Two Substrate Interaction Sites in Lignin Peroxidase Revealed by Site-Directed Mutagenesis†

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ABSTRACT: It has been shown recently that Trp171 of lignin peroxidase (LiP) is hydroxylated at the Cβ position [Blodig, W., Doyle, W. A., Smith, A. T., Winterhalter, K., Choinowski, T., and Piontek, K. (1998) *Biochemistry 37*, 8832−8838]. Comparative experiments, carried out on both wild-type fungal and recombinant LiP isoenzyme H8 (LiPH8*), indicate that the process of hydroxylation is autocatalytic and that Trp171 may be implicated in catalysis. The role of this residue has therefore been examined using site-directed mutagenesis to obtain recombinant enzymes with Trp171 substituted by Phe or Ser (W171F and W171S LiPH8*, respectively). The wild-type recombinant enzyme (LiPH8*) was analyzed in solution using ^{1}H NMR spectroscopy and its integrity confirmed prior to the kinetic and spectroscopic characterization of LiPH8* mutants. A charge neutralization mutation in the "classical heme edge" substrate access channel of LiP, in which Glu146 was substituted by Gly (E146G LiPH8*), showed substantial activity with respect to veratryl alcohol (VA) oxidation and a marked (2.4 pH units) increase in pK_a for the oxidation of a negatively charged difluoroazo dye. More surprisingly, the Trp171 LiPH8* mutants W171F and W171S LiPH8* were found to have lost all activity with VA as substrate, and compounds **I** and **II** were unable to react with VA. Both mutants, however, retained substantial activity with two dye substrates. These data provide the first direct evidence for the existence of two distinct substrate interaction sites in LiP, a heme-edge site typical of those encountered in other peroxidases and a second, novel site centered around Trp171 which is required for the oxidation of VA. Stopped-flow kinetic studies showed that all the mutants examined reacted normally with hydrogen peroxide to give a porphyrin cation radical (compound **I**). However, the rapid phase of spontaneous compound **I** reduction (2.3 s^{-1}), typical of wild-type LiP, was absent in the Trp171 mutants, strongly suggesting that an electron-transfer pathway must exist within the protein leading from the heme to a surface site in close proximity to Trp171. The kinetic competence of such a pathway is dependent on interaction of the enzyme with VA, at or near Trp171.

Lignin peroxidases are monomeric, heme proteins, classified in class II (*1*) of the plant peroxidase superfamily together with other peroxidases of fungal origin. They are secreted by wood-degrading fungi, the most well-known source being the white wood-rot fungus *Phanerochaete chrysosporium* (see ref *2* for review). In addition to their agricultural and environmental importance for the biodegradation of lignin (*3*), the enzymes are of interest to the biotechnology industry due to emerging applications in bioremediation and catalysis of difficult chemical transformations (*4*). Three independent high-resolution X-ray structures of lignin peroxidase (LiP)[1] isoenzymes have been solved (*5*−*7*); however, important questions remain to be answered concerning the generation and control of the high potentials required to oxidize electropositive substrates (*8*) without the autoxidation and destruction of the enzyme itself.

The overall catalytic cycle of LiP is similar to that of other peroxidases (*9*). Two-electron oxidation of the ferric enzyme by hydrogen peroxide yields the intermediate compound **I**, which is formally two oxidizing equivalents above the resting state enzyme. One of these oxidizing equivalents is stored in an oxyferryl iron(IV) species [Fe(IV)═O], but differences

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[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DFAD, 4-[(3,5-difluoro-4-hydroxyphenyl)azo]benzenesulfonic acid, sodium salt; LiP, lignin peroxidase; LiP (H8), lignin peroxidase (isoenzyme H8); PCR, polymerase chain reaction; VA, 3,4-dimethoxybenzyl alcohol (veratryl alcohol); VA$^{\bullet+}$, veratryl alcohol cation radical; LiPH8*, wild-type recombinant lignin peroxidase isoenzyme H8 including the seven-residue pro-sequence encoded in the original cDNA sequence (EMBL Accession No. Y00262); E146G LiPH8*, recombinant lignin peroxidase mutant in which Glu146 is replaced by Gly; W171F/S LiPH8*, recombinant lignin peroxidase mutants in which Trp171 is replaced by Phe and Ser, respectively; CIP/ARP *Coprinus cinereus* peroxidase; 6-c HS, six-coordinate high spin; CT1, heme iron charge-transfer electronic absorption band 1.

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exist between peroxidases with respect to the other. In lignin peroxidase, a porphyrin π cation radical has been shown to be present (*9*) and was recently confirmed by EPR spectroscopy (*10*). However, in cytochrome *c* peroxidase the radical resides on a Trp residue (W191), which is proximal to the heme and in contact with the proximal His (*11*). To complete the catalytic cycle, compound **I** is returned to the resting ferric enzyme, via compound **II** (a second, and distinct catalytic intermediate), by two sequential one-electron-transfer steps from substrate molecules. Uniquely, for lignin peroxidase, conversion of compound **I** to a "compound **II**-like" species occurs spontaneously and quite rapidly in the absence of added substrates (*12*).

A key role is played in LiP catalysis *in vivo* by veratryl alcohol (VA, 3,4-dimethoxybenzyl alcohol), a secondary metabolite produced at the same time as LiP by ligninolytic cultures of *P. chrysosporium* (*13*) and other wood-degrading fungi. It has been proposed that the VA cation radical ($VA^{\bullet+}$) might act as a diffusable oxidant or mediator, which is able to move from the enzyme surface and oxidize either a second VA molecule or another reducing substrate (*14*). This scheme is similar to the way in which Mn^{3+} is known to act as a mediator for manganese peroxidase (*15*, *16*) and is therefore attractive as it explains how oxidizing power from the enzyme could be made available to large insoluble substrates such as lignin. Recent studies have now confirmed that the VA-enhanced oxidation of four different substrates (chlorpromazine, guaiacol, 4-methoxymandelic acid, and dimethoxylated aromatics) is due to mediation by $VA^{\bullet+}$ (*17*−*20*). However, measurements of the stability of $VA^{\bullet+}$ have also led to suggestions that it would be unable to act as a diffusable oxidant without some form of stabilization. Khindaria et al. (*21*) have suggested that a LiP−$VA^{\bullet+}$ complex acts as the redox partner, with the cation radical being stabilized by a protein microenvironment of acidic character.

