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Irreversible Oxidation of Ferricytochrome *c* by Lignin Peroxidase[†]

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ABSTRACT: Lignin peroxidase (LiP) from *Phanerochaete chrysosporium* catalyzes irreversible oxidative damage to ferricytochrome *c* (Cc^{3+}) in the presence of H_2O_2 and 3,4-dimethoxybenzyl (veratryl) alcohol (VA). Atomic absorption analysis and UV/vis spectroscopy indicate that the oxidation of Cc^{3+} is accompanied by a loss of heme iron from the protein and probably oxidation of the porphyrin ring. At H_2O_2 concentrations of 7.5 μM or higher, this oxidation of Cc^{3+} by LiP is strictly dependent on the presence of VA. The latter is not oxidized to veratraldehyde at a significant rate in the presence of either ferrocytochrome *c* (Cc^{2+}) or Cc^{3+}, indicating it is not stimulating the reactions by specifically reducing LiP compound II. LiP is inactivated rapidly in 100 μM H_2O_2, and the presence of 500 μM VA protects LiP from this inactivation. Neither 20 μM Cc^{3+} nor 20 μM VA alone can protect LiP from inactivation; however, 20 μM each of VA and Cc^{3+} together protect LiP fully. This and other results strongly suggest that VA is acting as a protein-bound redox mediator in the oxidation of Cc^{3+}. SDS−PAGE analysis of the Cc^{3+} oxidation products demonstrates the formation of some covalently linked dimer of Cc^{3+} in addition to the oxidized Cc^{3+} monomer. Amino acid analysis of the dimeric and monomeric products indicates the presence of oxidized Met and Tyr residues. This suggests that Tyr residues on the surface of the protein are oxidized to Tyr radicals during LiP oxidation and that some of these radicals subsequently undergo intermolecular radical coupling, resulting in dimerization of some of the Cc^{3+} molecules. However, most of the Cc^{3+} molecules appear to be irreversibly oxidized without dimerization. These results demonstrate that Cc^{3+} can serve as a useful polymeric model of the lignin substrate in studying the enzymatic mechanism of lignin oxidation and the role of VA in the reaction.

White-rot basidiomycetous fungi are primarily responsible for initiating the depolymerization of lignin in wood, which is a key step in the earth's carbon cycle (*1*−*3*). The best-studied lignin-degrading fungus, *Phanerochaete chrysosporium*, secretes two extracellular heme peroxidases, manganese peroxidase and lignin peroxidase (LiP),[1] which, along with an H_2O_2-generating system, are apparently the major extracellular components of its lignin degradative system (*1*−*7*). Nucleotide sequences of a number of LiP cDNA and genomic clones (*8*−*11*), as well as several LiP crystal structures (*12*−*14*), demonstrate that important peroxidase catalytic residues, including the proximal and distal His, the distal Arg, and an H-bonded Asp, are all conserved within the heme pocket of LiP. Furthermore, neither LiP nor MnP contain Tyr residues (*8*−*11*). The crystal structure also indicates that the heme of LiP is buried and therefore unavailable for direct interaction with polymeric substrates such as lignin (*12*−*14*). The mechanism of long-range electron transfer between the heme of LiP and polymeric substrates has not been fully elucidated.

Veratryl (3,4-dimethoxybenzyl) alcohol (VA), a secondary metabolite secreted by *P. chrysosporium*, stimulates the rates of LiP-catalyzed oxidations of synthetic lignin and other polymers (*4, 15*) and a variety of nonphenolic lignin model compounds and aromatic pollutants (*16*−*19*) whose redox potentials are beyond the reach of other plant and fungal peroxidases. However, the mechanism of VA stimulation of LiP reactions is still unresolved. To explore the possible role of VA in long-range electron-transfer reactions by LiP, we are examining the LiP oxidation of model polymeric substrates. Previously, we used ferrocytochrome *c* (Cc^{2+}) as a model polymeric substrate and demonstrated that LiP oxidizes Cc^{2+} to ferricytochrome *c* (Cc^{3+}). We also observed that Cc^{3+} is a noncompetitive inhibitor of VA oxidation by LiP (*15*). This inhibition led us to examine the possible oxidation of Cc^{3+} by LiP. Herein, we demonstrate that, in the presence of VA, LiP catalyzes irreversible oxidative damage to Cc^{3+}, and VA appears to act as a redox mediator in the reaction. SDS−PAGE and amino acid analyses indicate that Cc^{3+} oxidation is accompanied in part by dimerization of the substrate, presumably mediated by the intermolecular coupling of Tyr residues in the protein.

EXPERIMENTAL PROCEDURES

Protein Preparation. LiP isoenzyme 2 (H8) was purified from cultures of *P. chrysosporium* strain OGC101 as

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[1] Abbreviations: Cc^{2+}, ferrocytochrome *c*; Cc^{3+}, ferricytochrome *c*; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LiP, lignin peroxidase.

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previously described (*20, 21*). The purified enzyme was electrophoretically homogeneous and had an R_Z value (A_{408}/A_{280}) of 5.0. The enzyme concentration was determined at 408 nm using an extinction coefficient of 133 mM^{-1} cm^{-1} (*21*). Horse heart Cc^{3+} and horseradish peroxidase (HRP) were obtained from Sigma. Cytochrome *c* peroxidase (CcP) was generously provided by Grant Mauk (University of British Columbia, Vancouver, Canada). Cc^{2+} was prepared by adding excess sodium dithionite to Cc^{3+} in water as described (*15, 22*). Excess sodium dithionite was removed by gel filtration on a Sephadex G-10 column with argon-saturated 10 mM potassium sulfate as the eluant. The concentrations of Cc^{3+} and Cc^{2+} were determined using extinction coefficients of 106 mM^{-1} cm^{-1} at 410 nm and 27 mM^{-1} cm^{-1} at 550 nm, respectively (*23*).

