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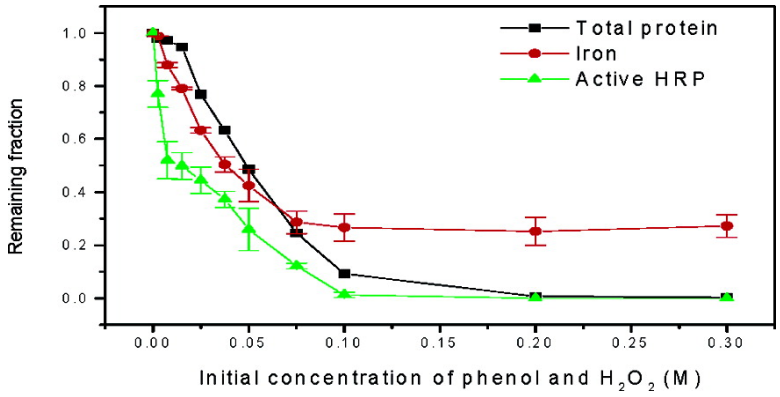

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Inactivation of Horseradish Peroxidase by Phenoxyl Radical Attack

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Abstract: To test the hypothesis that horseradish peroxidase (HRP) can be inactivated by phenoxyl radicals upon reaction with H_2O_2/phenol, we probed HRP-catalyzed phenol oxidation at various phenol/H_2O_2 concentrations. To this end the total protein, phenolic product, active protein, and iron concentrations in the aqueous phase were determined by protein assay, phenol-^{14}C isotopic labeling, resonance Raman and atomic absorption spectroscopy, respectively. Additionally, resonance Raman and FTIR measurements were carried out to probe possible structural changes of the enzyme during the reaction. The data obtained provide the first experimental support for the hypothesis that HRP can be inactivated by a phenoxyl radical attack. The heme macrocycle destruction involving deprivation of the heme iron occurs as a result of the reaction. An intermediate type of the active protein was observed by Raman difference spectra at low concentrations which features a stabilization of the quantum mixed state of the heme iron and a significant amount of phenoxylphenol-type oligomers in solution and probably also in the heme pocket. This work provides a basis for evaluating the relative contributions of different HRP inactivation mechanisms and is thus critical for optimizing engineering applications involving HRP reactions.

Introduction

Horseradish peroxidase (HRP) is a classic heme enzyme containing a ferric protoporphyrin IX prosthetic group.[1] HRP catalyzes the one-electron oxidation of phenolic substrates to form radicals via a Chance−George mechanism.[2] The enzyme is first oxidized by peroxide to compound I, after which compound I abstracts one electron from a phenol molecule to form compound II and generates a phenoxyl radical. Finally, compound II oxidizes a second phenol molecule, releasing another phenoxyl radical and returning the enzyme to its native state, thereby completing the cycle. The phenoxyl radicals generated react with each other to form oligomers, while the soluble coupling products can contribute to serve as phenolic substrates and undergo further oxidative coupling until larger polymers that precipitate from the solution are formed.[3] The apparent stoichiometric ratio between phenol and peroxide thus shifts from the theoretical value of 2:1 reflecting a catalytic cycle and approaches a value of 1:1 as the polymeric coupling products grow in size.[4]

Because polymerized precipitates formed from phenol coupling can be readily settled from water or become immobilized in soil/sediment systems, enzyme-enhanced oxidative coupling reactions have been proposed as potential tools for water treatment [5,6] and soil remediation.[7] However, such potentially important applications suffer from the fact that the enzyme becomes quickly inactivated during the phenol polymerization. Three possible pathways have been proposed for HRP inactivation. The first is by reaction with H_2O_2; i.e., both compounds I and II react with excessive peroxide to form different inactive species.[8] The second involves sorption/occlusion by polymeric products; i.e., HRP adsorbs on the precipitated products formed from phenol coupling. When large amounts of precipitate are formed, HRP becomes entrapped and its active sites occluded.[9] In the third possible pathway free phenoxyl radicals can react with HRP, leading to an inactive state.[5] While the first two