The presence of a substrate interaction site in plant peroxidases at the "exposed heme edge" is well established for horseradish peroxidase (*22*, *23*; reviewed in ref *24*), *Coprinus cinereus* peroxidase (CIP/ARP) (*25*), and ascorbate peroxidase (*26*). However, the substrate access channel and heme edge of LiP are relatively inaccessible (*5*−*7*, reviewed in ref *27*) compared to those of other peroxidases for which crystal structure data are available (*28*−*31*). This has prompted the suggestion that the classical heme-edge site may not be the only substrate interaction site present in LiP (*24*, *32*). In particular, Blodig et al. (*32*) have highlighted a possible alternative site on the basis of their observation from X-ray crystal structures and peptide analysis of fungal LiP isoenzymes that W171 is hydroxylated at the Cβ position (*32*) (Figure 1). This modification has also been shown to occur in wild-type recombinant enzyme [LiPH8*—expressed in *Escherichia coli* and recovered after controlled *in vitro* refolding (*33*, *34*)] only after treatment with 2−3 equiv of hydrogen peroxide (*32*). Addition of VA competed with but did not prevent this modification (*32*). In fact, examination of the LiP X-ray crystal structure (*5*−*7*) shows that the local environment of W171 is highly acidic (Figure 1) and could, therefore, contribute to the stabilization of a bound VA cation radical in a manner consistent with previous suggestions (*21*).

We have recently reported a system for the expression of recombinant LiP isoenzyme H8 (LiPH8*) that is capable of yielding, after controlled *in vitro* refolding, 0.5−1.0 mg of active, purified enzyme/L of *E. coli* (*33*, *34*). We report here the characterization of wild-type LiPH8*, including the use of ^{1}H NMR specroscopy to confirm that the recombinant enzyme is correctly folded, and three site-directed mutants designed to test current theories concerning putative substrate interaction sites in LiP (depicted in Figure 1). Trp171 has been substituted by Phe and Ser (W171F and W171S LiPH8*, respectively) in order to investigate the novel covalent modification site discussed above (*32*), while Glu146 has been substituted by Gly (E146G LiPH8*), a charge neutralization mutation located in the "classical" peroxidase active site access channel. The latter represents one of the more significant sequence differences between LiP and *C. cinereus* peroxidase (CIP) in this region; two peroxidases which are structurally very similar, yet which have very different biophysical and kinetic properties (*21*, *24*, *35*).

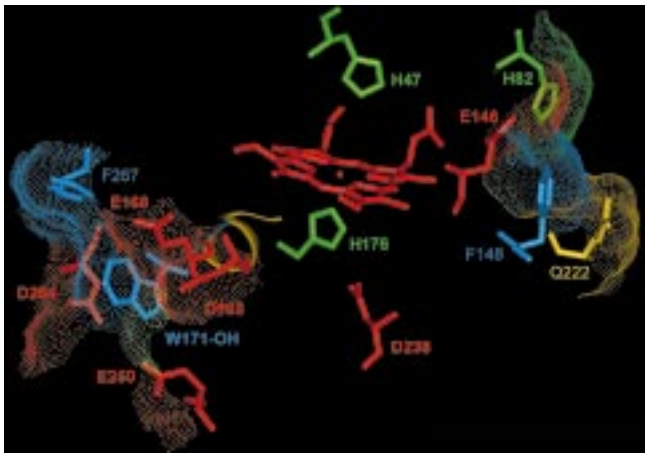

FIGURE 1: Selected active site architecture of *P. chrysosporium* lignin peroxidase (*5*−*7*). The two putative substrate interaction sites examined in this work are depicted. Molecular surfaces (colored dots) show the disposition of these sites with respect to solvent. The heme edge site is substantially occluded by E146, H82, and F148. The alternative VA oxidation site is centered on W171 depicted in blue (left) in its hydroxylated state. It is located at the end of helix F, five residues from the axial ligand and 11 Å from the nearest heme vinyl. The acidic nature of the W171 site can be seen. Coordinates (1QPA) (*7*) were obtained from the Brookhaven Protein Data Bank.

EXPERIMENTAL PROCEDURES

Materials. All chemicals used were reagent grade and were purchased from Sigma Aldrich Co. Ltd. or BDH Laboratory Supplies unless otherwise stated. Veratryl alcohol from Sigma was kindly vacuum-distilled by Dr. Luis Candeias (Gray Laboratory of the Cancer Research Campaign) to free it of a trace contaminant (*36*). Hydrogen peroxide solutions were prepared daily, and the concentration was determined using $\epsilon_{240nm} = 46.5\ mM^{-1}\ cm^{-1}$ (*37*). Molecular biology enzymes were supplied by either New England Biolabs Inc. or Boehringer Mannheim.

Recombinant LiP Expression. The generation of an engineered form of *P. chrysosporium* LiP H8 cDNA (EMBL Y00262), containing both the mature and prosequence regions, and its subsequent cloning into the commercially available expression vector pFLAG1 (International Biotechnologies Inc.) have been described previously (*33*). The resulting plasmid termed pFLAG1-LipP has already been

shown to express wild-type recombinant LiP H8 protein in *E. coli*, which can be refolded to the fully active enzyme LiPH8* (*33*, *34*).

Oligonucleotide Primers for Mutagenesis. For each mutant gene to be engineered, two primers were designed, one (the mutagenic primer) overlapped the area to be mutated and contained the intended base change(s) and the second (the reference primer) annealed to the other DNA strand so that the two primers sat exactly back to back on opposite strands of the template DNA, pFLAG1-LipP (*33*). Oligonucleotides are listed with the mutated codons in bold and any other base changes introduced to create additional restriction sites underlined: E146G mutagenic primer (WDLIG1), 5′AGTCCCC**GGC**CCCTTCCACAC3′; reference primer (WDLIG4), 5′AGTCCATCAGGAGCGGGCTG3′; W171F mutagenic primer (WDLIG13), 5′CTCGAACTTGTC**TTC**ATGCTCTCC3′; reference primer (WDLIG14), 5′CTCATCGAACTCGCCTGCGTC3′; W171S mutagenic primer (WDLIG12), 5′CTCGAACTTGTC**AGC**ATGCTCTCC3′; reference primer (WDLIG14), 5′CTCATCGAACTCGCCTGCGTC3′.

