ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 17 (2002) 49–57

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Veratryl alcohol binding sites of lignin peroxidase from *Phanerochaete chrysosporium*

Toru Johjima[1], Hiroyuki Wariishi*, Hiroo Tanaka

Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received 15 October 2001; received in revised form 14 December 2001; accepted 17 December 2001

Abstract

Possible binding sites of lignin peroxidase (LiP) for veratryl alcohol (VA) were investigated by determining the reactivity of three different chemically modified LiPs against VA when acting (i) as a reducing substrate, (ii) as a rescuing reagent for the rapid conversion of LiPIII* back to native LiP, and (iii) as an enzyme-bound redox mediator. The enzyme was chemically modified to alter its surface properties by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence and the absence of 2-aminoethanesulfonic acid to introduce sulfo and *N*-acylurea groups instead of carboxylic groups, respectively. LiP was also modified by *N*-bromosuccinimide (NBS) to yield Trp-modified enzyme. The spectral characteristics and compound I formation rates of modified LiPs were identical to those of unmodified LiP. The activities for VA oxidation by modified LiPs were significantly reduced but with almost unchanged pH dependencies. Several other substrates including phenolic, anionic, and polymeric substrates were also utilized to characterize the activity of the modified enzymes. Kinetic analysis of these reactions strongly suggests that LiP has at least two substrate oxidation sites, one is Trp 171 for VA oxidation and the other is for anionic substrate oxidation. For VA-supported oxidation of ferric cytochrome c (Cc^{3+}), the VA binding site was estimated to be the same as that of VA oxidation.

The reactivities of chemically modified LiPs with excess H_2O_2 were investigated, indicating the formation of compound III* species. Compound III* species of EDC–LiP and unmodified LiP were converted back to ferric enzymes by adding VA. However, there was a reduced recovery of ferric NBS–LiP, suggesting that the VA binding site for VA-derived rapid reversion of compound III* is located at a different site from that for VA oxidation.

Keywords: Chemical modification; Compound III; Lignin peroxidase; Steady-state kinetics; Substrate binding site; Transient-state kinetics; Veratryl alcohol

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate); AES, 2-aminoethansulfonic acid; Cc^{2+}, ferrous cytochrome c; Cc^{3+}, ferric cytochrome c; DMP, 2,6-dimethoxyphenol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HRP, horseradish peroxidase; LiP, lignin peroxidase; LiPI, II, and III, compounds I, II, and III of LiP; NBS, *N*-bromosuccinimide; S-LiP, sulfonated LiP; VA, veratryl alcohol

* Corresponding author. Tel.: +81-92-642-2993; fax: +81-92-642-2993.

E-mail address: hirowari@agr.kyushu-u.ac.jp (H. Wariishi).

[1]Present address: Japan Science and Technology Corporation (JST-ICORP), International Cooperative Research Project.

1381-1177/02/$ – see front matter
PII: S1381-1177(01)00079-0

1. Introduction

Lignin is the most abundant renewable aromatic polymer, and is known as one of the most recalcitrant biomaterials on Earth [1,2]. Its degradation plays a key role in the carbon cycle of the biosphere [2–6]. Only white-rot basidiomycetes are responsible for the complete mineralization of this polymer. *Phanerochaete chrysosporium*, the best-studied white-rot fungus, secretes two heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase under ligninolytic conditions [3–6]. Thus, these enzymes are believed to be involved in triggering lignin biodegradation.

Nucleotide sequences of a number of LiP cDNA and genomic clones [6–9] and crystal structures of LiP [10–12] have demonstrated that important peroxidase catalytic residues, including the proximal and distal His, the distal Arg, and an H-bonded Asp to proximal His, are all conserved. Therefore, LiP shares many structural and mechanistic features with other peroxidases, yet it has several unique properties [3–6,13–15]. The enzyme catalyzes the one-electron oxidation of nonphenolic aromatic compounds with a high redox potential through the formation of a substrate cation radical [16,17]. LiP can directly interact and oxidize polymeric substrates such as lignin and ferrous cytochrome c (Cc^{2+}) via a long-range electron transfer [18,19]. On the other hand, the oxidation of ferric cytochrome c (Cc^{3+}) is strictly dependent on the presence of veratryl alcohol (VA) as a cofactor [20]. In addition, the ready formation of LiPIII species occurs with considerably less H_2O_2 than is required with other peroxidases [21–23]. This compound III species is irreversibly inactivated in the presence of excess H_2O_2 via the intermediate formation of compound III* species, yet it can be converted back to the native enzyme by addition of the *P. chrysosporium* secondary metabolite VA to LiP III* [21,22,24]. Besides acting as a reducing substrate, multiple roles of VA in the LiP catalytic mechanism have been suggested [3–6,14,16–27]. Among them, the most controversial argument is also found in the role of VA for the oxidation of recalcitrant substrates by LiP [25–27]. Once widely accepted VA cation radical mediation mechanisms are now questionable [28–31].

