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Identification of the Veratryl Alcohol Binding Site in Lignin Peroxidase by Site-Directed Mutagenesis

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Site-directed mutagenesis was used to identify the veratryl alcohol binding site of lignin peroxidase. The cDNA encoding isozyme H8 was mutated at Glu146 to both an Ala and a Ser residue. The H8 polypeptide was produced by *E. coli* as inclusion bodies and refolded to yield active enzyme. The wild type recombinant enzyme and the mutants were purified to homogeneity and characterized by steady state kinetics. The kcat is decreased for both mutants of Glu146. The reactivity of mutants (kcat/Km) toward H_2O_2 were not affected. In contrast, the kcat/Km of the mutants for veratryl alcohol were decreased by at least half. The oxidation of guaiacol by these mutants were more significantly affected. These results collectively suggest that E146 plays a central role in the binding of veratryl alcohol by lignin peroxidase.

Lignin peroxidases (LP) are produced by lignin-degrading fungi and catalyze the oxidative depolymerization of lignin (1–3). These enzymes were first discovered in *Phanerochaete chrysosporium* (1,2) and now have been detected in many other wood-degrading fungi (4,5). Along with LP, ligninolytic fungi also produce manganese peroxidase (MnP); both the LPs and the MnPs are hemeproteins produced in isoenzyme forms. At least ten different LP isozymes have been detected in *P. chrysosporium* strain BKM-F-1767 (6) which are encoded by different genes (see 7). The LPs catalyze the oxidation of a number of phenolic and non-phenolic substrates (8,9). A physiologically-significant substrate is 3,4-dimethoxybenzyl (veratryl) alcohol which is oxidized to veratraldehyde (10). Veratryl alcohol is a physiological substrate, produced during secondary metabolism by *P. chrysosporium* (11). Harvey et al. (12) were the first to propose that the veratryl alcohol acts as a mediator for LP-catalyzed depolymerization of lignin. They suggested that the veratryl alcohol is first oxidized by a single electron to form the cation radical. This radical then diffuses away from the active site and oxidizes other substrates.

Little is known about the substrate binding site of the LP. Veratryl alcohol does not co-crystallize with the enzyme Edwards et al. (13) Attempts to locate the veratryl alcohol binding site by NMR spectroscopy have not been successful (14). Resolving the location and the structure of this site would help understand if the enzyme can directly interact with the lignin polymer or if it degrades the lignin through the action of low molecular weight mediators. Based on the crystal structure (13) and molecular dynamic simulations (15), a putative veratryl alcohol binding site has been proposed to be located along an access channel at the heme cavity. Poulos et al. (16) proposed that seven residues are involved in veratryl alcohol binding: Ile-85, Val-184, Gln-222, Phe-148, His-82, Glu-146 and Asp-183. The first five residues are proposed to directly interact with veratryl alcohol. His-82 is believed to form an ion pair or H-bond with Glu-146 and could be involved in the pH dependence.

To determine if this access channel is indeed the substrate binding site, we have mutated a large number of residues at this cavity. The present paper describes the initial results where Glu-146 has been mutated to Ala and Ser. Our results clearly demonstrate that Glu146 in involved in interaction of the enzyme with the substrate veratryl alcohol.

MATERIALS AND METHODS

Chemicals. Chloramphenicol, rifampicin, PMSF, Triton X-100, DTT, lysozyme, oxidized glutathione, bovine hemin and veratryl alcohol were purchased from Sigma Chemical Co. IPTG was purchased from Calbiochem. ABTS was purchased from Aldrich Chemical Co., and H_2O_2 from VWR Scientific Products. The H_2O_2 solution was prepared daily and the concentration was determined spectrophotometrically at 240 nm using an extinction coefficient of 39.4 $M^{-1}cm^{-1}$ (17).

Expression vector. The cDNA for LP isoenzyme H8 of *P. chrysosporium* (18) was cloned into the EcoRI site of pET21a(+) plasmid (Novagen, WI), yielding pET21aH8(+). All the bases on the vector

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TABLE I

Refolding and Purification Results for H8

Sample	Volume (ml)	Total activity (Units)	Percent recovery	Total protein (mg)	Specific activity (Units/mg)	Fold purification
Inclusion bodies†		—	—	326	—	—
Refolded†	1,200	11.2*	100	112	<0.1*	1×
DEAE¥	7.01	6.09	54.4	13	0.47	4.7×
MonoQ¥	2.4	2.53	22.6	0.079	32	30×

* Estimated values because the assay used was not sensitive enough to detect oxidation of VA at very dilute enzyme concentration. The values came from experiments using a more sensitive but less specific oxidizable substrate, ABTS.

