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Lignin Peroxidase: Resonance Raman Spectral Evidence for Compound II and for a Temperature-Dependent Coordination-State Equilibrium in the Ferric Enzyme†

Laura A. Andersson, V. Renganathan,‡ Thomas M. Loehr,* and Michael H. Gold
Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999
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ABSTRACT: Resonance Raman (RR) spectroscopy of lignin peroxidase (ligninase, diarylpropane oxygenase) from the basidiomycete *Phanerochaete chrysosporium* suggests two different coordination states for the native ferric enzyme. Evidence for a high-spin, hexacoordinate ferric protoporphyrin IX was presented by Andersson et al. [Andersson, L. A., Renganathan, V., Chiu, A. A., Loehr, T. M., & Gold, M. H. (1985) *J. Biol. Chem. 260*, 6080–6087], whereas Kuila et al. [Kuila, D., Tien, M., Fee, J. A., & Ondrias, M. R. (1985) *Biochemistry 24*, 3394–3397] proposed a high-spin, pentacoordinate ferric system. Because the two RR spectral studies were performed at different temperatures, we explored the possibility that lignin peroxidase might exhibit temperature-dependent coordination-state equilibria. Resonance Raman results presented herein indicate that this hypothesis is indeed correct. At or near 25 °C, the ferric iron of lignin peroxidase is predominantly high spin, pentacoordinate; however, at ≤2 °C, the high-spin, hexacoordinate state dominates, as indicated by the frequencies of well-documented spin- and coordination-state marker bands for iron protoporphyrin IX. The temperature-dependent behavior of lignin peroxidase is thus similar to that of cytochrome *c* peroxidase (CCP). Furthermore, lignin peroxidase, like horseradish peroxidase (HRP) and CCP, clearly has a vacant coordination site trans to the native fifth ligand at ambient temperature. High-frequency RR spectra of compound II of lignin peroxidase are also presented. The observed shifts to higher frequency for both the oxidation-state marker band ν_4 and the spin- and coordination-state marker band ν_{10} are similar to those reported for the compound II forms of HRP and lactoperoxidase and for ferryl myoglobin. These observations are consistent with a low-spin, hexacoordinate Fe(IV)=O structure for lignin peroxidase compound II.

Lignin is a complex, optically inactive, and random polymer that comprises 20–30% of woody plant tissue (Sarkanen, 1971; Crawford, 1981). Under secondary metabolic conditions the white rot fungus *Phanerochaete chrysosporium* produces at least two heme peroxidases (Gold et al., 1984; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985; Paszczynski et al., 1986) and efficiently degrades lignin to CO_2 and H_2O (Kirk et al., 1978; Gold et al., 1982). Lignin peroxidase (LiP,[1] ligninase, diarylpropane oxygenase) has been purified to homogeneity and shown to occur in multiple chromatographic forms (Gold et al., 1984; Renganathan et al., 1985; Kirk et al., 1986; Leisola et al., 1985), all of which contain a single iron protoporphyrin IX prosthetic group (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). Electronic absorption spectroscopy (Gold et al., 1984; Renganathan et al., 1985), EPR spectroscopy (Andersson et al., 1985), and

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* Author to whom correspondence should be addressed.
‡ Present address: Smith Kline & French Laboratories, Chemical Engineering L-25, Swedeland, PA 19479.

[1] Abbreviations: LiP, lignin peroxidase; RR, resonance Raman; CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; Mb, myoglobin; metMb, metmyoglobin; Hb, hemoglobin; dp, depolarized; p, polarized; ap, anomalously polarized; Me_2SO, dimethyl sulfoxide; LPO, lactoperoxidase; EPR, electron paramagnetic resonance; PP, protoporphyrin IX.

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resonance Raman spectroscopy (Andersson et al., 1985; Kuila et al., 1985) indicate that the heme iron in native lignin peroxidase is in the high-spin ferric state, most likely with a histidine fifth ligand. Resonance Raman and EPR experiments also support a *hexacoordinate* geometry for the native enzyme, with a weakly associated sixth ligand such as H_2O at ≤2 °C (Andersson et al., 1985). Subsequently, however, another resonance Raman (RR) spectral study of lignin peroxidase suggested that the native enzyme contains a high-spin, *pentacoordinate* iron (Kuila et al., 1985). Resolution of this discrepancy is of importance in understanding the lignin peroxidase mechanism, since a sixth ligand such as H_2O must be displaced by peroxide in the catalytic cycle.