Plasmid Amplification Mutagenesis. A novel PCR-based method, briefly described in ref *22* and similar to that described in ref *38*, was used to generate the desired E146G, W171F, and W171S site-directed mutations in the LiP H8 cDNA while cloned into the pFLAG1-LipP vector (*33*). For each mutant gene to be generated, a PCR reaction was carried out containing 10 ng of pFLAG1-LipP as template, the manufacturer's buffer for cloned *Pfu* (Stratagene) containing 2 mM Mg^{2+}, 100 μM each dNTP, and 1.0 μM appropriate mutagenic and reference primers, and 2.5 units of *Pfu* polymerase (Stratagene). After a "hot start" of 95 °C for 10 min and 25 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 20 min (Perkin-Elmer GeneAmp 9600), a linear DNA fragment was produced, whose size in each case was equal to that of linearized pFLAG1-LipP. To circularize this DNA, the PCR product was first cleaned by gel extraction (Geneclean II, BIO101 Inc.), phosphorylated using T4 polynucleotide kinase, and finally ligated using T4 DNA ligase (DNA manipulations were as described in ref *39*). The ligation mixture was then transformed into *E. coli* DH5α cells.

This new method of site-directed mutagenesis was found to have advantages over other methods in that it does not require the presence of a restriction enzyme site near the intended site of mutation. Primers were designed so that a new restriction enzyme site was generated or one already present was regenerated only when a ligation event produced an intact gene; i.e., the restriction enzyme's recognition site overlapped the 5′ end of each primer. This was necessary because of a spurious exonuclease activity, possibly arising from *Pfu*, which resulted in deletions at the ligation site in a high proportion of clones. For construction of the E146G LiPH8* mutant, oligonucleotide primers were designed so that a unique *Spe*I restriction enzyme site was generated only if a mutant PCR product ligated to give an intact gene (see underlined bases in WDLIG1/4 above). In this case wild-type plasmids and mutant plasmids bearing deletions were both excluded on the basis of their failure to digest with *Spe*I. The correct integrity of the W171F and W171S bearing genes was detected by the regeneration of a unique *Sac*I restriction enzyme site at the ligation site. In addition, the mutagenic primers contained a codon change (see underlined bases in WDLIG12/13 above) so that an *Xho*I site present in the wild-type plasmid was removed. All mutant genes (and the wild type) were completely sequenced. No extraneous mutations were introduced by the PCR procedure (*Pfu* is a high-fidelity polymerase). Mutational efficiencies were found to vary from 20% to 50% of the total clones resulting from a single ligation experiment.

Expression, Folding, and Purification of Recombinant LiP. Expression, folding, and purification of recombinant wild-type and mutant LiP was performed as in Doyle and Smith (*33*), except that the *E. coli* strain W3110 was substituted for DH5α. Typical yields of 0.5−1.0 mg of fully activated enzyme/L *E. coli* were obtained. The expected N-terminal sequence (minus the initiating Met) was confirmed and the molecular mass of LiPH8* determined to be 37 400 ± 100 Da (single peak obtained by MALDI-TOF mass spectrometry). Enzyme samples were stored in 10 mM sodium succinate, pH 6.0, at −80 °C. Absorption spectra were recorded on a Shimadzu UV 1601 spectrophotometer at 25 ± 0.5 °C, with a spectral bandwidth of 2 nm and a scan speed of 370 nm min^{-1}, in the same buffer. The concentrations of LiPH8* and the mutant enzymes were calculated from the absorption at 409 nm, using an extinction coefficient of 168 mM^{-1} cm^{-1}, determined previously for the native H8 enzyme (*40*). RZ (reinheitszahl) (A_{409}/A_{280}) values were always greater than 3.5 and generally above 4.0.

*^{1}H NMR of LiPH8**. ^{1}H NMR experiments were recorded using a Varian 500 MHz instrument. Sample preparation was as previously described using solution conditions of 20 mM KH_2PO_4, and D_2O, pH 7.0, to obtain the resting state or 20 mM KH_2PO_4, 15 mM KCN, and D_2O, pH 7.0, to obtain the cyanide-ligated state of the enzyme, respectively (*22*). The concentration of the recombinant LiPH8* sample used for NMR was 0.1 mM. Standard acquisition and processing parameters for one- and two-dimensional ^{1}H NMR of peroxidase samples were employed (*22*). All experiments were carried out at 30 °C and referenced to 1,4-dioxane as an internal standard with a resonance at 3.74 ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate.

Steady-State Enzyme Assays and K_m Determinations. All steady-state assays were carried out in 5 mM phosphate and 5 mM citrate, with the ionic strength maintained at 50 mM by the addition of sodium sulfate. The pH values of the standard buffers ranged from 2.5 to 7.0, at 0.5 or 1.0 pH intervals. Assays were conducted as follows, at 25 ± 0.5 °C, using 5−30 pmol of enzyme to initiate the reaction. Initial rates were taken over the first 5−20 s of the assay. The VA oxidation assay contained 2 mM VA and 400 μM H_2O_2; production of veratryl aldehyde was followed at 310 nm ($\epsilon_{310} = 9.3$ mM^{-1} cm^{-1}) (*40*). The ABTS oxidation assay contained 500 μM ABTS and 100 μM H_2O_2; accumulation of the ABTS cation radical was followed at 405 nm ($\epsilon_{405} =$ 36.6 mM^{-1} cm^{-1}) (*41*). The 4-[(3,5-difluoro-4-hydroxyphenyl)azo]benzenesulfonate (DFAD) bleaching assay contained 50 μM DFAD and 100 μM H_2O_2; bleaching was followed at 375 nm, an isosbestic wavelength for the protonated and deprotonated forms of the dye ($\epsilon_{375} = 14$ mM^{-1} cm^{-1}). Activities are expressed throughout as catalytic center activities (s^{-1}). The pH dependence of activity data was fitted to a single pK_a equation using Sigma Plot (Jandel Scientific).