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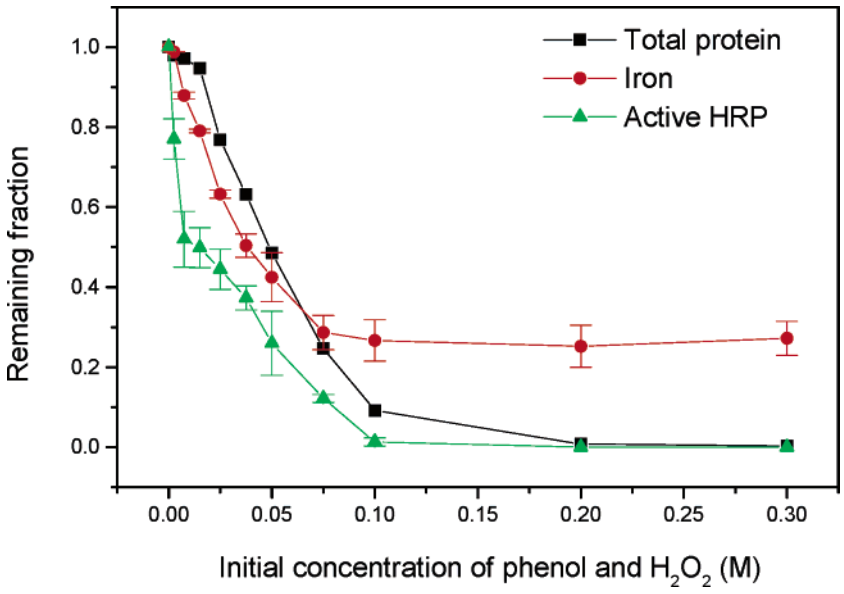

Figure 1. Fractions of total protein, iron, and heme content from active HRP remaining in the supernatant as a function of phenol/H_2O_2 concentrations. The initial HRP concentration was 0.1 mM. The error bars result from triplicate measurements of the iron content and from duplicate Raman measurements of the active protein, respectively. Triplicate measurements were also performed for the determination of the total protein fraction to determine the statistical errors which were found to be of the same size as the data points.

mechanisms have been corroborated by direct evidence, the third remains hypothetical, although it has been predicted from modeling of kinetic data.[10]

The aim of the present study was to obtain an experimental proof for such HRP inactivation by radical attack. To this end, we investigated HRP-catalyzed phenol oxidation at various phenol/H_2O_2 concentrations. Total protein, phenolic product, active protein, and iron concentrations in the aqueous phase were measured by protein assay, phenol-^{14}C isotopic labeling, resonance Raman and atomic absorption spectroscopy, respectively. Resonance Raman and FTIR measurements were also conducted to probe possible structural changes of the enzyme caused by a phenoxyl radical attack. The work provides a basis for evaluating the relative contributions of different HRP inactivation mechanisms and is thus critical for optimizing engineering applications that involve HRP reactions. The results are also of relevant for understanding inactivation mechanisms involving other heme-containing enzymes.

Material and Methods

Materials. High purity horseradish peroxidase isoenzyme C (code HRP 4B, *Reinheitszahl* = 3.4) was purchased from Biozyme Laboratories Ltd. Hydrogen peroxide (30.8%), phenol (99+%), 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (98%, in diammonium salt form), and phenol-UL-^{14}C (51.4 mCi/mmol) were obtained from Sigma Chemical Co. (St. Louis, MO). Phenol-d_6 (99atom % D) was purchased from Aldrich (Milwaukee, WI). ScintiSafe Plus 50% liquid scintillation cocktail were obtained from Fisher Scientific (Fairlawn, NJ). A Coomassie protein assay reagent kit was from Pierce Biotechnology (Rockford, IL).

Methods. A. Reaction Setup. Phenol coupling mediated by HRP/H_2O_2 was carried out in a phosphate buffer (10 mM, pH = 7.0) using airtight 5-mL borosilicate vials as reactors, with equimolar concentrations of phenol and peroxide ranging from 0 to 0.30 M. HRP concentration was fixed at 100 μM. After addition of peroxide, all vials were sealed and then agitated at 150 rpm for 1 h. After completion of the reaction, an aliquot of 2 mL was taken from each reaction vial and placed into Teflon-coated microcentrifuge tubes, which were centrifuged at 10 000 g for 10 min. The supernatants were used for determining protein, H_2O_2, dissolved iron, and dissolved phenolic product contents and for probing reaction products via Raman and FTIR measurements. All reactions and measurements were carried out at room temperature. We have checked the pH value of the solution and found it to be practically identical before and after reactions even with the highest substrate concentration (0.30 M).

B. Chemical Analyses. The Coomassie protein assay[11] was used to determine total protein concentration remaining in aqueous phase and ABTS as substrate[12] to measure HRP activity. One unit of HRP activity is defined as the amount catalyzing the oxidation of 1 μmol of ABTS per min. The concentration of dissolved iron was measured using atomic absorption at the wavelength 248.3 nm with a slit width of 0.2 nm. The concentration of residual hydrogen peroxide was determined using iodometric titration, in which 0.01 N thiosulfate was used as the titrant to reduce the iodine generated from the reaction of potassium iodide with the residual hydrogen peroxide catalyzed by ammonium molybdate in 1 M sulfuric acid. The detection limit was found to be 0.03 mM. Samples used for the determination of residual phenol and dissolved coupling products were prepared in a separate experiment by employing ^{14}C- labeled phenol at the exactly same series of conditions as those for other sample analyses. ^{14}C radioactivity was measured using a Beckman LS6500 liquid scintillation counter (Beckman Instruments, Inc.).