Because the two RR studies of LiP were performed at different temperatures [~2 (Andersson et al., 1985) and ~25 °C (Kuila et al., 1985)], we considered the possibility that temperature-dependent equilibria might exist for this enzyme, as were reported for cytochrome *c* peroxidase (Evangelista-Kirkup et al., 1985). Data supporting both temperature- and pH-dependent coordination-state equilibria for LiP are presented in this paper.

Like other peroxidases, LiP forms H_2O_2-oxidized complexes. Electronic absorption spectral evidence for the formation of LiP compounds I–III (Renganathan et al., 1985; Renganathan & Gold, 1986) has previously been reported. Here we also present a resonance Raman spectrum of compound II of lignin peroxidase and compare its spectral properties with those of the analogous higher oxidized forms of horseradish peroxidase (HRP), lactoperoxidase (LPO), cytochrome *c* peroxidase (CCP), catalase, and myoglobin (Mb).

Experimental Procedures

Lignin peroxidase was purified from acetate-buffered cultures of *P. chrysosporium* as previously described (Gold et al., 1984; Renganathan et al., 1985). For the pH- and temperature-dependent studies, the enzyme was concentrated to ~400 μM in either sodium succinate, pH 4.5, or 20 mM sodium phosphate buffer, pH 6.0. Raman samples of the ferric enzyme were prepared as previously described (Andersson et al., 1985) in buffer-rinsed melting point capillaries and were studied at a variety of temperatures, using either a 90° scattering geometry from a Varian variable-temperature accessory to obtain depolarization ratios or a backscattering geometry from a sample Dewar (Sjöberg et al., 1982).

For studies of compound II, 400 μM LiP [20 mM sodium phosphate buffer, pH 6.0] was treated with a 3-fold molar excess of H_2O_2, followed by mixing and spectroscopic characterization as described by Renganathan and Gold (1986). The compound II samples were immediately transferred to buffer-rinsed melting point capillaries, which were then quickly frozen in liquid nitrogen. Resonance Raman spectra of compound II were obtained from samples at ~90 K in a backscattering geometry from a sample Dewar (Sjöberg et al., 1982).

The resonance Raman data were obtained with 514.5-nm excitation from a Spectra-Physics 164-05 Ar ion laser with a computer-controlled Raman spectrophotometer, which has been previously described (Loehr et al., 1979). Green excitation was utilized both to minimize laser-induced reversion of compound II to the native ferric species and to ensure observation of the key spin- and coordination-state marker bands.

Results and Discussion

Resonance Raman spectroscopy provides detailed structural information for heme protein prosthetic groups and model porphyrin complexes (Kitagawa et al., 1986; Spiro, 1983). Extensive studies have established characteristic frequencies that are known to be diagnostic of the oxidation, spin, and coordination states of heme systems (Spiro, 1983; Felton & Yu, 1978). For example, the polarized oxidation-state marker band ν_4 [$\nu(C_a–N)$] occurs at ~1355 cm^{-1} for Fe(II)–porphyrins, at ~1370 cm^{-1} for Fe(III)–porphyrins, and at frequencies at or above 1378 cm^{-1} for the Fe(IV) compound II forms of HRP and LPO, and for ferryl myoglobin. The depolarized spin-state and coordination-state marker ν_{10} appears near 1610 cm^{-1} for high-spin hexacoordinate ferric systems, near 1625 cm^{-1} for high-spin pentacoordinate ferric systems, and at ~1640 cm^{-1} for low-spin hexacoordinate systems. These and other fundamental bands are readily detected both by their characteristic polarization properties and by their patterns of enhancement with different Raman excitation lines. Because there are several such marker bands, the assignment of the oxidation, spin, and coordination states of novel iron porphyrin systems is generally straightforward when RR spectra from several excitation lines and measured depolarization ratios are available.