† Determined by the BSA protein assay.

¥ Determined by the absorbance at 409 nm.

between the ATG codon and the GCT (Ala) codon, which encodes the first amino acid of the 7 amino acid pro-sequence (19), were removed by oligonucleotide-directed mutagenesis (20) in order to place the first codon of the polypeptide in frame with the ATG codon in the expression vector.

Mutagenesis. The mutagenesis of the pET21aH8(+) was carried out using pET21aH8(+) single strand DNA isolated from a dut^-ung^- strain of *E. coli*, CJ236 (20) and a 36 base oligonucleotide 5′-CTTCTCGATCACCGCAGCCATATGTATATCTCCTTC-3′, synthesized on a Beckman Oligo 1000 DNA Synthesizer. This initial mutagenesis removed the 5′ non-coding region and brought the LP cDNA in frame. Mutants were identified by the loss of an EcoRI site, yielding pET21aH8pro-looped(+) (pETH8). The looped-out construct was transformed into BL21(DE3)pLysS cells (Novagen, WI), which allowed expression of the LP polypeptide under IPTG induction.

To produce the E146A and E146S mutants, oligonucleotide-directed mutagenesis was used according to the procedure of Kunkel *et al* (19), using the pETH8 ssDNA isolated from CJ236. The oligonucleotide used to create the mutation were 5′GTGGAAGGG-CGCGGGGACAAG3′ and 5′-GGCCTTGTCCCCTCGCCCTTCC-ACACTGTC3′ for E146A and E146S respectively (the mutant codon is underlined). Mutations were confirmed by DNA sequence of the coding region. The mutated plasmids were then transformed into BL21(DE3)pLysS *E coli* cells for expression.

Expression and refolding of LiPs. The procedure for expression and refolding of LP is an adaptation of the method of Smith et al (21) and Whitwam et al (22). BL21 cells containing pETH8 or a mutant were grown to an absorbance of 0.6 at 600 nm then induced by IPTG (0.4 mM) as previously described (22). After one hour, rifiampicin was added to 20 mg/mL final concentration and the cells were incubated for an additional 4 hours. The cells were then centrifuged and then resuspended in 1/100 of the original culture volume with a nitrogen-purged solution of 2 mM EDTA, 10 mM DTT and 0.1 mM PMSF in 50 mM Tris-HCl, pH 8.0, and kept overnight at 4°C. Lysozyme and Triton X-100 were added to the resuspended cells to give final concentrations of 100 μg/ml and 1%, respectively. The cells were incubated at 30°C for 30 minutes and then cooled on ice for 10 minutes. The cells were then lysed by sonication with a Branson Sonifier 5 times for 30 sec at 3.5 power and 50% pulse (microtip) with a 2-min delay between each regimen. The lysed cells were centrifuged at 25,000xG for 15 minutes at 4°C. The pellet containing the LP inclusion bodies was resuspended in 1/100 of the original culture volume with nitrogen-purged denaturing buffer: 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 mM DTT and 6 M urea, and left on ice for 4–6 hours to fully solubilize the polypeptide. While the inclusion bodies were on ice in 6 M urea, the protein concentration of the preparation was determined using the Bio-Rad microassay (Bio-Rad, CA) with bovine serum albumin as a standard. Before refolding, the volume of the protein solution was adjusted with the denaturing buffer to give a protein concentration of 150 μg/ml.

After the incubation of 4–6 hours described above, 0.7 mM GSSG was added, which was then dialyzed overnight in 50 mM Tris-HCl pH 8.0 and 100 mM $CaCl_2$ at 4°C. This was followed by a second dialysis overnight in 20 mM Tris-HCl pH 8.0 and 10 mM $CaCl_2$. Upon dialysis a precipitate formed which was removed by centrifugation at 12,500×G for 10 minutes at 4°C. Heme was then added by the addition of 0.1 ml of 1 mM bovine hemin (in 0.01 N NaOH) per unit volume per unit OD_{280}; the solution was kept at 4°C for 4–5 hours then centrifuged at 12,500xG at 4°C for 15 minutes to remove any excess heme.