C. Spectroscopic Analyses. Resonance Raman spectra were measured with a triple-grating T64000 Raman spectrometer (Jobin-Yvon) using the 441.6 nm excitation provided by a He−Cd laser (Model IK 4601R-E, Kimmon Electric US) and the 457.9 nm excitation provided by an argon laser (Lexel). The heme content from active HRP was probed by determining the intensity of the ν_4 band at 1374 cm^{-1}, which was calibrated by the internal standard signal 983 cm^{-1} band from sodium sulfate. Using phenol-d_6, an isotopic Raman experiment was also conducted to assist the identification of reaction products. Fourier

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transform infrared (FTIR) spectra of the enzyme were measured with a Magna IR 560 optical bench (Nicolet). The amide I band was utilized for secondary structure identification. To this end lyophilized samples were dissolved in D_2O and incubated for 4 h before being inserted into a 12-μm spacer in a liquid cell with CaF_2 windows for measurement. The lyophilized protein was prepared by using a Labconco 6L lyophilizer at a condenser temperature of −45 °C and a pressure of <60 μm of Hg.

Results and Discussion

Figure 1 displays the fractions of total protein, iron, and active HRP remaining in the supernatant after 1 h of reaction as functions of the initial phenol/H_2O_2 concentration. In this figure, the relative active HRP content was estimated using the intensity of oxidation marker ν_4 at 1374 cm^{-1}.[13] It was observed that the characteristic heme bands of the solutions did not change after reactions, confirming that most of the remaining active HRP had returned to its native state through the catalytic cycle. The monotonic decay of the total protein fraction reflects the loss of dissolved HRP by means of sorption by precipitated products. The much smaller fraction of active HRP indicates that substantial amounts of HRP were inactivated by a mechanism different from HRP adsorption.

Reaction at High Phenol/H_2O_2 Concentrations. As shown in Figure 1, more than 50% of the protein precipitated from the supernatant when initial phenol and peroxide concentrations larger than 0.05 M were used. This significant loss of protein can be attributed to a larger amount of precipitate formation at higher substrate concentrations. Raising the substrate concentration even higher than 0.1 M yielded a nearly complete deprivation of proteins from solution. Interestingly, about 30% iron still remained in the supernatant, but no signal from intact hemes was detected. This indicates that the deactivation process eventually causes heme destruction, with iron ions deprived and tetrapyrrole fragments absorbed to the precipitate. Thus, our data provide the first evidence in support of the notion that heme chromophores are destroyed during HRP mediated phenol coupling reactions.

Reaction at Lower Phenol/H_2O_2 Concentrations. We paid special attention to reaction conditions below 0.0075 M, where minimal protein sorption (less than 6%) and severe heme loss (near 50%) occurred, to further elucidate the HRP inactivation pathways. This concentration level also corresponds to the reaction conditions most commonly encountered in environmental applications. We are able to rule out that the significant HRP inactivation at these conditions are caused by side reactions between compounds I and II with excessive H_2O_2, one mechanism that was shown to contribute at certain conditions.[14] Our additional experimental data shown in Figure 2 demonstrate that H_2O_2 was depleted during the first 90 s of the reaction with 7.5 mM initial concentrations of phenol and H_2O_2. In this time frame, the direct inactivation caused by H_2O_2 is negligible, as demonstrated by Figure 3, which shows that HRP inactivation is less than 2% in a system having 7.5 mM H_2O_2 and no phenol. It is known that H_2O_2-based HRP inactivation is largely suppressed in the presence of phenol, because phenol can compete with H_2O_2 for compounds I and II.[14c,e] Therefore, HRP

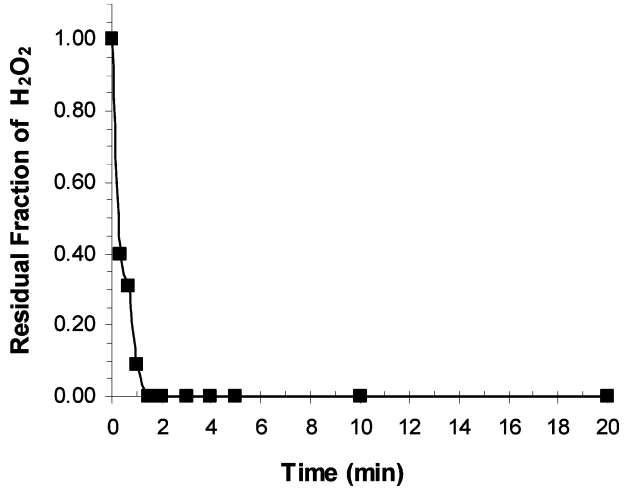

Figure 2. Rate of H_2O_2 depletion initiated by a reaction with 7.5 mM H_2O_2 and phenol and 0.1 mM HRP.