Recently, two distinct VA binding sites were proposed using the site directed mutagenesis technique [32,33]. One is Glu 146 located on the entrance of the distal heme pocket near the heme δ-*meso* edge [32], and the other is Trp 171 located on the protein surface, which is hydroxylated during the catalytic action [33,34]. Furthermore, recent reports claimed that LiP might possess more than two substrate binding sites [19,33]. In previous works, recombinant LiPs expressed in *Escherichia coli* were utilized; however, wild type recombinant LiP showed a different pH dependency for VA oxidation than fungal LiP. In this study, we utilize three types of chemically modified LiPs to investigate VA binding sites during: (i) its oxidation; (ii) the reaction with compound III*; (iii) the reaction with Cc^{3+}. VA most likely binds near Trp 171 during its oxidation and while acting as an enzyme-bound mediator for Cc^{3+} oxidation. A different binding site was suggested; however, when it reacted with LiPIII* to form native LiP.

2. Materials and methods

2.1. Proteins

LiP isozyme 2 (H8) was isolated from the extracellular culture medium of *P. chrysosporium* (ATCC 34541) and purified to electrophoretically homogeneous as previously described [22]. Horseradish peroxidase (HRP) (Grade IC) and Cc^{3+} (horse heart) were purchased from TOYOBO and Wako Pure Chem., respectively, and used without further purification. RZ values of LiP and HRP were 5.0 and 3.1, respectively. Cc^{2+} was prepared by adding excess sodium dithionite to Cc^{3+} in argon-saturated water. Excess sodium dithionite was removed by gel filtration on a Sephadex G-25 eluted with argon-saturated water as previously described [18]. LiPIII* was prepared by adding 50 eq. H_2O_2 to ferric LiP in 20 mM succinate, pH 3.0 [22]. Spectroscopic and kinetic measurements of LiPIII* were initiated 1 min after H_2O_2 addition.

2.2. Chemicals

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDC) was purchased from Dojindo. 2-Aminoethansulfonic acid (AES) and *N*-bromosuccinimide (NBS) were obtained from TCI. VA was purchased from Aldrich and purified using preparative HPLC equipped with an Inartsil PREP-ODS column

(GL Science Inc.). The 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate), ABTS and hydrogen peroxide (30% aqueous solution) were obtained from Wako Pure Chem. H_2O_2 stock solution was prepared daily and the concentration was determined as previously described [35]. All other chemicals were reagent grade. Solutions were prepared with deionized water obtained from Milli Q (Millipore) system.

2.3. Chemical modification of lignin peroxidase

An EDC aqueous solution (pH adjusted at 5.5 with HCl) was prepared immediately prior to use. The reaction mixture consisted of 3.0 μM LiP and 25 mM EDC. After a 3 h incubation, EDC modified LiP (EDC–LiP) was recovered using Sephadex G-25 column chromatography. A sulfo group was introduced to make sulfonated LiP (S-LiP). The reaction mixture consisted of 3.0 μM LiP, 0.5 M AES, and 25 mM EDC (pH 5.5). After a 3 h incubation, S-LiP was recovered using Sephadex G-25 column chromatography.

NBS was recrystallized from water. An NBS stock solution (5 mM) was prepared immediately prior to use. NBS–LiP was prepared as previously described [34] with slight modification. The reaction mixture contained 3.0 μM LiP and 50 mM NBS in 50 mM sodium acetate buffer, pH 4.0. After 30 min incubation, NBS–LiP was recovered using Sephadex G-25 column chromatography. The concentrations of all modified enzymes were calculated using an extinction coefficient of $133\,mM^{-1}\,cm^{-1}$ at 406.7 nm for unmodified LiP [36] since no spectral shift was observed upon any modifications described above.

2.4. Steady-state kinetic measurement

Initial oxidation rates were spectrophotometrically determined using a Perkin-Elmer Lambda 19 spectrophotometer at room temperature. All steady-state kinetic experiments were performed in 20 mM sodium succinate buffer, pH 3.0 except for Cc^{2+} oxidation performed at pH 4.0 because the LiP oxidation rate of Cc^{2+} in the absence of VA has been reported to be optimal at pH 4.0 [18]. The reactions were initiated by adding 0.1 mM H_2O_2.

The rate of VA oxidation (veratraldehyde formation) was determined using $\Delta\varepsilon_{310}$ of $9.3\,mM^{-1}\,cm^{-1}$ [37]. The rate of 2,6-dimethoxyphenol (DMP oxidation (quinone dimer formation) was determined using $\Delta\varepsilon_{469}$ of $49.6\,mM^{-1}\,cm^{-1}$ [38]. The rate of Cc^{2+} oxidation (Cc^{3+} formation) was determined using $\Delta\varepsilon_{555}$ of $18.7\,mM^{-1}\,cm^{-1}$ [39]. The rate of I^- oxidation (triiodide formation) was determined using $\Delta\varepsilon_{333}$ of $25.5\,mM^{-1}\,cm^{-1}$ [35]. The rate of ferrocyanide oxidation (ferricyanide formation) was determined using $\Delta\varepsilon_{420}$ of $1.02\,mM^{-1}\,cm^{-1}$ [40]. The rate of ABTS oxidation (ABTS radical cation formation) was determined using $\Delta\varepsilon_{415}$ of $36.0\,mM^{-1}\,cm^{-1}$ [41]. K_m and k_{cat} values were determined from a *Lineweaver–Burk* plot of the data.

