Articles

Autocatalytic Formation of a Hydroxy Group at Cβ of Trp171 in Lignin Peroxidase[†]

Wolfgang Blodig, Wendy A. Doyle,[‡] Andrew T. Smith,[‡] Kaspar Winterhalter, Thomas Choinowski, and Klaus Piontek*

Laboratory of Biochemistry I, Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland

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ABSTRACT: In the high-resolution crystal structures of two lignin peroxidase isozymes from the white rot fungus *Phanerochaete chrysosporium* a significant electron density at single bond distance from the Cβ of Trp171 was observed and interpreted as a hydroxy group. To further clarify the nature of this feature, we carried out tryptic digestion of the enzyme and isolated the Trp171 containing peptide. Under ambient conditions, this peptide shows an absorbance spectrum typical of tryptophan. At elevated temperature, however, the formation of an unusual absorbance spectrum with $\lambda_{max} = 333$ nm can be followed that is identical to that of *N*-acetyl-α,β-didehydrotryptophanamide, resulting upon water elimination from β-hydroxy tryptophan. The Trp171 containing tryptic peptide isolated from the recombinant and refolded lignin peroxidase produced from *Escherichia coli* does not contain the characteristic 333 nm absorbance band at any temperature. However, treatment with 3 equiv of H_2O_2 leads to complete hydroxylation of Trp171. Reducing substrates compete with this process, e.g., in the presence of 0.5 mM veratryl alcohol, about 7 equiv of H_2O_2 is necessary for complete modification. We conclude that the hydroxylation at the Cβ of Trp171 is an autocatalytic reaction which occurs readily under conditions of natural turnover, e.g., in the ligninolytic cultures of *P. chrysosporium*, which are known to contain an oxidase-based H_2O_2-generating system. No dependence on dioxygen was found for this oxidative process. Chemical modification of fungal lignin peroxidase with the tryptophan-specific agent *N*-bromo succinimide leads to a drastically reduced acitivity with respect to the substrate veratryl alcohol. This suggests that Trp171 is involved in catalysis and that electron transfer from this surface residue to the oxidized heme cofactor is possible under steady-state conditions.

Lignin peroxidases (LiP)[1] of the white rot fungus *Phanerochaete chrysosporium* are a group of extracellular heme containing isozymes that are able to degrade the plant cell wall constituent lignin and other organic compounds. Many of the latter have been shown to be oxidized via redox mediation involving the secondary metabolite veratryl alcohol (VA) (*1*−*3*). This is one of the unique properties of this enzyme, as is the unusually high redox potential that enables degradation of nonphenolic aromatic compounds and a remarkably low pH optimum of ∼3 (*4*).

The overall catalytic cycle is similar to that of other heme peroxidases and begins with the two electron oxidation of the ferric cofactor by hydrogen peroxide yielding compound I (*5*). These two oxidation equivalents are then sequentially transferred to substrate molecules. One of the oxidizing equivalents of compound I is stored as an oxyferryl iron-(IV). Differences, however, exist with respect to the second one. Whereas in most peroxidases, including LiP, UV−vis and Raman spectroscopy have provided evidence for a porphyrin π radical cation (*6*), in cytochrome *c* peroxidase this radical resides on a tryptophan residue in the vicinity of the proximal histidine (*7*).

There is an increasing number of reports on oxidative processes in redox enzymes that lead to modifications of the enzymes themselves. Among these are the activation via dihydroxylation of the heme and its covalent attachment to the apoprotein by hydrogen peroxide in lactoperoxidase (*8*). Furthermore, an oxidative conversion of heme b to d that is presumably connected with bond formation between the Cβ of the proximal tyrosine and an adjacent histidine in catalase HPII from *Escherichia coli* was reported (*9*). An observation regarding LiP which is not yet understood is the following: when 1 equiv of hydrogen peroxide is added to LiP in the absence of reducing substrate, the absorbance spectrum of the so formed compound I bleaches within seconds, much

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* To whom correspondence should be addressed. Tel: +41-1-632-3141. Fax: +41-1-632-1121. E-mail: klaus.piontek@bc.biol.ethz.ch.

[‡] School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, U.K.

[1] Abbreviations: HPLC, high-pressure liquid chromatography; LiP, lignin peroxidase; LiP465, LiP isozyme with pI 4.65; LiP415, LiP isozyme with pI 4.15; LipP*, recombinant LiP isozyme H8; NBS, *N*-bromo succinimide; VA, veratryl alcohol (3,4-dimethoxy benzyl alcohol).

