The Use of Nonaqueous Media To Probe Biochemically Significant Enzyme Intermediates: The Generation and Stabilization of Horseradish Peroxidase Compound II in Neat Benzene Solution at Room Temperature

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Abstract: UV–visible spectral evidence is presented demonstrating that the mechanistically significant horseradish peroxidase compound II intermediate can be generated and stabilized for spectroscopic study in virtually anhydrous (<1% H_2O) neat benzene solution at room temperature. In organic media, the intermediate exhibits remarkable photostability and thermostability in contrast to its well-known photolability and thermal lability in aqueous solution.

Introduction

It is now well-established that enzymes are catalytically active in a variety of nonaqueous media[1,2] such as organic solvents,[3] supercritical fluids,[4,5] and gases.[6] In these unconventional environments, enzymes have been shown to exhibit a number of intriguing new properties such as increased thermostability[7] and novel chemo-,[8] enantio-,[9,10] and regioselectivity.[3,11] While several factors including the nature of the organic solvent,[12] the solvent dryness,[13] and the aqueous pH[8] have been identified as critical for enzymatic activity in nonaqueous media, understanding of the molecular level mechanism of this phenomenon is in its infancy. Largely, the view of researchers in the field of nonaqueous enzymology appears to be that little, if any, structural change occurs in proteins in nonaqueous media.[1,2] Crystallographic work by Ringe *et al.*[14,15] on subtilisin, one of a very few enzymes which appear to function in both hydrophilic and hydrophobic organic solvents, suggests little, if any, structural change occurs in proteins in organic media.

In part, the current lack of structural information on enzymes and enzyme intermediates in nonaqueous media may be attributed to the difficulties usually associated with characterizing heterogeneous systems. Most enzymes are insoluble in the relatively hydrophobic solvents[16] which work best in nonaqueous enzymology, making the use of optical spectroscopy difficult at best.

Recently, it has been shown that homogeneous solutions of enzymes in organic solvents can be prepared by the covalent modification of free amino groups on the enzyme's surface with poly(ethylene glycol).[17] A variety of enzymes including horseradish peroxidase,[18–20] chymotrypsin,[21–24] and bovine liver catalase[25] have been modified in this way and have been shown to be highly soluble (>1 mg/mL) and catalytically active in relatively hydrophobic organic media. Unfortunately, no structural characterization has been reported for these pegylated enzymes.

One enzyme which has been the subject of much interest in nonaqueous enzymology is horseradish peroxidase (HRP).[26–30] The heme glycoprotein (av FW 44 000) is known to catalyze the selective one-electron oxidation of a rather wide range of organic substrates, usually phenols and anilines[31] in the presence of hydrogen peroxide. Interesting industrial applications (polymerizations) already have been shown to exist for the chemistry of this enzyme in nonaqueous media.[26,27] Recently, Klibanov *et al.*[3] investigated the one-electron oxidation of phenols and aromatic amines by peroxidase in organic media and found that HRP catalyzes identical reactions in both aqueous and nonaqueous (enzyme suspensions) media. Klibanov's work suggests that the mechanism of enzyme catalysis should be

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independent of the reaction medium for horseradish peroxidase. Thus, the considerable body of information available on the chemical mechanism of HRP catalysis and catalytically active enzyme intermediates in aqueous media should be relevant in approaching the study of HRP catalysis in nonaqueous media.

The aqueous reaction mechanism for HRP catalysis is fairly well understood and involves two highly oxidized enzyme intermediates, compounds I and II.[31] The addition of 1 equiv of hydrogen peroxide converts the resting ferric enzyme to compound I. The addition of 1 equiv of an oxidizable substrate, RH_2, reduces compound I by one electron to produce compound II. The rate-limiting step in the enzyme mechanism appears to be the addition of more substrate, which converts compound II back to its native form and allows the catalytic cycle to begin again. Both intermediates are believed to contain an oxyferryl center. The extra oxidizing equivalent in compound I is known to be centered on the porphyrin.[32,33] Both intermediates are thermally labile and photoreactive.[34,35] These characteristics have slowed efforts to structurally characterize these mechanistically important enzyme intermediates. For example in recent years, several groups[36-38] have attempted to probe the active site structure of compound I yet not even these efforts have led to a consensus concerning the correct spectrum for this intermediate.