The oxidation reaction of Cc^{3+} was performed as previously described [20]. The reaction mixture contained 10 μM Cc^{3+}, 30 nM enzyme in the presence of VA (0, 0.5, 1.0, 2.0, and 4.0 mM) in 20 mM sodium succinate, pH 3.0. The reactions were initiated by adding 0.1 mM H_2O_2. The rate of Cc^{3+} oxidation was determined by following the decrease in absorbance at 408 nm, the Soret maximum.

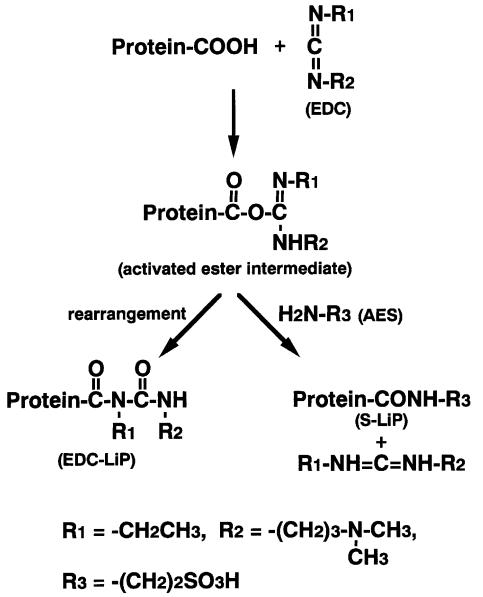
2.5. Transient-state kinetic measurement

Kinetic measurements were conducted using the Photal RA 401S Rapid Reaction Analyzer (Otsuka Electronics Co. Ltd.) equipped with a 1 cm observation cell at 25±0.5 °C. One reservoir contained the enzyme (2 μM) in water, while the other reservoir contained H_2O_2 (40–160 μM) in a succinate buffer ($\mu = 0.08$). LiPI formation was monitored at the isosbestic point between LiPI and LiPII [42]. The pseudo-first-order rate constants were determined by a nonlinear least-squares computer analysis of exponential traces.

3. Results

3.1. Chemical modification of LiP

Three chemically modified LiPs were prepared. EDC is known as a carboxylate modification reagent, introducing a bulky *N*-acylurea substituent which carries a tertiary amino group [43]. Thus, the surface charge properties of the enzyme are altered in EDC–LiP. In the presence of excess AES during EDC treatment, activated ester intermediate has been known to react with the amino group of AES, resulting in the introduction of the sulfo group [44]. Therefore, S-LiP



Scheme 1. Procedures for chemical modification of LiP.

carries sulfo groups instead of carboxyl groups. Actually, ion-exchange chromatography and isoelectric focusing revealed that EDC–LiP possesses a much higher p*I* than S-LiP (data not shown). Scheme 1 summarizes the substitutions occurred during chemical modification of LiP using EDC in the present study. NBS is a well-known Trp modification reagent [34,45].

Upon the treatment of LiP with EDC, the VA oxidation activity was reduced (Fig. 1). The decrease of LiP activity was saturated at an EDC concentration of 25 mM. Thus, 25 mM EDC was utilized to prepare EDC–LiP in this study. On the other hand, under the same preparation conditions, the DMP oxidation activity of HRP was not affected (Fig. 1). In a similar manner, the conditions to cause the saturation of activity reduction were determined for preparing S- and NBS–LiP. Chemically modified LiPs exhibited an identical Soret and visible absorptions to those of unmodified native LiP. Spectra of EDC–LiP and native LiP are shown in the inset of Fig. 1.

3.2. Effect of pH on the activity of modified LiPs

The effect of pH on VA oxidation activity of EDC-, S-, and NBS–LiP was investigated at pH ranging from

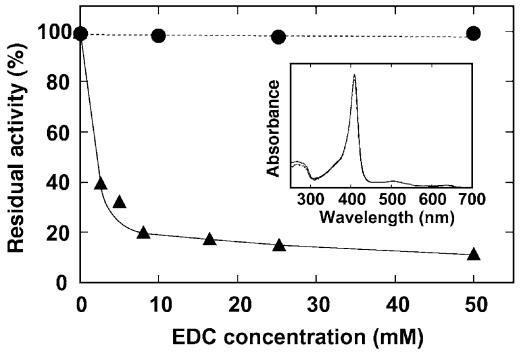


Fig. 1. Effect of [EDC] on LiP (▲) and HRP (●) reactivity. Reaction mixture contained LiP (30 nM) and VA (0.5 mM) in 20 mM sodium succinate, pH 3.0 or HRP (15 nM) and DMP (0.5 mM) in 50 mM sodium malonate, pH 6.0. The reactions were initiated by adding H_2O_2 (0.1 mM). Inset shows the absorption spectra of unmodified LiP (dashed line) and EDC–LiP (solid line).

3.0 to 6.0. Three different types of chemically modified LiPs utilized in this study exhibited very similar pH dependencies for VA oxidation to unmodified LiP (Fig. 2). Basically, the lower the pH, the higher the activity.