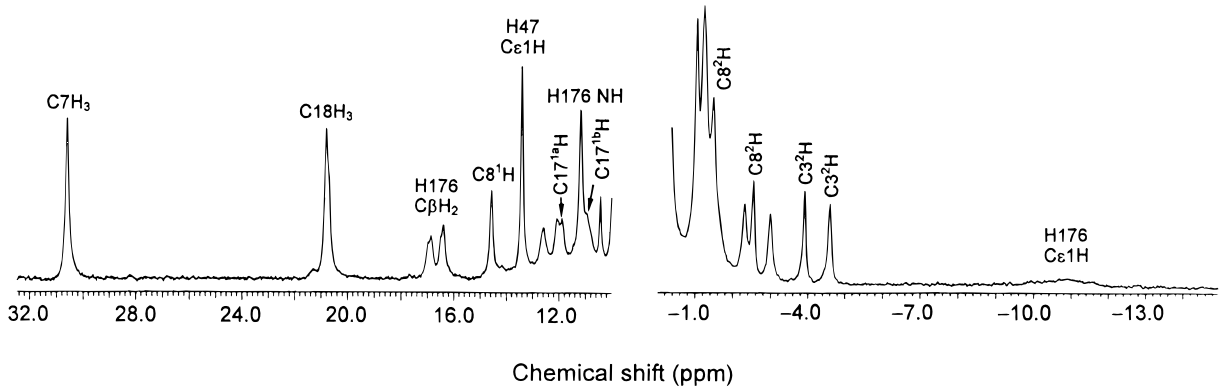

FIGURE 2: One-dimensional ^{1}H NMR spectrum of cyanide-ligated wild-type recombinant LiP isoenzyme H8. Specific assignments of the hyperfine-shifted resonances were obtained using standard two-dimensional experiments and are as follows (ppm): $C7H_3$ (30.62), $C18H_3$ (20.81), His176 $C\beta H_2$ (16.88, 16.41), $C8^1H$ (14.58), His47 $C\epsilon 1H$ (13.42), $C17^1H_2$ (11.88, 10.94), His176 NH (11.20), $C8^2H_2$ (−1.47, −2.54), $C3^2H_2$ (−3.91, −4.60), and His176 $C\epsilon 1H$ (−10.9). The spectrum was acquired at 500 MHz with solution conditions of 20 mM KH_2PO_4, 15 mM KCN, and D_2O, at pH 7.0 and 30 °C.

For the wild-type and E146G LiPH8* enzymes, apparent k_{cat} and K_m values for VA oxidation were determined by varying the VA concentration from 62.5 μM to 2 mM, at a fixed H_2O_2 concentration of 100 μM. Assay conditions were as above, pH 3.0. Curves were fitted to a Michaelis−Menten equation using FigP (Elsevier Biosoft).

Pre-Steady-State Experiments. Transient kinetics were monitored on an SX19MV stopped-flow spectrophotometer (Applied Photophysics), fitted with a diode array detector, at 25 ± 0.2 °C. Buffer conditions were 5 mM phosphate and 5 mM citrate, pH 4.0; the ionic strength was kept constant at 50 mM. Compound **I** formation was followed at 400 nm and its decay at 412 nm. The resulting time-dependent spectra were analyzed using the manufacturer's software (Pro-Kineticist, Applied Photophysics).

RESULTS AND DISCUSSION

^{1}H NMR of Wild-Type Recombinant Lignin Peroxidase H8 (LiPH8).* The procedure for successful recovery of active wild-type recombinant lignin peroxidase (LiPH8*) requires controlled *in vitro* refolding of inclusion body material produced by overexpression of the protein in *E. coli*. To confirm that LiPH8* produced in this way was correctly folded and that the integrity of the heme binding site was maintained, one- and two-dimensional ^{1}H NMR spectra were obtained and compared with data available for wild-type fungal LiP isoenzymes (*42*, *43*). This approach has previously been valuable as a method of comparing recombinant and natural source wild-type peroxidases from horseradish (*44*, *45*) and *C. cinereus* (*46*). Analysis of the one-dimensional ^{1}H NMR spectra of both the resting and cyanide-ligated states of recombinant LiP H8 indicated that the enzyme was correctly folded. The hyperfine-shifted resonances (Figure 2), representing protons of the heme group and heme pocket residues, were similar to those reported previously for wild-type fungal LiP isoenzymes H1 and H2 (*42*, *43*, *47*). The great sensitivity of these resonances to even small structural or electronic changes in the heme region makes them excellent indicators of heme site integrity. Some modest chemical shift differences for the heme-linked resonances were noted among the low-spin cyanide-ligated states of the three LiP isoenzymes H1, H2, and H8. These may reflect subtle alterations in heme pocket architecture or electronic structure (the H8 isoenzyme shows 85% and 91% amino acid sequence identity with the H1 and H2 isoenzymes, respectively). Chemical shifts recorded for the proximal His176 $C\epsilon 1H$ proton, at −8.4 (LiP H1, 28 °C) (*46*), −8.8 (LiP H2, 25 °C) (*43*), and −10.9 ppm (LiPH8* H8, 30 °C), show the greatest variation, which is significant even when the slightly different temperatures at which the data were collected are taken into consideration. The greater upfield shift of this resonance in LiPH8* may be taken as an indication that this isoenzyme has a slightly (10−20 mV) more electronegative heme Fe^{2+}/Fe^{3+} redox potential compared to the H1 and H2 isoenzymes (*42*).

Expression and Refolding of LiPH8 Mutants.* Expression levels for all three mutant genes were found to be similar to that of the wild type. The folding efficiencies and yields of W171S LiPH8* and W171F LiPH8* mutants were also similar to the wild type (∼1% conversion of inclusion body material to active enzyme). However, E146G LiPH8* folded less efficiently under the standard conditions used, with routinely only 0.2−0.5% of the protein folding to active enzyme. The ion-exchange properties of all three site-directed mutants during purification were not noticeably affected.

Resting State Electronic Absorption Spectra. Figure 3a shows the resting state spectra of wild-type recombinant lignin peroxidase H8 (LiPH8*) and W171F LiPH8*. They are identical except for a decreased extinction at 280 nm for the mutant, attributable to the loss of one of the three tryptophan residues (W171S LiPH8* was identical in this respect; data not shown). This increases the RZ (reinheitszahl) value of the purified enzyme from 4.4 in the wild-type enzyme to 5.7 in the W171F LiPH8* mutant. A spectrum of authentic fungal lignin peroxidase isoenzyme H2 was also found to be identical to that of LiPH8*, as was that obtained for E146G LiPH8* (data not shown). The sharp Soret maximum at 409 nm, high extinction coefficient (168 mM^{-1} cm^{-1}), lack of a shoulder at 385 nm, and a charge-transfer band 1 (CT1) maximum at 632 nm indicate a six-coordinate high spin (6-c HS) heme state. The spectrum of a substantially 6-c HS mutant of horseradish peroxidase (F41V), at the same enzyme concentration and 95% saturated with the aromatic donor benzhydroxamic acid, is shown for comparison (Figure 3b) as an example of an