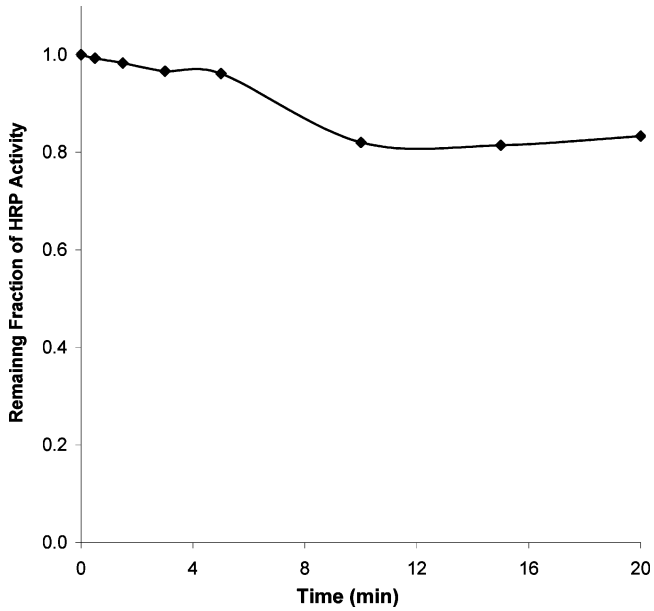

Figure 3. Rate of active HRP depletion initiated by a reaction with 7.5 mM H_2O_2 and 100 μM HRP.

inactivation at 90 s (less than 2%) in Figure 3 would represent the worst scenario that H_2O_2-related side reactions can contribute to the overall HRP inactivation in the reaction system with 7.5 mM initial concentrations of phenol and H_2O_2. This leads to the conclusion that H_2O_2-based HRP inactivation at 7.5 mM initial concentration is far less than 2%. It follows therefore that the heme loss at low phenol/H_2O_2 concentration is predominantly caused by a radical attack.

Spectroscopic Investigation. To investigate how the phenoxyl radical would interact with the heme site, we analyzed the Raman spectra of the reaction products in more detail. Figure 4 presents the Raman difference spectra for samples taken at different phenol/H_2O_2 concentrations. The red line in Figure 4 displays the difference spectrum obtained by subtracting the

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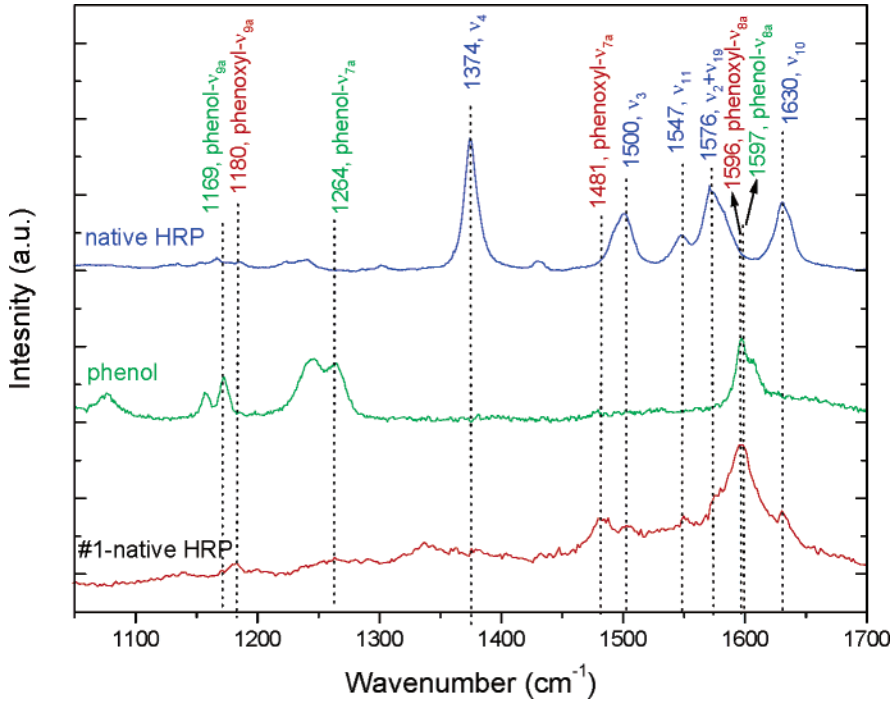