The Ferric Enzyme. In our previous resonance Raman study of lignin peroxidase, we reported that the native ferric enzyme is high spin and hexacoordinate on the basis of its electronic absorption, EPR (~4 K), and resonance Raman (~2 °C) spectral properties (Andersson et al., 1985). In the RR spectra, we observed both a polarized band at 1627 cm^{-1} and a depolarized band at 1612 cm^{-1}. The presence of two such bands assigned as $\nu(C{=}C)_{vinyl}$ and ν_{10} ($C_a–C_m$), respectively, has been shown to be diagnostic of high-spin hexacoordinate ferric protoporphyrin IX systems (Spiro, 1983), such as lactoperoxidase (Manthey et al., 1986) and metMb (Kitagawa et al., 1976; Andersson et al., 1985). Subsequent to our study, Kuila et al., (1985) reported that native ferric LiP was a high-spin pentacoordinate species. Because their RR study was limited to Soret and near-Soret excitation and no depolarization ratios were reported, it seemed possible that they failed to observe the ν_{10} feature. This depolarized band is maximally enhanced with excitation in the visible region and is often obscured or only weakly present with violet and blue excitation lines. However, the RR spectrum of native LiP obtained with 406.7-nm excitation (Kuila et al., 1985) is very similar in both frequencies and intensities to that which we obtained by using the same excitation line (Andersson et al., 1985). Until now, the difference in the assignments between the two reports was unresolved.

Comparable discrepancies appeared to exist for cytochrome *c* peroxidase. X-ray crystallography of the ferric enzyme indicated a high-spin, hexacoordinate structure with H_2O trans to histidine (Poulos et al., 1980) analogous to that of the oxygen-binding heme proteins hemoglobin and myoglobin, although the Fe–H_2O bond length observed for ferric CCP (2.4 Å; Finzel et al., 1984) was shown to be considerably longer than that of methemoglobin (2.0 Å; Takano, 1977). In contrast, RR spectroscopy of ferric CCP suggested a high-spin, pentacoordinate state for the iron (Sievers et al. 1979), a structure more similar to that of ferric HRP. A recent study by Evangelista-Kirkup et al. (1985) provided evidence that, in fact, both structures are correct for CCP, since the ferric enzyme undergoes temperature-dependent equilibria involving changes in coordination state. Thus, at 17 °C, CCP consists of a mixture of 5- and 6-coordinate species; at lower temperatures, stabilization of the bound water occurs and the 6-coordinate form of ferric CCP becomes dominant. Because the two RR studies of native LiP (Andersson et al., 1985; Kuila

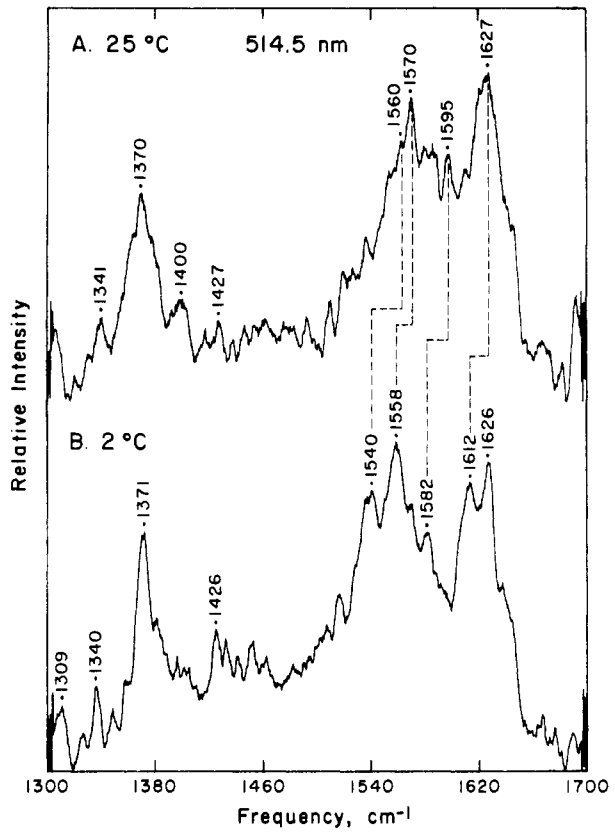

FIGURE 1: Effect of temperature on RR spectra of ferric lignin peroxidase. (A) 450 μM enzyme, pH 4.5 sodium succinate buffer, at ~25 °C. (B) Same enzyme sample, maintained at ~2 °C. Conditions: backscattering geometry from sample Dewar; 514.5-nm excitation (120 mW); scan rate 2 cm^{-1}/s; slit width 5 cm^{-1}.

et al., 1985) were performed at different sample temperatures, we considered the possibility that the reported structural differences for LiP might also result from a temperature-dependent coordination-state equilibrium.