Polyethylene glycolated HRP has been prepared using a variety of coupling strategies by a number of groups.[18-20] Studies of pegylated HRP have largely focussed on the preparation of the pegylated enzyme and on demonstration of its catalytic activity in organic solvents. Good nonaqueous catalytic reaction rates have been reported for these derivatives in a wide variety of organic solvents. For these reasons, pegylated HRP represents a good model system with which to investigate the relationship between enzyme structure and reactivity in organic media.

In view of the aforementioned need for structural studies of enzymes in nonaqueous media, the continued interest in physicochemical characterization of HRP and its enzyme intermediates, the demonstrated solubility, and catalytic activity of polyethylene glycolated HRP, we wish to report our initial findings related to the optical spectroscopic study of pegylated HRP and its intermediates in organic media.

Experimental Section

Materials. Horseradish peroxidase (Sigma; Type VI) was used as received without further purification. Sigma Type VI peroxidase, the highest specific activity formulation of peroxidase, is a mixture of basic isozymes—principally isozyme C (>75%) based on SDS PAGE assay.[39]

The following materials were obtained commercially as analytical grade or better and used as received: potassium phosphate monobasic (Fisher), methoxypoly(ethylene glycol) activated with cyanuric chloride (Sigma), sodium tetraborate decahydrate (Aldrich), pyrogallol (Sigma), benzene (Fisher), 30% (w/w) hydrogen peroxide (Fisher).

Preparation of HRP-PEG. HRP-PEG was prepared as follows: Commercial methoxypoly(ethylene glycol) activated with cyanuric chloride (Sigma; 181 mg; 36.2 μmol) and HRP Type VI (Sigma; 87 mg; 2.2 μmol) were stirred together in 20 mL of 0.1 M sodium borate buffer, pH 9.2, for 3 h at 5 °C. The reaction was quenched by the addition of 20 mL of 76 mM sodium phosphate buffer, pH 7.0. Unreacted horseradish peroxidase, poly(ethylene glycol), and sodium borate were removed by the repeated concentration (>3 times) from an initial volume of 50 mL to less than 5 mL of the sample in an Amicon ultrafiltration cell (YM 30 membrane) using distilled water and subsequent overnight dialysis (Spectra Por 7 MWCO 50 000) against 1 L of distilled water. HRP-PEG in the dry state was prepared by the lyophilization of this solution.

Lyophilization. Dilute aqueous solutions (<1 mM) were frozen in dry ice using the shell freeze technique. Frozen solutions were lyophilized overnight on a Virtis VacuFreeze freeze dryer equipped with a Welch Model 8905 direct drive vacuum pump. Freeze drying vials were kept submerged in dry ice to ensure the protein solutions were kept frozen throughout the freeze drying process. Lyophilized protein was stored at −10 °C in a freezer until use.

Determination of the Extent of Modification. The average extent of modification of amino groups in HRP was determined to be 2 ± 1 using a combination of UV−vis and ^{1}H NMR. A calibration curve relating the concentration of PEG (δ = 3.49 ppm) to that of the internal NMR standard tetramethylsilane, at constant known concentration, in DMSO-d_6 was constructed and used to determine the concentration of PEG in actual HRP-PEG samples. The average concentration of HRP in the NMR samples was subsequently determined by UV−vis, assuming that the molar absorptivities of the Soret band ($\epsilon = 9.1 \times 10^4$ M^{-1} cm^{-1}), a porphyrin-centered $\pi-\pi^*$ transition, is unaffected by PEG modification. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of the sample dissolved in 0.5% trifluoroacetic acid confirmed that the modified protein was a mixture of singly- (*m/z* 49 160), doubly-, and triply-polyethylene glycolated horseradish peroxidase.