Figure 4. Raman measurements to identify the phenoxyl radical associated with the inactivated HRP. The red line is the difference spectrum which emerged from subtracting the resting-state HRP spectrum from that of sample 1 (2.5 mM phenol/H_2O_2) by using the ν_4 band as reference. The green and blue lines are Raman spectra for native HRP and neat phenol, respectively. All spectra were taken with 442 nm excitation.

resting HRP spectrum from that of sample 1 (2.5 mM phenol/H_2O_2) by using the ν_4 band as reference. Weak signals at 1500, 1547, 1576, and 1630 cm^{-1} are identified as the ν_3, ν_{11}, ν_2 overlapping with ν_{19} and ν_{10} modes of the quantum mixed spin state of HRP, respectively.[15] Three bands appear at 1180, 1481, and 1596 cm^{-1}, which cannot be assigned to modes of the heme macrocycle. These bands correlate well with the CH bending vibration ν_{9a}, the C−O stretching mode ν_{7a}, and the C−C ring mode ν_{8a} of a phenoxyl radical.[16] The corresponding Raman bands of phenol appear at 1169, 1263, and 1595 cm^{-1}.[17] Particularly the absence of bands in the region between 1250 and 1300 cm^{-1} indicates that phenol bands are not detected under our experimental conditions. However, since it is unlikely that phenoxyl radicals are stable enough to be probed in a steady-state experiment, we have to invoke either oligomeric products such as *p*-phenoxylphenol or phenoxyphenoxyphenol[3] or coupling with either the heme (iron) or the protein matrix. An interaction with the heme can be ruled out by the above sample #1—resting-state HRP difference spectrum. Binding to any group in the protein matrix is certainly less likely than the coupling of phenoxyl. It is therefore reasonable to assign the three bands to phenoxyphenol-type oligomers. One might argue that the band at 1481 cm^{-1} could also arise from a hexacoordinated high-spin (hc-hs) species. However, this assignment can be ruled out by the following reasoning. First, 1481 cm^{-1} is somewhat low for an hc-hs state of the heme iron. Second, a change of the coordination and spin state would give rise to a difference signal with a minimum at 1500 cm^{-1}, reflecting the depletion of the corresponding pentacoordinated quantum mixed state. The spectrum argues to the contrary in that it shows a residual intensity of ν_3 at the wavenumber, indicating an increased population of this iron state. Moreover, one would also expect a difference signal at the ν_{10} position, with the minimum at 1630 cm^{-1}. Again, we obtained instead a slight increase of the intensity at this wavenumber, indicative of a more populated quantum mixed state. It is also impossible to assign the 1596 cm^{-1} band to any heme band for the following reasons. Since the band is polarized, the only possible candidate would be ν_2, but the highest possible wavenumber for this mode is 1592 cm^{-1}, which would be diagnostic of a ferrous low spin-hexacoordinated iron state.[13] In this case ν_{10} would appear at 1638 cm^{-1}, in contrast to our observation (1630 cm^{-1}).[13] Another striking argument can be inferred from the polarized Raman spectra shown in Figure 5. The upper panel exhibits the difference of the polarized Raman spectra of sample 1 and native HRP taken with 457 nm excitation. The 1596 cm^{-1} band exhibits a depolarization ratio of 0.4, which is significantly higher than what has been obtained for the ν_2 band of a variety of HPR states at this excitation wavelength.[15c] This band is not assignable to the ν_8-phenol mode either, since the absence of the ν_{7a} band at 1263 cm^{-1} indicates that phenol bands are not detectable under our experimental conditions. As a matter of fact, the ν_8-phenol is hardly detectable at low phenol concentrations, as demonstrated by the Raman spectrum of a HRP/phenol mixture in the absence of H_2O_2 in the lower panel of Figure 5. Moreover, the ν_8 band of neat phenol is depolarized (middle panel of Figure 5). Its 40 cm^{-1} upshift from the literature values

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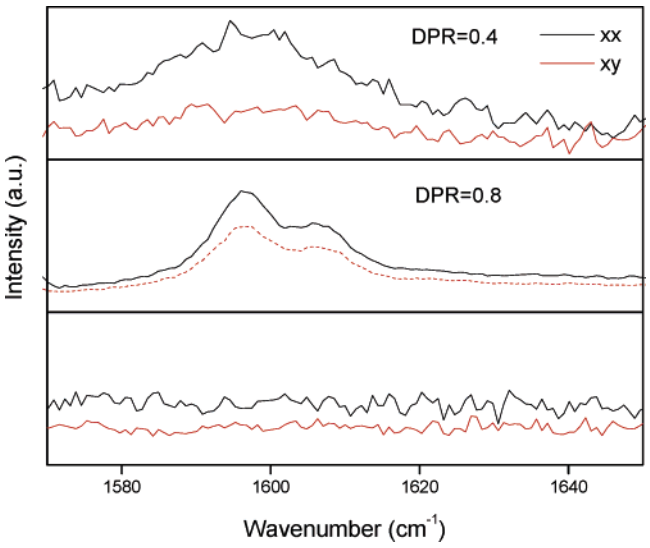