Purification of recombinant LP. Recombinant LP was loaded onto a DEAE-BioGel A column (BioRad) (1.5 × 20 cm) in 20 mM Tris-HCl pH 8.0 and 10 ml $CaCl_2$. The enzyme was eluted with 800 ml of a linear 10-100 mM $CaCl_2$ gradient in 10 mM Tris-HCl pH 8.0. Fractions exhibiting the highest activity were pooled and concentrated in a Amicon YM10 filter (Millipore, MA) to approximately 2 ml. Enzyme activity was determined ABTS assay by oxidation of veratryl alcohol in 50 mM sodium tartrate pH 2.5, 2 mM veratryl alcohol and 0.4 mM H_2O_2 (23). Peak fractions were then loaded onto an FPLC Mono-Q column (Pharmacia) equilibrated with 10 mM $CaCl_2$ in 10 mM sodium acetate, pH 6.0 and eluted with a linear gradient of 0.01 to 1 M sodium acetate pH 6.0 with 10 mM $CaCl_2$. Protein expression was monitored by SDS-PAGE and Western blotting using affinity-pure fungal H8 antibody.

Kinetics. Steady-state kinetic data was measured by oxidation of veratryl alcohol in 50 mM sodium tartrate pH 2.5, 2 mM veratryl alcohol and 0.4 mM H_2O_2 (10).

RESULTS

Expression and purification of recombinant H8. Our recombinant LP preparations were obtained as inclusion bodies as described by Doyle and Smith (21). Due to the low yield of refolding, we attempted a number of modifications to maximize the yield, most being unsuccessful. A couple of modifications are worthy of mention. Rather than refolding by dilution of the 6 M urea, the preparation was dialyzed in 50 mM Tris-HCl and 100 mM $CaCl_2$. This allowed for a more gradual decrease in urea concentration which resulted in a slight increase in the yield of the recombinant LP (data not shown). We also found that the optimal enzyme activity was obtained when hemin was added after refolding as opposed to adding the heme in the refolding buffer. Not only was the yield slightly increased but

TABLE II

Steady-State Kinetic Parameters of Fungal LP, Recombinant H8 (rH8), and Two Mutants

Sample	$K_m(H_2O_2)$ μM	$K_m(VA)$ μm	k_{cat} s^{-1}	$k_{cat}/K_m(H_2O_2)$ $M^{-1}s^{-1}$	$k_{cat}/K_m(VA)$ $M^{-1}s^{-1}$
Fungal LP	26	450	6.4	2.4×10^5	1.4×10^4
rH8	28	445	5.6	2.0×10^5	1.3×10^4
E146A	8.5	470	2.6	3.1×10^5	5.5×10^3
E146S	7.2	340	1.2	1.7×10^5	3.5×10^3

time required for refolding was much shorter without the heme.

The results of the purification procedure are shown in Table I. Two chromatography steps based on the same principle (anion exchange) were used. The first step with DEAE was used mainly for removal of excess heme which became tightly bound to the resin. It did not result in a very large fold in purification of the protein suggesting that DEAE did not separate active LP from inactive LP. The more significant purification was obtained with the Mono Q column. The specific activity was increased by more than 30 fold. We found that the calcium was necessary to stabilize the enzyme, thus it was used as the salt for the chromatography steps. The final preparation had an R_Z value (A407/A280) of 2.2. The absorption spectrum of the recombinant H8 is similar to the fungal H8 in the Soret (409 nm) and visible region.

The two mutant proteins E146A-H8 and E146S-H8 were produced by similar methodology. They both exhibited high Rz values similar to the recombinant H8.

Steady-state kinetic studies of recombinant H8 and its mutants. The steady-state kinetic values for the native LP isolated from *P. chrysosporium*, the wild-type recombinant H8 and the two recombinant mutants are listed in Table II. Incubations were at pH 3.5 which is not the pH for maximum activity but one which LP is stable. The recombinant H8 exhibited steady-state kinetic properties identical to those of the native fungal LP. Within experimental error, recombinant H8 had the same K_m for veratryl alcohol and H_2O_2. It also exhibited the same k_{cat} as that of the native fungal enzyme. The kinetic parameters obtained here are similar to those obtained by Tien et al (10) except for that the Km for veratryl alcohol is slightly higher. The Km for veratryl alcohol of the mutants, within experimental error are similar to those of fungal and recombinant wild-type H8 enzymes. The Km for H_2O_2 showed a decrease for both mutants. Both mutants had lowered kcat values, with a decrease more pronounced for the E146S mutant. The $k_{cat}/K_m(H_2O_2)$ and k_{cat}/K_m (VA) values which are measures of the reactivity of the enzyme with the substrates is not changed for H_2O_2, however, for veratryl alcohol, there is a decrease of at least 50% for both mutants.