Moisture Determination. The coulometric Karl Fischer titration method which provides ppm sensitivity levels was used to determine the water content in the nonaqueous protein solutions studied in this work.[40] In each case, the moisture determination was made using a Cosa Instruments CA-06 moisture meter, immediately following the optical spectroscopic measurement based on three independent measurements using the same aliquot used in the optical analysis.

Determination of Hydrogen Peroxide Concentration in Neat Benzene Solution. Saturated solutions of hydrogen peroxide in benzene were prepared by shaking mixtures of 5 mL of 30% hydrogen peroxide and 20 mL of benzene vigorously for a period of 5 min. The mixture was then allowed to sit for 5−10 min at room temperature to bring about the complete separation of the aqueous and benzene solution layers. Aliquots of the top benzene layer were then carefully removed for use via gas-tight microliter Hamilton syringes.

The molar absorptivity of purpurogallin at 420 nm and room temperature was determined to be (1.12 ± 0.05) × 10^3 M^{-1} cm^{-1} by UV−vis spectroscopy: Excess 30% hydrogen peroxide was added to a 0.1 M potassium phosphate buffer solution, pH 6.0, containing a known and limiting concentration of pyrogallol and several milligrams of horseradish peroxidase. The molar absorptivity of purpurogallin at room temperature at 420 nm was then determined from the absorbance at 420 nm using Beer's law, assuming a two-to-one stoichiometry between the number of moles of pyrogallol consumed and the number of moles of purpurogallin produced.

The concentration of hydrogen peroxide in the aforementioned benzene solutions was determined to be 2.91 ± 0.50 mM based on the following procedure: 5 μL of saturated hydrogen peroxide in benzene solution were added to 2 mL of an aqueous 0.1 M potassium phosphate buffer solution, pH 6.0, containing an excess amount of pyrogallol and several milligrams of horseradish peroxidase. The concentration of hydrogen peroxide in the benzene solution was then determined from the absorbance of purpurogallin at 420 nm using Beer's law, assuming a three-to-one stoichiometry between the number of moles of hydrogen peroxide consumed and the number of moles of purpurogallin produced.

Optical Absorption and Circular Dichroism Measurements. UV−vis spectra were recorded at room temperature on a Perkin-Elmer Lambda 9 UV−vis/NIR spectrophotometer using 0.1 or 1.0 cm path length rectangular supracil cells (Hellma).

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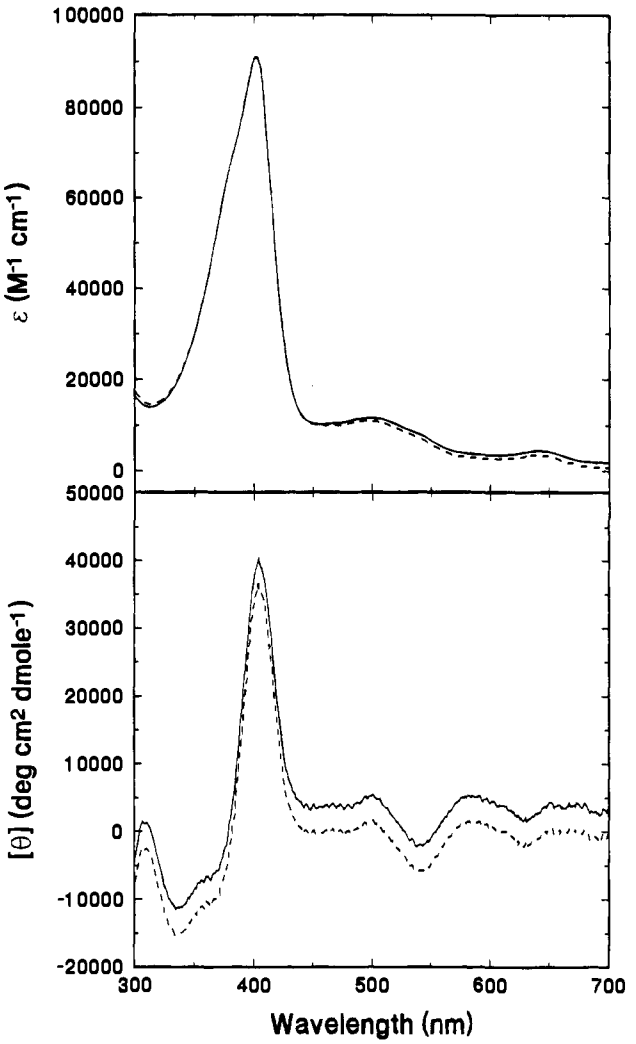

