivation of Compound I toward oxo transfer by R38 stabilization.

The change of reaction mechanism in benzene has important implications for the use of HRP and other peroxidases in industrial applications, since it greatly expands the possible repertoire of substrates. Is oxo transfer relevant to the biological role of HRP? In aqueous solution, the answer is no, but HRP may find itself in environments of low water activity in plants (cell wall) and soils.[45] In such environments, oxo transfer is a possible pathway for reactivity with otherwise unsuitable substrates.

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