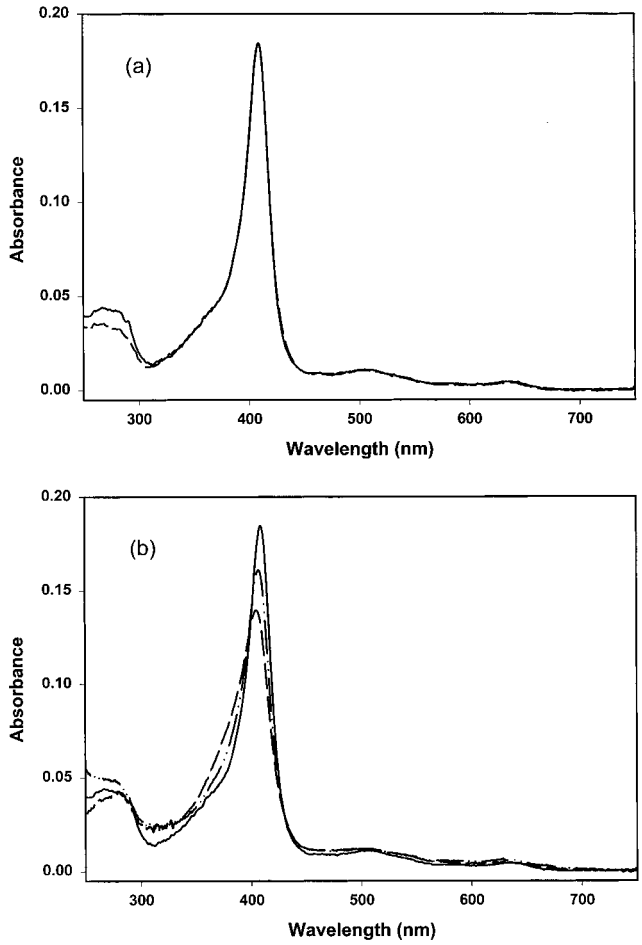

FIGURE 3: (a) UV/visible absorption spectrum of wild-type recombinant lignin peroxidase, LiPH8* (—), compared with that of W171F LiPH8* (- - -). Spectra were recorded in 10 mM sodium succinate, pH 6.0, and normalized at 409 nm to an enzyme concentration of 1.1 μM. (b) UV/visible absorption spectrum of LiPH8* (—) compared to that of the HRP mutant, F41V HRPC*, in the presence (···), and absence (- - -) of 20 μM benzhydroxamic acid (BHA) as an example (*48*) of an almost pure six-coordinate high-spin heme protein. Buffer conditions and enzyme concentrations as above.

almost pure 6-c HS heme protein (*48*). LiPH8* is undoubtedly substantially 6-c HS in solution as is also the H2 isoenzyme. This may in part account for the somewhat lower (30-fold) second-order rate constant for compound **I** formation observed for most lignin peroxidases as compared to other plant peroxidases, since water must be displaced from the sixth coordination position during the reaction of the enzyme with hydrogen peroxide.

Steady-State Kinetic Comparison of LiPH8, E146G LiPH8*, W171S LiPH8*, and W171F LiPH8**. Three different reducing substrates, with the molecular structures shown in Figure 4, were used to identify specific changes in the activities of the LiPH8* mutants as compared to the wild-type enzyme. (i) One is veratryl alcohol (VA) which can be directly oxidized by LiPH8* and whose half-potential for the formation of its respective cation radical ($VA^{\bullet+}$) has been measured as 1.4 V (*8*). $VA^{\bullet+}$ is thought to act either as a mediator in free solution or in complex with the enzyme or as a kind of "cofactor" in the oxidation of other substrates (*17*−*20*), most notably lignin (*3*). (ii) ABTS is a large negatively charged aromatic disulfonate, which is relatively poorly oxidized by wild-type LiP, in comparison to many

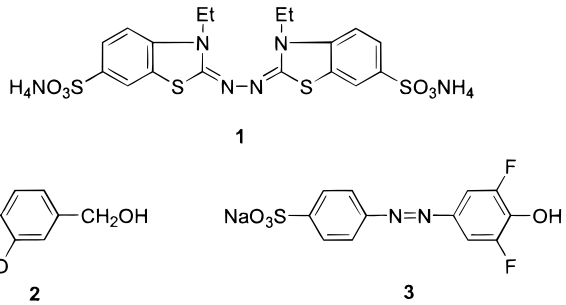

FIGURE 4: Structures of lignin peroxidase substrates used to elucidate the two substrate interaction sites: **1**, ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (*41*); **2**, VA, veratryl alcohol (3,4-dimethoxybenzyl alcohol) (*40*); **3**, DFAD, 4-[(3,5-difluoro-4-hydroxyphenyl)azo]benzenesulfonic acid, sodium salt (*50*).

Table 1: Steady-State Parameters Determined for LiPH8* and the Site-Directed Mutants E146G LiPH8*, W171S LiPH8*, and W171F LiPH8*

	steady-state turnover at optimum pH[a] (s^{-1})			pK_a
peroxidase	VA	ABTS	DFAD	DFAD
LiPH8* (WT)	29.7 ± 1.0	27.0 ± 0.9	31.3 ± 1.2	3.5 ± 0.1
E146G LiPH8*	8.9 ± 1.3	35.8 ± 1.5	71.6 ± 8.2	5.9 ± 0.1
W171S LiPH8*	<0.02	29.0 ± 0.1	12.2 ± 1.7	3.4 ± 0.2
W171F LiPH8*	<0.02	29.0 ± 0.2	19.7 ± 0.3	3.4 ± 0.3

[a] The optimum pH for each assay was identical for LiPH8*, W171S LiPH8*, and W171F LiPH8* enzymes but was higher in each case for E146G LiPH8*. In detail, the VA assay pH optimum increased from 2.5 to 3.0, the ABTS assay pH optimum increased from 3.0 to 3.5, and the DFAD assay pH optimum increased from 2.5 to 3.5. Assay conditions were as described in Experimental Procedures.

other peroxidases (*49*), to a stable cation radical ($E^{\circ\prime} = 0.68$ V) (*41*). (iii) The difluoroazo dye, DFAD, is identified in ref *50* as being a direct substrate for LiP ($E^{\circ\prime} = 0.88$ V, obtained by differential pulse voltametry, unpublished observation).

