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Resonance Raman Spectra of Extracellular Ligninase: Evidence for a Heme Active Site Similar to Those of Peroxidases

Debasish Kuila,[‡] Ming Tien,[§] James A. Fee,[‡] and Mark R. Ondrias*

Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131, Los Alamos National Laboratory, University of California, Los Alamos, New Mexico 87545, and Forest Products Laboratory, U.S. Department of Agriculture, Forest Service, Madison, Wisconsin 53705

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ABSTRACT: The first resonance Raman spectra of the heme active site of the lignin-degrading enzyme ligninase are described. The strong correspondence between the spectra of ligninase and those of animal and plant peroxidases indicates that the local heme environment of ligninase greatly resembles those of peroxidases. By analogy with other heme-containing proteins it is likely that both the ferric and ferrous forms of ligninase are five-coordinate and high spin. The addition of cyanide to ferriligninase results in the formation of a low-spin six-coordinate heme active site. An iron-histidine stretching mode at ~244 cm^{-1} is suggested for ferroligninase in analogy with those of other peroxidases.

Lignin is a polymer of phenylpropanoid subunits that makes up 15-35% of lignocelluloses. The biodegradation of cellulose and hemicelluloses in woody plant tissues is hindered by the presence of lignin. The carbon cycle of this planet is most likely governed by lignin biodegradation (Millet et al., 1975; Kirk, 1983). In nature, this amorphous and complex polymer is decomposed mainly by higher basidiomycetous fungi that cause white rot of wood (Ander & Eriksson, 1978; Crawford, 1981). The major reaction involves the oxidative cleavage of the lignin propyl backbone between C_α and C_β. The lignin-degrading enzyme (ligninase) of the white-rot fungus *Phanerochaete chrysosporium* Burds. has been shown to catalyze this reaction in model compounds as well as spruce and birch lignins (Tien & Kirk, 1983). Ligninase also catalyzes the hydroxylations of benzylic methylene groups, the oxidation of phenols, and the oxidation of benzyl alcohols to their corresponding aldehydes or ketones. Very recently, this enzyme has been purified and partially characterized (Tien & Kirk, 1984; Gold et al., 1984). It has a molecular weight of ~42000 and requires H_2O_2 for its activity. The enzyme contains a single protoporphyrin IX, and EPR spectra (M. Tien and J. A. Fee, unpublished results) indicate that the iron is present as high-spin Fe^{3+}.

The mechanism by which this enzyme functions and the molecular origin of its ability to display optimal activity at extremely low pH (~2.5) are not yet completely understood. Recent studies have shown that aryl cation radicals are formed from the oxidation of substrates (Kersten et al., 1985; Hammel et al., 1985), and unpublished observations suggest that intermediates similar to those of compounds I and II of classical peroxidase reactions occur (M. Tien, C. Bull, and J. A. Fee, unpublished results).

Resonance Raman scattering studies of different hemoproteins have established that the vibrational spectra of the heme group provide a powerful means of characterizing the active sites of enzymes (Rousseau & Ondrias, 1983; Spiro, 1983). In this paper we describe the resonance Raman spectra of the heme group of ligninase. Our data show that the active site of this fungal protein is very similar to those of peroxidases and suggest that the high-spin Fe^{3+} of the native enzyme is five-coordinate. The reduced protein displays a mode (~250 cm^{-1}) that may be the analogue of the iron-proximal histidine stretching mode previously identified in horseradish peroxidase (Teraoka & Kitagawa, 1981).

Materials and Methods

Phanerochaete chrysosporium, strain BKM-1767 (ATTC 24725), was grown, and ligninase was purified to homogeneity according to Tien & Kirk (1984). All spectra were obtained from samples in ~0.2 M phosphate buffer at pH ~6.7 or in ~0.2 M tartrate buffer at pH 3-3.5. Reduced samples were

* Address correspondence to this author at the University of New Mexico.
‡ Los Alamos National Laboratory.
§ Forest Products Laboratory.