Figure 1. UV−vis (top) and CD (bottom) spectra for Fe^{3+} HRP (—) and Fe^{3+} HRP-PEG (- - -) in 50 mM phosphate buffer, pH 7.0.

CD spectra were recorded at room temperature on a Jasco J200 spectropolarimeter using 0.1 or 1.0 cm path length rectangular supracil cells (Hellma).

ESR Measurements. ESR spectra were obtained for frozen solutions contained in quartz ESR tubes at 4 K on a Bruker ECS106 spectrometer. All spectra were recorded at low power (2.0 mW) to avoid saturation effects. UV−vis spectra of the ESR samples were recorded immediately afterward.

Raman Spectroscopy. Raman measurements were made on a home-built instrument consisting of a Coherent INNOVA 306 argon ion laser (with UV option), 599 dye laser, SPEX Triplemate spectrometer (0.6 m, f/6.3), and intensified photodiode array detector. Data acquisition and signal processing is controlled by an 80386 computer running SPEX DM3000 software. A broadband depolarizer (Optics for Research) was used in all measurements. Samples contained in melting point capillary tubes (1-mm i.d.) were typically excited with 100 mW or less of 514.5-nm light. Fenchone was used to calibrate the Raman frequencies.[41] All observed intensities are relative and not true intensities and have not been corrected for the spectral sensitivity of the instrument.

Results and Discussion

A. Polyethylene Glycolated Horseradish Peroxidase in Aqueous Solution. Figure 1 shows the UV−vis and CD spectra for poly(ethylene glycol) (av FW 5000)-modified Fe^{3+} horseradish peroxidase together with that for native horseradish peroxidase. The absorption and CD spectra for pegylated horseradish peroxidase are dominated by the intense Soret and Q-band features which arise from $\pi-\pi^*$ transitions of the heme active site. The energy, intensity, and spectral bandwidth of the UV−vis and CD features for the pegylated enzyme are indistinguishable from that of the native enzyme at pH 7.0. The similarity of the UV−vis and CD spectral data of the pegylated and native horseradish peroxidase suggests that modification of the enzyme by poly(ethylene glycol) does not affect the heme active site structure.

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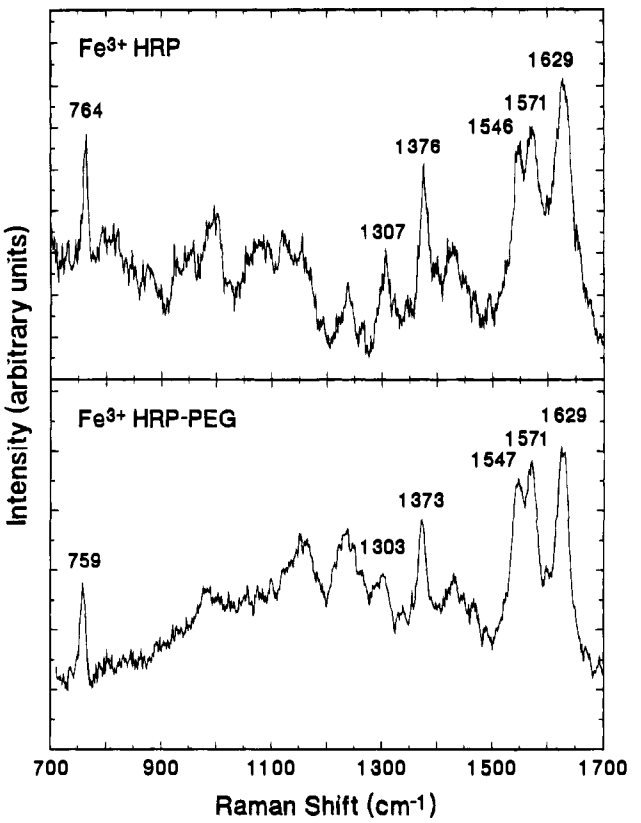