A comparison of the catalytic center activities at the optimum assay pH for wild-type LiPH8* and the three mutants is shown in Table 1. In addition, pH versus activity profiles obtained for the LiPH8*, E146G LiPH8*, and W171S LiPH8* enzymes are shown in Figure 5. All the enzymes exhibit increasing activity with decreasing pH in all assay systems, reflecting the well-known low pH optimum of LiP. However, a true optimum is never reached, as activity decreases very sharply below pH 2.5, presumably due to acid denaturation. Two results stand out from the data. First, the pH profiles for the oxidation of ABTS (Figure 5b) and DFAD (Figure 5c) by LiPH8* and W171S LiPH8* were found to be very similar ($pK_a \sim 3.5$), while E146G LiPH8* exhibited a marked increase in pH optimum for the oxidation of DFAD (Figure 5c). Specifically, the pK_a for this reaction increases by 2.4 pH units, and the catalytic center activity doubles relative to that of the wild type (Table 1). This strongly indicates that the low pK_a for the oxidation of this substrate by the wild-type enzyme can be assigned to E146. Some degree of steric hindrance of DFAD access to the heme-edge oxidation site may also have been moderated by this mutation (Figure 1). Two alternative explanations for the new observed pK_a (5.9 ± 0.1) for the oxidation of DFAD by E146G LiPH8* are possible. It could be attributable to the pK_a of the dye itself (measured in solution to be 5.1 ± 0.1), providing that the protonated form of the

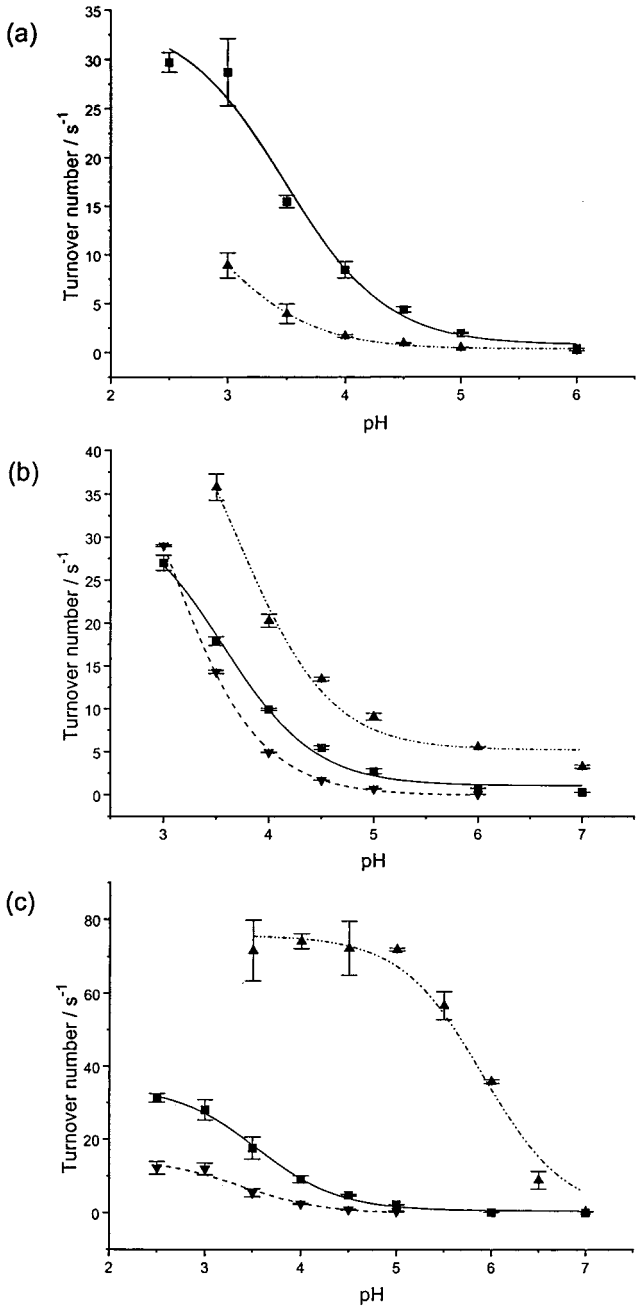

FIGURE 5: pH/activity profiles of wild-type recombinant lignin peroxidase, LiPH8*, and the site-directed mutant enzymes, E146G LiPH8* and W171S LiPH8*, in the (a) veratryl alcohol (VA), (b) ABTS, and (c) DFAD assay systems. For each graph the symbol ■ denotes LiPH8*, ▲ denotes E146G LiPH8*, and ▼ denotes W171S LiPH8*. Experimental conditions were as described in Experimental Procedures.

dye is the preferred substrate. However, since phenolate species are generally more reactive toward peroxidase high-oxidation state intermediates (*9*), it is possible that the apparent pK_a for the oxidation of DFAD by E146G LiPH8* is explained by the presence of an additional negative charge in the heme access channel. A possible candidate for the source of this negative charge in the local environment is D183, which hydrogen bonds to the heme propionate at pyrrole ring A. This is an unusual interaction, since in all other peroxidases the equivalent linkage is provided by a neutral, polar or basic residue (*5*, *6*). Interaction of these two negatively charged groups in LiP could result in a significant increase in the apparent pK_a observed for D183. Unambiguous assignment of the new observed pK_a "uncovered" in the E146G LiPH8* mutant will require further mutagenesis experiments. E146 does, however, control the low pH optimum for the oxidation of small negatively charged dyes such as DFAD, strongly suggesting that the "heme-edge site" is the preferred site for their oxidation.

The Alternative Substrate Oxidation Site. The second, and more surprising, result from Table 1 is the selective loss of VA oxidation activity by both W171F LiPH8* and W171S LiPH8*. In contrast, E146G LiPH8* retains 30% of the wild-type VA oxidation activity, and its catalytic effectiveness (k_{cat}/K_m, Table 2) is unchanged. This is therefore direct evidence for the existence of a second novel substrate interaction site in LiP, which must be located near to the surface of the enzyme and close to Trp171 (see Figure 1). In particular, this site would seem to be involved principally in the oxidation of VA (and presumably other methoxybenzene derivatives) and adds to the significance of the unusual covalent modification observed at this site (*32*).

Compound I Formation and Stability. Figure 6a shows the rapid reaction of wild-type LiPH8* with excess hydrogen peroxide to form a "classical" compound **I** spectrum, similar to that described for other peroxidases and indicative of the formation of a porphyrin π cation radical (*10*). Substitutions at either W171 or E146 have no significant effect on this reaction (Table 2). Lignin peroxidase porphyrin π cation radical decays rapidly and spontaneously in a biphasic manner (Table 2) to a species with a compound **II**-like spectrum. This is in contrast to the behavior exhibited by other peroxidases, where compound **I** is stabilized by axial ligands with greater imidazolate character (*42*). The extinction coefficient of this intermediate (calculated from Figure 6a to be 108 mM^{-1} cm^{-1} at 420 nm) and its general spectral features are known to be very similar to those of classical horseradish peroxidase compound **II** (*9*). The oxidation state of this species has not been assigned unambiguously but has been assumed by analogy with other peroxidases that form a porphyrin π cation radical during compound **I** formation to be the same as horseradish peroxidase compound **II**, i.e., one electron less than that of the resting state. Figure 7 shows the biphasic nature (at pH 4.0) of the spontaneous reaction of compound **I** to give the compound **II**-like species. The rapid phase of this reaction is eliminated in the W171F LiPH8* and W171S LiPH8* mutants, strongly implicating Trp171 as one source of reducing equivalents within the enzyme. The presence of an additional slower phase (still present in Trp171 mutants) suggests that a second slower pathway of electron transfer may also exist within LiP.