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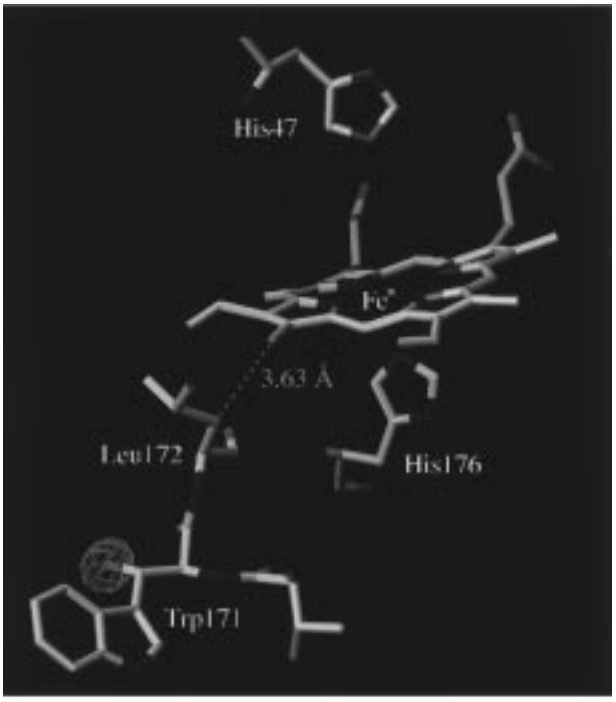


FIGURE 1: Omit $F_o - F_c$ electron density map of the hydroxy group at Trp171, contoured at 4σ. The neighboring residue Leu172 is with its Cβ atom in van der Waals contact to the heme suggesting a possible electron pathway between Trp171 and the heme. The proximal (His176) and distal (His47) histidines are also shown.

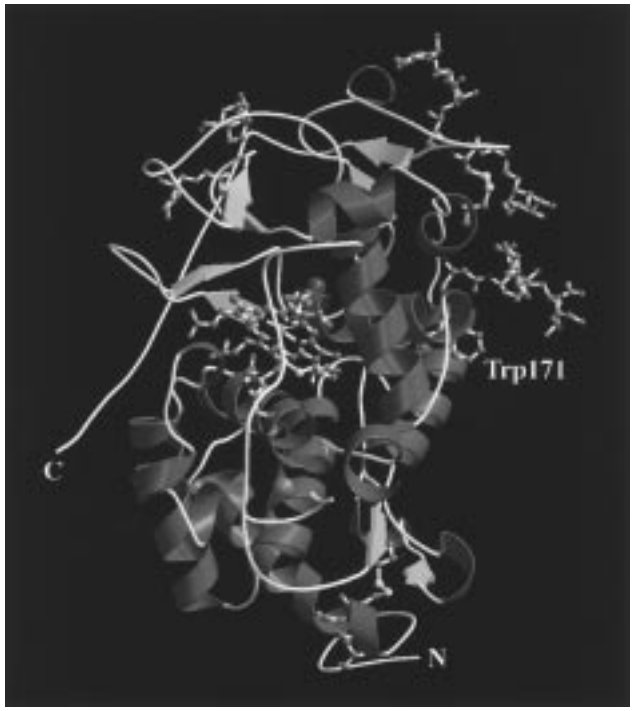


FIGURE 2: Ribbon diagram of lignin peroxidase isozyme LiP465, prepared using the program MOLSCRIPT (*30*). Helices are in blue and β-strands in orange. The heme, the proximal and distal histidines, Trp171, the carbohydrate molecules, and the disulfide bridges (sulfur atoms in yellow) are depicted as ball-and-stick models. The two calcium ions are represented by purple spheres.

faster than that of related peroxidases, and the optical spectrum of an oxyferryl heme known as compound II is obtained (*10*, *11*). Redox-active protein residues such as tyrosines and tryptophans would be conceiveable as reducing agents which could in turn serve to oxidize substrates. Nothing specific about the source and location of this reductant is known so far. In metmyoglobin, treatment with hydrogen peroxide gives rise to protein radicals located on tryptophan and tyrosine residues (*12*, *13*), resulting in cross-linking of protein molecules (*14*) and oxidation of the heme to a hydroxychlorin (*15*).