Chemicals. H_2O_2 (30% solution) was obtained from Sigma, and the concentrations of stock solutions were determined as described (*24*). VA was obtained from Aldrich and purified by silica gel column chromatography (ethyl acetate/hexane, 1:1, v:v) before use. All other chemicals were reagent grade. All solutions were prepared using deionized water from a Milli-Q 50 system (Millipore).

Spectrophotometric Measurements. The oxidations of Cc^{3+} and VA were measured at room temperature with a Shimadzu UV-260 spectrophotometer in cells with a 1-cm light path. Reaction mixtures for Cc^{3+} oxidation contained Cc^{3+} (10 μM), LiP [1 μg/mL (0.024 μM) or as indicated], with or without VA (500 μM), in 20 mM sodium succinate, pH 3.0 or as indicated in the legends to the figures. The ionic strength was adjusted with potassium sulfate. The reactions were initiated by the addition of H_2O_2 (100 μM). The rate of Cc^{3+} oxidation was determined by following the decrease in absorbance at 408 nm, the Soret maximum. Cc^{2+} oxidations were carried out as described previously (*15*). LiP oxidations of VA to veratraldehyde were performed as described previously and followed at 310 nm (extinction coefficient, 9.3 mM^{-1} cm^{-1}) (*1, 2*). CcP and HRP reactions with Cc^{3+} were carried out at both pH 3.0 (sodium succinate, 20 mM) and pH 7.0 (sodium phosphate, 20 mM) containing Cc^{3+} (10 μM), H_2O_2 (100 μM), and enzymes (10 μg/mL).

Atomic Absorption Spectrometry. A Perkin-Elmer 630 atomic absorbance spectrometer was used to determine the protein-bound iron at 249 nm, using ferrous chloride as a standard. Reaction mixtures (7 mL), as described above, were incubated at room temperature for 15 min. Aliquots (1 mL) were removed to measure the decrease in the Soret band; the remaining 6 mL was concentrated and washed with water by ultrafiltration (Amicon-10) and analyzed by atomic absorption spectrometry for protein-bound iron.

LiP Inactivation by H_2O_2. Reaction mixtures consisted of LiP (100 μg/mL, ~2.4 μM) and H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0. Reactions were carried out at 25 °C, with or without VA and/or Cc^{3+}, as indicated. Aliquots (20 μL) were removed at intervals, and LiP activity was assayed using VA (5 mM) and H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0, as previously described (*2, 9*).

HPLC Analysis of Veratraldehyde Formation. High-performance liquid chromatography (HPLC) was carried out with an HP Lichrospher 100 RP-18 column. Veratraldehyde formation was analyzed at 280 nm using a linear gradient of acetonitrile in 0.05% phosphoric acid (0−50% over 22 min). The veratraldehyde was quantitated using a calibration curve obtained with a standard.

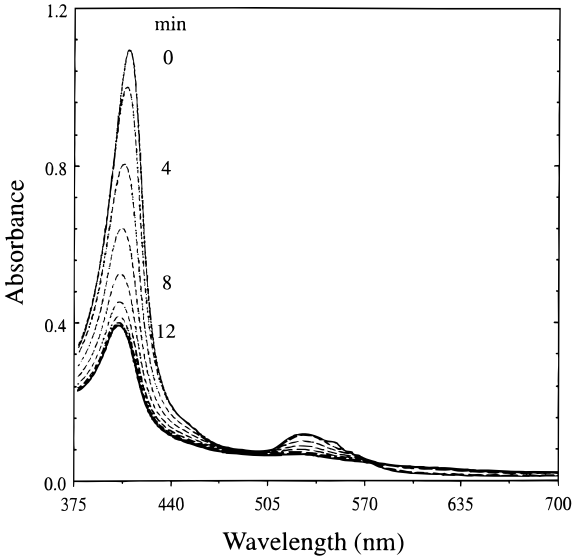

FIGURE 1: Cc^{3+} oxidation by LiP. Electronic absorption spectra were recorded at 2-min intervals. The reaction mixture consisted of Cc^{3+} (10 μM), LiP (1 μg/mL), and VA (500 μM) in 20 mM sodium succinate, pH 3.0. The reaction was initiated by the addition of H_2O_2 (100 μM).

SDS−PAGE. Reaction mixtures (1 mL), as described above, were incubated at room temperature for 15 min. The reaction mixtures were concentrated to 100 μL by ultrafiltration (Amicon-10), and aliquots (20 μL) were analyzed by SDS−PAGE (Mini-PROTEIN, BioRad) (*25*).

Isolation and Amino Acid Analysis of Cc^{3+} Reaction Products. Reaction mixtures, as described above, were concentrated by ultrafiltration to a final volume of 500 μL, heated in loading buffer without 2-mercaptoethanol, and subjected to SDS−PAGE. The SDS−PAGE pattern for native Cc^{3+} is essentially identical whether heated in the presence or absence of 2-mercaptoethanol (data not shown). The monomer and dimer bands were cut from the gel, and the proteins were eluted in Tris−glycine buffer, pH 8.0, and dialyzed exhaustively against deionized water. Amino acid analysis was performed at the Beckman Institute, Stanford University, Stanford, CA.