3.3. Steady-state kinetics

Steady-state kinetics of VA oxidation by unmodified and modified LiPs were investigated. The kinetic parameters are presented in Table 1. The k_{cat}/K_m values of EDC- and NBS–LiP were ∼10% of that

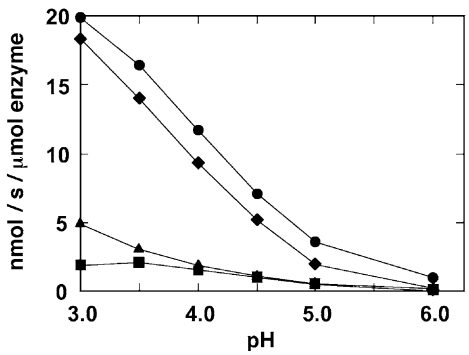


Fig. 2. The pH dependence of VA oxidation by unmodified (●), EDC- (▲), S- (◆), and NBS–LiP (■). Reaction mixtures contained enzyme (30 nM), VA (5 mM) and H_2O_2 (0.1 mM) in sodium succinate ($\mu = 0.04$).

Table 1
Steady-state kinetic parameters of VA oxidation by chemically modified LiPs[a]

	K_m for VA (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$) × 10^3
Unmodified LiP	110	13.0	118.0
NBS–LiP	314	3.9	12.4
EDC–LiP	680	6.4	9.4
S-LiP	370	12.0	32.4

[a] Reaction mixture contained enzyme (30 nM native LiP, 100 nM NBS–LiP, 70 nM EDC–LiP, 34 nM S-LiP), VA (25-2000 μM), and H_2O_2 (0.1 mM) in 20 mM succinate, pH 3.0.

observed for unmodified LiP. The K_m for VA of S-LiP was 3.4-fold higher than that of unmodified LiP, whereas the k_{cat} was almost the same.

Besides VA, several other substrates, ABTS, DMP, ferrocyanide, KI and Cc^{2+} were utilized to evaluate the effect of chemical modification on LiP catalytic actions. Likewise, the catalytic activities of all three modified LiPs against DMP and Cc^{2+} were reduced compared to that of unmodified LiP (Table 2). On the other hand, the behavior against anionic substrates was different. Decrease amounts for I^- oxidation activities of the modified LiPs over unmodified LiP were much smaller compared to those against the other substrates, such as VA and DMP. Furthermore, against ABTS and ferrocyanide, EDC- and NBS–LiP shows higher activities than unmodified LiP (Table 2).

Table 2
Relative activities for the oxidation of various reducing substrates by modified LiPs

Substrates[a]	Relative activity (%)[b]		
	NBS–LiP	EDC–LiP	S-LiP
ABTS	213.0	157.0	57.3
KI	69.4	73.0	77.9
$Fe(CN)_6$	131.0	245.0	69.2
VA	10.5	8.0	27.0
DMP	7.7	44.1	34.3
Cc^{2+}	15.7	27.4	27.9

[a] Substrate used was 5–75 μM for ABTS, 0.75–7.5 mM for KI, 12.5–250 μM for ferrocyanide, 25–2000 μM for VA, 15–500 μM for DMP, and 15–75 μM for Cc^{2+}.

[b] (k_{cat}/K_m) of modified LiP/(k_{cat}/K_m) of unmodified LiP × 100.

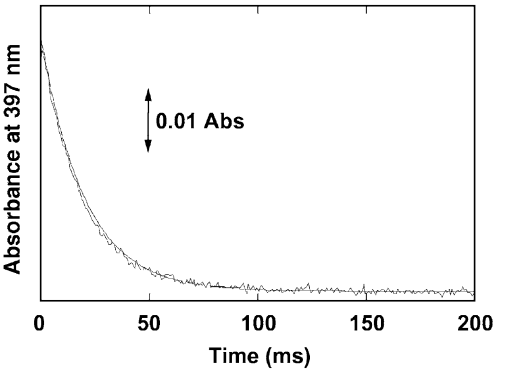


Fig. 3. Time course for the reaction of EDC–LiP and H_2O_2. Reactions were followed at 397 nm. One reservoir contained enzyme (2 μM) in water, and the other reservoir contained H_2O_2 (60 mM) in sodium succinate, pH 3.0 ($\mu = 0.08$).

3.4. Compound I formation rate

Fig. 3 shows the time course of the change in absorbance at 397 nm, which was caused after the addition of H_2O_2 to EDC–LiP. The curve exhibits a single exponential character, from which the pseudo-first-order rate constant (k_{obs}) for compound I formation was determined. The plots of k_{obs} versus [H_2O_2] are linear with zero-intercept for either EDC-, NBS–LiP or unmodified LiP (Fig. 4). Thus, compound I formation rates of EDC–LiP ($5.6 \times 10^5\ M^{-1}\ s^{-1}$) and NBS–LiP ($5.9 \times 10^5\ M^{-1}\ s^{-1}$) were almost identical as that of unmodified LiP ($5.4 \times 10^5\ M^{-1}\ s^{-1}$) and these values were similar to the values previously reported for two LiP isozymes [42,46]. At pH

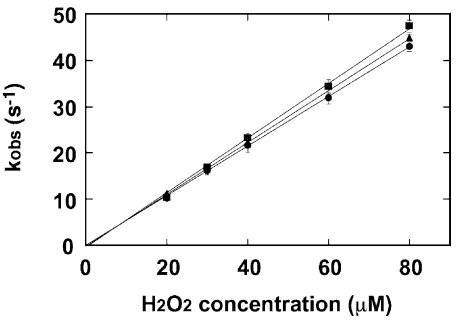


Fig. 4. Plot of k_{obs} against H_2O_2 concentration for the reaction of unmodified (●), EDC- (▲), and NBS–LiP (■) with H_2O_2. The experiment was conducted as described in Fig. 3 at various [H_2O_2]. Each determination of k_{obs} is the mean of five traces. Lines are a linear lease-squares fit of the data.

