Structural and functional investigations on the mechanism of lignin peroxidase

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Structural and Functional Investigations on the Mechanism of Lignin Peroxidase

Academic dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY Zürich for the degree of Doctor of Natural Sciences

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Summary

Lignin peroxidases (LiPs) are extracellular heme containing enzymes of white-rot fungi which participate in the biodegradation of the plant cell wall constituent lignin. LiPs catalyse the oxidation by hydrogen peroxide of a vast variety of compounds with reduction potentials exceeding 1.4 V (versus NHE) by single-electron abstraction. In the present study, two isozymes purified from cultures of Phanerochaete chrysosporium were employed along with a third recombinant one and two mutants. These LiP variants were investigated by protein crystallography and various biochemical methods with the aim of improving the understanding of the reaction mechanism of this enzyme.

Previously, a modification of the surface residue Trp171 was detected by the observation of a strong electron density at single bond distance to its Cβ atom. Here, tryptic digestion of the fungal enzymes was carried out and the Trp171 containing peptide was isolated by HPLC. Under ambient conditions this peptide shows an absorbance spectrum typical of tryptophan. At elevated temperature or under acidic conditions, however, the formation of an unusual absorbance spectrum with λ_max=333 nm can be followed that is identical to that of N-acetyl-α,β-didehydrotryptophanamide which must result from water elimination from β-hydroxy tryptophan. In accordance with the geometric situation seen in the crystal structure the modification is interpreted as a hydroxy group covalently attached to the Cβ atom of Trp171.

The corresponding tryptic peptide from the recombinant and refolded LiP which has not yet reacted with hydrogen peroxide lacks the 333 nm absorbance under any conditions. However, treatment with three equivalents of hydrogen peroxide prior to proteolytic digestion leads to complete hydroxylation as was judged from the detection and quantification of the didehydro elimination product. Reducing substrates compete with this process, e.g. in the presence of 0.5 mM of the natural substrate veratryl alcohol (VA), about seven equivalents of hydrogen peroxide is necessary for complete modification. We conclude that the hydroxylation at Cβ of Trp171 is an autocatalytic oxidative reaction which must occur readily also under conditions of natural turnover, e.g. in ligninolytic cultures of P. chrysosporium which are known to contain an oxidase-based hydrogen peroxide-generating system.

Chemical modification of the fungal enzyme with the tryptophan-specific agent N-bromo succinimide leads to a drastically reduced acitivity with VA. A site-directed mutant which has Trp171 replaced by serine is completely inactive with VA and its high oxidation states, compound I and II, are inert with this substrate. These results show that Trp171 is an essential residue for the oxidation of VA. Other artificial substrates are still oxidised by W171S LiP indicating a specialised interaction site for VA at Trp171 in contrast to the previous assumption that the heme access channel is the only place of VA oxidation.
Compound I of LiP is very unstable and is reduced by a previously unidentified electron donor. The present finding that compound I of W171S LiP has a thirteen-fold longer lifetime at pH 4.5 than that of the wild-type enzyme indicates that Trp171 can act as an intramolecular electron donor for the heme thereby giving rise to a tryptophan radical cation. Independent evidence for such a species has been obtained by spin-trapping with methyl nitroso propane (MNP). When LiP was treated with this spin-trap and hydrogen peroxide, a novel absorbance spectrum was detected in the Trp171 containing tryptic peptide. A very similar spectrum could be generated by treating the tryptophan model compound N-acetyl tryptophanamide with MNP and a one-electron oxidising system. We thus conclude that Trp171 carries a transient radical that can be trapped by MNP. The precise site the spin-trap is attached to could be located by protein crystallography as the C6 atom of Trp171.

Crystal structures of the pristine (non-hydroxylated at Trp171) and the oxidatively processed (hydroxylated at the Cβ of Trp171) recombinant LiP forms were solved by molecular replacement and were refined to R-factors of about 0.185 (free R-factors =0.225) at resolutions of 1.75 and 1.73 Å, respectively. The presence of the hydroxy group leads to only subtle structural differences and no functional need for it could be derived on a pure structural basis. However, the origin of the absolute stereospecificity of the hydroxylation can be explained from these data. In addition, the structure of a triple mutant which has an enlarged heme access channel has been determined which can serve as a structural basis for the interpretation of kinetic data.

A reaction mechanism leading to the Cβ hydroxylation of Trp171 is proposed whose starting point is the tryptophan radical cation. The possible effect of this modification on the redox properties of the indole system of this residue is discussed. Based on previous work and the new data from this study a reaction mechanism for VA oxidation is put forward whose central points are the redox activity of Trp171 and stabilisation of the reactive intermediate VA+ in immediate proximity to this residue by a striking accumulation of acidic residues. The oxidation of natural substrates at this surface site provides a solution to the long-sought way how electrons are transferred from the buried heme to bulky substrates like lignin which cannot approach the cofactor.
Zusammenfassung

Lignin Peroxidasen (LiPs) sind extrazelluläre Hämenzyme von Weissfäulnispilzen, die am Abbau des pflanzlichen Zellwandbestandteils Lignin beteiligt sind. LiPs katalysieren die Einelektronenoxidation einer Vielzahl von Verbindungen, wobei diese Redoxpotentiale von mehr als 1.4 V (NHE) haben können. In dieser Arbeit wurden zwei Isoenzyme aus Kulturen von *Phanerochaete chrysosporium* benutzt, sowie ein rekombinantes Isoenzym und zwei Mutanten. Diese wurden mittels Proteinkristallographie und verschiedener biochemischer Methoden untersucht mit dem Ziel, den Reaktionsmechanismus besser zu verstehen.


Die Kristallstrukturen der ursprünglichen, nicht hydroxylierten, und der oxidativ prozessierten, d.h. am Cβ von Trp171 hydroxylierten rekombinanten LiP-Formen wurden mit der Methode des molekularen Ersatzes gelöst und zu R-Faktoren um 0.185 (free R-Faktoren um 0.225) bei Auflösungen von 1.75 und 1.73 Å verfeinert. Die Gegenwart der Hydroxygruppe führt nur zu marginalen strukturellen Veränderungen. Die Ursache der absoluten Stereospizität am Cβ von Trp171 kann anhand der Strukturen erklärt werden. Ausserdem wurde die Kristallstruktur einer Dreifachmutante, die einen stark vergrösserten Hämkanal aufweist, bestimmt. Diese kann als strukturelle Grundlage zur Interpretation kinetischer Daten dienen.

Abbreviations and Symbols

ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)
CcP, cytochrome c peroxidase
5-c, 5-fold coordinated heme
6-c, 6-fold coordinated heme
CIP, coprinus cinereus peroxidase
DFAD, 4-[3,5-difluoro-4-hydroxyphenyl)azo]-benzenesulfonic acid, sodium salt
equiv., equivalents
Fo, observed structure factor amplitude
Fc, calculated structure factor amplitude
HPLC, high-pressure liquid chromatography
HRP, horse radish peroxidase
HS, high spin
IEF, isoelectric focussing
LiP, lignin peroxidase
LiP*, recombinant lignin peroxidase
LiP H8, lignin peroxidase isozyme H8
LiP415, fungal lignin peroxidase isozyme with pI 4.15
LiP465, fungal lignin peroxidase isozyme with pI 4.65
LiPI, LiP compound I
LiPII, LiP compound II
LiPMNP, MNP/H2O2-treated LiP
LS, low spin
MMA, p-methoxy mandelic acid
MnP, manganese dependent peroxidase
MNP, methyl nitroso propane
NBS, N-bromo succinimide
NCS, non-crystallographic symmetry
NHE, normal hydrogen electrode
PEG, polyethylene glycol
rms, root mean square
rmsd, root mean square displacement
RR, resonance raman spectroscopy
VA, veratryl alcohol
VAld, veratryl aldehyde
vs, versus
Leer - Vide - Empty
1. Introduction

1.1 Lignin structure, function and biosynthesis

Lignin is a three-dimensional heterogeneous biopolymer which constitutes, together with cellulose and hemicellulose, the cell walls of plants. It accounts for 20-30% of the dry weight of wood and is, after cellulose, the second most abundant biopolymer on earth. Its main function is to give structural support to woody plants and allow them to stand upright (Lewis et al., 1998). Moreover, it provides the vasculature which is necessary for water conduction. Its specific disposition and composition determine largely the texture of plants and affect properties like wood colour, durability, rot resistance and even their fragrance (Lewis et al., 1998). Figure 1.1 gives a structural model of lignin. It is composed of phenylpropanoid units interlinked by various C-C and C-O bonds among which the β-O-4 bond (boxed in Figure 1.1) is the most prevalent (> 50%). Covalent linkages exist also to the hemicelluloses via ether, ester and glycosidic bonds.