Figure 5. Polarized Raman spectra measured between 1570 and 1650 cm^{-1}, taken with 457.9 nm excitation. The black and red dashed line represent the scattered light measured parallel and perpendicular to the polarization of the exciting laser beam, respectively. (Upper panel) Difference Raman spectra obtained by subtracting the spectrum of resting-state HRP from sample 1. (Middle panel) Raman spectra of neat phenol. (Lower panel) Difference Raman spectra obtained by subtracting the signal of native HRP from that of a sample containing native HRP and phenol prior to the addition of H_2O_2.

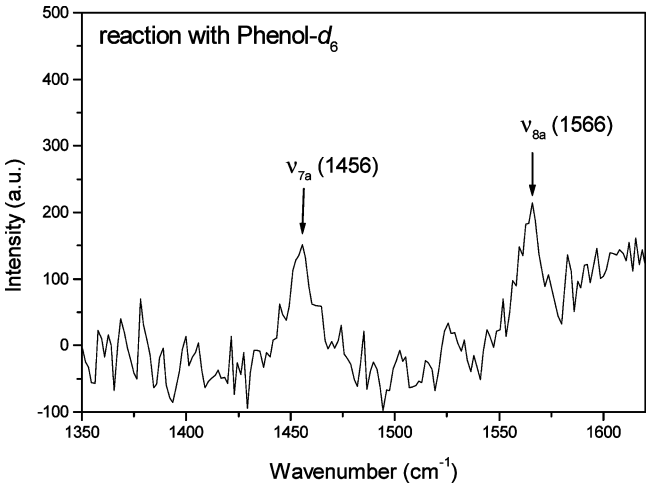

Figure 6. Difference spectrum obtained by subtracting the Raman spectrum of resting HRP from that of HRP in the presence of 25 mM phenol-d_6/H_2O_2. The same substrate concentration was used to obtain the Raman difference spectrum of sample 1 shown in Figure 4. Both spectra were recorded with 457.9 nm excitation.

between 1550 and 1560 cm^{-1} obtained for *free* radicals[18] may be explained by different environments (aqueous solution and heme pocket) and the above-discussed incorporation into oligomers. The sensitivity of this mode is underscored by its much higher (1613 cm^{-1}) wavenumber in the spectrum of, e.g., benzosemiquinone.[16c]

To further corroborate our assignments of the bands at 1481 and 1596 cm^{-1}, we measured the Raman spectrum of HRP C in the presence of 2.5 mM H_2O_2/phenol-d_6. Figure 6 shows the difference spectrum obtained from the subtraction of the Raman spectrum of resting HRP from that of HRP in the presence of H_2O_2/phenol-d_6. Both spectra were taken with 457 nm excitation. The two bands dominating the difference spectrum at 1456 and 1566 cm^{-1} are assignable to downshifted ν_{7a} and ν_{8a} bands of the respective isotopically labeled phenoxyl-d_6 radical.[16a]

The spectral changes assignable to heme spin marker bands in the above difference spectrum may be caused by a strengthening of the proximal histidine ligand owing to subtle structural changes due to phenol oligomers located in the heme pocket. It is well-known from earlier investigations that substrates and aromatic molecules can reside in close proximity to the heme chromophore.[15c]

The high relative intensity of both the two phenoxyl Raman bands deserves some further consideration. As mentioned above the depolarization ratio of the 1596 cm^{-1} is ca. 0.4. For the 1481 cm^{-1} band we obtained a depolarization ratio of ca. 0.3, while the corresponding phenol band in the spectrum is totally polarized (data not shown). These depolarization ratios are close to 0.33, which is consistent with a predominant resonance enhancement due to Franck−Condon transitions into a nondegenerate excited state.[19] In free phenoxyl radicals the two phenoxyl modes are vibronically coupled to two different $\pi \rightarrow \pi^*$ transitions ($^2A_2$ and $^2B_1$ for ν_{8a} and ν_{7a}, respectively) which both give rise to the strong absorption bands in the 400 nm region. Though certainly modified by intramolecular electronic coupling these transition are certainly still present in phenoxyl-phenol-type oligomers.