Previously, we determined high-resolution crystal structures of LiP isozymes LiP415 (manuscript in preperation) and LiP465 (*16*) based on diffraction data collected with synchrotron radiation. Both enzyme models[2] include the whole polypeptide chain of the enzyme and obey good stereochemistry with an estimated coordiante error of about 0.2 Å. The final crystallographic *R*-factors are 16.4 and 18.4% in the 10−1.7 Å and 10−1.8 Å resolution shells for LiP415 and LiP465, respectively. Difference Fourier maps of both crystal structures revealed a strong spherical electron density 1.4 Å distant from the Cβ of Trp171 (Figure 1). On the basis of the geometry and the hydrogen-bonding network, a hydroxy group was assigned to it and we reasoned that it might have evolved from the reaction of a tryptophyl radical with some oxygen species similarly as in metmyoglobin mentioned above. We therefore considered that oxidative processes other than those that have been taken into account so far in LiP may also exist and may play a role in lignin degradation. Trp171 is located close to the heme and in contact with solvent (Figure 2). We assumed that the modification originated from an autocatalytic self-oxidation and that an electron pathway may exist between this residue and the nearby heme.

In this study, we confirm the previous crystallographic finding of the Cβ hydroxy group at Trp171 by the detection of an elimination product in a tryptic peptide of the fungal enzyme. We then examine the formation of the hydroxylation with hydrogen peroxide using recently available recombinant LiP isozyme H8 (LipP*), which has not previously reacted with hydrogen peroxide. Our results suggest that electron transfer from Trp171 is one of the factors responsible for the short lifetime of LiP compound I. The catalytic significance of this site was addressed by chemical modification of the solvent-accessible tryptophan residues which revealed a marked effect on the specific activity of the enzyme.

EXPERIMENTAL PROCEDURES

Chemicals. Veratryl alcohol was purchased from Fluka and was vacuum distilled. TPCK-treated trypsin was from Sigma. NBS was from Aldrich and was recrystallized from water. The concentration of hydrogen peroxide solutions was determined by titration with potassium permanganate. *N*-acetyl-α,β-didehydrotryptophanamide was prepared as described (*17*).

Enzymes. The fungal LiP isozyme with pI 4.65 was purified from the extracellular culture medium of *Phanerochaete chrysosporium*, strain BKM-F-1767 as described (*18*). It had a specific activity of 42 μmol of VA oxidized/min/mg in the standard assay (see below). The enzyme is obtained in the ferric high-spin form as judged from its UV−

[2] The crystallographic coordinates referred to have been deposited with the Protein Data Bank, Brookhaven National Laboratory, entry codes 1llp and 1qpa for LiP415 and LiP465, respectively.

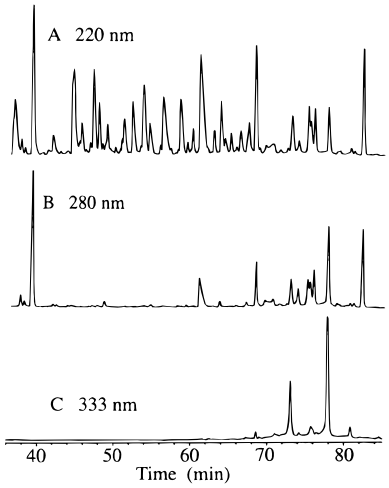


FIGURE 3: Section of the HPLC peptide map of fungal LiP465 at 60 °C after tryptic digestion. (Panel A) Elution was followed at 220 nm. (Panel B) followed at 280 nm. (Panel C) followed at 333 nm.

vis spectrum. Recombinant LiP H8 was expressed in *E. coli*, refolded as described earlier (*19*), and shown to be pure by mass spectroscopy (MALDI). It had a specific activity of 34 μmol of VA oxidized/min/mg under the conditions reported in ref *19*, which compares well to the fungal enzyme. Concentrations of both enzymes were determined using $\epsilon^{408} = 168$ mM^{-1} cm^{-1}, and 38 kDa was taken as molecular weight. Standard assays were performed by following the formation of veratryl aldehyde ($\epsilon^{310} = 9.3$ mM^{-1} cm^{-1}) with 0.6 mM H_2O_2 and 2 mM VA at pH 3 and 25 °C in 100 mM sodium tartrate buffer.

Tryptic Digestion. The enzyme was reduced and carboxamidomethylated in 6 M urea essentially as described in ref *20*. After dialysis against 0.1 M ammonium bicarbonate, it was incubated for 20 h at 37 °C with 1:30 (w:w) trypsin. The resulting solution was subjected directly to HPLC analysis.

HPLC Analysis. A HP liquid chromatograph 1090 equipped with a diode array detector and a 125/4 Nucleosil 120-5 C18 reversed-phase column was used. Chromatograms were recorded at 220, 280, and 333 nm, and UV−vis spectra were recorded of the more intense peaks. Eluent A was 0.1% trifluoro acetic acid in water and eluent B 0.05% trifluoro acetic acid in acetonitrile. Elution was performed with a linear gradient from 0 to 50% B in 100 min and a flow rate of 0.5 mL/min either at room temperature or at 60 °C.