Lignin precursors are the three monolignols p-coumaryl, coniferyl and sinapyl alcohol (Figure 1.1 b) which are thought to couple non-specifically, i.e. without enzymatic control, after the peroxidase or laccase catalysed generation of the corresponding phenoxy radicals to form the polymer (Brunow et al., 1998). However, this so-called non-specific dehydrogenative coupling hypothesis has also been questioned on the basis that the non-specificity has no counterpart elsewhere in biochemistry and that coupling patterns from in vitro reactions have never matched satisfactorily those found in vivo (Lewis et al., 1998).

The biosynthesis of the monolignols starts from phosphoenolpyruvate, an intermediate of glycolysis, and erythrose-4-phosphate, from the pentose phosphate pathway, employing the shikimate-chorismate pathway. Nitrogen is introduced into prephenate to afford arogenate (Figure 1.2) that can be transformed to the aromatic amino acids phenylalanine and tyrosine. When these are conscripted into the phenylpropanoid pathway rather than for protein synthesis, the nitrogen is immediately removed in reactions catalysed by phenylalanine and tyrosine ammonia lyases to give cinnamic acid or p-coumaric acid, respectively (Figure 1.2). There are essentially four types of transformations leading to the monolignols, namely cytochrome P-450 dependent aromatic hydroxylations, O-methyltransferase reactions, coenzyme A ligations and NADPH-dependent reductions. The exact order of these reactions is still unclear in some cases as are the regulatory and rate-limiting steps and the way the monolignols are transported from the cytoplasm into the lignifying cell wall. A recent study identified the expression level of ferulate-5-hydroxylase (compare step 11 in Figure 1.2) as a determinant of the lignin monomer composition (Meyer et al., 1998) which in turn influences
Figure 1.1 a) Structural model of lignin adopted from Adler (1977). One β-O-4 bond is boxed.
b) Structures of the three monolignols which are the monomeric precursors used for lignin biosynthesis.
Figure 1.2 Main elements of monolignol biosynthesis starting from chorismate (adopted from Lewis et al., 1998). The enzymes involved are: 1 chorismate mutase, 2 prephenate amino transferase, 3 arogenate dehydratase, 4 arogenate dehydrogenase, 5 tyrosine ammonia lyase, 6 phenylalanine ammonia lyase, 7 cinnamate-4-hydroxylase, 8 CoA ligases involving AMP and CoA ligation, 9 cinnamoyl-CoA:NADP oxidoreductases, 10 cinnamyl alcohol dehydrogenase, 11 hydroxylases, 12 O-methyltransferases.
the properties of wood. Whereas softwood lignin is composed of coniferyl and some p-coumaryl alcohol but no sinapyl alcohol, hardwood lignin consists mainly of coniferyl and sinapyl alcohol (Sakakibara, 1980). It should be noted that a number of other plant natural products are also synthesised via the phenylpropanoid pathway, e.g. flavonoids and coumarines.

Before the onset of lignification, the overall structure of the cell wall is established by the deposition of cellulose, hemicellulose and structural proteins (Donaldson, 1994). The latter are thought to provide initiation sites for the oxidative coupling of the monolignols and thereby determine the assembly of lignin.

Approximately 80-90% of the phenolic hydroxy groups of the monomeric lignin precursors form intermolecular linkages which explains the largely non-phenolic nature of lignin. One peculiarity of it is that these bonds are not susceptible to hydrolytic cleavage mechanisms as employed for the degradation of other biopolymers like carbohydrates, proteins and nucleic acids.

1.2 Biodegradation of wood and lignin

The most effective wood rotters in nature are filamentous fungi of the genus basidiomycete which are essential contributors to biomass recycling and soil fertility in forest ecosystems. According to the types of degradation they bring about they are classified into three specific decay groups: brown-rot and soft-rot fungi are mainly soft wood degraders growing primarily on conifers. They preferentially attack the wood carbohydrates leaving behind a brownish mass of modified lignin. Soft-rot is generally attributed to ascomycetes. White-rotters decompose both the carbohydrate and the lignin of hard wood from deciduous trees which results in a white colour of rotting wood due to the loss of lignin. Although they are able to mineralise it completely to carbon dioxide, they cannot rely on it as the only carbon source (Kirk and Farrell, 1987). White-rot fungi are thought to degrade lignin in order to get easier access to the nitrogen and carbon sources of proteins and the cellulose of wood.

The ligninolytic system of Phanerochaete chrysosporium

The white-rot fungus Phanerochaete chrysosporium is the best studied lignin degrading organism from which all enzymes used in this study originate. What will be said in the following relates mainly to this fungus but will also be true to a large extent for other white rotters.
The primary reactions of lignin breakdown are of oxidative nature and take place extracellularly up to the stage of soluble monomeric or small oligomeric fragments which can be taken up by the cells for further processing (Tuor et al., 1995). The extracellular ligninolytic system consists of many components whose number increases as research proceeds.

**The oxidative enzymes** involved are lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and in many cases, but not in *P. chrysosporium*, laccase, a copper containing phenol oxidase. The latter is not considered any further. LiP and MnP are actually mixtures of several isoenzymes, encoded by various genes and differentially expressed depending on the conditions. For instance, six related lip genes were identified in *P. chrysosporium* (Stewart et al., 1992) and excessive glycosylation is thought to be the main additional reason for the splitting in many isoenzymes. At least 21 peroxidases were found in cultures of this organism (Leisola et al., 1987) and only minor differences between the isoenzymes are found (Glumoff et al., 1990). They are all heme containing glycoproteins with molecular weights of 41-45 kDa, low isoelectric points between 2.8 and 5.3 (Leisola et al., 1987) and low pH optima of around 3 for LiP (Tien and Kirk, 1984) and 4.5 for MnP (Kuan et al., 1993). MnP oxidises Mn$^{2+}$ to Mn$^{3+}$ which can diffuse away from the enzyme in complex with organic acids like oxalate and can in turn oxidise other compounds (Wariishi et al., 1992; Kuan and Tien, 1993). Thus, MnP together with manganese ions works via redox mediation, which is an important concept for the understanding of lignin biodegradation since it explains how the peroxidases interact with large and bulky substrates like lignin. LiP will be described in detail below.

**Hydrogen peroxide-generating system.** An essential component of ligninolysis is a hydrogen peroxide-generating system that reduces dioxygen to hydrogen peroxide which is needed as a cosubstrate for the peroxidases. Various oxidases like the flavin containing glucose oxidases as well as aryl alcohol oxidases and the single-copper containing glyoxal oxidases (Whittaker et al., 1996) have been identified to participate (Kersten, 1990). Substrates for glyoxal oxidase are methylglyoxal, acetaldehyde, glyoxal, glyoxylic acid, dihydroxyacetone and glycolaldehyde which are fragments resulting from lignin degradation. Glycolaldehyde is a product of LiP-catalysed C$_{\alpha}$-C$_{\beta}$-bond cleavage of arylglycerol-β-aryl ethers (Ander and Marzullo, 1997) which are dimeric lignin model compounds. As another source of H$_2$O$_2$ dismutating superoxide has been suggested which results from the reaction of dioxygen with organic radicals which in turn are products of peroxidase-catalysed reactions (Kuan and Tien, 1993). In this scheme, dioxygen is the terminal electron acceptor and only catalytic amounts of hydrogen peroxide are needed to keep the cycle running. This situation is
reminiscent of the oxidation of indole acetic acid among other compounds by horseradish peroxidase in the absence of H$_2$O$_2$ (see e.g. Krylov and Dunford, 1996).