As shown in Figure 1, native (ferric) lignin peroxidase clearly exhibits a structural change from a predominantly high-spin, pentacoordinate species to a predominantly high-spin, hexacoordinate complex as the temperature of the sample is lowered. The spectrum in Figure 1A of the native enzyme at ~25 °C (pH 4.5)[2] exhibits the characteristic spectral pattern of a high-spin, pentacoordinate Fe(III)–protoporphyrin IX system with a vacant coordination site trans to the fifth ligand, as reported for the model complex $ClFe^{III}$–protoporphyrin IX (Table I). For example, the depolarized ν-(C_a–C_m) mode, ν_{10}, appears at 1627 cm^{-1}, with an almost equally intense ν_{19} mode at 1570 cm^{-1}, and ν_{37} appears at ~1595 cm^{-1}. These data suggest that, at ~25 °C, LiP has a vacant coordination site available for the binding of peroxide trans to the presumed histidine fifth ligand. Nonetheless, some hexacoordinate form of LiP may be present, on the basis of both the observation of a second ν_{37} band at ~1580 cm^{-1} and the presence of a weak feature at ~1610 cm^{-1} (ν_{10}), frequencies typical of high-spin hexacoordinate Fe(III)–protoporphyrin IX systems.

In contrast, when the spectrum of the identical enzyme sample was obtained at ~2 °C (Figure 1B), two bands above

[2] The spectra shown in Figure 1 are relatively noisy, due to a marked propensity of lignin peroxidase to denature when exposed to the laser at the nominal sample temperature of ~25 °C. This observation contrasts with the recently reported laser stability of LiP at room temperature (Kuila et al., 1985).

Table I: Resonance Raman Frequencies (cm^{-1}) Characteristic of 5- vs. 6-Coordination for Ferric Heme Proteins and Model Complexes

	ν_{10} (dp)	ν(C=C)$_{vinyl}$ (p)
5-coordinate		
LiP,[a] 25 °C	1625	1625
catalase[b,c]	1624,[b] 1621[c]	1623[c]
CCP,[d] 17 °C	1625	
$ClFe^{III}PP$[e]	1626	1626
HRP[f,g]	1630	1622[g]
6-coordinate		
LiP,[a] 2 °C	1613	1625
metMb[a,h]	1614	1620
$(Me_2SO)_2Fe^{III}PP$[e]	1610	1621
CCP,[d] 9 K	1615	
LPO,[i] 25 °C	1616	1623

[a] This work and Andersson et al. (1985). [b] Felton and Yu (1978). [c] K. Sharma, J. Terner, L. A. Andersson, and T. M. Loehr, unpublished results. [d] Evangelista-Kirkup et al. (1985). [e] Choi et al. (1982). [f] Rakhit and Spiro (1974). [g] Terner and Reed (1984). [h] Kitagawa et al. (1976). [i] Manthey et al. (1986).

1600 cm^{-1} were observed: ν_{10} at 1612 cm^{-1} (depolarized) and ν(C=C)$_{vinyl}$ at 1626 cm^{-1} (polarized; depolarization data not shown). In addition, the strong ν_{19} band observed at 1570 cm^{-1} in Figure 1A has shifted to 1558 cm^{-1} in Figure 1B, as expected for a hexacoordinate ferric heme. The frequencies of the spectral features in Figure 1B are in good agreement with those observed for the high-spin, hexacoordinate model complex $(Me_2SO)_2Fe^{III}$–protoporphyrin IX (Choi et al., 1982) and for high-spin, hexacoordinate LPO, CCP, and metMb (Table I). These data clearly confirm the spin-state and coordination number of native ferric LiP at ~2 °C that we reported previously (Andersson et al., 1985). A small amount of the pentacoordinate form of ferric LiP remains, as evidenced by the shoulder at ~1570 cm^{-1}, a frequency representative of ν_{19} for pentacoordinate systems.