A question arises for wild-type LiP as to the precise oxidation state of the protein in the compound **II**-like form (depicted in Figure 6a) 20 s after treatment with excess peroxide. If the protein radical were stable, then clearly this species would be more akin to a cytochrome *c* peroxidase type compound **I**. However, as no obvious protein radical has been detected (*10*) and yet electron transfer, at least between LiP compound **I** and Trp171, undoubtedly occurs, we therefore infer that this radical must be labile to have escaped detection, perhaps reacting rapidly with oxygen or water (*32*). The amount of radical remaining at any point in time would determine the observed oxidation state of the compound **II**-like species and hence its strict definition as

Table 2: Rates of Compound **I** Formation and Decay and Steady-State Parameters for VA Oxidation for LiPH8* and the Site-Directed Variants E146G LiPH8*, W171S LiPH8*, and W171F LiPH8*

	steady-state parameters for VA oxidation[a]					
peroxidase	k_{cat} (s^{-1})	K_m (μM)	effectiveness k_{cat}/K_m ($10^5\ M^{-1}\ s^{-1}$)	rate of compound **I** formation[b] k_1 ($M^{-1}\ s^{-1}$)	rates of compound **I** decay[c] k_f (s^{-1})	k_s (s^{-1})
LiPH8* (WT)	22.5 ± 0.4	170 ± 10	1.32 ± 0.11	$(5.6 \pm 0.1) \times 10^5$	2.3 ± 0.1	0.15 ± 0.01
E146G LiPH8*	7.7 ± 0.1	68.6 ± 5.5	1.12 ± 0.10	$(6.0 \pm 0.1) \times 10^5$	3.0 ± 0.2	0.20 ± 0.2
W171S LiPH8*	n/a	n/a	n/a	$(5.8 \pm 0.2) \times 10^5$	n/a	0.15 ± 0.02
W171F LiPH8*	n/a	n/a	n/a	$(5.6 \pm 0.2) \times 10^5$	n/a	0.15 ± 0.01

[a] Assay conditions were as described in Experimental Procedures. [b] The final enzyme concentration in each case was 1 μM; H_2O_2 concentrations ranged from 5 to 30 μM (intervals of 5 μM). Other conditions were as described in Experimental Procedures. [c] Conditions were as described for Figures 6 and 7.

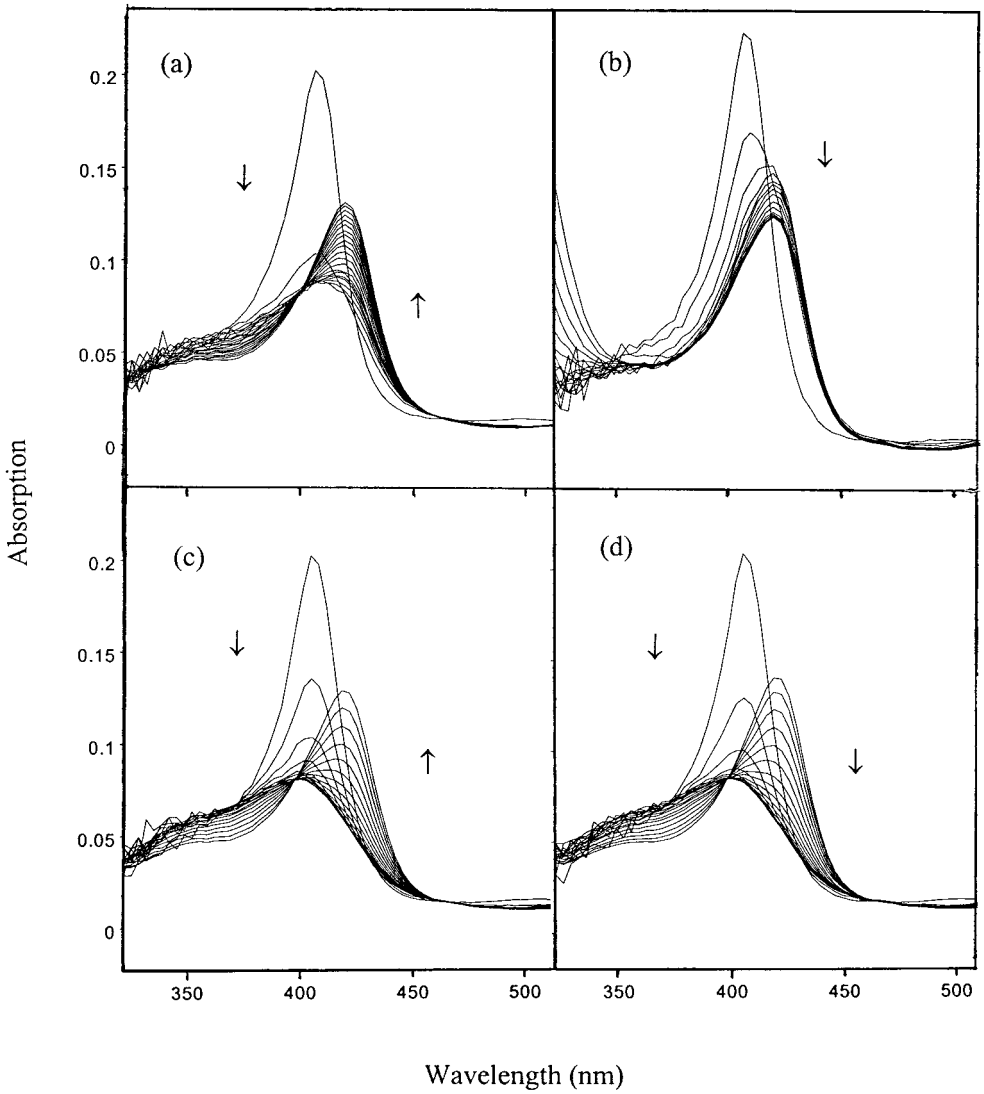

FIGURE 6: Effect of W171F mutation on compound **I** stability in the presence and absence of veratryl alcohol. Stopped-flow rapid scan diode array spectra showing the reaction of LiPH8* variants with a 50−60-fold molar excess of hydrogen peroxide at pH 4.0 in the absence (a, c) and presence (b, d) of veratryl alcohol. Panels: (a) 1.3 μM LiPH8* + 65 μM H_2O_2; (b) 1.3 μM LiPH8* + 65 μM H_2O_2, 1 mM VA; (c) 1.1 μM W171F LiPH8* + 65 μM H_2O_2; (d) 1.1 μM W171F LiPH8* + 65 μM H_2O_2, 1 mM VA. All data shown are for 20 scans between 1.2 ms and 19.6 s, the first 10 scans are at intervals of 26 ms, except in (a) where they are 52 ms apart, and remaining spectra are distributed logarithmically.

either a cytochrome *c* peroxidase type compound **I**, with the radical located on the protein (*11*), or an HRP-type compound **II**, with no protein radical (*9*). What seems more likely in view of the rates in Table 2 is a mixture of these two states, with a labile protein radical being only a minor species. Both species would give rise to the same optical spectrum, but one might expect their kinetic behavior to be quite different.