Quantification of β-Hydroxytryptophan. To estimate the content of β-hydroxy-trp171 in LiP, the HPLC fractions with 333 nm absorbance were collected and quantified spectroscopically using $\epsilon^{333} = 19.8$ mM^{-1} cm^{-1} (*17*) for the model compound *N*-acetyl-α,β-didehydrotryptophanamide. For the data of Figure 7, the areas of all peaks of the 333 nm trace were determined by integration and divided by the area of one well-resolved peak in the 220 nm trace of the same run. For the maximal ratio that was obtained after increasing additions of H_2O_2, the total amount of didehydro compound corresponded to that found in fungal LiP and was set to 100%. Lower ratios after treatment with less H_2O_2 were calculated relative to this value.

Edman sequencing of tryptic peptides was done by the Protein Chemistry Laboratory of the ETH Zürich.

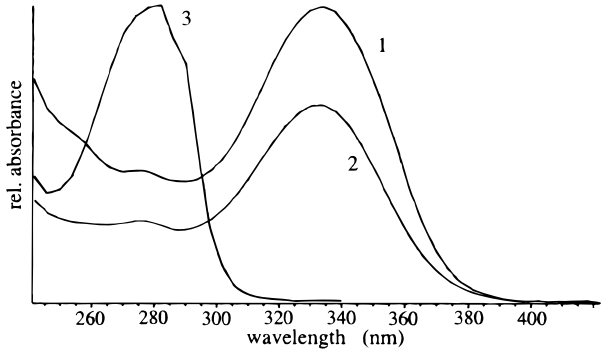


FIGURE 4: Absorbance spectra of peptides from the chromatogram in Figure 3 and of the model compound. Line 1 corresponds to the peak eluting at 78 min and line 2 to the model compound *N*-acetyl-α,β-didehydrotryptophanamide. For comparison a typical tryptophan spectrum of the peptide eluting at 82 min is also shown (line 3). Note that the spectra are scaled arbitrarily.

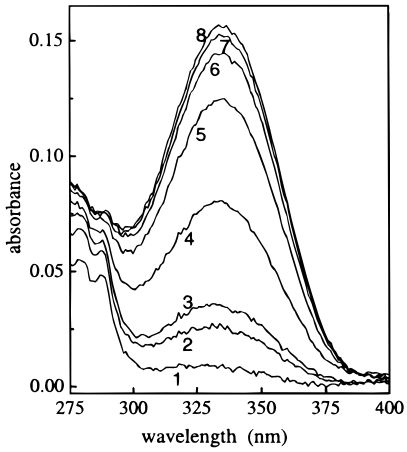


FIGURE 5: Progress of the water elimination in the Trp171 containing tryptic peptide from LiP465 monitored by the increasing absorbance around 330 nm. The peptide was isolated at ambient temperature by HPLC and a spectrum was recorded immediately (line 1). Spectra 2 and 3 were recorded 60 and 90 min later. Then the solution was made strongly acidic by adding 4% trifluoro acetic acid and spectra were run after 1 min each (traces 4−8). Scan speed: 400 nm/min.

Reaction of LipP with Hydrogen Peroxide.* LipP* (20 μM) was incubated in 50 mM sodium tartrate, pH 4.5, that contained the indicated concentrations of VA with the indicated amounts of hydrogen peroxide for 10 min and was then subjected to tryptic digestion. To test the dependence of the modification of LipP* on dioxygen, the reaction was carried out anaerobically as follows. The enzyme was deoxygenated by blowing a stream of water-saturated oxygen-free argon on the surface of the buffered enzyme solution for 1 h. Hydrogen peroxide was made anaerobic using the syringe-bubbling technique with argon, and a constant amount of 3 equiv relative to enzyme was transferred to the latter with a Hamilton syringe. After 10 min of anaerobic incubation, the amount of Trp171 present as the hydroxy derivative was determined as above.

NBS Modification of LiP465. To follow the activity decrease with increasing amounts of NBS, 1.6 nmol of LiP465 in 100 μL of 100 mM sodium acetate, pH 4.5, was treated with the calculated volume of 1.5 mM NBS in water. The reaction was stopped after 2 min by addition of 10 mmol of tryptophan in water. The reaction mixture was dialyzed excessively against 20 mM sodium tartrate, pH 4.5, at 4 °C