A more significant change is obtained for the oxidation of guaiacol by these two mutants. Where the Km for guaiacol for the wild type recombinant H8 enzyme is 44 μM, the Km for the E146A mutant is 9,000 μM and for the E146S mutant is 11,000 μM.

Effect of pH on oxidation of veratryl alcohol. The effect of pH on activity for the mutants, wild type fungal LP and recombinant wild type LP is shown in Fig. 1. The activity of wildtype recombinant enzyme is not decreased by lower pH which is in contrast to the both the wild type fungal and the mutant proteins.

DISCUSSION

The location of the substrate binding site of LP has not been experimentally determined. Attempt to crystallize LP with veratryl alcohol were not successful (13, 16) and ^{1}H-nmr studies (14) have been equally unsuccessful in identifying the binding site. Two substrate binding sites have been proposed. One binding site, shown in Fig. 2, was proposed by Poulos et al. (16) based on crystallographic data and by Du et al. (15) based on molecular dynamic calculations. This first site is the only channel where aromatic substrates can gain access to the heme active site. This site, however, is not large enough to accommodate large polymeric

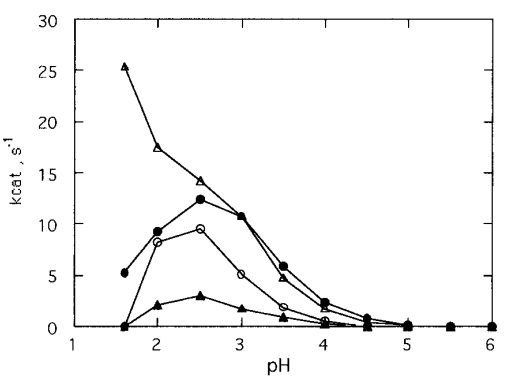

FIG. 1. Effect of pH on veratryl alcohol oxidation by wild type and mutant LP. Wild type fungal (open triangle), recombinant wild type (closed circles), the E146A mutant (open circles) and the E146S mutant (closed triangles) were assayed for veratryl alcohol oxidation at various pH values as described in Materials and Methods. Sodium tartrate was used as the buffer.

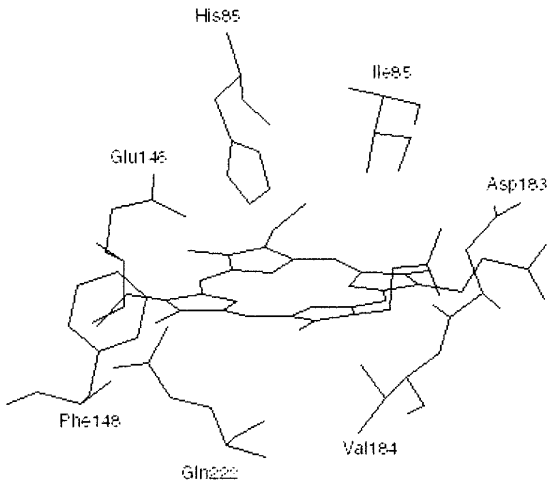

FIG. 2. Proposed substrate binding site for veratryl alcohol. The figure shows the proposed access channel showing the heme and the residues involved in veratryl alcohol binding. See text for details.

lignin substrates unless the substrate is bound from the terminal residue. A second site was proposed by Du et al. (15) that is large enough to accommodate polymeric substrates.