RESULTS

Cc^{3+} Oxidation by LiP. Figure 1 shows the oxidation of Cc^{3+} by LiP in the presence of H_2O_2 (100 μM) and VA (500 μM). Bleaching of the Cc^{3+} Soret band was observed, but there were no major shifts in the peaks. The absorbance at 695 nm, indicative of the iron−sulfur bond in Cc^{3+} (*26*), also did not shift (data not shown). In the absence of VA, Cc^{3+} was oxidized at a very slow rate either in the presence of H_2O_2 alone or in the presence of LiP plus H_2O_2 (Figure 2). In contrast, in the presence of 100 μM VA, the rate of Cc^{3+} oxidation by LiP was approximately 12-fold faster (Figure 2A). Under similar conditions, neither CcP nor HRP was able to oxidize Cc^{3+} either at pH 3.0 or at 7.0 (data not shown). In the presence of VA, the initial rate of Cc^{3+} oxidation by LiP increased with increasing LiP concentrations between 0.5 and 6.0 μg/mL (Figure 2B). Within the pH range of 2.5−6.0, the initial rate of Cc^{3+} oxidation in the presence of LiP and VA decreased with increasing pH.

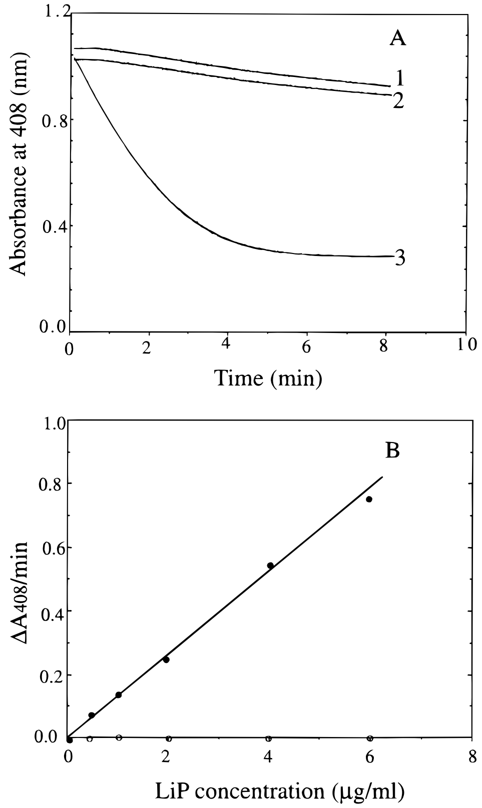

FIGURE 2: Effect of VA and enzyme concentration on the oxidation of Cc^{3+}. Reactions were monitored by following the decrease in absorbance at 408 nm. (A) All reactions contained Cc^{3+} (10 μM) in 20 mM sodium succinate, pH 3.0. Trace 1, H_2O_2 (100 μM); trace 2, H_2O_2 (100 μM) and LiP (1 μg/mL); trace 3, H_2O_2 (100 μM), LiP (1 μg/mL), and VA (500 μM). (B) The initial rates of Cc^{3+} oxidation at various LiP concentrations. Reactions contained Cc^{3+} (10 μM), LiP (as indicated), and H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0, with (●) or without (○) VA (500 μM).

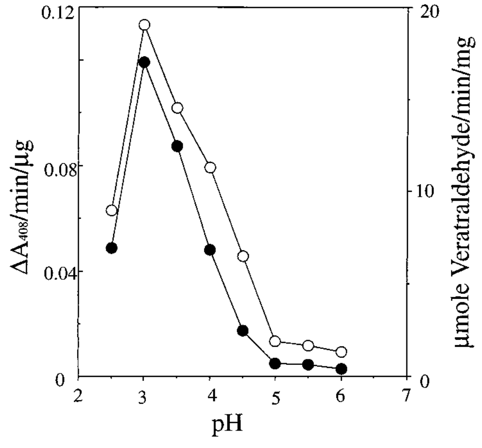

FIGURE 3: pH dependence of the oxidation of Cc^{3+} and VA. Cc^{3+} oxidation reactions (●) contained Cc^{3+} (10 μM), H_2O_2 (100 μM), VA (500 μM), and LiP (1 μg/mL) in 20 mM sodium succinate at the indicated pH. VA oxidation reactions (○) contained VA (500 μM), H_2O_2 (100 μM), and LiP (1 μg/mL) in 20 mM sodium succinate at the indicated pH.

Table 1: Fe Content and Soret Absorbance of Cc^{3+}

reaction components[a]	Cc^{3+}	Cc^{3+}, H_2O_2	Cc^{3+}, LiP, H_2O_2	Cc^{3+}, LiP, H_2O_2, VA
Soret absorbance	100[b]	96	100	40
Fe content	100[c]	97	104	56

[a] Reactions were as described in the text. [b] Iron concentrations were normalized to 100 for the control with Cc^{3+} alone. [c] Soret absorbance was normalized to 100 for the control with Cc^{3+} alone.