**Low molecular weight compounds.** The vital role for MnP of manganese which is naturally present in wood has already been mentioned. Of the same importance for LiP is veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA), a metabolite of *P. chrysosporium* (Lundquist and Kirk, 1978). It can be biosynthesised from glucose and is produced and secreted at the same time as LiP when the fungus switches to secondary metabolism. Intermediates of its biosynthetic pathway are phenylalanine, cinnamate, benzaldehyde, benzoate and ring hydroxylated and O-methylated derivatives (Jensen et al., 1994; Harper et al., 1996). Besides, degradation products from lignin can be used as precursors for VA which is a good substrate for LiP. VA can act as a redox mediator for the oxidation of compounds which are no direct substrates themselves and it protects LiP from H$_2$O$_2$-induced inactivation (see below). Although its importance for LiP is undisputed, its exact role in ligninolysis is still unclear, probably because it has several functions.

Oxalate is a metabolite of rot fungi which derives from oxaloacetate and glyoxylate and is secreted during secondary metabolism. White-rotters accumulate concentrations between 0.04-10 mM oxalate (Dutton et al., 1993), which has been correlated with a pH drop during cultivation (Shimada et al., 1997). The action of glyoxylate oxidase produces oxalate and H$_2$O$_2$ (Shimada et al., 1997). Among the well-established functions of oxalate is the promotion of MnP activity by chelating manganese ions (Kuan and Tien, 1993). In addition, it may act as an acid catalyst and as an electron donor for peroxidases to produce formate radicals. The latter can give rise to diverse radical chemistries like activation of dioxygen to form superoxide, dismutation to H$_2$O$_2$ and reduction of ferric to ferrous iron, and the production of hydroxyl radicals from Fenton chemistry (Barr et al. 1992; Gieger, 1997). The H$_2$O$_2$ produced from the reaction of oxalate-derived radicals with dioxygen can support the catalytic cycle of peroxidases (Kuan and Tien, 1993).

**Reductive system.** In studies investigating the lignin depolymerising activity of LiP significant polymerisation of phenoxy radicals has also been observed (Sarkanen et al., 1991). The enzymes cellobiose:quinone oxidoreductase and veratryl alcohol oxidase have been reported to have phenoxy radical reducing activities and to suppress the coupling reactions of radicals which are produced by peroxidases (Marzullo et al., 1995; Ander and Marzullo, 1997). The reductive ability of superoxide has been mentioned in the previous paragraph.
Mechanism of lignin breakdown

The mechanism of lignin degradation by rot fungi is largely still a mystery in spite of a lot of research done during the last two decades ranging from in vivo studies to the molecular level of the components involved. The main reason for this elusiveness is the complexity of the natural systems, which does not (yet) allow a classification of ligninolysis into a few simple principles, and the oversimplification introduced by model studies. One has to keep in mind that the natural delignifying process, optimised by evolution, is extremely slow, at least compared to the needs of the wood-utilising pulp and paper industry. The time it takes all natural rotting organisms together to remove a fully-grown tree is comparable to the time it takes the tree to grow.

Among the few components that are known for sure to participate in lignin breakdown are LiP and MnP which act together with their cofactor/mediator VA and manganese ion, respectively. The primacy of LiP over MnP in the white-rot process derives from the fact that only LiP can oxidise non-phenolic aromatics due to its higher redox potential. Based on work on the oxidation of non-phenolic lignin model compounds, the initial event is thought to be electron abstraction from the aromatic rings of lignin which is followed by uncatalysed bond cleavages at the propyl side chains leading ultimately to depolymerisation (Harvey et al., 1985; Kersten et al. 1985; Hammel et al., 1985; Schoemaker, 1990). LiP catalyses the cleavage of a synthetic lignin preparation to give soluble low molecular weight products provided that VA was also present (Hammel et al., 1993). In a recent study using insoluble poplar lignin, MnP produced some lignin-derived hydrochinones and LiP produced lignin-derived p-hydroxybenzoate but only LiP and MnP together decreased the solid mass and the lignin content substantially (Thompson et al., 1998).

1.3 Peroxidases

An overview

Peroxidases oxidise various compounds at the expense of hydrogen peroxide. They are widely distributed among living organisms and many are known from mammals, plants, fungi and bacteria. The removal of the potentially hazardous hydrogen peroxide or organic peroxides is generally stated as one of their functions although catalases are much more efficient in this respect. In the majority of the cases the specific substrate oxidation they bring about appears to be the essential point. Most peroxidases contain a heme as redox cofactor. Some exceptions are the selenium containing glutathion peroxidase, vanadium containing haloperoxidases from fungi and seaweeds, and an interesting novel type of mammalian
peroxidases referred to as peroxiredoxins. The latter rely either on a single cysteine or on two cysteine residues undergoing redox cycles and which can use thioredoxin as electron donor (Kang et al., 1998; Choi et al., 1998). Peroxiredoxins have been connected with signalling cascades in which H$_2$O$_2$ acts as an intracellular messenger (Kang et al., 1998). In general, the functional roles of the mammalian peroxidases are better known than those of the plant and fungal ones. Some of the former enzymes are involved in antimicrobial defense like myeloperoxidase, eosinophil peroxidase and lactoperoxidase. The first two enzymes and also chloroperoxidase have redox potentials high enough to oxidise chloride to hypochlorous acid. Lactoperoxidase is present in secretory fluids like milk and others. It oxidises SCN$^-$ to OSCN$^-$ which inhibits microbial growth (Reiter and Perraudin, 1991). Thyroid peroxidase catalyses two steps in thyroxine synthesis, iodination of tyrosyl residues and their coupling. Glutathion peroxidase is essential for the reduction of organic peroxides like those derived from lipid peroxidation.

**Peroxidases from the plant peroxidase superfamily**

LiP, the topic of this thesis, belongs to the plant peroxidase superfamily which comprises enzymes from plants, fungi and bacteria. Welinder (1992) has divided them into three classes according to sequence similarities. Class I includes yeast mitochondrial cytochrome c peroxidase (CcP), plant cytosol and chloroplast ascorbate peroxidases (APXs), and gene-duplicated bacterial peroxidase. Secretory fungal peroxidases like LiP, MnP, *Coprinus cinereus* (CIP) and *Arthromyces ramosus* peroxidase (ArP; CIP and ArP are essentially identical) make up class II. Horseradish peroxidase (HRP) and other secretory plant peroxidases belong to class III. The classic peroxidase substrates for class II and III are phenols and anilines. The substrate specificities are usually broad. Among the functional roles of class III enzymes are lignification of the cell wall, suberisation of wound tissue and plant hormone catabolism (Welinder, 1992).

**Common structural features**

All members of the plant peroxidase superfamily are globular and mostly helical proteins with only a few short $\beta$-sheets and contain an iron protoporphyrin IX cofactor. The heme divides the protein into a proximal and a distal domain. The proximal one provides a conserved histidine as the fifth ligand for the iron. Furthermore, the members of class II and III contain two structural calcium ions, one in each domain. They have disulfide bridges (four in the case of LiP) and are glycosylated.
Chapter 1

\[
\begin{align*}
\text{Enz-Fe}^{III}, \text{por} + \text{H}_2\text{O}_2 & \rightarrow \text{Enz-Fe}^{IV}=\text{O}, \text{por}^{++} + \text{H}_2\text{O} \quad (1) \\
\text{Enz-Fe}^{IV}=\text{O}, \text{por}^{++} + \text{S} & \rightarrow \text{Enz-Fe}^{IV}=\text{O}, \text{por} + \text{S}^{++} \quad (2) \\
\text{Enz-Fe}^{IV}=\text{O}, \text{por} + \text{S} & \rightarrow \text{Enz-Fe}^{III}, \text{por} + \text{S}^{++} + \text{H}_2\text{O} \quad (3)
\end{align*}
\]

Scheme 1.1 The three basic steps of the catalytic cycle of heme peroxidases. Enz, por and S denote the protein part of the enzyme, the porphyrin and a substrate, respectively. In Ccp the radical of compound I resides on a tryptophan residue close to the heme rather than on the porphyrin.