Figure 2. Resonance Raman spectra in 50 mM sodium phosphate buffer, pH 7.0, of 0.15 mM Fe^{3+} HRP (top) and 1.0 mM Fe^{3+} HRP-PEG (bottom). Spectral conditions: wavelength, 514.5 nm; power at sample, 100 mW; integration time, 5 s/scan. The spectra are composites of 200 scans.

Supporting evidence is provided by the resonance Raman spectra (700−1700 cm^{-1}) for native horseradish peroxidase and HRP-PEG upon laser excitation at 514.5 nm (Figure 2). At this excitation wavelength, resonant enhancement is observed primarily for in-plane heme vibrations.[42] Several of these in-plane heme vibrations are well-known to be extremely useful markers of the oxidation state, spin state, and core size of the heme active site.[42,43] As shown in Figure 2, the frequencies of the marker bands for HRP-PEG [1373 cm^{-1} (I); 1571 cm^{-1} (IV); 1629 cm^{-1} (V)] are identical to those of HRP [1376 cm^{-1} (I); 1571 cm^{-1} (IV); 1629 cm^{-1} (V)] within experimental error. Thus, the Raman data for HRP-PEG support the conclusion drawn from the UV−vis and CD data that pegylation of surface lysines does not affect the heme active site structure in horseradish peroxidase.

Furthermore, the specific activity of HRP-PEG for the oxidation of 42.3 mM pyrogallol to purpurogallin in 0.01 M potassium phosphate buffer, pH 6.0, in the presence of 7.84 mM hydrogen peroxide is 15% lower than that of the native protein (239 ± 15 units/mg vs 282 ± 5 units/mg), consistent

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with findings previously reported for other PEG-modified enzymes.[44] *Together the spectroscopic (UV–vis, CD, and resonance Raman) and specific activity data provide strong evidence that PEG modification of lysine residues on the surface of HRP does not affect the structure or reactivity of the heme active site in HRP.*

These findings are in direct contrast to recent findings for the structure and reactivity of poly(ethylene glycol)-modified horse cyt *c*.[45] Mabrouk studied the effects of different degrees of pegylation on the structure and function of horse cyt *c* and found that the heme active site structure and redox function of cyt *c* were significantly affected by the extent of pegylation. Likely the insensitivity of horseradish peroxidase to pegylation is due to the fact that there are few (three) lysine residues on the surface of HRP as well as the fact that the heme active site in horseradish peroxidase is buried deeply within the glycoprotein.[46] In contrast, there are 19 lysine residues on the surface of horse cyt *c*.[47] Furthermore, the heme active site in horse cyt *c* lies near the surface of the protein in a crevice surrounded by a large number of these surface lysine residues.[48,49]

B. Polyethylene Glycolated Horseradish Peroxidase in Neat Benzene Solution. Figure 3 shows the UV–vis and CD spectra of homogeneous solutions of HRP-PEG dissolved in neat benzene solution. As expected, the characteristic Sorent band and Q-band are observed in the UV–vis and CD spectra of HRP-PEG in benzene. However, the intensity of the Soret band in both the UV–vis and CD spectra recorded in benzene is significantly lower compared to that observed in aqueous solution. On the other hand, the intensity of the absorption bands at 500 and 640 nm does not appear to be affected by the solvent. This solvent-induced decrease in the Soret band intensity signals a change in the heme active site microenvironment. Enzyme denaturation with the generation of free heme can be ruled out since the presence of free heme would result in a shift of the Soret band to higher energy and a corresponding decrease in signal intensity for the Q-bands that is not observed experimentally.[50]

The ESR spectrum of HRP-PEG in neat benzene solution (Figure 4) further supports the conclusion that the enzyme remains structurally intact. The spectrum exhibits the low-temperature ESR signature characteristic of the somewhat rhombic high spin ferric iron center in HRP, specifically, two principal signals at $g = 2$ and 6, with significant rhombic splitting evidenced for the $g = 6$ signal.[51] The change of solvent appears to produce a minor increase in the rhombicity of the heme iron for the enzyme in benzene.