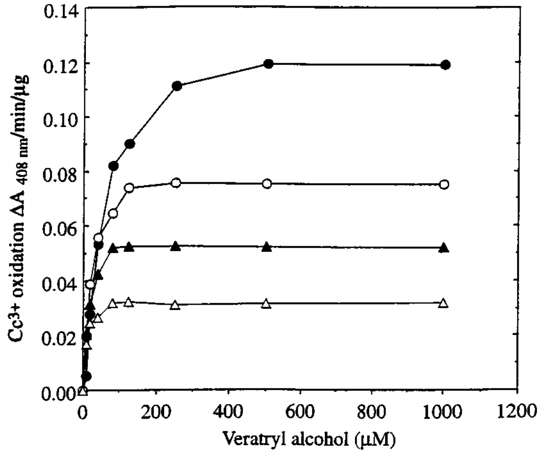

FIGURE 4: Effect of H_2O_2 concentration on the stimulation of Cc^{3+} oxidation by VA. Reaction mixtures contained LiP (1 μg/mL), VA (as indicated), and Cc^{3+} (10 μM) in 20 mM sodium succinate, pH 3.0. Reactions were initiated by the addition of H_2O_2 to a final concentration of 7.5 (△), 15 (▲), 30 (○), and 100 (●) μM, and Cc^{3+} oxidation was monitored by following the decrease in absorbance at 408 nm, as described in the text.

Likewise, the initial rate of LiP-catalyzed VA oxidation to veratraldehyde decreased with increasing pH (Figure 3), confirming our earlier work (*27*).

The results of atomic absorption spectrometric analysis indicated that approximately 50% of the iron was released from Cc^{3+} following a 15-min incubation with LiP, H_2O_2 (100 μM), and VA (500 μM). Similarly, there was a decrease of approximately 60% in the intensity of the Cc^{3+} Soret band. If the reaction was carried out in either the absence of VA or the absence of LiP, neither a significant loss in the iron content nor a decrease in the Soret band of Cc^{3+} was observed (Table 1; Figure 2).

The oxidation of Cc^{3+} was strictly dependent on the presence of VA at H_2O_2 concentrations of 7.5 μM or higher (Figure 4). Furthermore, at higher concentrations of H_2O_2, more VA was required to attain maximal oxidation of Cc^{3+}. For example, in the presence of 100 μM H_2O_2 and 10 μM Cc^{3+}, approximately 500 μM VA was required to obtain maximal activity. However, even VA concentrations as high as 1 mM did not inhibit the oxidation of 10 μM Cc^{3+} (Figure 4).

The effects of Cc^{3+} and VA on the H_2O_2-induced inactivation of LiP also were investigated directly (Figure 5). When LiP (2.4 μM) was incubated with 100 μM H_2O_2 at pH 3.0 and 25 °C, almost all LiP activity was lost within 30 min. Although 20 μM VA did not prevent this inactivation, essentially all of the LiP activity was retained at 500 μM VA. Likewise, in the presence of 20 μM Cc^{3+} alone, 70% of the LiP activity was lost within 30 min. However, in the presence of 100 μM H_2O_2, 20 μM Cc^{3+}, and 20 μM VA, maximal LiP activity was retained after 30 min. In the presence of 20 μM Cc^{3+}, as little as 5 μM VA protected LiP from inactivation by H_2O_2 to a significant extent (Figure 5).

HPLC Analysis of VA Oxidation. The LiP oxidation of VA to veratraldehyde in the presence and absence of Cc^{3+} and Cc^{2+} was measured by HPLC. When the ratio of VA to Cc^{3+} in the reaction was high (500:10 nmol), only 7 nmol

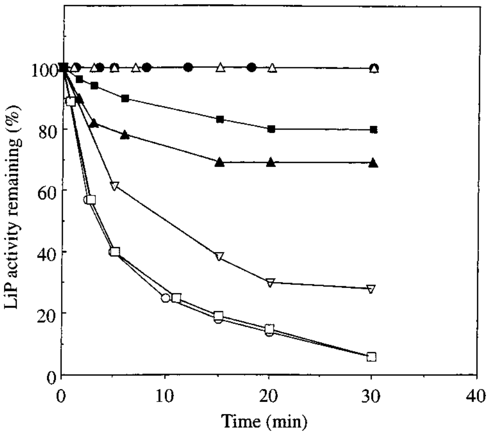

FIGURE 5: Effects of VA and Cc^{3+} on LiP inactivation by H_2O_2. LiP (100 μg/mL, 2.4 μM) was incubated with H_2O_2 (100 μM) in 20 mM sodium succinate, pH 3.0, alone (□), with 20 μM VA (○), with 500 μM VA (●), with 20 μM Cc^{3+} (▽), with 5 μM VA and 20 μM Cc^{3+} (▲), with 10 μM VA and 20 μM Cc^{3+} (■), and with 20 μM VA and 20 μM Cc^{3+} (△). At intervals, aliquots (20 μL) were removed and assayed for LiP activity, as described in the text.

Table 2: Veratraldehyde Formation in Reactions Containing Cc^{3+} or Cc^{2+} [a]

time (min)	VA (500 nmol), Cc^{3+} (10 nmol)		VA (50 nmol), Cc^{3+} (10 nmol)		VA (500 nmol), Cc^{2+} (20 nmol)	
	+	−	+	−	+	−
1	2	20	<0.1	5	0	20
4	5	50	<0.1	13	0	50
10	6	90	<0.1	23	0	90
15	7	110	<0.1	30	0	110

[a] Reactions contained LiP (1 μg/mL), H_2O_2 (100 μM), and VA (50 or 500 nmol, as indicated), with or without Cc^{3+} (10 nmol) or Cc^{2+} (20 nmol), in 20 mM sodium succinate, pH 3.0. Veratraldehyde formation was measured by HPLC at the indicated times as described in the text.