Redox states and the catalytic cycle

In its most simplistic form, the overall catalytic cycle can be described by the three steps depicted in Scheme 1.1. Firstly, \( \text{H}_2\text{O}_2 \) oxidises the ferric resting state enzyme by two redox equivalents to compound I which consists of an oxoferryl \( \text{Fe}^{IV} \) species and a radical cation on the heme porphyrin. In the special case of CcP this radical resides on a nearby tryptophan, Trp191, that is located next to the proximal histidine. Then two electrons are sequentially abstracted from substrate molecules to restore the resting state. The intermediate compound II is still one redox equivalent above the ferric state. It consists of the oxoferryl heme but has no more porphyrin/protein radical. The typical heme absorbance spectra in these three redox states are shown in Figure 1.3 using LiP as an illustrative example. Another redox state that is frequently observed but is not part of the normal turnover cycle is compound III, also called oxy-peroxidase, an inactive form that can be reactivated upon oxidation e.g. by product cation radicals. It can be described as a ferrous-oxy or as a ferric-superoxy complex and is thus electronically analogous to oxy-hemoglobin. According to its redox state, compound III can be prepared from ferrous peroxidase and oxygen, from ferric enzyme and superoxide, and from compound II and hydrogen peroxide.

A number of conserved residues are present in the heme environment to carry out the catalytic steps in the redox cycle and a lot of mutagenical and structural work, mostly on CcP and HRP, has been dedicated to assign their roles. Briefly, the distal histidine and arginine carry out the acid-base catalysis required for O-O bond cleavage of \( \text{H}_2\text{O}_2 \) and compound I formation (Poulos and Fenna, 1994; Miller et al., 1994a). On the proximal side there is an aspartate that hydrogen bonds to the essential proximal histidine and stabilises the high oxidation states of peroxidases by imparting a partial imidazolate character to the histidine (Goodin and McRee, 1993). This aspartate is not present in globins which have a considerably higher redox potential of the \( \text{Fe}^{3+}/\text{Fe}^{2+} \) couple than peroxidases. Some other factors which are
Figure 1.3 Qualitative absorbance spectra of lignin peroxidase in the resting ferric state (continuous line), compound I (dotted line) and compound II (dashed line). The spectra were obtained from a rapid-scanning stopped-flow experiment in which 1.1 μM LiP465 was reacted with 200 μM H₂O₂ at pH 4.5 and 25°C. The resting state spectrum is 3-4 ms, that of compound I 14 ms, and that of compound II 4 s after mixing. The resting state spectrum contains some contribution from compound I formed before the first spectrum was recorded and the compound II spectrum contains a slight contribution from compound III as indicated by a weak shoulder around 580 nm.

thought to control the redox potential of peroxidases are the distance between the iron and the proximal histidine (Piontek et al., 1993), more generally the electron density at the iron (He et al., 1996), and charges in the heme environment (Varadarajan et al., 1989; Miller et al., 1994b). The considerable differences in the redox potentials of the ferric/ferrous couple that exist among peroxidases have not yet been explained satisfactorily. The redox potentials of the high oxidation states compound I and II are known in some cases e.g. 0.92 V and 0.94 V for the HRPI/HRPII and HRPII/HRP couples, respectively, at neutral pH (Hayashi and Yamazaki, 1979). For LiP, however, their short lifetimes have not allowed to measure these redox potentials.

1.4 Lignin Peroxidase

LiP (E.C. 1.11.1.7) from P. chrysosporium is a glycoprotein consisting of about 344 amino acids, depending on the particular isozyme, and a mass of 38 kDa (protein only). It belongs to class II of the plant peroxidase superfamily and the general descriptions given above are also valid for it. Ligninases were discovered in the early eighties independently in
several laboratories which explains the different nomenclature systems that are in use for the different isozymes. In this study, two LiP isozymes purified from fungal cultures are used which are denoted according to their isoelectric points, namely LiP415 and LiP465 for the isozymes with pI 4.15 and 4.65, respectively. The recombinant enzyme is called LiPH8* (the asterisk indicates the recombinant origin) because its cDNA stems from the gene that codes for the fungal enzyme eluting from an anion exchange column as heme protein No. 8 (Kirk and Farrell, 1987).

Figure 1.4 shows the electronic absorbance spectrum of resting state LiP465. The bands at 409, 502 and 635 nm are typical for a ferric high-spin (HS) heme. LiP samples tend to have a variable small fraction of the heme in a low-spin (LS) state, probably compound III, which can be easily discerned in the absorbance spectra by bands around 540 and 580 nm. The spectrum in Figure 1.4 also reveals a weak shoulder at ≈545 nm which is due to a small fraction of the enzyme present as LS species. It has also been well-established that LiP forms compound III from compound II much easier than HRP (Wariishi et al., 1990). The compilation of the most important redox states and their interconversions of Scheme 1.2 is valid for all heme peroxidases and to some extent also for globins but differences exist in the relative stabilities of these states and in the rates of their formation.

The most striking peculiarities of LiP are its high redox potential which enables it to oxidise non-phenolic aromatics like VA and various methoxybenzenes and its low pH.

**Figure 1.4 Absorbance spectrum of fungal LiP465 (2.8 μM) at pH 4.5.**
optimum of below 3 (Marquez et al., 1988). Especially the high redox potential and its control by the enzyme has attracted much attention. One should keep in mind that the redox potential of the VA+/VA couple of about 1.4 V (Khindaria et al., 1996) is considerably higher than that for the oxidation of water to dioxygen of 0.99 V at pH 4 or 1.05 V at pH 3. From a purely thermodynamic point of view one would therefore expect oxygen evolution rather than VA oxidation. This net catalase-type reaction, however, does not appear to occur to any significant extent.

There is reason to assume that the low pH optimum and the high redox potential of LiP are correlated with one another and also with the unusual short lifetime of compound I: when one equiv. of hydrogen peroxide is added to LiP in the absence of reducing substrates, the absorbance of the so formed compound I bleaches within seconds, much faster than that of related peroxidases, and the electronic spectrum of compound II is obtained (Tien et al., 1986; Harvey et al., 1989). Redox-active protein residues like tyrosines and tryptophans would be conceivable as reducing agents which could in turn serve to oxidise substrates. Nothing specific about the source and location of this reductant was known at the beginning of this work. An important consequence of this behaviour is that the true overall redox state of the enzyme cannot be judged from the heme absorbance alone. Therefore, in this thesis the terms 'compound II' or 'LiPII' refer only to the redox state of the heme but do not state anything regarding the true overall redox state of the enzyme.

Two independent crystal structures of the same LiP isozyme have been solved (Piontek et al., 1993; Poulos et al., 1993). The overall fold of LiP is shown in Figure 1.5 for LiPH8*. In contrast to what one would expect for an enzyme with a polymeric substrate the heme cofactor is very much buried inside the protein and there is only a relatively small opening giving access to the heme (see also Figures 3.13 and 4.1). This so-called active site channel is smaller than those in related proteins like CIP and will prevent the direct interaction of the
bulky polymeric lignin with the heme. Long distance electron transfer from lignin bound somewhere on the surface of LiP to the heme is unlikely since the driving force for the oxidation of non-phenolic compounds is too small to allow such a mechanism (Schoemaker et al., 1994).

A possible solution to this problem comes from the concept of redox mediation. In 1985 it was proposed that the high oxidation states of LiP oxidise substrates by single electron abstractions (Harvey et al., 1985) and ten years later direct evidence for the LiP-generated VA cation radical (VA⁺⁺) was found by ESR spectroscopy (Khindaria et al., 1995). Long before the determination of the crystal structures of LiP it had been suggested that VA⁺⁺ generated by LiP compound I (LiP I) or compound II (LiP II) may mediate the oxidation of p-methoxy mandelic acid (MMA) and anisyl alcohol (Harvey et al., 1986). Since the latter two compounds have only one methoxy group at the aromatic ring their redox potentials are supposed to be higher than that of VA. In recent years redox mediation by VA has been substantiated for the LiP oxidation of MMA (Tien and Ma, 1997), chloropromazine (Goodwin et al., 1995), and guaiacol (Koduri and Tien, 1995). Anisyl alcohol, however, reacts only with LiP compound I and VA is needed to reduce compound II and to complete the catalytic cycle (Koduri and Tien, 1994).