3.0 and 6.0, the rates were almost the same within experimental error (data not shown).

3.5. Compound III* of modified LiP and their behavior against VA

The addition of 50 eq. H_2O_2 to ferric form of EDC- and NBS–LiP resulted in the formation of compound III* species with Soret peak at 419 nm and visible peaks at 543 and 578 nm (Fig. 5). Upon the addition of VA (0.35 mM), the immediate recovery of ferric form was observed with EDC–LiP and unmodified LiP. Recovery of ferric enzyme 2 min after VA treatment was 58.2 and 63.9%, respectively. In contrast, recovery of ferric enzyme was only 24.8% with NBS–LiP (Fig. 5).

3.6. Cc^{3+} oxidation by modified LiP in the presence of VA

The effect of the chemical modification on LiP oxidation of Cc^{3+} was determined. With all modified LiPs, the decreased amount of VA oxidation activity caused by modification was parallel to that of VA-supported Cc^{3+} oxidation activity (Table 3). Especially, with EDC- and NBS–LiP, the relative activities for VA oxidation and Cc^{3+} oxidation were almost identical. But with S-LiP, the relative activities for VA oxidation were always higher than that for Cc^{3+} oxidation by 20 points (Table 3).

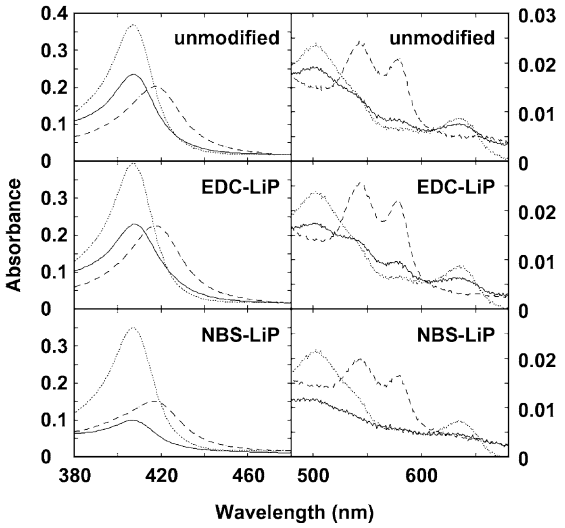


Fig. 5. Formation of Compound III* species by adding H_2O_2 to ferric modified and unmodified LiPs and their reversion back to ferric forms by adding VA. Spectra were recorded before (dotted line) and after (dashed line) the addition of 50 eq. H_2O_2 in 20 mM sodium succinate, pH 3.0. After 1 min incubation with H_2O_2, VA (350 μM) was added and 2 min later, spectra were recorded (solid lines).

Table 3
Relative activities (%) of Cc^{3+} and VA oxidation by modified LiPs[a]

[VA] (mM)	NBS–LiP		EDC–LiP		S-LiP	
	VA[b]	Cc^{3+}[c]	VA	Cc^{3+}	VA	Cc^{3+}
0.5	11.6	19.5	12.5	14.3	46.1	29.4
1.0	13.8	11.6	15.1	14.1	59.0	37.5
2.0	14.7	13.2	19.6	17.6	67.0	45.8
4.0	15.2	14.0	23.9	21.2	69.6	50.7

[a] (Activity of modified LiP/Activity of unmodified LiP) × 100.
[b] VA oxidation at the concentration indicated.
[c] Ferric cytochrome c oxidation in the presence of VA at the concentration indicated.

4. Discussion

It was recently reported that VA oxidation activities of Glu 146 mutants were reduced to 50% of wild type recombinant LiP [32] and that Glu 146 is located at the entrance of the distal heme pocket, which is close to the δ-*meso* edge of the heme [11]. This edge has been thought to be the oxidation site of phenolic substrates in HRP [48]. Then, Ambert-Balay et al. proposed that Glu 146 may play a central role in VA oxidation [32]. On the other hand, Doyle et al. proposed Trp 171, located on the protein surface and 11 Å apart from the heme vinyl group, as a VA oxidation site, based on their observations that W171S and W171F mutants totally lost their VA oxidation activities [33]. They also reported that the k_{cat} values of these mutants for the oxidation of ABTS and 4-[(3,5-difluoro-4-hydroxyphenyl)azo]benzene-sulfonic acid, which are anionic substrates, were comparable to those of wild type recombinant LiP. Combining these observations, they claimed that LiP might possess two substrate binding sites, one for VA and the other for anionic substrates [33]. More recently, we reported that LiP possesses a lignin binding site at His 239 on the protein surface where lignin is oxidized by one electron via a long range electron transfer [19].