In the present study, we have investigated whether the site proposed by Poulos et al. (16) and Du et al. (15), shown in Fig. 2, is the veratryl alcohol binding site by site directed mutagenesis. We have mutated Glu146, which is hydrogen bonded to His85, to a non-charged residue Ala and also to Ser. Poulos et al. (16) proposed that His85 is hydrogen bonded to the hydroxyl oxygen of veratryl alcohol. Both of these mutant enzymes are active but show altered kinetic properties. Both of these mutations caused a decrease in the Km for H_2O_2 and a decrease in the kcat without any change in the Km for veratryl alcohol. Examined only on kcat and Km, these results would incorrectly suggest that the mutations in this channel only affects the affinity of the enzyme for H_2O_2 and also the maximal velocity. However, examination of kcat/Km indicates little or no change for H_2O_2 and more substantial decrease for veratryl alcohol. This indicates that mutation at Glu146 indeed does affect the interaction of the enzyme with veratryl alcohol but does not affect its reactivity with H_2O_2. Whereas at best, these is a 15% decrease in kcat/Km for the E146 mutant for H_2O_2, the kcat/Km value for veratryl alcohol for the mutants is at least half that of the recombinant wild type H8. This is not a very large decrease in kcat/Km for veratryl alcohol, however, if the proposed role for E146 is correct, mutation at this residue would not be predicted to have a very large effect. However, the results obtained with guaiacol, which will be further characterized in a subsequent manuscript, indicates a much larger change in Km and kcat/Km. These data collectively suggest that the residues described by Poulos et al. (16) and by Du et al. (15) do define the aromatic substrate binding site. Studies, to be described elsewhere, will further detail the role of the other residues of this channel in veratryl alcohol oxidation.

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REFERENCES

1. Tien, M., and Kirk, T. K. (1983) *Science* **221,** 661–663.
2. Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983) *Biochem. Biophys. Res. Commun.* **114,** 1077–1083.
3. Hammel, K. E., and Moen, M. A. (1991) *Enzyme Microb. Technol.* **13,** 15–18.
4. Orth, A. B., Royse, D. J., and Tien, M. (1993) *Appl. Environ. Microbiol.* **59,** 4017–4023.
5. Nerud, F., Zouchova, Z., and Misurcova, Z. (1991) *Biotechnol. Lett.* **9,** 657–660.
6. Kirk, T. K., Coran, S. C., Tien, M., Murtaugh, K., and Farrel, R. (1986a) *Enzyme Microb. Tech.* **8,** 27–32.
7. Gaskell, J., Dieperink, E., Cullen, D. (1991) *Nucleic Acids Res.* **19,** 599–603.
8. Kirk, T. K., Tien, M., Kersten, P. J., Mozuch, M. D., and Kalyanaraman, B. (1986b) *Biochem. J.* **236,** 279–287.
9. Paszczynski, A., Hynh, V.-B., and Crawford, R. (1986) *Arch. Biochem. Biophys.* **244,** 750–765.
10. Tien, M., Kirk, T. K., Bull, C., and Fee, J. A. (1986) *J. Biol. Chem.* **261,** 1687–1693.
11. Lundquist, K., and Kirk, T. K. (1978) *Phytochemistry* **17,** 1676.
12. Harvey, P. J., Schoemaker, H. E., and Palmer, J. M. (1986) *FEBS Lett.* **195,** 242–246.
13. Stevens, S. L., Raag, R., Wariishi, H., Gold, M. H., and Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 750–754.
14. Banci, L., Bertini, I., Bini, T., Tien, M., and Turano, P. (1993) *Biochemistry* **32,** 5825–5831.
15. Du, P., Collins, J. R., and Loew, G. H. (1992) *Protein Eng.* **5,** 679–691.
16. Poulos, T. L., Edwards, S. L., Wariishi, H., and Gold, M. H. (1993) *J. Biol. Chem.* **268,** 4429–4440.
17. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem.* **49,** 474–478.
18. Tien, M., and Tu, C.-P. D. (1987) *Nature* **326,** 520–523; 328, 742.
19. Ritch, Jr., T. G., Nipper, V. J., Akileswaran, L., Smith, A. J., Pribnow, D. G., and Gold, M. H. (1991) *Gene* **107,** 119–126.
20. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Meth. Enzymol.* **154,** 362–387.
21. Doyle, W. A., and Smith, A. T. (1996) *Biochem. J.* **315,** 15–19.
22. Whitwam, R. E., Gazarian, I. G., and Tien, M. (1995) *Biochem. Biophys. Res. Comm.* **216,** 1013–1917.
23. Archibald, F. S. (1992) *Appl. Environ. Microbiol.* **58,** 3110–3116.