Figure 6b shows the reaction of LiPH8* with excess hydrogen peroxide (65 μM) in the presence of VA (1 mM). There is rapid accumulation of the rate-limiting steady-state intermediate (within 200 ms), predominantly the compound **II**-like form. The product veratryl aldehyde can also be seen

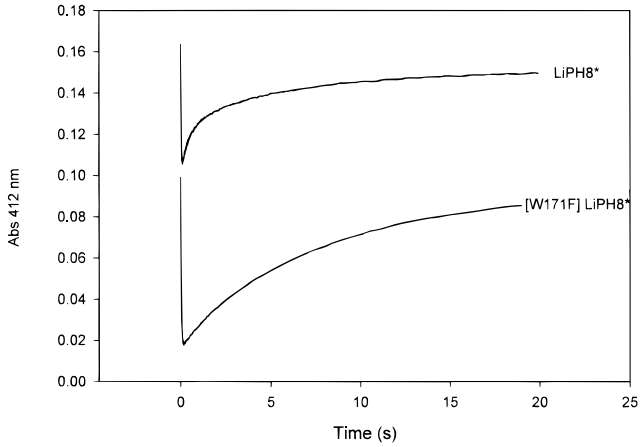

FIGURE 7: Single wavelength stopped flow traces showing the conversion of compound **I** to a compound **II**-like species. Time slices at 412 nm (an isosbestic point for the conversion of compound **II** to the resting state) are shown for (a) LiPH8* (1.3 μM) and (b) W171F LiPH8* (1.1 μM). These were fitted to double or single exponentials, respectively, as appropriate. Traces are offset for display purposes.

accumulating in the near-UV region of the spectra during the same time course. Figure 6d shows the equivalent experiment conducted with the W171F LiPH8* mutant. The presence of VA has no effect at all (compare panels d and c of 6). VA is therefore unable to react with either the W171F LiPH8* compound **I** or the compound **II**-like form, presumably because the interaction site or elecron transfer pathway is defective.

CONCLUSIONS

Wild-type recombinant lignin peroxidase (LiPH8*), produced by controlled *in vitro* refolding of insoluble polypeptide after expression in *E. coli*, is fully competent catalytically, both in turnover and in combination with VA in mediating the oxidation of chlorpromazine to the sulfoxide (data not shown). From ^{1}H NMR experiments, the recombinant protein has been found to be correctly folded, and heme is incorporated correctly. There is also no evidence from kinetic studies for the presence of any catalytically inactive enzyme forms. In all respects it appears to be functionally equivalent to the enzyme derived from fungal sources. In addition, the recombinant enzyme, like fungal LiP, is 6-c HS in solution. This is entirely consistent with a recent review of the UV/visible and resonance Raman data for this enzyme (*51*).

The data for Trp171 LiPH8* mutants reported here confirm the recently reported suggestion that Trp171 or Cβ hydroxy Trp171 (recent experiments (*32*) and herein do not distinguish between these two possibilities) is essential for the oxidation of VA. The negative charge in the classical substrate access channel provided by E146 accounts for the very low pH optima for the oxidation of small negatively charged dyes. Taken together, LiPH8* can clearly interact with substrates at two possible sites; indeed, preliminary indications suggest that some substrates such as chlorpromazine are capable of interacting at both sites (unpublished work).

Trp171 is completely conserved in all lignin peroxidase sequences described to date and in one unusual managanese peroxidase which oxidizes VA efficiently (*52*). Figure 1 shows the local environment of W171. It is surrounded by a cluster of conserved negative charges, including E250 (which hydrogen bonds to the N−H of the indole ring) and E168 (which hydrogen bonds to the Cβ hydroxy W171 when modified by the enzyme during its first few turnovers). E163, E166, and D165 are also very highly conserved and are within a 5 Å radius of Trp171. This is consistent with the requirement for a negatively charged environment at low pH to stabilize bound VA$^{\bullet+}$ (*21*); the low pH is presumably required to raise the redox potentials of compounds **I** and **II**. Protein stabilization of VA$^{\bullet+}$ could result in a significant decrease in the half-potential for the formation of an enzyme-bound VA$^{\bullet+}$, perhaps decreasing it from the 1.4 V reported in solution studies (*53*) to a less electropositive value (<1.1 V), more consistent with the known redox potentials of other peroxidase high oxidation state intermediates ($E^{o\prime}$ for HRP compound **I**/compound **II** = 0.898 V and compound II/ferric = 0.869 V) (*53*), thereby minimizing autooxidative damage to the heme center.

The precise mechanistic role of Trp171 is still intriguing, since the kinetic competence of the alternative VA oxidation site under steady-state conditions exceeds by some 10-fold the rapid phase of spontaneous compound **I** reduction attributed to electron transfer from Trp171. VA also competes with the Trp171 covalent modification reaction (*32*). However, this does not entirely exclude the possibility that Trp171 undergoes a redox cycle itself that is directly coupled to VA oxidation. Nevertheless, interaction of VA at this alternative site promotes rapid, through-protein, electron transfer over a distance of 11 Å (measured from the Cα of Trp171 to the nearest heme vinyl or 10 Å to the Cα of the axial His ligand) at catalytically competent rates (23 s^{-1}). The possibility that the electron-rich π system of W171 could provide a vital component of a binding site through a stacking interaction with VA, resulting in an enhancement of the rate of electron transfer, is an intriguing one.

In summary, these findings may necessitate some reinterpretation of past kinetic studies and a careful search for a labile tryptophan radical. If such a radical exists (which seems likely), then its kinetic competence during catalytic turnover with VA will have to be established.

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