Veratryl alcohol is synthesized de novo by *P. chrysosporium* [47] and is one of the most preferred substrates for LiP [3–6]. VA has been proposed as a rescuing reagent to protect LiP from H_2O_2-derived inactivation [21,22]. Furthermore, VA was once reported as a diffusible radical mediator involved in the oxidation of recalcitrant substrates which are not directly oxidized by LiP [25]. Recently, the oxidation of Cc^{3+} was reported to be strictly dependent on the presence of VA as an enzyme-bound redox mediator [20]. Thus, it is not difficult to assume that VA plays a key role in LiP catalytic action. In the present work, we attempted to obtain the information on VA binding sites for a series of reactions involving VA as described above. Instead of LiP mutants, chemically modified LiP was utilized here, since wild type recombinant LiP expressed in *E. coli* exhibited different pH dependencies for VA oxidation from fungal wild type LiP [32]. Three types of chemically modified LiPs utilized in this study exhibited very similar pH dependencies for VA oxidation to unmodified LiP (Fig. 2).

4.1. VA oxidation mechanism

VA oxidation activity was remarkably reduced by chemical modification using EDC which is known as carboxylic group modification reagent. Whereas, HRP oxidation of DMP was not affected by EDC modification (Fig. 1), strongly suggesting that the environment of substrate binding sites and/or electron transfer routes of LiP are different from those of HRP. The binding site of aromatic compounds in HRP has been well characterized using chemical modification [48], NMR spectroscopic [49], and X-ray crystallographic [50] techniques, indicating that it is found in the vicinity of heme 8-methyl and δ-*meso* carbons. Recently, the crystal structure of HRP was determined [51], showing that no acidic amino acid is present in this region.

In the vicinity of Trp 171 proposed as a VA binding site for LiP [33], three acidic amino acid residues, Glu 168, Glu 250, and Asp 264, were found on the protein surface [11]. If these residues are modified by EDC, the accessibility of VA to Trp 171 should be decreased and oxidation activity would consequently be reduced. Glu 146 which was also proposed as a VA binding site [32], could be modified by EDC treatment, causing the reduction of VA oxidation activity. However, this residue is located at the entrance of the heme distal pocket [11]. Therefore, compound I formation rate should be simultaneously affected, since a bulky *N*-acylurea ($C_7H_{15}N_3$) is introduced to Glu located at the heme access channel. In addition, if an opposite charge (tertiary amino group) is introduced to carboxylate of Glu near the heme, the absorption in the Soret and visible regions should be affected. However, identical spectra and compound I formation rates were obtained upon chemical modification using EDC (Figs. 1 and 4). These observations strongly suggest that Glu 146 is hardly modified under the conditions utilized in this study. Then, it is concluded that Trp 171 is the most favorable candidate for a VA binding site. Reduced activity observed with NBS–LiP itself may indicate Trp as a VA binding site, since NBS is a Trp modifying reagent [34,45]. Although NBS is also known to react with Tyr, Met, Cys and His [45], still the observation with NBS–LiP (Table 1) strongly suggests the involvement of Trp residue in VA binding as previously discussed [34].

The k_{cat} of S-LiP for VA oxidation was almost the same as that of unmodified LiP. On the other hand, the k_{cat} of EDC–LiP, in which the same amino acids should be modified as S-LiP, was one half of unmodified LiP. Opposite charges were introduced into those enzymes. In EDC–LiP, tertiary amino groups are theoretically introduced, whereas sulfo groups are introduced into S-LiP [44]. This result may be indicative of the important role of the anionic charges in VA oxidation by LiP. It has been suggested that a LiP-bound VA cation radical might be more stable than an enzyme-free form and that the stabilization of VA cation radical might be due to the acidic microenvironment at the enzyme active site [52]. Our finding is in agreement with the hypothesis. Before concluding this, however, further study is required to obtain more solid evidence for the enzyme-bound VA cation radical.

The importance of surface carboxylate residues of cytochrome c peroxidase for Cc^{2+} binding has been reported [53,54]. If this is also true in LiP/Cc^{2+} complex formation, the data shown in this study is of interest. The extents of activity reductions for Cc^{2+} oxidation by EDC- and S-LiP were the same (Table 2). Since EDC- and S-LiP possessed opposite charges on the surface, these results may suggest that carboxylate modification mainly inhibits the electron

transfer rather than a complex formation between Cc^{2+} and LiP oxidized intermediate.

4.2. Reaction of VA with compound III* of modified LiP

It has been shown that in the absence of a reducing substrate, LiP readily forms LiPIII in the presence of considerably less H_2O_2 than is required for other peroxidases and that LiPIII also reacts with H_2O_2, resulting in irreversible inactivation via the intermediate formation of LiPIII* [21,22,24]. Furthermore, it has also been shown that LiPIII* is rapidly converted to ferric LiP by the addition of VA [21–24]. Compound III* species were also obtained with EDC- and NBS–LiP after adding 50 eq. H_2O_2 to ferric forms. Those spectra were almost identical to LiPIII* derived from unmodified LiP (Fig. 5). Again, the modification of LiP was shown not to cause a change in reactivity with H_2O_2 as seen in compound I formation (Fig. 4).