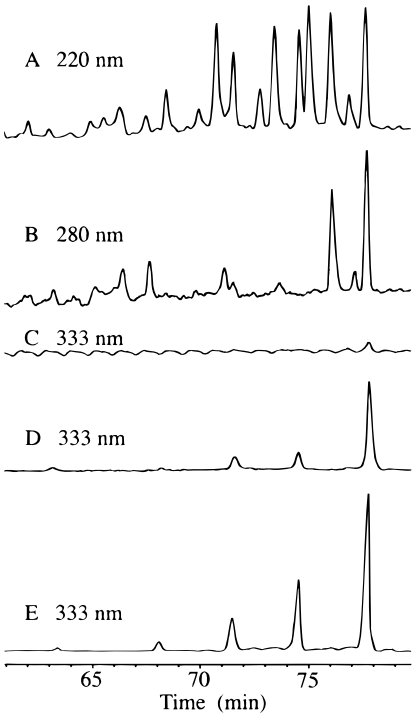


FIGURE 6: HPLC traces of tryptic digests of recombinant LipP* at 60 °C. Panels A−C show the tryptic digest of pristine LipP* followed at 220, 280, and 333 nm, respectively. Panels D and E show 333 nm traces after incubation of LipP* with 2 and 10 equiv of H_2O_2 in the presence of 2 mM VA.

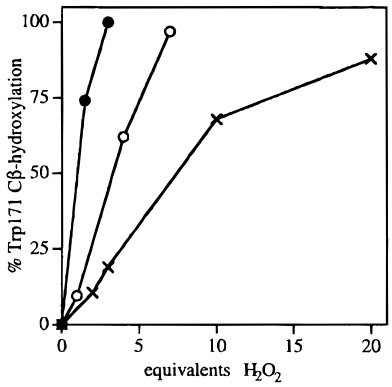


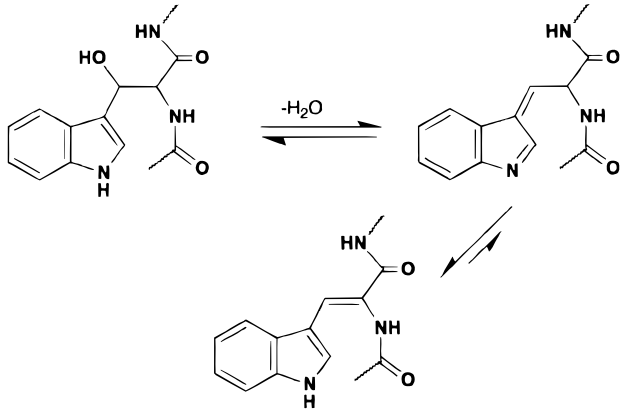
FIGURE 7: Formation of the Cβ hydroxylation at Trp171 of LipP* on addition of hydrogen peroxide. The reactions with H_2O_2 were carried out in the absence of reducing substrate (●), in the presence of 0.5 mM VA (○), and in the presence of 2 mM VA (×). The enzyme concentration was 20 μM in 50 mM sodium tartrate, pH 4.5. The amount of Trp171 hydroxylation was quantified as described in Experimental Procedures.

to remove substances that could interfere with the assay. To follow the decrease of tryptophan fluorescence, to a solution of 2 mL of 1.5 μM LiP465 in 100 mM sodium acetate, pH 4.5, increasing amounts of 1 mM NBS were added and emission spectra were recorded 1 min after the addition using an Aminco SPF-500 instrument. Excitation wavelength, 285 nm; excitation slit, 8 nm; emission slit, 5 nm.

RESULTS AND DISCUSSION

Hydroxy-Adduct at the Cβ of Trp171. On the basis of the crystal structures of two LiP isozymes isolated from the extracellular medium of *P. chrysosporium*, evidence was found by us for a Cβ hydroxylation of Trp171. To test this finding chemically, LiP465 was denatured, reduced, alkylated, and trypsin digested. The resulting peptide mixture was then analyzed by reversed-phase HPLC and the products detected at 220, 280, and 333 nm and by their absorbance spectra. LiP465 contains four residues that will absorb at 280 nm: three tryptophans which are conserved among the family of isozymes and one nonconserved tyrosine. From the sequence (*21*), all four residues reside on different tryptic peptides. Figure 3 shows a typical chromatogram carried out at 60 °C.