Furthermore, the specific activity of HRP-PEG for the oxidation of 42.3 mM pyrogallol to purpurogallin in 0.01 M potassium phosphate buffer, pH 6.0, in the presence of 7.84 mM hydrogen peroxide was found to be 7% lower when the enzyme was recovered from benzene solution by vacuum (231 ± 33 units/mg vs 215 ± 12 units/mg). The demonstration of retention of peroxidase activity following exposure of HRP-PEG to benzene provides additional evidence that the enzyme active site retains its structural integrity in benzene. *Together,*

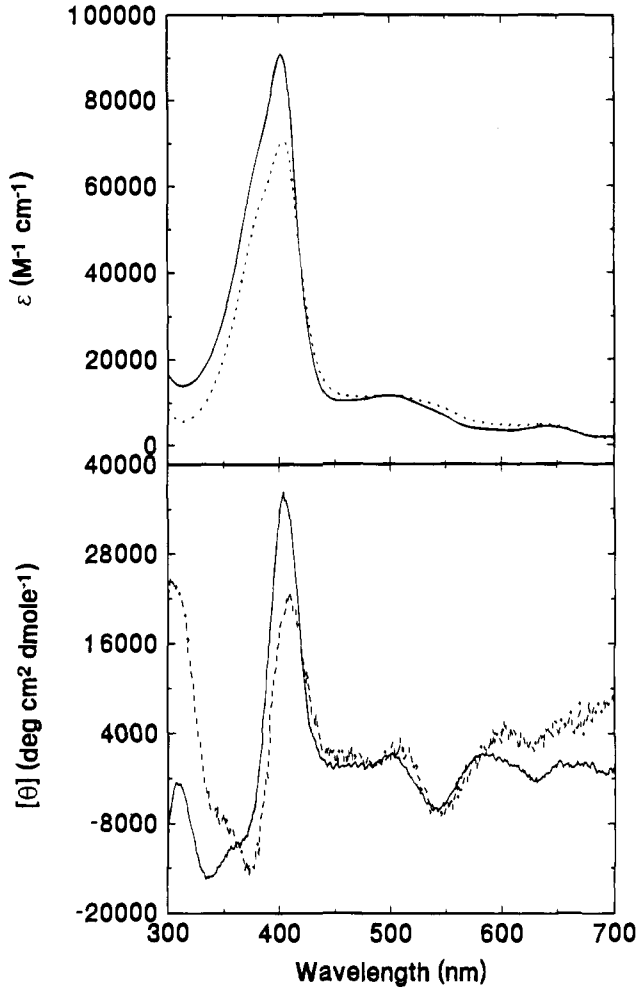

Figure 3. UV–vis (top) and CD (bottom) spectra for Fe^{3+} HRP-PEG in 50 mM phosphate buffer (—), pH 7.0, and neat benzene (- - -) (UV–vis sample: 264 ppm H_2O; CD sample: 136 ppm H_2O).

the spectroscopic (UV–vis, CD, and ESR) and specific activity data strongly suggest that the heme active site of HRP-PEG remains structurally intact in benzene.