The mechanism of mediation, however, has remained unclear. In one study the lifetime of chemically generated VA⁺⁺ was found to be long enough for VA⁺⁺ to act as a diffusible oxidant and to diffuse up to 7 µm in solution (Candeias and Harvey, 1995). The chemically generated VA⁺⁺ did not oxidise MMA or anisyl alcohol which led the authors to propose that in the enzymatic system VA⁺⁺ is bound to and stabilised by LiP so that its lifetime increases permitting the oxidation of monomethoxylated compounds (Candeias and Harvey, 1995).
Mechanism of VA oxidation

Khindaria et al. (1995) presented experimental evidence for the proposal (Harvey et al., 1989) that VA$^+$* formed by the reduction of LiPI by VA is stabilised by the enzyme and that a second molecule of VA reacts with the LiPII-VA$^+$* complex to form veratryl aldehyde (VAld, Scheme 1.3) and the resting state enzyme. These authors determined the first order decay rates of VA$^+$* to be 1.85 s$^{-1}$ in the LiPII-VA$^+$*-complex and 1200 s$^{-1}$ free in solution (Khindaria et al., 1996). They suggested what seems now to be generally accepted, that an acidic microenvironment in the active site will prevent the loss of a benzylic proton of VA$^+$* and the subsequent reaction with oxygen (Khindaria et al., 1996). In their scheme, VA would not act as a diffusable oxidant but rather as a transiently protein bound cofactor or as an electron shuttle. Recently, they reported to have found direct EPR-spectroscopical evidence for the LiPII-VA$^+$* complex (Khindaria et al., 1997). Free in solution at acidic pH, VA$^+$* decays by deprotonation from the benzylic carbon followed by nearly diffusion controlled reaction with oxygen to give superoxide and veratryl aldehyde (Steenken, 1998). Oxygen consumption has also been observed (Barr and Aust, 1994).

The above mentioned access channel to the heme is generally believed to be the site of VA oxidation due to its suitable size to accomodate VA in the proximity of the heme (Poulos et al., 1993; Schoemaker et al., 1994; Khindaria et al., 1995; Khindaria et al., 1997) but convincing evidence is missing. Several attempts have been undertaken to cocrystallise LiP with VA and other methoxybenzenes but no electron density attributable to VA has been obtained indicating that VA does not bind in a well-ordered way to the resting state enzyme. We face therefore the unfortunate situation that it is neither known how the enzyme interacts with VA nor how it does with lignin.

Until recently, no expression system for LiP was available and research progress was hampered because site-directed mutagenesis could not be used to study enzyme variants. In
Figure 1.6 Overall fold of LiP using pristine recombinant LiPH8 as an example. The distal domain is above and the proximal one below the heme plane. Helices are in blue-green and β-strands in gold. The heme is in red, the calcium ions in green, Trp171 in cyan and the sulfur atoms of disulfide bridges in yellow. The nitrogen atoms of the axial iron ligands are in blue. The N and C termini are also labelled.

Figure 1.7 Orientation of Trp171 with respect to the heme and omit Fo-Fc electron density map of the hydroxy group at Trp171 contoured at 4σ. The distal His47, the proximal His176, and Leu172 which is in van-der-Waals contact to the heme are also shown. The structure from the fungal enzyme LiP465 was used. Picture by T. Choinowski.
1996 the first successful expression of LiP isozyme H8 in *E. coli* was reported (Doyle and Smith, 1996). Fully active enzyme was obtained only after in vitro refolding. Now that mutant enzyme forms are at hand the understanding of the reaction mechanism of LiP can be expected to improve a lot in the near future.

Another novel aspect in LiP research has been disclosed by the detection of a strong electron density at single bond distance to the Cß of Trp171 in the crystal structures of the two fungal isozymes LiP415 and LiP465 (Choinowski, 1996). This density was tentatively interpreted as to derive from a covalently attached hydroxy group. Figure 1.6 shows the location of Trp171 with respect to the heme. The closest distance between an indole carbon of Trp171 to a porphyrin carbon is 11 Å. Since Trp171 is conserved among LiP isozymes a role comparable to Trp191 in CcP, the radical site in compound I of this peroxidase, has been proposed (Choinowski, 1996).

### 1.5 Aims of this work

The aim of this work was to study the formation and functional significance of the unusual Trp171 modification and, in general, to improve the understanding of the reaction mechanism of lignin peroxidase. Thanks to a collaboration with the group of Dr. A. T. Smith at the University of Sussex a quite broad range of methods like mutagenesis, protein chemistry, enzyme kinetics and protein crystallography could be combined.
2. Protein chemistry and kinetics

2.1 Materials and Methods

**Chemicals.** Veratryl alcohol was purchased from Fluka and was vacuum distilled. TPCK-treated trypsin was from Sigma. NBS was from Aldrich and was recrystallised from water. Storage in vacuo (=10 kPa) increased its stability. The concentration of hydrogen peroxide solutions was determined by titration with potassium permanganate or spectroscopically using $\varepsilon_{240} = 46.5 \text{ M}^{-1}\text{cm}^{-1}$.

$N$-acetyl-$L$-tryptophanamide was prepared as follows. 2.4 g (12 mmol) L-tryptophan was dissolved in 70 ml acetic anhydride and stirred for 1 hour in the presence of 1 g sodium bicarbonate in a distillation apparatus. Then a moderate vacuum (about 10 kPa) was applied and slowly heated to boiling. Within an hour the solvent was completely removed. After cooling down, the dry residue was suspended in 100 ml of dry ether and NH$_3$ (25%) was added dropwise. A colourless material is formed. Then 50 ml of water was added, cooled in ice and the product separated, washed with water and methanol, and dried in vacuo (yield 65%). The material is homogeneous by HPLC and $^1$H-NMR (DMSO-d$_6$). $\delta$=1.75 (s, CH$_3$); $\delta$=4.45 (s, C$_2$H); $\delta$=2.95 (dd, C$_3$H$_1$); $\delta$=3.12 (dd, C$_3$H$_2$).

$N$-acetyl-$\alpha,\beta$-didehydrotryptophanamide. To obtain an optical reference spectrum for the peptide bound $\alpha,\beta$-didehydrotryptophan, $N$-acetyl-$\beta$-keto-tryptophanamide in water was treated with sodium borohydride (Noda et al., 1978). The elimination of water from the so formed $\beta$-hydroxy compound took place immediately under the basic conditions created by the borohydride addition. An optical spectrum of the didehydro compound was obtained from a fraction of the reaction mixture eluting from a reverse phase HPLC column but it was not isolated.

$N$-acetyl-$\beta$-keto-tryptophanamide was obtained from $N$-acetyl-$L$-tryptophan ethyl ester (Sigma) by oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (Fluka) and subsequent ammonolysis of the ester as described (Oikawa and Yonemitsu, 1977).

**Enzymes**

The fungal LiP isozymes with pI 4.15 and 4.65 were purified from the extracellular culture medium of *Phanerochaete chrysosporium*, strain BKM-F-1767 (Leisola and Fiechter, 1985). Briefly, enzyme production during the secondary metabolism of the fungus under carbon-limited conditions was induced by adding 1.5 mM VA to the culture medium. After concentration of the medium the extracellular proteins were separated and LiP415 and LiP465 purified by preparative flat-bed isoelectric focussing (IEF) using equal amounts of ampholyte 4/6 and 3.5/5 (Pharmacia). Ampholytes were removed afterwards from the enzyme by gel-filtration on a Biogel P-60 column (1.5 x 70 cm) in 20 mM sodium tartrate pH 4.5. The
enzyme was concentrated in the same buffer to 2-8 mg/ml, flash-frozen in liquid nitrogen and stored at -70° C. In such a way, it remained fully active and crystallisable for at least a year. Typically, 1-2 mg of LiP415 and 10-20 mg of LiP465 were obtained from a preparation consisting of 16 1 l flasks, 3 consecutive inductions and a total of 7 l medium. It had a specific activity of 42-47 mmol VA oxidised per minute per mg in the standard assay (see below).