Scheme 1: Proposed Mechanism of Water Elimination from Peptide Bound β-Hydroxy-tryptophan



N-Terminal partial sequencing was performed to identify the peptides with respect to the known primary structure (*21*). The peak eluting at 39.5 min was shown to be due to the tryptic peptide Thr247−Lys260, containing Trp251 which is buried in the proximal domain of the native enzyme (amino acid numbering according to isozyme LiP415). The intense peak at 82 min was identified as the tryptic peptide Val0−Arg43, containing the surface residue Trp17. These two peptides gave absorbance spectra typical of tryptophan (Figure 4, spectrum 3). The peaks at 73 and 78 min were assigned to the peptide starting with Met158 and containing Trp171. The absorbance spectra of these peptides, recorded by the diode array detector of the HPLC system, showed the same $\lambda_{max} = 333$ nm and shape as that of the model compound *N*-acetyl-α,β-didehydrotryptophanamide (Figure 4). Peptides that consist of the 20 standard amino acids do not absorb above 300 nm. Free tryptophan that was kept for 2 h at pH 2 and 60 °C did not show any heat or acid-induced alterations of its absorbance spectrum. These results indicate that the α,β-didehydro derivative of Trp171 is formed upon water elimination from the β-hydroxy compound. If the HPLC run was carried out at room temperature, only trace amounts of the 333 nm signal could be detected, suggesting that the hydroxy group is hardly lost under these conditions and allowed the isolation of the β-OH Trp171 containing peptide. Spectra 1−3 in Figure 5 show that the elimination in this peptide proceeds on an hour time scale in the eluent of the HPLC run but substantially quicker, on a minute time scale, under much stronger acidic conditions (spectra 4−8 in Figure 5). Hydroxy groups in α-position to conjugated systems are known to eliminate easily driven by the formation of a more delocalized electron system (*22*). We propose the two-step reaction sequence depicted in Scheme 1 for the case of a β-hydroxytryptophan, both of which can be acid or base-catalyzed. During the first step, water is lost in a process involving the indole π-system. This

provides an explanation for the low activation barrier, i.e., the fact that the elimination can be induced by a moderate increase in temperature. A subsequent tautomerization then leads to the final α,β-didehydro product whose π-system extends up to and includes the peptide bonds to the adjacent amino acids and is therefore very stable. Consequently, the equilibrium of the overall reaction will be entirely on the right as was shown for the model compound (*17*).

Taken together and in accordance with the crystal structures, Trp171 is present in its β-hydroxy form in the native enzyme and can be isolated as such under mild conditions. Contrarily, conditions necessary for efficient elimination of water, namely high acidity or temperature, will denature the enzyme but are used in this study for analytical purposes since they allow an easy indirect detection of the modification.

When all peptides with 333 nm absorbance from an HPLC run were collected and pooled, about 80% of the starting enzyme was recovered using $\epsilon^{333} = 19.6\ mM^{-1}\ cm^{-1}$ (*17*). Taking losses during the applied procedure especially the dialysis into account we conclude that almost 100% of the Trp171 is present in the Cβ hydroxylated form, which is consistent with the strong electron density and the low-temperature factor found in the crystal structure (*16*). Analogous results were obtained for a second isozyme (LiP415, results not shown) and with enzyme from several independent cultivations of the fungus/protein purifications.

Hydroxylation of Trp171 in Recombinant LipP.* In contrast to fungal LiP isozymes, which have undergone numerous turnover cycles in the culture medium from which they are purified, LipP* can be investigated in a pristine state. When LipP* was digested and analyzed in the same way as LiP465 above, no absorbance above 300 nm could be detected in any tryptic peptide (Figure 6). This finding excludes the possibility that the 333 nm absorbance recorded with the fungal enzyme was an artifact of the applied procedure. Since LipP* is not glycosylated and is a different isozyme to LiP465, the peptide map is not identical but similar to the one from LiP465. Again, the three major peptides with 280 nm signals were assigned to the three tryptophan-containing tryptic peptides in the sequence (*23*) by partial Edman sequencing. The peptide at 76 min contains Trp17, and the one at 77.5 min Trp171 (Figure 6). The time window in Figure 6 was chosen so as to show the 70−80 min area in detail and the Trp251-containing peptide eluting at 39 min is omitted.