The temperature-dependent coordination-state equilibrium observed for ferric LiP is similar to that observed for CCP (Evangelista-Kirkup et al., 1985). For ferric LiP, a further decrease in temperature to 90 K yields a RR spectrum that indicates a complete shift to the hexacoordinate species, again in agreement with the data reported for CCP. The latter, however, has a larger proportion of the 6-coordinate form present at ambient temperature. Thus, for CCP, HRP, and LiP at ~25 °C there is apparently a vacant coordination site trans to the fifth ligand. The fifth ligand of CCP was established to be a normally hydrogen bonded histidine (Finzel et al., 1984; Poulos & Finzel, 1984), whereas a strongly hydrogen bonded histidine is the accepted fifth ligand for HRP (La Mar & de Ropp, 1982; Teraoka & Kitagawa, 1981; Stein et al., 1980). More recently, Evangelista-Kirkup et al., (1985) showed that at 77 K the histidine fifth ligand of ferric HRP becomes less strongly hydrogen bonded and is, accordingly, more like the histidine ligand of ferric CCP or metMb. Kuila et al. (1985) suggested that the fifth ligand of ferrous LiP at room temperature is an anomalous histidine similar to that of HRP on the basis of similar low-frequency RR spectra for the Fe(II) complexes of the two enzymes. However, the degree of hydrogen bonding of the histidine fifth ligand of native Fe(III) LiP is less clear. Given the spectral similarities between native LiP, CCP (Evangelista-Kirkup et al., 1985), and LPO (Manthey et al., 1986) and the differences between these three enzymes and HRP, it is likely that ferric LiP has a histidine ligand that is less strongly hydrogen bonded than that of HRP. It is also apparent that LiP, like CCP, is capable of binding a weakly coordinated water ligand at the sixth coordination site upon a decrease in temperature to ~2 °C or lower. In contrast, the temperature-dependent equilibrium

Table II: Comparative Resonance Raman Spectral Features of H_2O_2-Oxidized Forms of Lignin Peroxidase and Other Heme Systems

	ν_4	ν_3	ν_{10}
compound II LiP[a]	1379	1503	1642
compound II HRP[b-e]	~1380	1508	~1642
peroxy-Mb[f]	1381	1513	1642
compound II LPO[g]	1379	1507	1640
compound II catalase[b]	1376		1641
compound ES CCP[b]	1375		1641

[a] Data for LiP from this work. [b] Felton et al. (1976). [c] Rakhit et al. (1976). [d] Van Wart and Zimmer (1985). [e] Terner and Reed (1984). [f] Sitter et al. (1985). [g] Manthey et al. (1986).

behavior of HRP indicates only a partial occupation of the distal coordination site by H_2O, and then only at much lower temperatures (Evangelista-Kirkup et al., 1985).

Lignin peroxidase only undergoes the temperature-dependent coordination-state equilibrium when buffered at pH 4.5. The pH 6.0 buffered enzyme is high spin, hexacoordinate between 298 and 90 K, as evidenced by the presence of both ν_{10} at 1612 cm^{-1} and $\nu(C{=}C)_{vinyl}$ at 1626 cm^{-1}. Since the enzyme has a greatly decreased catalytic activity at pH 6.0 (Gold et al., 1984; Tien et al., 1986), these observations may suggest the necessity for a protonated group to participate in the oxidation of substrates by LiP compound I (Tien et al., 1986) or may suggest structural or conformational changes in the active-site pocket. Such changes cannot simply reflect a tighter ligation of the sixth ligand, however, because at pH 6.0 LiP is still capable of forming both the compound I and the compound II intermediates (Renganathan & Gold, 1986).