It is known that the brown colour of LiP which is characteristic of ferric HS heme changes to the red during the purification step of preparative IEF (Glumoff, 1991; Glumoff et al., 1990) and that this process is more pronounced in LiP465. This change is associated with the appearance of bands around 580 and 540 nm, respectively, in the optical spectrum which is indicative of a LS species, most probably compound III. After the IEF the enzyme spontaneously but slowly converts back to the ferric HS state, LiP415 virtually completely but LiP465 always keeps a variable fraction of compound III. To obtain homogeneous LiP preparations the separated isozymes were treated with about 100 equiv. VA and 5 equiv. H2O2 after the IEF step and before the gel-filtration which results in an almost homogeneous ferric HS enzyme form as judged by the absence of bands at 540 and 580 nm in the uv/vis spectrum.

Recombinant LiPH8* (LiP*, the asterisk indicates the recombinant origin) expressed in E. coli and refolded as described earlier (Doyle and Smith, 1996) as well as a number of mutants were generously provided by these authors. The wild-type enzyme had a specific activity of 34 mmol VA oxidised per minute per mg under the conditions reported (Doyle and Smith, 1996) which compares well to the fungal enzyme.

All enzyme concentrations were determined using ε408=168 mM⁻¹cm⁻¹ of the ferric enzyme and 38 kDa was taken as molecular weight for all enzyme forms. Standard assays were performed by following the formation of veratryl aldehyde (ε310=9.3 mM⁻¹cm⁻¹) with 0.6 mM H2O2 and 2 mM VA at pH 3 and 25 °C in 100 mM sodium tartrate buffer.

**Redox mediation assay**

The formation of anisyl aldehyde from 4-methoxy mandelic acid (MMA, Aldrich) by a system consisting of one of various LiP forms, VA and H2O2 in 50 mM sodium tartrate pH 3 or 50 mM sodium succinate pH 4.5 was followed by HPLC. In a typical experiment, 1 mM MMA, 0.2 mM VA, 20 μM enzyme and 1 mM H2O2 were incubated in buffer for 20 minutes and then subjected to HPLC analysis. Various ratios of MMA:VA were also tried. Elution was performed on a 125/4 Nucleosil 120-5 C18 reverse phase column with the same eluents as those used for peptide mapping (see below), a gradient from 13% B to 75% B in ten minutes, a flow rate of 1.5 ml/min and detection at 270 nm. The peaks were assigned by comparing the retention times and absorbance spectra to those of the authentic compounds.
Tryptic digestion

The enzyme was reduced and carboxamidomethylated essentially as described by Stone et al. (1989): A solution of 8 M urea, 0.1 M sodium bicarbonate was added to the enzyme (typically 1-5 nmol) to achieve a urea concentration of at least 6 M. For the subsequent HPLC analysis, the protein concentration should not drop much below 5 μM which corresponds to 1.25 nmol protein for the maximal injection volume of 250 μl of the HPLC instrument. The protein was concentrated by an acetone precipitation (80 % acetone left for half an our at -20° C) if its concentration was insufficient and after centrifugation the pellet was redissolved in urea. Then 20 μl 30 mM DTT was added and heated to 50 °C for 15 minutes. After cooling to room temperature, 40 μl of 100 mM jodoacetamide was added and incubated for half an hour in the dark. To remove excess urea the mixture was dialysed against 0.1 M ammonium bicarbonate and then incubated for 20 h at 37 °C with 1:30 (w:w) trypsin. The resulting solution was subjected directly to HPLC analysis.

HPLC analysis of tryptic peptide mixtures

A HP liquid Chromatograph 1090 equipped with a diode-array detector and a 125/4 Nucleosil 300-5 C18 reverse phase column (Machery und Nagel) 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% (w:w) trifluoro acetic acid in water and eluent B was acetonitrile with the trifluoro acetic acid content adjusted so that its absorption at 220 nm matched that of eluent A, about 0.05% (w:v) trifluoro acetic acid. Elution was performed with a linear gradient from 0-50% B in 100 minutes and a flow-rate of 0.5 ml/min either at room temperature or with the column compartment thermostated 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 ε333=19.8 mM⁻¹cm⁻¹ (Noda et al., 1978) for the model compound N-acetyl-α,β-didehydrotryptophanamide. 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 (the peak eluting at 46 min in a run carried out at 60 °C). For the maximal ratio that was obtained after increasing additions of H₂O₂ the total amount of didehydro compound corresponded to that found in fungal LiP and was set to 100%. Lower ratios after treatment with less H₂O₂ were calculated relatively to this value.

Edman sequencing of tryptic peptides was done by the Protein Chemistry Laboratory of the ETH Zürich.
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Reaction of LiP* with hydrogen peroxide

20 μM LiP* 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 minutes and was then subjected to tryptic digestion. To test the dependence of the modification of LiP* 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 one hour. Hydrogen peroxide was made anaerobic using the syringe-bubbling technique with argon and a constant amount of three equivalents 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 100 mM sodium acetate pH 4.5 was treated with the calculated volume of 1.5 mM NBS in water. If the NBS was not freshly recrystallised the protein precipitated due to the content of hypobromite. The reaction was stopped after two minutes by adding 10 mmol of aqueous tryptophan. The reaction mixture was dialysed overnight against 20 mM sodium tartrate pH 4.5 at 4 °C to remove substances that could interfere with the subsequent activity assay. To follow the decrease of tryptophan fluorescence, to a solution of 2 ml 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 one minute after the addition using an Aminco SPF-500 instrument. Excitation wavelength: 285 nm, excitation slit: 8 nm, emission slit: 5 nm. The data were analysed by comparison of the fluorescence intensities at selected wavelengths.

Kinetic comparison of LiP465 and NBS-treated LiP465

NBS-LiP was prepared by treating the required amount of enzyme with 30 equiv. NBS in 100 mM sodium acetate pH 4.5. After 1 min incubation the reaction mixture was applied on a PD-10 Sephadex G-25 column that had been equilibrated with 50 mM sodium succinate pH 4.5. Then the enzyme concentration was adjusted to typically 2.5 μM. Native LiP465 was rebuffered on PD-10 and its concentration was adjusted to the same value. For stopped-flow kinetics, these enzyme preparations were shot against a mixture of 100 μM H2O2 (after mixing) and variable concentrations of VA in 50 mM sodium succinate pH 4.5 in a RSM 1000 two-syringe rapid scanning stopped-flow apparatus (On-Line Instrument Systems, Inc.) at 20 °C. The pseudo first order rates of LiPI and LiPII formation were determined with the software provided by the producer by global analysis of the spectral data recorded in the soret region and sometimes also by single wavelength fitting of time cuts. In the first case, a model with 3 species and 2 sequential first order reactions was used for compound I formation and
its subsequent reduction by VA. In the latter case compound I formation was followed at 397
nm and compound II formation at 426 nm. Traces were fit to single exponential functions to
obtain pseudo-first order rates. The data from at least 2 shots were averaged.

Steady-state kinetics were measured by following VA oxidation in 50 mM succinate pH
4.5 with a Cary Bio-3 spectrophotometer. Solutions and spectrophotometer cells were
thermostated at 20 °C. The kinetic constants were obtained by fitting initial velocities plotted
against VA concentrations to a hyperbolic function with the program Origin (Microsoft).

The steady-state control with ferro-cytochrome c (cyt c) as substrate was carried out as
follows: 0.6 mM horse heart ferri-cyt c (SIGMA) was treated with 1.3 mM sodium dithionite
and excess dithionite was removed by chromatography on a PD-10 Sephadex G-25 column
equilibrated with 10 mM argon saturated sodium succinate pH 5. Activity assays were carried
out with 16 μM ferro-cyt c, 120 μM H₂O₂ in 10 mM sodium succinate pH 5 at 25 °C and the
reaction was followed at 550 nm using ε₅₅₀=19.5 mM⁻¹cm⁻¹ (Wariishi et al., 1994).