In an effort to identify the factors affecting the intensity of the Soret band for HRP-PEG in neat benzene, several experiments were performed. The concentration of water in the local microenvironment and the aqueous pH have been identified as two factors important in determining the catalytic activity of enzymes in nonaqueous media.[1,2] In order to investigate the significance of added water on the intensity of the Soret band for HRP-PEG in benzene, 1 μL of distilled water, pH 6.96, was added to a 1.0-cm cuvette containing HRP-PEG in benzene. The intensity of the Soret band relative to that of the 503-nm Q-band was observed to increase only slightly (factor of 1.1). On the other hand, the addition of HRP-PEG to a benzene solution saturated with 0.1 M potassium phosphate buffer, pH 6.0, was observed to produce a dramatic increase (factor of 2.0) in the intensity of the Soret band relative to that of the 503-nm Q-band. However, no measureable effect was observed when simple nonbuffer salts such as NaCl were added to HRP-PEG in benzene. These results suggest that the pH in the microenvironment of the heme is an important factor governing the intensity of the Soret band of HRP-PEG in nonaqueous media.

C. Polyethylene Glycolated Horseradish Peroxidase–H_2O_2 Compound II Intermediate in Neat Benzene Solution. Figure 5 shows the striking result that an HRP-H_2O_2 intermediate can be generated and spectrophotometrically probed on a

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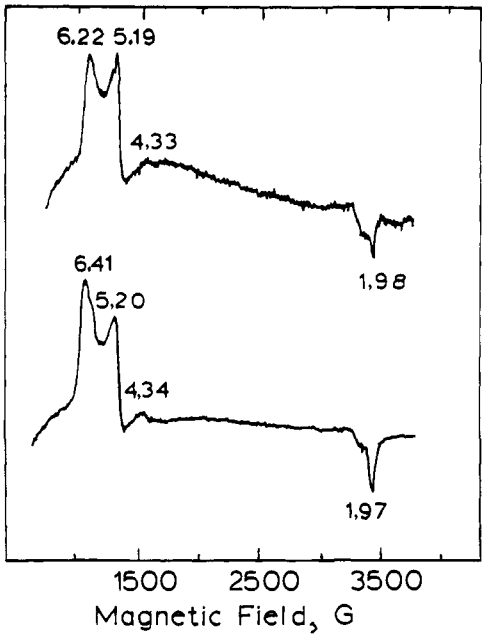

Figure 4. ESR spectra at 8 K (frozen solution) for Fe^{3+} HRP-PEG in 25 mM Tris (top) and neat benzene (333 ppm H_2O) (bottom). In both cases, the microwave frequency was 9.45 GHz and the microwave power was 2.0 mW. The signal at $g = 4.3$ in these spectra reflects the presence of a small amount of rhombic iron.

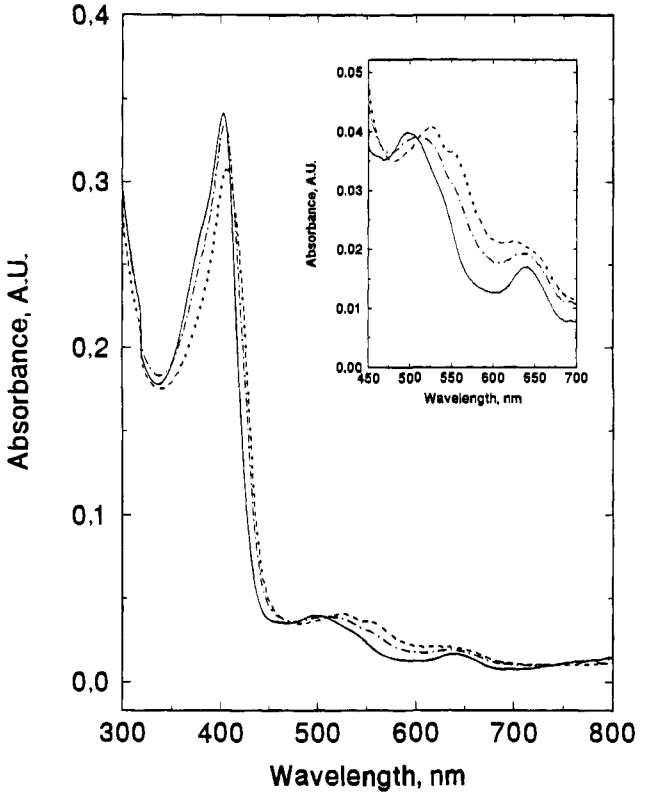

Figure 5. UV–vis spectra spectra obtained by the addition of a total of 0 (—), 24 (- · -), and 48 (- - -) μL of 3.0 mM H_2O_2 in benzene to a 1.0-cm cuvette containing 2.5 mL of 9.2 μM Fe^{3+} HRP-PEG in benzene saturated with 0.1 M potassium phosphate buffer, pH 6.0 (505 ppm H_2O).