Upon the addition of VA, LiPIII* species were converted to ferric forms, but with different extents of inactivation. From EDC–LiP and unmodified LiP, 58.2 and 63.9% recovery of ferric forms over starting ferric enzymes were obtained, respectively. In contrast, from NBS–LiP, only 24.8% recovery was observed (Fig. 5). However, VA oxidation activities of EDC- and NBS–LiP were almost the same; furthermore, their reactivities were only ∼10% of unmodified LiP (Table 1), indicating that VA-derived rapid conversion of LiPIII* back to native LiP does not require a VA oxidation step. A difference in the recovery efficiency of compound III* of EDC- and NBS–LiP suggests that the VA binding site for LiPIII* conversion reaction is most likely different from that for its oxidation. For LiPIII* reversion, VA binds to the site which is not modified by EDC but modified by NBS. It is known that NBS reacts with Tyr, Met, Cys and His besides Trp, but LiP has no free Cys and no Tyr. Therefore, Met and/or His on the protein surface or their vicinity are possible candidates for the VA binding site which is involved in the rapid LiPIII* conversion reaction.

4.3. Reaction of modified LiP with Cc^{3+}

Recently, Sheng and Gold reported that LiP is capable of oxidizing of Cc^{3+} and that this reaction is strictly dependent on the presence of VA. The role of VA in this reaction was proposed to be a protein-bound redox mediator [20]. The oxidation reactions of Cc^{3+} were also observed in all modified LiPs only in the presence of VA but the reactivities were reduced, compared to unmodified LiP (Table 3). The similarity of the decrease extent of Cc^{3+} oxidation in the presence of VA and that of VA oxidation was observed with every modified LiPs (Table 3). EDC- and NBS–LiP exhibited the almost identical relative activities for Cc^{3+} and VA oxidations, suggesting that the binding site of VA is most likely the same when acting as a reducing substrate and as a protein-bound mediator during Cc^{3+} oxidation.

5. Conclusion

The binding site of VA was investigated by determining the reactivity of three different chemically modified LiPs against VA acting as: (i) a reducing substrate; (ii) as a rescuing reagent for the rapid conversion of LiPIII* back to native LiP; (iii) as an enzyme-bound redox mediator. As previously proposed [19,33], LiP likely possesses more than two substrate binding sites for VA. VA probably binds to Trp 171 or its vicinity as a reducing substrate and as an enzyme-bound mediator. It is also strongly suggested that VA binds to a different site when it reacts with LiPIII* for the reversion reaction. One of the reasons, which make the determination of a VA binding site difficult, is that there are very few inhibitors reported which cause typical and classical inhibition patterns such as competitive and noncompetitive inhibition. A search for such inhibitors has been carried out and a series of inhibitory studies are now under way. Furthermore, LiP mutants which exhibit the same reactivity with H_2O_2 as wild type LiP are definitely required for further determination of substrate binding mode. Such studies are also underway.

Acknowledgements

This research was supported by Kyushu University Interdisciplinary Programs in Education and Projects in Research Development (to H.W.) and by the Sasakawa Scientific Research Grant from the Japan Scientific Society (to T.J.). T.J. was supported