We have to mention in this context that the dissolved phenolic residues measured using ^{14}C-labled phenol were found to increase with increasing phenol/H_2O_2 concentration (Figure 7). One would therefore expect a concomitant increase of the relative intensity of the phenoxyl bands in Raman spectra taken at higher substrate/H_2O_2 concentrations. However, the difference spectrum in Figure 8 shows that more phenoxylphenol-type species existed in Sample 1 than in Sample 2 (7.5 M phenol/H_2O_2). This contradiction can be rationalized by assuming that other types of phenol oligomers such as biphenols become predominant at high phenol concentrations.[3] Since these species do not contain phenoxyl groups, their spectral properties are different. They do not exhibit absorption bands in the near UV region so that its Raman lines are not resonance enhanced at the excitation wavelength used for our experiments.

Our Raman spectroscopic investigation did not indicate any direct interactions between phenoxyl radicals and heme-iron. This suggests that the phenoxyl radical may attack on protoporphyrin leading to heme destruction, which has caused the much larger losses of active protein than total protein in the solution phases at lower substrate concentrations and the remaining fraction of dissolved iron at higher substrate concentrations that are evident in Figure 1.

The native HRP is a monomeric protein with thirteen α-helices and two antiparallel β-strands.[20] It is interesting to examine if heme destruction is accompanied by structural changes of the protein. Figure 9 displays the amide I spectrum of the protein remaining in sample 2 (initial phenol/H_2O_2 concentration 7.5 mM) after the reaction, compared with the spectrum of native HRP. The amide I bands were decomposed

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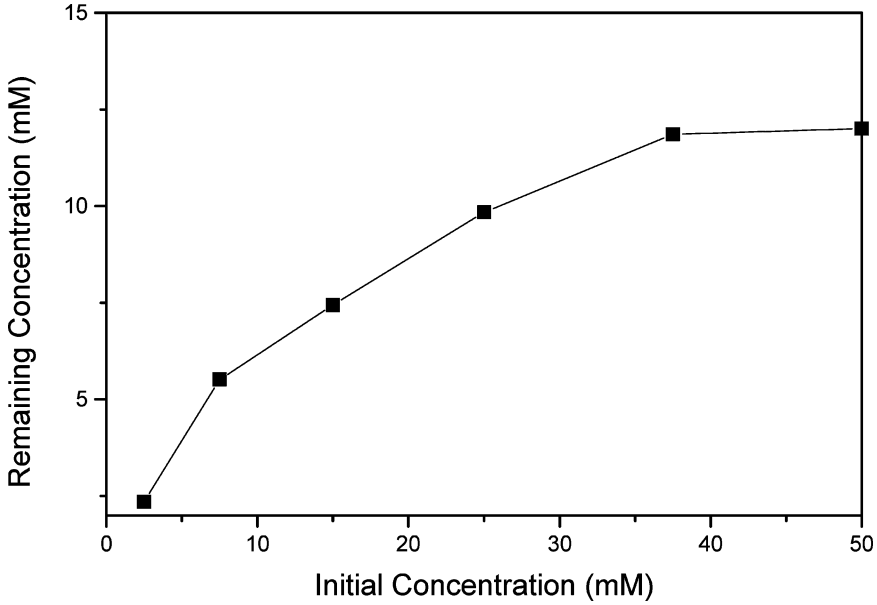

Figure 7. Concentration of phenolic products in the supernatant as a function of the initial phenol concentration up to 50 mM.

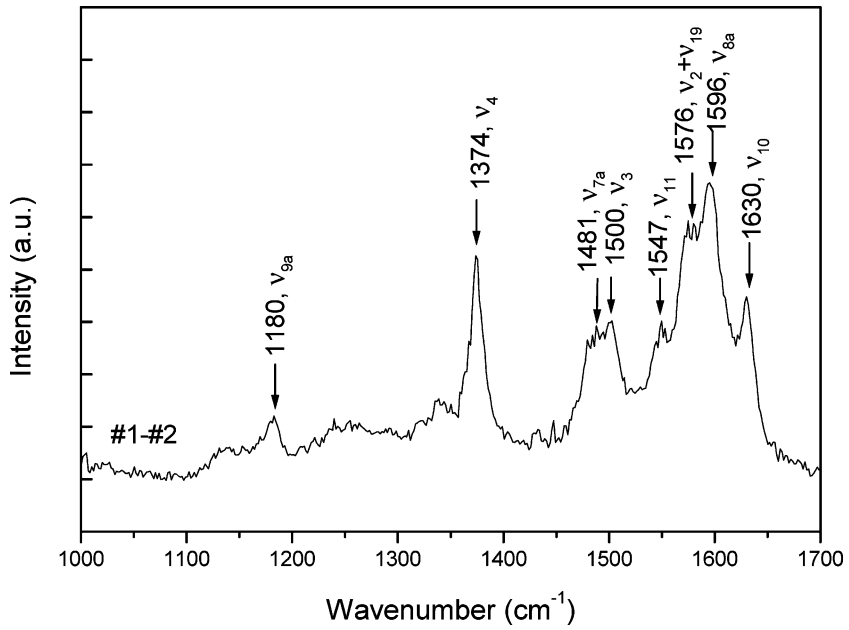

Figure 8. Raman difference spectrum between samples 1 and 2. Initial concentrations of phenol/H_2O_2 for samples 1 and 2 were 2.5 mM and 7.5 M, respectively. The subtraction was conducted by using the internal standard as reference.