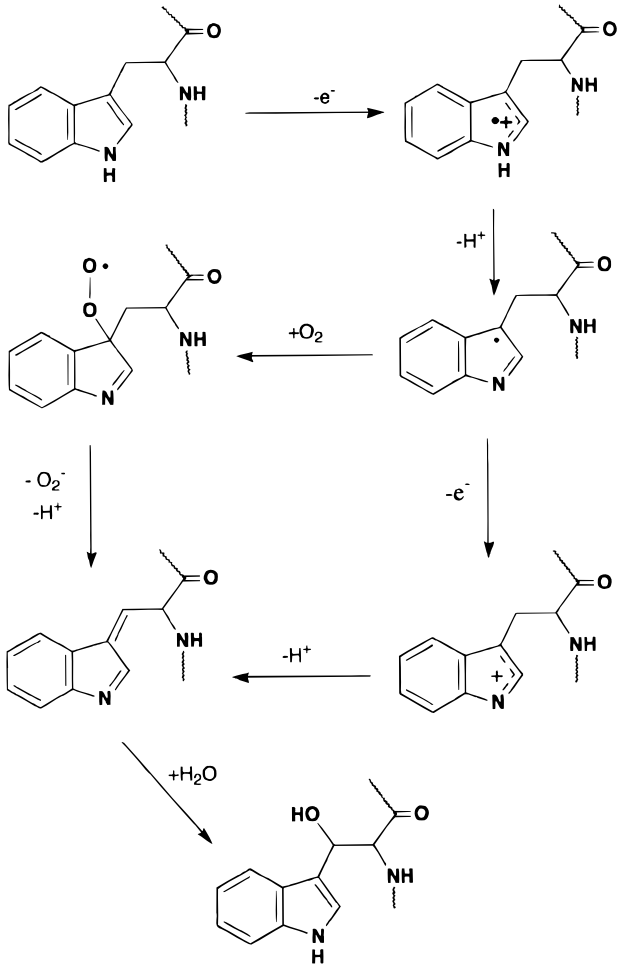
LipP* was treated with hydrogen peroxide to see whether hydroxylation of Trp171 could be achieved. These experiments were carried out at pH 4.5, the pH at which the fungus for the production of the fungal enzyme was grown. Addition of 1 equiv of hydrogen peroxide led to a significant 333 nm signal from the Trp171-containing peptide. Its absorbance was identical to spectrum 2 in Figure 4. This suggests that the hydroxylation is an autocatalytic process. Figure 7 displays the increasing extent of the covalent modification with increasing amounts of hydrogen peroxide. The data show that, in the absence of reducing substrate, addition of 1 equiv of H_2O_2 leads to the hydroxylation of about 60% of the enzyme molecules and 3 equiv is enough for completion. Addition of the substrate VA to the reaction mixture, however, competed with the self-oxidation but could not prevent it (Figure 7). At 2 mM VA, a saturating concentration in steady-state kinetics, about 30 turnovers were necessary to modify all the enzyme molecules. It is important to note that the conditions applied in these experiments do not lead to a deactivation of the enzyme as judged by its specific activity and by the unaltered absorbance spectra before and after the reaction (data not shown).

As in the case of the fungal enzyme, there is more than one peptide in the 333 nm traces. Because they all have spectra identical to the one of didehydrotryptophan they were included in the quantification of the hydroxylated enzyme fraction for Figure 7. Since the digestion steps cannot be expected to be completely specific, the presence of multiple Trp171-containing peptides is not surprising. No indications for the modification of the other two tryptophans were found since the intensities of their absorbances at 280 nm remained unchanged and no related 333 nm signals appeared.

These results show that the autocatalytic modification of Trp171 is very efficient and specific requiring only a small excess of H_2O_2 (2−3 equiv, Figure 7) or a quite small number of turnover cycles. It follows that, in a typical activity assay of pristine LipP*, the enzyme is modified during the first few turnovers so that one effectively obtains the activity of the hydroxylated enzyme form. The lignolytic medium from which fungal LiP is purified contains also a H_2O_2-generating system and VA (*31*). This H_2O_2 will be responsible for the hydroxylation during cultivation of the fungus as well as under physiological conditions. Until now, all kinetic and functional studies reported on LiP in the literature were done using enzymes from fungal cultures. From that, the intriguing conclusion can be drawn that all these data must have been obtained for Trp171 hydroxylated enzyme forms.

Proposed Mechanism for the Modification. Trp171 is located about 6 Å above the plane defined by the heme in the proximal domain of the LiP molecule (Figure 2) and approximately opposite to the narrow opening that gives access to the δ-mesocarbon of the heme (*24*, *25*). The shortest distance of an indole carbon of Trp171 to a pyrrole carbon of the heme is ∼11 Å. Trp171 is, thus, two to three times as distant from the heme as a molecule of VA modeled into the heme edge channel (*4*, *25*), but, nevertheless, has a redox potential about 0.25 V lower than VA at pH 4.5 as estimated for the free compounds (*26*, *27*). Long-distance electron transfer from Trp171 to the oxidized heme, possibly via residue 172, which is in van der Waals contact to the heme, could initiate the sequence of reactions leading finally to hydroxylation of Trp171 (Figure 1). On the basis of work on the degradation of tryptophan derivatives after one-electron oxidation (see, e.g., ref *28*), two routes from the indole radical cation that has a p*K* of 4.3 (*29*) can be envisaged depending on the pH (Scheme 2). One route involves molecular oxygen, but both lead to the incorporation of an oxygen atom from water into the hydroxy group. To discriminate between the two routes, we carried out the autocatalytic process anaerobically. However, no difference in the amount of Trp171 hydroxylation could be detected as compared to the aerobic control in any case (data not shown). This result provides indirect evidence that the right-hand reaction sequence in Scheme 2 is the preferred one and that dioxygen is not involved in the modification under the conditions applied. In principle, a similar reaction sequence to that in Scheme 2 could lead to the β-keto compound of

Scheme 2: Proposed Reaction Sequences for the Autocatalytical Cβ Hydroxylation at Trp171 of LiP[a]



[a] The electrons are abstracted by the oxidized heme. As described in the text, the right hand sequence which does not require dioxygen is the preferred one.