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Table I: Comparison of Resonance Raman Bands of Ligninase with Those of Horseradish and Intestinal Peroxidases and Mb[a]

mode assignment	IPO[b]	HRP[b,c]	ligninase[d]	Mb[e]	IPO–CN$^-$[f]	HRP–CN$^-$[g]	ligninase–CN$^-$[h]	Mb–CN$^-$[i]
ν_{10}	1622	1630	1628	1613		1642	1636	1644
ν_{37}		1608	1610–1615		1616	1625		
ν_2	1586	1575	1580	1562		1598	1594	1587
ν_{11}, ν_{19}	1564	1548	1562		1577	1590	1578	
						1562		
					1552	1555		
ν_3	1485	1500	1486	1481	1500	1497	1507	1507
ν_{20}		1430	1425	1395	1439	1437		
		1405				1403	1402	1410
ν_4	1375	1374	1373	1370	1377	1375	1375	1374
	1344		~1340	1340		1345	1343	
	1306	1302	1312	1305	1277	1312	1314	
ν_{13}		1238	~1240				1231	
ν_5, ν_{18}			1220				1183	
ν_{30}		1170	1169			1167		
ν_6, ν_8		1140	1135				1135	
	677							
		592						
	488		496	500				
		441	467	443				
	416	408	406	413				
		380	379	379				
		350	355	346				
		321	327					
		292		307				
		274	273	270				
	260			248				
	223			223				

[a] All in the ferric oxidation state. [b] Kimura et al., 1981; Kitagawa et al., 1983; Rakshit & Spiro, 1974; Teraoka & Kitagawa, 1981. [c] pH 5.2; 100–600 cm^{-1}. [d] This work. pH 3.3; 100–600 cm^{-1}. [e] Osaki et al., 1976; Teraoka & Kitagawa, 1981. pH 7.8; 100–600 cm^{-1}. [f] Kimura et al., 1981. [g] Rakshit & Spiro, 1974. [h] This work. [i] Kitagawa et al., 1976.

prepared by adding a slight excess of sodium dithionite (Baker purified grade) to a degassed solution of the protein at pH ~6.7. Resonance Raman spectra were recorded in a 3-mm quartz tube equipped with a stopper to provide anaerobiosis; instrumentation was described, in part, elsewhere (Findsen & Ondrias, 1984). Excitation wavelengths of 440 or 406 nm (10-ns pulse width, 0.4–0.6 mJ/pulse) from a Molectron DL 14/UV 24 nitrogen-pumped dye laser were used to generate the spectra. A backscattering geometry was used in all cases. All spectra were obtained at room temperature. No signs of sample degradation were evident during the course of the experiment.

Results and Discussion

Figure 1 shows the resonance Raman spectra of ligninase, ligninase + CN$^-$, and ferroligninase (deoxy). Tables I and II summarize spectral data for two peroxidases, myoglobin, and ligninase under different conditions. The resonance Raman spectra confirm previous suggestions (Tien & Kirk, 1984; Gold et al., 1984) that the active site of ligninase is a iron protoporphyrin IX species. The presence of vinyl substituents is indicated by the weak modes at 1312 and 1342 cm^{-1} in both the ferric and ferrous enzymes. A prominent mode at 1626 cm^{-1} in the ferrous enzyme is analogous to the 1621-cm^{-1} mode of human deoxyhemoglobin (Rousseau et al., 1983; Choi et al., 1982a,b) and the model iron(II) protoporphyrin–2-methylimidazole complex (Desbois et al., 1984a), which has been unambiguously assigned to a vinyl group frequency. There is no evidence in our data for the existence of anomalous heme substituents such as formyl groups (Tsubaki et al., 1980; Babcock & Salmeen, 1979; Babcock & Callahan, 1983).

The spectrum of ferric ligninase is qualitatively similar to that of ferric intestinal peroxidase (Kimura et al., 1981) (Table I). The prominent Raman lines in the upper trace of Figure 1 are at 1628, 1580, 1562, 1486, 1425, and 1373 cm^{-1}, which