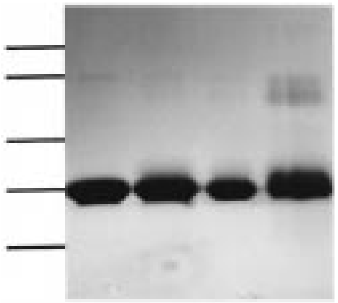

FIGURE 6: SDS−PAGE of Cc^{3+} oxidation products. Reactions and SDS−PAGE were as described in the text. Lane 1 (left), untreated Cc^{3+}; lane 2, reaction products of Cc^{3+} and H_2O_2; lane 3, reaction products of Cc^{3+}, H_2O_2, and LiP; lane 4, reaction products of Cc^{3+}, H_2O_2, LiP, and VA. The molecular mass markers at far left are lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.0 kDa).

Table 3: Amino Acid Analysis of Native Cc^{3+} and Cc^{3+} Oxidation Products

amino acid	theoretical[a]	native Cc^{3+} [b]	native Cc^{3+} [c]	monomeric product[d]	dimeric product[e]
Asx	8	7.9	8.2	8.1	8.1
Thr	10	9.3	9.4	9.8	9.1
Glx	12	12.5	12.4	12.5	11.7
Pro	4	4.2	4.1	4.1	4.1
Gly	12	11.6	12.2	12.4	12.6
Ala	6	5.8	6.1	6.0	6.0
Cys	2	nd[f]	nd	nd	nd
Val	3	2.8	3.1	3.1	3.1
Met	2	1.9	1.8	1.5	0.7
Ile	6	6.0	6.4	6.2	6.1
Leu	6	5.5	5.9	5.8	5.3
Tyr	4	3.9	3.5	2.0	0.7
Phe	4	4.0	4.1	4.0	3.7
His	3	3.8	5	4.7	5.6
Lys	19	16	15.9	15.8	15.0
Trp	1	nd	nd	nd	nd
Arg	2	2.0	2.2	2.2	2.2

[a] Theoretical amino acid composition. [b] Native Cc^{3+}. [c] Native Cc^{3+} eluted from SDS−PAGE. [d] Oxidized Cc^{3+} monomer eluted from SDS−PAGE. [e] Oxidized Cc^{3+} dimer eluted from SDS−PAGE. The amino acid yields were divided by 2 to correlate with values for the monomer. [f] nd = not determined.

of veratraldehyde was formed after 15 min, as compared with 110 nmol formed in the absence of Cc^{3+}. In addition, only trace amounts of veratraldehyde (<0.1 nmol) were formed at a lower ratio of VA to Cc^{3+} (50:10 nmol) (Table 2). No veratraldehyde formation was detected from 500 nmol of VA during the complete oxidation of 20 nmol of Cc^{2+} (Table 2).

Dimerization of Cc^{3+}. SDS−PAGE experiments indicated that, in the presence of VA, LiP oxidatively polymerized some of the Cc^{3+} molecules. As shown in Figure 6, SDS−PAGE analysis of the reaction products obtained from the LiP oxidation of Cc^{3+}, in the presence but not in the absence of VA, revealed a 26.6-kDa protein, corresponding in size to the dimer form of Cc^{3+}. In addition to the Cc^{3+}-oxidized monomer, trace amounts of products corresponding in molecular weight to a Cc^{3+} trimer and tetramer also were observed (Figure 6, lane 4). To examine further the dimerization of Cc^{3+}, amino acid analysis was carried out on native Cc^{3+}, on native Cc^{3+} eluted from SDS−PAGE, and on the monomeric and dimeric reaction products of the LiP oxidation of Cc^{3+} eluted from SDS−PAGE (Table 3). Comparison of the amino acid content of the reaction products from the LiP oxidation of Cc^{3+}, with those of the native Cc^{3+} proteins and with the theoretical amino acid composition of Cc^{3+} (*28*), indicated that the Met and Tyr content decreased in the monomeric and dimeric oxidized products, but that the amino acid compositions of both products were otherwise similar to native Cc^{3+}. In the oxidized monomeric product, the Met content was reduced by 25%, and in the dimeric product the Met content was reduced by 65% with respect to native Cc^{3+}. Methionine sulfoxide was found in the oxidized monomer, and methionine sulfoxide and methionine sulfone were detected in the oxidized dimer by amino acid analysis (data not shown). The Tyr content decreased by 50% in the oxidized monomeric product and by 83% in the oxidized dimeric product. There also was a slight decrease in the Tyr content of native Cc^{3+} recovered from SDS−PAGE, probably due to autoxidation during electrophoretic separation (Table 3).

DISCUSSION

LiP is a well-characterized heme peroxidase that is secreted by *P. chrysosporium* under ligninolytic conditions (*1−7, 9*). Considerable work has been reported on the structure and mechanism of LiP (*9, 20, 29−36*). In particular, the crystal structure reveals that the heme of LiP is buried (*12−14*) and, therefore, unavailable for direct interaction with polymeric substrates such as lignin. It has been suggested that the mechanism of LiP may involve long-distance electron transfer between the heme and substrate at the protein surface (*15*). It also has been proposed that LiP oxidizes polymeric substrates via a diffusible oxidized intermediate, the VA cation radical (*31, 37, 38*).