steady state basis in neat benzene at room temperature. The UV–vis spectrum for the HRP–H_2O_2 intermediate in benzene solution displays the same general characteristics that have been previously reported for HRP compound II in aqueous media.[52] The UV–vis spectrum of compound II is of the normal porphyrin type and is characterized by an intense Soret band at 420 nm and two Q-bands at 527 and 554 nm. These characteristics compare favorably with those of the intermediate produced in benzene, as shown in Figure 5, specifically, the Soret shifts to slightly longer wavelength, the 500-nm band disappears, and two new bands appear at longer wavelengths: 525 and 550 nm, respectively. In addition, the intermediate displays the characteristic red-brown color that compound II is known to exhibit in aqueous solution.[53]

A solution of HRP-PEG in 0.1 M potassium phosphate buffer, pH 6.0, saturated benzene was titrated with microliter aliquots of a 3.0 mM solution of hydrogen peroxide in benzene. Complete conversion of the protein to the intermediate was achieved by the addition of a 6-fold molar excess of hydrogen peroxide. The addition of a small amount of hydrogen peroxide in excess of this molar ratio, e.g., 7.5:1 molar ratio of H_2O_2 to HRP-PEG, was found to produce no detectable change in the UV–vis spectrum for this intermediate. This suggests that the spectrum shown in Figure 5 corresponds to "pure" intermediate. We believe that the six-to-one stoichiometry requirement of HRP-PEG for hydrogen peroxide in benzene likely reflects the inability of hydrogen peroxide in benzene to gain ready access to the horseradish peroxidase heme active site which is buried deep within the enzyme.

In aqueous solution, compound II is typically generated by the one-electron reduction of compound I.[31] Thus, our ability to generate this intermediate in benzene solution in the absence of an obvious reducing substrate is intriguing. At this point, the identity of the reducing agent is unknown. However, we propose that one possible mechanism for the formation of this intermediate in benzene could be the immediate reduction of 1 equiv of the compound I intermediate by 1 equiv of the resting form of the protein with the formation of 2 equiv of compound II. Studies are currently underway to further probe the mechanism of formation of this intermediate in benzene solution.

In benzene, PEG-modified HRP compound II is surprisingly photo- and thermostable. The intermediate can be maintained at room temperature or be irradiated with 200 mW of 514.5-nm light for more than 2 h without detectable change in the intensity or frequency of the Soret band in the UV–vis absorption spectrum. This behavior is in marked contrast to the well-known thermal lability and photolability of aqueous HRP compound II which has made rigorous spectroscopic characterization of the intermediate challenging. Compound II is known to undergo photochemical reduction at room temperature in aqueous solution to completely regenerate HRP in approximately 1 h.[34] The thermo- and photostability of compound II in benzene means that detailed spectroscopic examination of this intermediate and possibly other biochemically significant enzyme intermediates may now be feasible in organic media (studies in progress). We believe that our ability to stabilize compound II is likely a consequence of decreased flexibility and reactivity of the intermediate in benzene.

In summary, these studies have demonstrated the feasibility of investigating mechanistically significant enzyme intermediates in organic media as well as the possible benefits of nonaqueous media in facilitating the stabilization of enzyme intermediates for rigorous structural study. We propose that the use of nonaqueous media in enzyme structure–function studies may

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provide an unparalleled opportunity to probe intermediates in complex biochemical mechanisms. For this reason, we are at present extending this experimental protocol to probe the bacterial P450 enzymatic pathway and will report our findings in future publications.

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