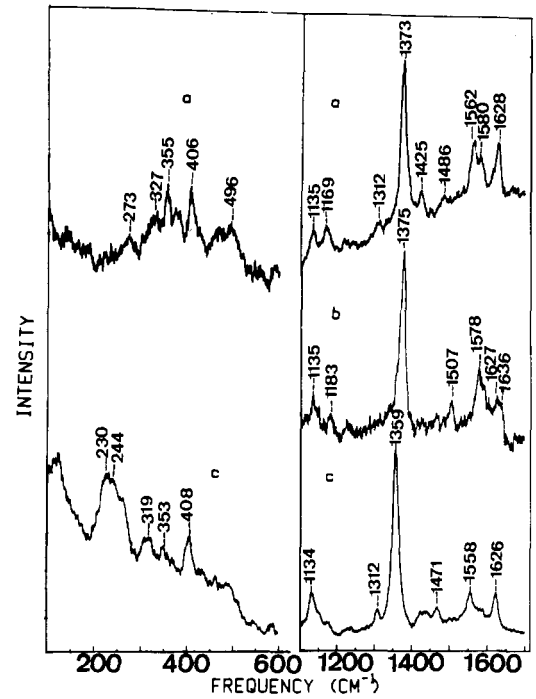

FIGURE 1: Resonance Raman spectra of ligninase. (a) The upper trace is the spectrum of native ferriligninase: pH 3.3; ~125 μM; excitation frequency, 406 nm. (b) The middle spectrum is that of cyanoferriligninase: [KCN]/[ligninase] = ~100; pH 6.7; ~200 μM; excitation wavelength, 440 nm. (c) The lower trace is the spectrum of reduced ligninase: pH ~6.7; ~250 μM; excitation frequency, 440 nm. In all cases, the spectral slit width was 10–12 cm^{-1} and spectra were scanned at 15 cm^{-1}/min. All spectra are unsmoothed sums of multiple scans.

can be assigned respectively to ν_{10}, ν_2, a mixture of ν_{11} and ν_{19}, ν_3, ν_{20}, and ν_4 of the porphyrin skeletal modes in analogy with those of octylethylporphyrin derivatives (Abe et al., 1978). The

Table II: Comparison of Bands of Ferroligninase with Those of Reduced Intestinal Peroxidase, Horseradish Peroxidase, and Deoxy-Mb

mode assignment	IPO^{2+} [a]	HRP^{2+} [a]	ligninase^{2+} [b]	deoxy-Mb[c]
vinyl	1617	1627	1626	1618
ν_{10}		1605	1607	
ν_{37}, ν_{11}		1587	1584	1586
ν_2	1559	1565	1558	1564
ν_3	1472	1472	1471	1473
	1427	1427	1437	
ν_4	1358	1358	1359	1356
		1308	1312	1303
ν_{13}		1232	1240	1210
ν_{30}		1180	1176	1170
ν_6, ν_8		1133	1134	1117
		591	589	
	430	435	436	
ν_{34}, ν_{35}	410	407	408	407
$2\nu_{35}$		375	377	373
		352	353	341
ν_{50} or ν_{51}	319	312	319	303
		296		
	269	270	262	
				242
Fe–His	254	244	244	221
	232	221	230	

[a] Kitagawa et al., 1983. [b] This work. [c] Carson and Ondrias, unpublished results.

position of ν_4 (1373 cm^{-1}) is that expected for a ferric protoheme species. The frequency of the ν_{10} mode of ferric ligninase is close to that of ferric horseradish peroxidase (Rakshit & Spiro, 1974). Teraoka & Kitagawa (1980) and Spiro et al. (1979) have shown that a band at 1629–1631 cm^{-1} indicates a five-coordinate high-spin heme in the hemoproteins. On the basis of this empirical rule, a five-coordinate high-spin ferric heme is suggested in ferric ligninase. While the band at 1486 cm^{-1} (ν_3) resembles that of six-coordinate ferric heme of intestinal peroxidase (1485 cm^{-1}) (Kimura et al., 1981), the addition of excess KF (data not shown) shifts the ν_{10} mode to 1624.5 cm^{-1}, and thus ferric ligninase is most likely five-coordinate. Variation of pH (6.7, 5.5, 3.3) does not affect the high-frequency region of ferric ligninase (data not shown).

The low-frequency region of the resonance Raman spectrum of ferric ligninase (100–600 cm^{-1}) shows prominent peaks at 273, 327, 355, and 406 cm^{-1} (see Table I) at pH 3.3. When the pH is changed to 5.5, these peaks appear at 276, 322, 354, and 408 cm^{-1}, respectively. The 273-cm^{-1} band may be assigned as an iron–histidine stretching frequency by analogy to a similar assignment in ferric horseradish peroxidase (Teraoka & Kitagawa, 1981); this, however, remains speculative. The 322-cm^{-1} peak is also observed in horseradish peroxidase, and its intensity varies with pH (Teraoka & Kitagawa, 1981). The 322-cm^{-1} peak observed in ligninase at pH 5.5 shifts to 327 cm^{-1} and broadens at pH ~3.3. Since this is the only substantive pH-dependent shift in the low-frequency spectrum, it may be related to the increased activity of the enzyme at low pH (M. Tien, C. Bull, and J. A. Fee, unpublished results).