Compound II. The application of RR spectroscopy to the H_2O_2-oxidized forms of horseradish peroxidase, catalase, cytochrome *c* peroxidase, and myoglobin has demonstrated the facility of this spectral technique to characterize such systems. For example, the frequency of the oxidation-state marker ν_4 at ~1370 cm^{-1} for ferric systems is shifted above 1375 cm^{-1} for the compound II forms of HRP and LPO and for feryl-Mb. Because ν_4 is known to be sensitive to the oxidation state of the central metal ion in porphyrins, these observations were interpreted to indicate an Fe(IV) species for these heme systems (see below). A second characteristic of the higher oxidation states of these proteins is the shift of ν_{10} to ~1640 cm^{-1}. This high frequency for ν_{10} provides evidence that the iron moiety of the H_2O_2-oxidized forms of these proteins is in the low-spin hexacoordinate state, with a sixth ligand such as an oxo group trans to the native fifth ligand.

The electronic absorption spectral properties of the H_2O_2-oxidized forms of lignin peroxidase (compounds I–III) have been documented and compared with those of other peroxidases (Renganathan & Gold, 1986). The absorption maxima of LiP compound II were shown to be very similar to those of HRP compound II, and hence, analogous RR spectra of these two proteins in their high oxidation states would be predicted. Resonance Raman spectra of LiP compound II and the native ferric enzyme are shown in Figure 2. Selected frequencies are presented in Table II, along with the corresponding values for the H_2O_2-oxidized forms of other heme systems.

As shown in Figure 2, the oxidation-state marker band ν_4 shifts from 1371 cm^{-1} for native ferric LiP to 1379 cm^{-1} following the addition of a 3-fold molar excess of H_2O_2. This 8-cm^{-1} shift is of the order of those observed for HRP compound II [7 cm^{-1} (Felton et al., 1976; Rakhit et al., 1976); 5 cm^{-1} (Terner et al., 1985; Van Wart & Zimmer, 1985)], lactoperoxidase [8 cm^{-1} (Manthey et al., 1986)], and ferryl-Mb [11 cm^{-1} (Sitter et al., 1985)]. In contrast, ν_4 of catalase

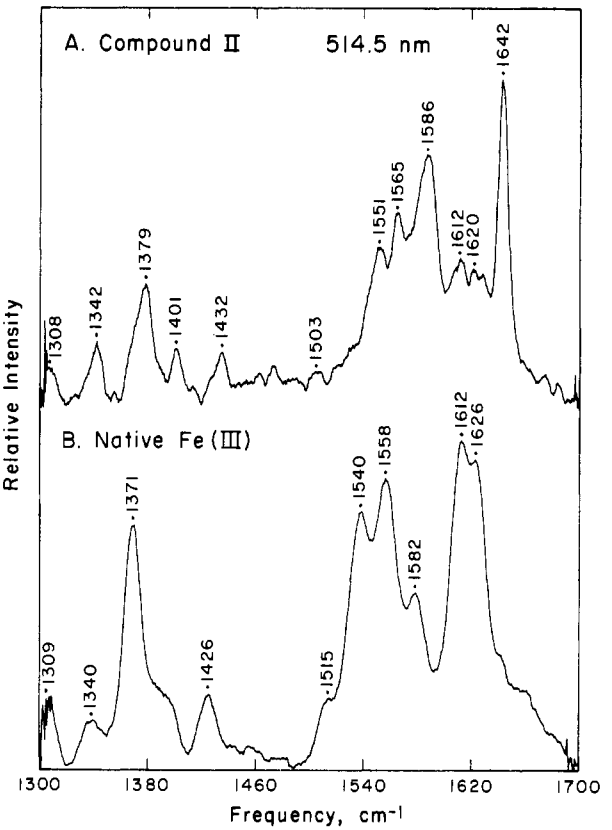

FIGURE 2: High-frequency RR spectra of lignin peroxidase, in pH 6.0 sodium phosphate buffer. (A) Compound II, prepared from 393 μM ferric enzyme plus a 3-fold molar excess of H_2O_2; final [LiP] = 346 μM. (B) Ferric enzyme, 393 μM. Conditions: Backscattering geometry from sample Dewar; samples maintained at liquid nitrogen temperature (~90 K); 514.5-nm excitation (150 mW); scan rate 2 cm^{-1}/s; slit width 5 cm^{-1}.

increases by only 2 cm^{-1} between the ferric and compound II forms (Felton et al., 1976), whereas for CCP, it increases by 4 cm^{-1} between the ferric and compound ES forms (Felton et al., 1976; Evangelista-Kirkup et al., 1985).