by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

References

[1] K.V. Sarkanen, C.H. Ludwig, Lignins: Occuurrence, Formation, Structure and Reactions, Wiley, New York, 1970.
[2] R.L. Crawford, Lignin Biodegradation and Transformation, Wiley, New York, 1980.
[3] M.H. Gold, H. Wariishi, K. Valli, in: J.R. Whitaker, P. Sonnet (Eds.), Proceedings of the ACS Symposium Series 389 on Biocatalysis in Agricultural Biotechnology, American Chemical Society, Washington, DC, 1989, p. 127.
[4] T.K. Kirk, R.L. Farrell, Annu, Rev. Microbiol. 41 (1987) 465.
[5] J.A. Buswell, E. Odier, CRC Crit. Rev. Biotechnol. 6 (1987) 1.
[6] M. Tien, CRC Crit. Rev. Microbiol. 15 (1987) 141–168.
[7] M. Tien, C.-P.D. Tu, Nature 326 (1987) 520.
[8] M.H. Gold, M. Alic, Microbiol. Rev. 57 (1993) 605.
[9] T.G. Ritch Jr., V.J. Nipper, L. Akileswaran, A.J. Smith, D.G. Pribnow, M.H. Gold, Gene 107 (1991) 119.
[10] S.L. Edwards, R. Raag, H. Wariishi, M.H. Gold, T.L. Poulos, Proc. Natl. Acad. Sci. USA 90 (1993) 750.
[11] T.L. Poulos, S.L. Edwards, H. Wariishi, M.H. Gold, J. Biol. Chem. 268 (1993) 4429.
[12] K. Piontek, T. Glumoff, K.H. Winterhalter, FEBS Lett. 315 (1993) 119.
[13] T. Higuchi, Wood Sci. Technol. 24 (1990) 23.
[14] H.E. Schoemaker, Recl. Trav. Chim. Pays-Bas Belg. 109 (1990) 255.
[15] H.B. Dunford, J.S. Stillman, Coord, Chem. Rev. 19 (1976) 187.
[16] P.J. Kersten, M. Tien, B. Kalyanaraman, T.K. Kirk, J. Biol. Chem. 260 (1985) 2609.
[17] H.E. Schoemaker, P.J. Harvey, R.M. Bowen, J.M. Palmer, FEBS Lett. 183 (1985) 7.
[18] H. Wariishi, D. Sheng, M.H. Gold, Biochemistry 33 (1994) 5545.
[19] T. Johjima, N. Itoh, M. Kabuto, F. Tokimura, T. Nakagawa, H. Wariishi, H. Tanaka, Proc. Natl. Acad. Sci. USA 96 (1999) 1989.
[20] D. Sheng, M.H. Gold, Biochemistry 37 (1998) 2029.
[21] H. Wariishi, M.H. Gold, FEBS Lett. 243 (1989) 165.
[22] H. Wariishi, M.H. Gold, J. Biol. Chem. 265 (1990) 2070.
[23] D. Cai, M. Tien, Biochem. Biophys. Res. Commun. 162 (1989) 464.
[24] H. Wariishi, L. Marquez, H.B. Dunford, M.H. Gold, J. Biol. Chem. 265 (1990) 11137.
[25] P.J. Harvey, H.E. Schoemaker, R.M. Bowen, J.M. Palmer, FEBS Lett. 183 (1985) 13.
[26] M. Tien, D. Ma, J. Biol. Chem. 272 (1997) 8912.
[27] K.E. Hammel, M.A. Moen, Enzyme Microbial. Technol. 13 (1991) 15.
[28] K. Valli, H. Wariishi, M.H. Gold, Biochemistry 29 (1990) 8535.
[29] L.P. Candeias, P.J. Harvey, J. Biol. Chem. 270 (1995) 16475.
[30] A. Khindaria, T.A. Grover, S.D. Aust, Biochemistry 34 (1995) 6020.
[31] P.J. Harvey, R. Floris, T.K. Lundell, J.M. Palmer, H.E. Schoemaker, R. Wever, Biochem. Soc. Trans. 20 (1992) 345.
[32] K. Ambert-Balay, S.M. Fuchs, M. Tien, Biochem. Biophys. Res. Commun. 251 (1998) 283.
[33] W.A. Doyle, W. Blodig, N.C. Veitch, K. Piontek, A.T. Smith, Biochemistry 37 (1998) 15097.
[34] W. Blodig, A. Doyle, A.T. Smith, K. Winterhalter, T. Choinowski, K. Piontek, Biochemistry 37 (1998) 8832.
[35] M.L. Cotton, H.B. Dunford, Can. J. Chem. 51 (1973) 582.
[36] M.H. Gold, M. Kuwahara, A.A. Chui, J.K. Glenn, Arch. Biochem. Biophys. 234 (1984) 353.
[37] M. Tien, T.K. Kirk, Proc. Natl. Acad. Sci. USA 81 (1984) 2280.
[38] H. Wariishi, K. Valli, M.H. Gold, J. Biol. Chem. 267 (1992) 23688.
[39] E. Margoliash, N. Frohwirt, N. Biochem. J. 71 (1959) 570.
[40] K.A. Schellenberg, L. Hellerman, J. Biol. Chem. 231 (1958) 547.
[41] B.S. Wolfenden, R.L. Willson, J. Chem. Soc. Perkin. Trans. II (1982) 805.
[42] L. Marquez, H. Wariishi, H.B. Dunford, M.H. Gold, J. Biol. Chem. 263 (1988) 10549.
[43] N. Nakajima, Y. Ikada, Bioconjugate Chem. 6 (1995) 123.
[44] T.-Y. Lin, D.E. Koshland Jr., J. Biol. Chem. 244 (1969) 505.
[45] T.F. Spande, B. Witkop, in: C.H.W. Hirs (Ed.), Method in Enzymology, Vol. 11, Academic Press, New York, 1967, p. 498.
[46] A. Andrawis, K.A. Johnson, M. Tien, J. Biol. Chem. 263 (1988) 1195.
[47] K. Lundquist, T.K. Kirk, Phytochemistry 17 (1978) 1676.
[48] M. Ator, P.R. Ortiz de Montellano, J. Biol. Chem. 262 (1987) 1542.
[49] N.C. Veith, Biochem. Soc. Trans. 23 (1995) 232.
[50] A. Henriksen, D.J. Schller, K. Meno, K.G. Welinder, A.T. Smith, M. Gajhede, Biochemistry 37 (1998) 8054.
[51] M. Gajhede, J.S. Schuller, A. Henriksen, A.T. Smith, T.L. Poulos, Nat. Struct. Biol. 4 (1998) 1032.
[52] A. Kindaria, I. Yamazaki, S.D. Aust, Biochemistry 35 (1996) 6418.
[53] J.E. Erman, G.C. Kresheck, L.B. Vitello, M.A. Miller, Biochemistry 36 (1997) 4054.
[54] H. Pelletier, J. Kraut, Science 258 (1992) 1748.