P. chrysosporium produces VA under ligninolytic conditions. VA is a substrate for LiP and also stimulates the LiP-catalyzed depolymerization of synthetic lignin (*4*) and the oxidation of a variety of recalcitrant aromatic substrates (*18, 19, 31 39−41*); however, the precise role(s) of VA in the mechanism of LiP remains to be clarified. It has been reported that VA protects LiP from irreversible inactivation by excess H_2O_2, both by reducing LiP compound II, thereby preventing the formation of compound III, and by directly converting LiP compound III* back to the native enzyme (*20, 29, 30*). It also has been reported that VA is obligatory for reducing LiP compound II, thus preventing LiP compound II from reacting with H_2O_2 to form LiP compound III (*29, 33, 42*). In addition, it has been proposed that VA functions as a diffusible mediator, wherein the cation radical produced via the one-electron oxidation of VA diffuses away from the active site of LiP and oxidizes the terminal substrate at a distance from the enzyme surface (*31, 37, 38*); however, no direct proof for this diffusible mediator hypothesis has been reported. In contrast, recent chemical and spectroscopic evidence argues against the diffusible mediator hypothesis, indicating that the VA cation radical is too short-lived to diffuse away from the enzyme active site and oxidize the terminal substrate (*43−45*). For example, the LiP oxidation of dimethoxytoluene leads to dimer formation via the coupling of a cation radical with a neutral species. In contrast, the LiP oxidation of VA does not lead to dimer formation, strongly suggesting that the VA cation radical loses a benzylic proton extremely rapidly (*43*). In addition, fast-flow electron spin resonance studies indicate that the free VA cation radical is very unstable ($t_{1/2} \sim 0.6$ ms), whereas the enzyme-bound VA cation radical is much more stable ($t_{1/2} \sim 370$ ms) (*44, 45*). Another possibility exists for the mechanism of VA activation of LiP. VA could act as an enzyme-bound redox cofactor, essentially as was reported recently for Trp191 of CcP (*46−49*).

We have been studying the involvement of VA in the LiP oxidation of model polymeric substrates. Previously, we demonstrated that LiP oxidizes Cc^{2+} to Cc^{3+} in the presence and absence of VA (*15*). However, the redox potential of the Cc^{2+}/Cc^{3+} couple (250 mV) is low compared to that required for lignin oxidation. Therefore, in this study we have examined the involvement of VA in the LiP-catalyzed oxidation of Cc^{3+}.

LiP-Catalyzed Bleaching of Cc^{3+}. The results shown in Figures 1 and 2A demonstrate that LiP oxidation of Cc^{3+}, in the presence of H_2O_2 and VA, is accompanied by a decrease in the Soret band at 408 nm. Atomic absorption spectrometric analysis (Table 1) demonstrates that this decrease in the Soret, representing the bleaching of the heme in Cc^{3+}, is accompanied by a loss of iron from the protein.

Several factors could be contributing to the loss of the iron from the heme. First, all of the bands in the Cc^{3+} absorption spectrum decrease without observable wavelength shifting. This suggests that the porphyrin itself is being oxidatively cleaved. If the iron was lost from an intact porphyrin, some shift in the spectrum might be expected. Second, the iron in Cc^{3+} is low spin and hexacoordinated with the sulfur of Met80 as the sixth ligand; thus, the iron does not readily react with H_2O_2 to form higher redox states as occurs in the peroxidase catalytic cycle (*50*). In addition, the heme in Cc^{3+} is covalently attached to the protein by thioether bridges to two cysteine residues (*28*). Our amino acid analysis of the oxidized monomer and dimer of Cc^{3+} detects oxidized Met and Tyr residues with no other significant changes in the amino acid composition (Table 3). Methionine sulfoxide is found in the oxidized monomer, and both methionine sulfoxide and methionine sulfone are found in the oxidized dimeric product. However, the formation of methionine sulfoxide may not be directly responsible for the bleaching, since Cc^{3+} with Met80 sulfoxide as the sixth ligand exhibits an increase in the extinction coefficient of the Soret band, as compared with native Cc^{3+} (*26*). Furthermore, the presence of Met80 sulfoxide should be associated with a decrease in the absorbance at 695 nm, since the 695-nm absorbance is believed to be indicative of Met coordination to the heme (*26*). In contrast, the Soret intensity of LiP-oxidized Cc^{3+} decreases (Figure 1), and the 695-nm absorption remains unchanged (data not shown). In addition to porphyrin oxidation, the formation of Met sulfone could partially explain the heme bleaching, since the fully oxidized form of Met80 would not be a ligand for the iron. Third, oxidation of Tyr also might result in heme bleaching. The formation of Tyr radicals from either free Tyr or Tyr residues in certain proteins is known (*51*). In addition, Catalano et al. (*52*) have demonstrated that in myoglobin a H_2O_2-generated Tyr103 radical couples with the meso carbon of the heme prosthetic group. The distance between Tyr103 and the heme meso carbon in myoglobin is about 6 Å. The distance between the only buried Tyr (Tyr67) and the heme meso carbon in Cc is between 4.5 and 6 Å, depending on the orientation (*28*); thus, a similar reaction between a Tyr radical and the heme might occur in Cc^{3+}. The formation of a Tyr−heme adduct may distort the heme environment and result in the release of iron and bleaching of the heme. Additional work is required to elucidate the exact chemical mechanism of heme bleaching in this reaction.

The nonenzymatic reaction between Cc^{3+} and H_2O_2 (Figure 2A) has been reported previously (*53*). It is believed to involve the direct reaction between the heme iron of Cc^{3+} and H_2O_2, forming a bound hydroxy radical. The latter and H_2O_2 react with the porphyrin to generate carbon monoxide and liberate oxygen, leading to the opening of the porphyrin ring and loss of the Soret absorbance (*53*). However, to obtain a nonenzymatic reaction rate, as measured by the decrease in the Soret, that is equivalent to the reaction rate obtained in the presence of LiP and VA (Figure 2) would require 15 mM H_2O_2 (*53*), whereas the LiP reactions contain only 100 μM H_2O_2. Furthermore, in the presence of VA, the initial rate of Cc^{3+} oxidation increases with increasing LiP concentration (Figure 2B). Finally, oxygen evolution is not observed following the LiP-catalyzed oxidation of Cc^{3+} (data not shown), indicating that the oxidation of Cc^{3+} observed here is due to LiP and not to a nonenzymatic reaction between Cc^{3+} and H_2O_2.