Additional information about compound II of LiP can be gained from ν_{10} which shifts from 1612 cm^{-1} in the 90 K ferric enzyme to 1642 cm^{-1} in the compound II form (Figure 2). Similar shifts were reported for the H_2O_2-oxidized forms of HRP, LPO, catalase, CCP, and Mb, indicative of changes from high-spin ferric to hexacoordinate, low-spin species (Table II). These data indicate that compound II of LiP, like that of HRP, is a low-spin hexacoordinate species in which the central metal ion is oxidized above Fe(III), presumably to the ferryl, Fe(IV)=O, state. This assumption is strongly supported by the fact that Mössbauer spectroscopy of HRP compound II indicates that the iron is a low-spin Fe(IV) species (Schulz et al., 1984). La Mar et al. (1983) provided NMR evidence for a ferryl group in both HRP compound II and ferryl myoglobin. Recently Terner and co-workers (Terner et al., 1985; Sitter et al., 1985) provided clear evidence that both HRP compound II and ferryl myoglobin are Fe(IV)=O complexes on the basis of ^{18}O isotope exchange experiments. Kitagawa and co-workers have confirmed these conclusions (Hashimoto et al., 1984).

Concluding Remarks. We have shown that the enzyme lignin peroxidase exhibits spectral properties that agree well with those of other peroxide-dependent enzyme systems. The native ferric enzyme (pH 4.5) undergoes a temperature-dependent coordination-state equilibrium from high spin, pentacoordinate at ~25 °C to high spin, hexacoordinate at ≤2 °C. A similar temperature-dependent phenomenon was ob-

served for CCP. These observations clearly indicate that native LiP at ambient temperatures has a vacant coordination site available to bind peroxide or other ligands. However, like CCP and LPO, LiP may also have a weak-field ligand such as water in the active-site environment which is capable of binding to the heme iron under certain conditions. Because pH 6.0 buffered LiP is high spin, hexacoordinate, regardless of temperature, the coordination equilibrium appears also to be pH-dependent. The spectral similarities between ferric LiP (≤ 2 °C) and ferric CCP, LPO, and metMb suggest that the histidine fifth ligand of LiP may be less strongly hydrogen bonded than the histidine of HRP. Indeed, since the native Fe(III) peroxidases listed above can be either penta- or hexacoordinate, whereas native HRP is always pentacoordinate, perhaps the coordination state is controlled by the degree of protonation of the proximal histidine ligand.

We have shown that the compound II form of lignin peroxidase has similar electronic absorption and RR spectra to those of horseradish peroxidase. For both enzymes, the observation of an $\sim 6\text{-cm}^{-1}$ increase in ν_4 and the presence of ν_{10} at 1642 cm^{-1} indicate structural similarities. Thus, compound II of LiP is suggested to be a low-spin, hexacoordinate ferryl species. Furthermore, the compound II forms of LiP and HRP are spectrally distinct from that of catalase and suggest a structural difference; one obvious difference is the tyrosinate ligand of catalase (Fita & Rossmann, 1985). The structural difference between HRP and catalase was predicted from their spectroscopic properties by Hanson et al. (1981).

Lignin peroxidase has been shown to have spectral properties resembling those of other peroxidases. Ferric LiP undergoes a temperature-dependent change in coordination number (five/six) similar to that of CCP. Like HRP, the highest oxidation state of LiP is the green compound I species, generally believed to be a porphyrin π-cation radical (Renganathan & Gold, 1986), whereas the analogous form of CCP is the red compound ES, which is known to be a protein-centered radical. In addition, like HRP, LiP forms the intermediate compound II, and its RR spectra resemble those of HRP rather than those of CCP or catalase. These observations demonstrate that LiP has properties both corresponding to and differing from other peroxidases, further indicating that each peroxidase can be differentiated by careful study of its spectral characteristics. Further studies will be required to elucidate the structural parameters that ultimately account for the different mechanisms of these peroxidases.

Registry No. Fe, 7439-89-6; lignin peroxidase, 42613-30-9; protoporphyrin IX, 553-12-8.