Rapid kinetics with LiPH8* variants

All experiments of chapter 2.2.5 were carried out with an Applied Photophysics
SX19MV stopped-flow spectrometer at 25 °C. The buffer was 5 mM phosphate, 5 mM citrate
pH 4.5 and the ionic strength was adjusted to 50 mM with sodium sulfate. One syringe
contained enzyme in buffer and the other one hydrogen peroxide with or without VA in
buffer. Rate constants were obtained by fitting kinetic traces at selected wavelengths with the
program xmgr (http://www.teleport.com/~pturner/acegr/).

Spin trapping experiments

Methyl nitroso propane (MNP) was from Aldrich. It was dissolved and converted from
the dimer to the monomer (fresh daily) by heating an aqueous suspension in an Eppendorf
tube for one hour in the dark to 50 °C. It was then put on ice in the dark (Makino et al., 1985).

3.5 μM LiP465 (typically about 4 nmol) in 60 mM sodium tartrate pH 3.0 containing
50 % (v/v) of the MNP solution prepared as above was treated with a total of 60 equiv. of
H₂O₂ in three portions in 5 min intervals. HPLC-peptide mapping was done as described
above. For crystallisation, only 5 equiv. of H₂O₂ were added at a time to reduce oxidative
stress to the enzyme, in this case LiP415.

In an attempt to produce a small molecular model compound of the spin-trapped
tryptophan, 5 mM N-acetyl tryptophanamide and 20 % (v/v) MNP solution in 70 mM sodium
tartrate pH 3.0 was treated with a one-electron oxidating system consisting of 4 mM VA, 3
μM LiP465 and 2 mM H₂O₂ (added in several portions). The mixture turns quickly
intensively yellow. An analytical separation of the reaction mixture could be achieved by thin
layer chromatography on cellulose and 5% ammonia as liquid phase giving an R₇ of ≈ 0.3 for
the yellow product. Analogous conditions were tried for a preparative purification by column
chromatography. However, too little pure product for spectroscopic structure elucidation could be obtained. From an HPLC analysis of a relatively pure fraction of the yellow material from the column chromatography an absorbance spectrum was obtained that is reported in the results section. It is assumed that the yellow spin-adduct has a very high absorbance pretending a higher amount during these experiments as was actually produced.

**LiP415MNP crystals and structure**

Crystals of fungal LiP415 were obtained with 30% ammonium sulfate, 0.1 M sodium citrate pH 4.0 as precipitant using micro and macro seeding techniques (Choinowski, 1996). Those of LiP415MNP were obtained from MNP/H₂O₂-treated LiP415 by mixing 10 mg/ml protein in 5 mM sodium tartrate pH 4.0 with the same precipitant. Since de novo crystals could not be obtained, micro crystals from smashed LiP415 crystals were used as seeds. Room temperature diffraction data from a 0.5 · 0.3 · 0.2 mm crystal were collected at the beamline BW7B of the EMBL outstation in Hamburg with the following quality (numbers in brackets are for the outmost of 20 resolution bins): resolution 18-1.9 Å (1.93-1.9 Å), I/σ(I) 14.9 (5.7), redundancy 4.5, completeness 81% (72%), Rsym 0.059 (0.15).

The complete LiP415 structure (pdb entry 1lhp) was used as an almost perfect starting model. After 15 cycles with REFMAC the refinement had essentially converged. Then a nitrogen atom was placed into the rest density near the indole C6 of Trp171 and a few water molecules in the Trp171 vicinity were remodelled. Then ten more refinement cycles were carried out with the van der Waals cutoff radius in PROTIN set to 0.1.
2.2. Results and Discussion

2.2.1 Redox mediation with fungal and recombinant lignin peroxidases

In 1985 it was proposed that veratryl alcohol (VA) mediates the oxidation of lignin (Schoemaker et al., 1985; Harvey et al., 1986). This was based on the observation that in the presence of catalytic amounts of VA or 1,4-dimethoxybenzene LiP oxidised the monomethoxylated compounds p-methoxy mandelic acid (MMA) and anisyl alcohol, which are poor substrates for LiP in the absence of VA, to the corresponding benzaldehydes. The redox potential of the aryl radical cation/arene redox couples decreases with increasing number of methoxy groups. Consequently, VA$^{+*}$ can oxidise compounds with higher redox potentials than his own. In the case of MMA the overall driving force must stem from the subsequent C$_{\alpha}$-C$_{\beta}$ bond cleavage of the MMA radical cation resulting in the exergonic liberation of carbon dioxide (see Scheme 2.1 for a structural representation). With the determination of the crystal structures of LiP (Piontek et al., 1993; Edwards et al., 1993) the prime point of interest became the way by which the enzyme interacts with VA to accomplish redox mediation. The presence of a carbohydrate moiety close to a molecule of VA modelled into the active site channel of the LiP crystal structure led to the proposal that VA$^{+*}$ could be stabilised by the nucleophilic attack of a carbohydrate hydroxy group at a phenyl carbon of VA$^{+*}$. The so formed cyclohexadienyl radical-sugar adduct would then diffuse into the solvent where VA$^{+*}$ would be regenerated and react with bulky substrates like lignin (Schoemaker et al., 1994; Schoemaker and Piontek, 1996). Thus, the essential involvement of a carbohydrate for the function of the enzyme was predicted.

Scheme 2.1 Oxidation of 4-methoxy mandelic acid by LiP-generated veratryl alcohol cation radical to anis aldehyde.
With the availability of recombinant LiP expressed in *E. coli* it became possible to test this hypothesis because *E. coli* does not produce glycosylated proteins. As a test case the mediation of the LiP oxidation of MMA by VA was chosen. VA was incubated with fungal or recombinant LiP and H₂O₂ in the absence and presence of MMA and the reaction mixture was analysed by reverse phase HPLC as it had been originally done by Harvey et al. (1986). The results of Figure 2.1 a) and b) using fungal glycosylated LiP465 are essentially a confirmation of what was done by these authors. In the absence of MMA the formation of veratryl aldehyde (VAld) from VA is observed (Figure 2.1 panel a). If MMA is present only anis aldehyde, the product of MMA oxidation, is formed but no VAld (panel b) in Figure 2.1) although MMA has a higher redox potential than VA. In panel c) the conditions were as those of panel b) but fungal LiP was replaced by recombinant and therefore not glycosylated LiP. The pattern is qualitatively the same as that obtained with the fungal enzyme. Similar experiments carried out with various VA/MMA ratios (data not shown) can be summarised as follows: As long as there is still MMA present in the reaction mixture no VAld is produced. VAld formation starts only after all MMA has been consumed and transformed to anis aldehyde.

These results are not compatible with the prediction of the above cited hypothesis that a sugar chain of fungal LiP is an essential component for efficient redox mediation by VA.
2.2.2 Chemical detection of the Trp171 hydroxylation

Based on the crystal structures of two LiP isozymes isolated from the extracellular medium of *P. chrysosporium* evidence was found for a Cβ hydroxylation of Trp171 (Choinowski, 1996). To test this finding chemically, LiP465 was denatured, reduced, alkylated and trypsin digested. The resulting peptide mixture was then analysed by reverse 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 non conserved tyrosine. From the sequence (deBoer et al., 1987), all four residues reside on different tryptic peptides. Figure 2.2 shows a typical chromatogram carried out at 60 °C.

N-terminal partial sequencing was performed to identify the peptides with respect to the known primary structure (deBoer et al., 1987). 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 2.3, 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
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Figure 2.3 Absorbance spectra of peptides from the chromatogram in Figure 2.2 and of a 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). The spectra are scaled arbitrarily.

The diode array detector of the HPLC system, showed the same λₘₐₓ = 333 nm and shape as that of the model compound N-acetyl-α,β-didehydrotryptophanamide (Figure 2.3). 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 2.4 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 2.4). Hydroxy groups in α-position to conjugated systems are known to eliminate easily if a β-hydrogen is also present driven by the formation of a more delocalised electron system (March, 1992). The two-step reaction sequence depicted in Scheme 2.2 is proposed for the case of a β-hydroxytryptophan, both of which can be acid or base-catalysed. 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 tautomerisation 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 (Noda et al., 1978).
Figure 2.4 Course of 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.