Table 4: Steady-State Kinetic Parameters for the LiP Oxidation of Cc^{2+}, Cc^{3+}, and VA[a]

substrates	$K_{m\ app}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{m\ app}$ ($M^{-1}\ s^{-1}$)	reference
Cc^{2+}	68	25	3.7×10^5	*15*
Cc^{2+}, VA	1.4	40	2.8×10^7	this work
Cc^{3+}, VA	0.4	1	2.5×10^6	this work
VA	113	19	1.6×10^5	*15*

[a] Reaction conditions were as described in the text and in ref *15*.

Effects of VA on the LiP-Catalyzed Oxidation of Cc^{3+}. In previous studies, we demonstrated that VA stimulates the LiP-catalyzed oxidation of Cc^{2+} only at low pH and relatively high concentrations of H_2O_2, indicating that, in these experiments, VA is stimulating the reaction by rescuing LiP from inactivation by H_2O_2 at low pH (*15, 20, 30, 54*). However, the LiP-catalyzed oxidation of Cc^{3+} is strictly dependent on the presence of VA in the pH range of 2.5−6.0 at a H_2O_2 concentration of 100 μM (Figure 2). The redox potential required for Cc^{2+} oxidation (250 mV) is far lower than that required for tyrosine oxidation, which partially explains why VA is required for Cc^{3+} oxidation but not for Cc^{2+} oxidation. The results in Figure 4 demonstrate that, even at H_2O_2 concentrations as low as 7.5 μM, the rate of Cc^{3+} oxidation is strictly dependent on the presence of VA. Furthermore, at higher H_2O_2 concentrations, increasing amounts of VA are required for maximal Cc^{3+} oxidation. This suggests that the H_2O_2 is being consumed during the reaction and that more VA is required to consume higher concentrations of H_2O_2. Finally, the results in Figure 4 demonstrate that concentrations of VA as high as 1 mM do not inhibit the rate of Cc^{3+} oxidation, suggesting that VA does not compete with Cc^{3+} for a binding site on LiP, nor is VA specifically reducing LiP compound II. Rather, the oxidation of VA is required as an intermediate step in the oxidation of Cc^{3+}.

As with CcP oxidation of Cc^{2+} (*22*), the initial rate of LiP oxidation of Cc^{2+} decreases with increasing ionic strength (*15*), implying that an electrostatic interaction between LiP and Cc may be a rate-limiting step in the reaction. At any rate, it implies some interaction between the acidic LiP protein and the basic Cc protein (*15, 55*).

The steady-state kinetic parameters shown in Table 4 indicate that the rates of oxidation of Cc^{2+} and VA are similar even though a direct interaction between the heme of LiP and the polymeric Cc^{2+} is not possible. In contrast, computational modeling indicates that VA binds close to the heme (*13*). This suggests that the fast oxidation rate with Cc^{2+} is likely due to the lower redox potential of the Cc^{2+}/Cc^{3+} couple (250 mV) as compared with that of VA (1.45 V) (*56*). However, the highest rates of Cc^{2+} oxidation occur in the presence of VA. In particular, the $K_{m\ app}$ for Cc^{2+} oxidation decreases by ~50-fold in the presence of VA. In addition, we calculate that the second-order rate constant for Cc^{2+} oxidation in the presence of VA is $\sim 2.8 \times 10^7\ M^{-1}\ s^{-1}$. This is 170-fold higher than the rate for VA oxidation. This large difference in the overall rate as calculated from steady-state kinetic data suggests that the rate of electron transfer between the VA cation radical and Cc^{2+} may be faster than the rate for deprotonation of the VA cation radical. The micromolar values for the $K_{m\ app}$ for the oxidation of Cc^{3+} in the presence of VA and for the K_i for Cc^{3+} inhibition of VA oxidation suggest an interaction or weak binding between the enzyme and this polymeric substrate. In particular, the low K_i value would not be expected if the VA cation radical was acting as a diffusible redox mediator. In that case, significant amounts of veratraldehyde would be expected, even in the presence of Cc^{3+}.

The stoichiometry of Cc^{2+} oxidation as compared to H_2O_2 consumption is exactly 2:1 in the presence VA (data not shown). Consistent with these results, HPLC analysis shows that there is negligible VA oxidation while Cc^{2+} is being oxidized (Table 2). These observations indicate that VA is not an obligatory substrate for the reduction of LiP compound II during Cc^{2+} oxidation, as proposed for the oxidation of anisyl alcohol (*33*). If VA was a required reductant, 0.5 equivalent of veratraldehyde should be formed for each equivalent of Cc^{2+} oxidized. Owing to the complexity of products formed in the LiP-catalyzed oxidation of Cc^{3+} (Figure 6), it is difficult to determine the stoichiometry between Cc^{3+} oxidation and H_2O_2 consumption. However, VA is absolutely required for the oxidation of Cc^{3+} in the presence of 100 μM H_2O_2. In this case, a negligible amount of veratraldehyde is formed when LiP oxidizes Cc^{3+} in the presence of a 5-fold excess of VA. Although small amounts of veratraldehyde are formed when LiP oxidizes Cc^{3+} in the presence of a 50-fold excess of VA, these amounts are not nearly stoichiometric. Taken together, these results indicate that VA is not acting as a required reductant for compound II in these reactions. Indeed, the results suggest strongly that the VA cation radical oxidizes Cc^{3+} while still in its enzyme-binding site. In the time it takes to diffuse away, it would lose a proton to form the benzylic radical.