Trp171, although, according to simple chemical considerations on substituent effects, the redox potential of β-hydroxytryptophan will be slightly higher than that of tryptophan. In our new crystal structures of two LiP isozymes, the Cβ atom of Trp171 is strictly tetrahedrical and all atoms of Trp171 including the hydroxy oxygen have low-temperature factors. Therefore, no other adducts appear to be present. For recombinant LipP* no indication of the β-ketotryptophan absorbance (*17*) was found during turnover with hydrogen peroxide and VA for up to 100 turnover cycles. For this reason, the further oxidation of Trp171 does not seem to occur.

Implications of the Covalent Modification of Trp171 for the Redox Cycle. It is possible that this modification has no influence on enzyme function and that as a result no evolutionary pressure for the elimination or substitution of this residue has been operating. Alternatively, Trp171 could play an active role in the redox chemistry of lignin degradation. As a first approach to probe the functional role of Trp171, we undertook chemical modification of the fungal enzyme using the tryptophan-specific agent *N*-bromo succinimide (NBS). Out of the three tryptophans in LiP isozymes, two, including Trp171, are solvent accessible and should be amenable to oxidation to the corresponding oxindoles by NBS. If one of these residues is needed for catalysis, an effect on the activity would be expected. Figure 8A shows the decreasing activity of the fungal LiP depending on increasing amounts of NBS. The activity changes most between a 10- and 25-fold molar excess to reach a constant value of ~15% residual activity at ~30-fold excess of reagent. The change in activity is accompanied by a diminishing tryptophan fluorescence (Figure 8B) in accordance with oxindoles being nonfluorescent. Five equivalents of NBS lead to the loss of about $^1/_3$ of the initial fluorescence intensity while the activity is only slightly affected. We attribute this to the modification of the surface residue Trp17 that is about 20 Å away from the heme in the distal domain. The second stage of the modification, which requires higher amounts of NBS, includes the loss of most of the activity and of roughly another third of the initial fluorescence at 315 nm. The remaining emission is due to Trp251, which is buried in the proximal domain. This interpretation was confirmed by spectral analysis of the tryptic peptides of the NBS-treated LiP: (i) the peptide of the buried Trp251 did not show any significant change of its 280 nm absorbance as compared to nontreated enzyme; (ii) the peptides containing the two solvent-accessible tryptophans revealed a marked decrease in their 280 nm signals, and the 333 nm signals characteristic for the didehydro derivative of Trp171 were essentially gone. These data indicate that a tryptophan is important at least for the oxidation of VA. The slight increase of the redox potential of Trp171 by the introduction of an electron withdrawing OH substituent at Cβ should modulate its reactivity. Trp171 is accessible to high molecular mass substrates and could be reduced at least by the phenolic moieties of lignin. For direct electron transfer from a substrate bound near Trp171 to the heme, however, the influence of the new Cβ substituent is not easy to predict.

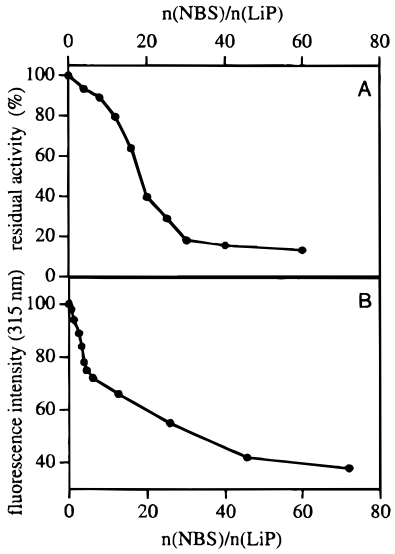


FIGURE 8: Chemical modification of LiP465 by NBS. (A) Dependence of the LiP activity on increasing amounts of NBS determined in the standard VA assay. (B) Effect of NBS treatment of LiP on the tryptophan fluorescence at 315 nm. The resulting oxindoles are non fluorescent.

The kinetic competence of Trp171 to mediate substrate oxidations is under further investigation.

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