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Affinity Alkylation of 3-Oxo-Δ^5-steroid Isomerase by Steroidal 3β-Oxiranes: Identification of the Modified Amino Acid by Reduction with Hydroxyborohydride†

Patricia L. Bounds and Ralph M. Pollack*
Laboratory for Chemical Dynamics, Department of Chemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228

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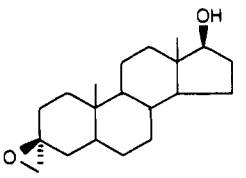
ABSTRACT: The steroidal 3β-oxirane (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1β**) is an active site directed irreversible inhibitor of the 3-oxo-Δ^5-steroid isomerase from *Pseudomonas testosteroni*. Two steroid-bound peptides (TPS_1 and TPS_2) were isolated by high-performance liquid chromatography (HPLC) from the trypsin digest of enzyme inactivated with **1β**. The modified tryptic peptides (residues 14–45 of the enzyme) were further digested with chymotrypsin, each giving rise to a single steroid-containing product (CPS_1 and CPS_2, respectively) derived from residues 31 to 45 of the enzyme. The modified chymotryptic peptides were isolated by HPLC, and the peptide–steroid ester linkage was reduced with sodium hydroxyborohydride. Amino acid analysis of the reduced peptides gave ca. 0.5 residue of homoserine and one less residue of aspartic acid than the corresponding unreduced peptides. Sequence analysis of both reduced chymotryptic peptides revealed that homoserine was located at position 8 in the peptide sequence, corresponding to residue 38 of the enzyme. The finding that the steroidal 3β-oxirane, like the 17β-oxiranes, inactivates the isomerase via esterification of aspartic acid-38 is strong evidence that this enzyme binds steroids in at least two orientations.

The 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1) of the soil bacterium *Pseudomonas testosteroni* catalyzes the conversion of a variety of 3-oxo-Δ^5-steroids to their corresponding Δ^4-isomers (Talalay & Benson, 1972; Batzold et al., 1976). This enzyme is of particular interest because it is a remarkably potent catalyst, having a turnover number (4.4×10^6 min^{-1}) that places it among those enzymes that may be classified as "diffusion controlled" (Fersht, 1985). Unlike its mammalian counterpart, which is an integral membrane protein (Murota et al., 1971; Ford & Engel, 1974), the bacterial isomerase is soluble in water, is readily obtainable in pure form, and has been extensively characterized. The isomerase exists in dilute solution as a dimer of identical subunits having a monomer M_r of 13 394 (Benson et al., 1971). The primary structure of the enzyme has been reported by Benson et al. (1971) and was partially confirmed by Ogez et al. (1977), although there is some disagreement on the assignment of residues near the N-terminus. Westbrook et al. (1984) have recently reported the crystal structure of the isomerase at 6-Å resolution and demonstrated that the steroid binding site of each monomer is located near the contact interface of the dimer.

The catalytic mechanism of the bacterial isomerase has been the focus of much research in recent years, and several workers have employed active site directed irreversible inhibitors to identify amino acid residues that may play a role in catalysis. Talalay and co-workers have located asparagine-57 at the active site on the basis of their work with the suicide inactivator 5,10-secoestr-5-yne-3,10,17-trione (Penning et al., 1981, 1982; Penning & Talalay, 1981). Benisek and co-workers have shown that photoinactivation of the isomerase by 3-oxo-4-estren-17β-yl acetate is accompanied by chemical modification of aspartic acid-38 (Martyr & Benisek, 1975; Ogez et al., 1977; Hearne & Benisek, 1985). In recent work from our laboratory on the active site directed inactivation of the isomerase by 3β- and 17β-steroidal oxiranes (Pollack et al., 1979; Bevins et al., 1980), we have shown that inactivation occurs by formation of an ester linkage between the steroid and a carboxylic acid residue of the enzyme (Kayser et al., 1983; Bevins et al., 1984). In addition, we have identified aspartate-38 as the nucleophile involved in the reaction with the 17β-oxiranes (Kayser et al., 1983).

We report here that aspartate-38 is also the amino acid residue esterified by the 3β-oxiranyl steroid (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1β**). Specifically esterified



1β

enzyme was digested with trypsin and chymotrypsin, and the

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