into several primary components which represent different secondary structures in the protein. The peaks were assigned to the corresponding secondary structures as described in previous work.[21] The bands at ca. 1659 cm^{-1} and 1647 cm^{-1} were assigned to long helices (having at least 10 residues) and short helices, respectively. The bands at 1627 cm^{-1} and 1690−1697 cm^{-1} were assigned to β-sheet, and all other bands, to other secondary structures, such as, β-turns, nonrepetitive ("random coil") secondary structure, and extended chains. Our analysis for active and inactivated HRP regarding the major secondary structures, i.e., α-helices and β-sheet, is listed in Table 1. It shows that the secondary structure remains intact even after the inactivation of the protein. This is not unreasonable because even a mostly folded or molten globule state of the apo-protein could maintain a secondary-like structure.[22] With heme out of the pocket, the inactivated HRP may remain as a folded apo-protein.

Conclusions

The work described here provides the first experimental data supporting the hypothesis that HRP is inactivated by phenoxyl radical attack. The heme macrocycle destruction, which involves deprivation of the heme iron, occurs already at low substrate/

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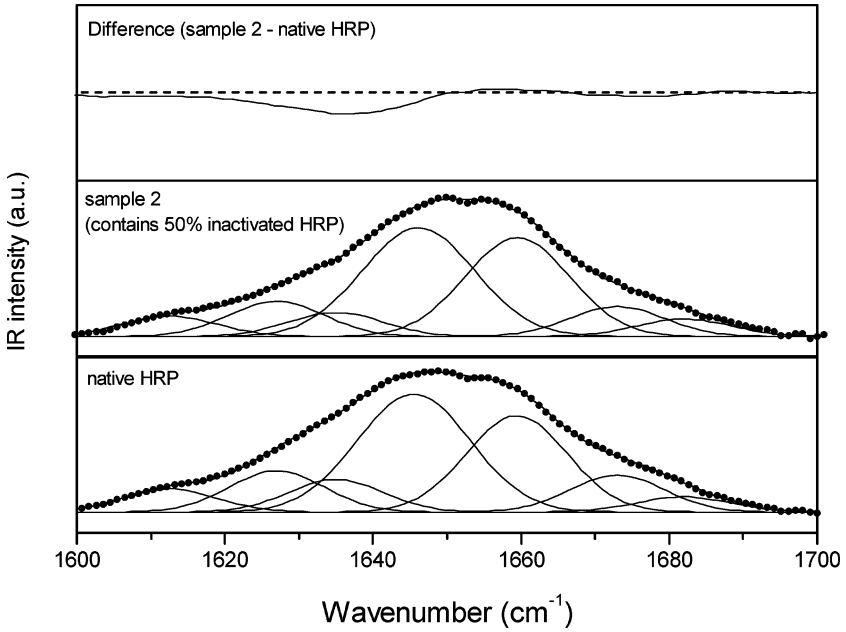

Figure 9. Amide I′ region of the FTIR spectra of native HRP (upper panel) and a 50:50 mixture of native and inactivated HRP (sample 2, initial phenol/H_2O_2 concentration 7.5 mM)) (middle panel). The respective HRP concentration was 22 and 20 mg/mL. The lower panel depicts the difference of the two spectra.

Table 1. Secondary Structure Composition of Native HRP and a 50:50 Mixture of Active and Inactive HRP Obtained from the Respective Amide I′ Band Profile Shown in Figure 8

	native hrp (all active) (%)	hrp in sample 2 (50% inactive) (%)
long α-helices (1659 cm^{-1})	28	29
short α-helices (1647 cm^{-1})	35	35
β-sheet (1629 cm^{-1})	10	8

H_2O_2 concentrations and is completed at high substrate concentrations above 0.1 M. The differences between the Raman spectra taken at different substrate/H_2O_2 concentrations suggest that phenoxylphenol-type oligomers are predominantly formed at low phenol concentrations, while phenol oligomers dominate at high concentrations. Phenoxylphenol-type oligomers located in the heme pocket might be responsible for an increased population of the quantum mixed state of the heme iron observed at low substrate concentrations.

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