The results in Figure 5, which directly measure the effects of Cc^{3+} and VA on H_2O_2-induced LiP inactivation, support this latter alternative. In the presence of 100 μM H_2O_2, 500 μM VA is required to stabilize LiP and 20 μM VA exhibits no protective effect. However, with 20 μM Cc^{3+} and 20 μM VA together, full LiP activity is retained. The simplest explanation for this result is that VA is oxidized to a cation radical which rapidly oxidizes Cc^{3+}, enabling the enzyme to complete its catalytic cycle. Since there are several oxidizable Tyr and Met residues in Cc^{3+}, only 20 μM Cc^{3+} is required. The stoichiometry of this protective effect suggests that the VA cation radical rapidly oxidizes Cc^{3+}, implying close proximity of the LiP and Cc^{3+}, rather than losing a proton to form the benzylic radical which would lead to the formation of veratraldehyde. The results in Table 2 confirm that, in the presence of Cc^{3+}, very little VA is converted to veratraldehyde. Inactivation of LiP is due to the oxidation of LiP compound II by H_2O_2 to form LiP compounds III and III* with subsequent bleaching (*20*). VA protects LiP from inactivation by reducing LiP compound II to native LiP, preventing the formation of LiPIII and LiPIII*. If the VA cation radical is reduced by Cc^{3+} while still in its binding site, then the VA produced by this recycling reaction could immediately reduce LiP compound II. In contrast, if the VA cation radical were to diffuse away, another VA molecule would be required to reduce compound II. This latter situation would be essentially identical with having VA as the lone substrate. However, the results in Figure 6 show that 20 μM VA alone does not protect the enzyme from inactivation, while 20 μM VA and 20 μM Cc^{3+} do afford complete protection.

The results described here lead us to propose a role for VA in these reactions. We propose that VA binds to LiP between the heme and the surface of the enzyme as predicted by molecular modeling (*13*). This location for VA would facilitate the oxidation of a polymer located at the surface of LiP. Thus, LiP oxidizes VA to the cation radical which is reduced back to VA during the oxidation of Cc^{3+}. In this sense, VA functions as an electron transfer cofactor between the LiP heme and the surface of the enzyme. VA may act analogously to Trp191 in CcP reactions (*46*−*49*). This accomplishes two things: it ensures the rapid oxidation of the terminal substrate, and it ensures that the enzyme completes its catalytic cycle without being shunted into compounds III and III*.

Polymerization of Cc^{3+} and Formation of Dityrosine. In addition to heme bleaching, some of the Cc^{3+} molecules are polymerized by LiP in the presence of VA and H_2O_2 (Figure 6). It has been reported that both free tyrosine and Tyr residues in a variety of proteins can be oxidized to Tyr radicals by peroxidases (*51*), and two Tyr radicals can undergo phenolic coupling to form dityrosine. The coupling of Tyr radicals in proteins can be intramolecular, intermolecular, or both. Intermolecular coupling can lead to dimerization of proteins (*51*). For example, the reaction of sperm whale myoglobin with H_2O_2 decreases the Tyr content of the protein and results in the formation of dimeric myoglobin (*57*).

The formation of dityrosine within proteins has been confirmed by amino acid analysis (*51*). Amino acid analyses of the monomeric and dimeric products of LiP-catalyzed Cc^{3+} oxidation demonstrate that the Tyr content of the dimer decreases during the reaction, suggesting the formation of dityrosine. The crystal structure of Cc^{3+} (*28*) suggests that the Tyr residues on the surface of the protein (Tyr48, Tyr74, and Tyr97) might be susceptible to oxidation, resulting in dimerization of the protein.

In vitro studies with pure LiP have demonstrated the partial polymerization of synthetic lignin containing free phenolic groups. This polymerization does not occur when exhaustively methylated synthetic lignin is used (*4*), indicating that LiP oxidizes phenols in synthetic lignin to form phenoxy radicals which can couple to form polymerized products. A similar LiP mechanism may result in the coupling of phenoxy radicals in synthetic lignin and in lignin model compounds, as apparently occurs in the coupling of Tyr radicals in Cc^{3+}. The ability of LiP to oxidize Tyr residues in proteins may explain the complete absence of Tyr residues in both LiP and MnP (*8*−*11*, *13*).

Conclusions. LiP catalyzes a novel VA-dependent irreversible oxidation of Cc^{3+}, leading to bleaching of the Cc^{3+} heme and polymerization of some of the Cc^{3+} molecules. Our results suggest that VA acts as an enzyme-bound radical mediator in this reaction. Cc^{3+} heme oxidative cleavage may contribute to bleaching of the heme with subsequent loss of heme iron. The polymerization reaction appears to result from coupling of Tyr radicals formed by LiP oxidation. Cc^{3+} has a higher redox potential than Cc^{2+} and, therefore, is a better model polymer for studying the LiP-catalyzed oxidation of lignin. In addition, the LiP-catalyzed oxidation of Cc^{3+} is easily measured via the decreasing Soret band intensity. We are planning additional studies to further probe the role of VA in these reactions.

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