The ligation of CN^- to ferric forms of myoglobin and hemoglobin and to peroxidases results in low-spin forms (Rousseau & Ondrias, 1983; Spiro, 1983). Our data suggest that CN^- also binds to the ferric form of ligninase, causing spectral changes similar to those observed in other hemoproteins. ν_3 and ν_{10} shift to higher frequencies upon CN^- addition to Mb and various peroxidases (see Table I). Similar shifts are evident in the ligninase spectra upon CN^- addition and are thus consistent with cyanoligninase having a low-spin configuration.

However, the overall spectrum of ferroligninase does not correlate well with those of deoxymyoglobin and hemoglobin, and thus its heme environment is surely different. In particular, the positions of the modes at 1134 ($\nu_6 + \nu_8$), 1240 (ν_{13}), and 1627 cm^{-1} (vinyl stretch) are more comparable to those of peroxidases. The oxidation state marker, ν_4, also appears to be significantly different between the two classes of proteins (Desbois et al., 1984b). Its position in ferroligninase (1359 cm^{-1}) is indicative of reduced π^* electron density relative to the hemes of Hb and Mb (1356–1357 cm^{-1}) and is similar to those of peroxidases. The modes ν_{10}, ν_{19}, and ν_3 are used as porphyrin core size indicators. [See, for instance, Spaulding et al. (1975).] On the basis of an empirical relation, $\nu = K(A - d)$ cm^{-1}, the core expansion and contraction can be calculated [see Spiro et al. (1979) for details]. The porphyrin center to nitrogen distance d of the native ferric enzyme is calculated to be 2.043 Å (2.028, 2.053, and 2.049 Å on the basis of ν_{10}, ν_3, and ν_{19}, respectively). The Δd values upon coordination of CN^- are −0.056, −0.035, and −0.019 Å, respectively, when the following empirical relation is used: $\Delta d = -\Delta\nu_i/K_i$, where $\Delta\nu_i$ is the frequency shift upon the coordination of CN^- and K_i is an empirical parameter calculated for each individual mode, 375.5, 555.6, and 423.7 for ν_3, ν_{19}, and ν_{10}, respectively [see Huong & Pommier (1977)].

The spectrum of the reduced protein in the low-frequency region (100–600 cm^{-1}) is also quite analogous to those of peroxidases (Teraoka & Kitagawa, 1981; Desbois et al., 1984b; Kitagawa et al., 1983). This suggests that the peak at 244 cm^{-1} may be an iron–proximal histidine stretching mode. It was found that, for pH ≤7.0, CN^- does not bind to ferroligninase. In the absence of a definitive low-frequency spectrum of low-spin ferroligninase (in which the Fe–His mode would be expected to be absent), our assignment of the 244-cm^{-1} mode must be regarded as speculative. However, Teraoka and Kitagawa have assigned the 244-cm^{-1} line of horseradish peroxidase isozyme C on the basis of an ^{54}Fe isotope frequency shift (Teraoka & Kitagawa, 1981). Other plant peroxidases display prominent modes in the 240–250-cm^{-1} region that have been assigned as Fe–His stretches (Teraoka et al., 1983). In lactoperoxidase this band occurs at 248 cm^{-1} (Kitagawa et al., 1983). All these data indicate that the 244-cm^{-1} band of ferroligninase may arise from an iron–histidine stretching mode.

In summary, the data from our preliminary investigations clearly show that ligninase contains a peroxidase-like active site. We find no evidence to suggest that any unusual substituents are bound to the heme. The low-frequency spectra of ferroligninase are suggestive of an iron–histidine bond strength similar to those of other peroxidases. Finally, we infer that the hemes in both the ferric and ferrous resting enzymes are five-coordinate and high spin.

Added in Proof

After our paper was accepted, a communication by Andersson et al. (1985) appeared. On the basis of resonance Raman data in the 1000–1700-cm^{-1} range, these authors concluded that the heme environment in ligninase is similar to that in myoglobin. However, the Raman spectra reported here (100–600 and 1100–1700 cm^{-1}) show compelling similarities to those of previously studied plant and animal peroxidases. This is particularly ture for the low-frequency vibrational modes that are sensitive to the heme environment.

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