Scheme 2.2 Proposed mechanism of water elimination from peptide bound β-hydroxytryptophan.

Taken together and in accordance with the crystal structures (Choinowski, 1996 and this study, chapter 3) 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 here 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 ε_{333}= 19.6 mM\(^{-1}\)cm\(^{-1}\) (Noda et al., 1978). 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 structures. Analogous results were obtained for a second isozyme (LiP415, results not shown) and with enzyme from several independent cultivations of the fungus/protein purifications.
2.2.3 Hydroxylation of Trp171 in recombinant LiPH8*

In contrast to fungal LiP isozymes which have undergone numerous turnover cycles in the culture medium from which they are purified, LiPH8* can be investigated in a pristine state. When LiPH8* was digested and analysed in the same way as LiP465 above, no absorbance above 300 nm could be detected in any tryptic peptide (Figure 2.5 trace C). This finding excludes the possibility that the 333 nm absorbance recorded with the fungal enzyme was an artefact of the applied procedure. Since LiPH8* 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 (Tien and Tu, 1987) by partial Edman sequencing. The peptide at 76 min contains Trp17 and the one at 77.5 min Trp171 (Figure 2.5). The time window in Figure 2.5 was chosen so as to show the 70 - 80 minute area in detail and the Trp251 containing peptide eluting at 39 min is omitted.

LiPH8* 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 one equiv. of hydrogen
Figure 2.6 Formation of Cβ hydroxylation at Trp171 of LiPH8* on addition of hydrogen peroxide. The reactions with H₂O₂ 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 Materials and Methods.

peroxide led to a significant 333 nm signal from the Trp171 containing peptide (compare Figure 2.5 traces D and E). Its absorbance was identical to spectrum 1 in Figure 2.3. This suggests that the hydroxylation is a H₂O₂-induced autocatalytic process. Figure 2.6 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 H₂O₂ leads to the hydroxylation of about 60% of the enzyme molecules and 3 equiv. are enough for completion. Addition of the substrate VA to the reaction mixture, however, competed with the self oxidation but could not prevent it (Figure 2.6). 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 or compound III formation 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 2.6. 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.

In order to study whether the hydroxylation of Trp171 is oxygen-dependent, the treatment of LiPH8* with H₂O₂ was carried out anaerobically. However, no difference in the amount of Trp171 hydroxylation could be detected compared to the aerobic control in any case (no data shown).
These results show that the autocatalytic modification of Trp171 is very efficient and specific requiring only a small excess of H$_2$O$_2$ (2-3 equivalents, Figure 2.6) or a quite small number of turnover cycles. It follows that, in a typical activity assay of pristine LiP*, the enzyme is modified during the first few turnovers so that one effectively obtains the activity of the hydroxylated enzyme form. The ligninolytic medium from which fungal LiP is purified contains also a H$_2$O$_2$-generating system and VA (Kersten, 1990). This H$_2$O$_2$ 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.

2.2.4 Chemical modification using $N$-bromo succinimide

Activity and fluorescence behaviour of NBS-treated LiP

It is possible that Trp171 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 chemical modification of the fungal enzyme was undertaken using the tryptophan-specific agent $N$-bromo succinimide (NBS) since mutant enzymes were not yet available (see Scheme 2.3 for the mechanism of NBS modification). 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 2.7 A shows the decreasing activity of fungal LiP465 depending on increasing amounts of NBS. The activity changes most between a 10- and 25-fold molar excess to reach a constant value of $\approx15\%$ residual activity at $\approx30$ fold excess of reagent. The change in activity is accompanied by a diminishing tryptophan fluorescence (Figure 2.7) in accordance with oxindoles being non fluorescent. Five equiv. of NBS leads to the loss of about 1/3 of the initial fluorescence intensity at 315 nm while the activity is only slightly affected. We attribute this to the modification of the surface residue Trp17 that is about 20 Å

![Scheme 2.3 Modification of indole derivatives by $N$-bromo succinimide to oxindoles.](image-url)
Figure 2.7 Chemical modification of LiP465 by N-bromo succinimide (NBS) in sodium acetate pH 4.5.

(A) Dependence of the LiP activity on increasing amounts of NBS determined in the standard VA assay.

(B) Effect of NBS treatment of LiP465 on the tryptophan fluorescence at 315 and 343 nm. The resulting oxindoles are non-fluorescent.

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 in the HPLC eluent of the NBS-treated LiP (compare Figure 2.8): i) the peptide of the buried Trp251 did not show any significant change of its 280 nm absorbance as compared to non-treated enzyme. ii) the peptides containing the two solvent accessible tryptophans revealed a marked decrease in their 280 nm signals since the absorbance of oxindoles is only 30% of that of tryptophan at this wavelength. (iii) the 333 nm signals characteristic for the α,β-didehydro derivative of Trp171 were essentially gone. These data indicate that a tryptophan is important at least for the oxidation of VA.

Since the emission characteristics of tryptophan depend on the particular environment the pattern of the fluorescence decrease at 343 nm in Figure 2.7 B is somewhat different. The buried Trp251 will contribute only weakly to the fluorescence at this wavelength because tryptophans in the hydrophobic medium of the protein interior emit at shorter wavelengths.
Figure 2.8 Comparison of HPLC tryptic peptide maps of native LiP465 (a) and LiP465 treated with 30 equiv. N-bromo succinimide (b). The separation was carried out at 60 °C and the elution was followed at 280 nm.

This dependence of the emission maximum on the electrostatic response of the medium is due to the fact that the excited states of indoles have a larger dipole moment than the ground state (Callis, 1997). The fluorescence of Trp171 is not very intense either due to effective resonance energy transfer to the nearby heme (see e.g. Chen and Barkley, 1998 for a recent review on tryptophan fluorescence and quenching mechanisms). Consequently, Trp17 alone accounts for about two thirds of the initial emission intensity at 343 nm and only about 10 %, attributed to Trp251, remain after the modification of Trp17 and Trp171.

Kinetic analysis of NBS-treated LiP

At this point, the mechanistically interesting question arose whether the effect of NBS on LiP could be attributed to one of the reductive steps in the turnover cycle, namely compound I or compound II reduction. The second order rates of compound I reduction (k_2) by VA of native LiP465 and LiP465 pretreated with 30 equiv. NBS (NBS-LiP) were determined by the stopped-flow method under pseudo-first order conditions (Figure 2.9, left). In single-mix experiments the enzyme was shot against a mixture of H_2O_2 and VA in buffer. An excess of H_2O_2 was employed to render compound I formation and its subsequent reduction sufficiently separated. This condition was reasonably fulfilled up to a concentration of about 50 μM VA at 100 μM H_2O_2. k_{obs} at 70 μM VA for native LiP is already slightly biased because compound I formation is partially rate-limiting. From the data compiled in Table 2.1 a 14-fold decrease in the second-order rate of compound I reduction upon NBS-treatment of the enzyme is calculated indicating an effect on binding of VA.

The second order rates of compound I formation (k_1) were not determined explicitly by varying the H_2O_2 concentrations but roughly estimated at a single one using k_{obs} = k_1[H_2O_2]. No difference in k_1 between native LiP and NBS-LiP was detected indicating that NBS-
Figure 2.9 Kinetics of native and NBS-treated (30 equiv.) LiP465 with VA at pH 4.5 and 20 °C. Left: rates of compound I reduction from pre-steady-state kinetics. The straight lines were obtained by linear regressions. Right: steady-state kinetics at saturating H2O2 concentration. The lines are hyperbolic fits to the data points.

Table 2.1 Kinetic comparison of native and NBS-treated (30 equiv.) LiP465 at 20 °C pH 4.5. $k_1$ is the second order rate of compound I formation calculated from the pseudo-first order rate at 100 μM H2O2 assuming irreversible second order kinetics. $k_2$ is the second order rate of compound I reduction by VA. $k_{cat}$ and $K_M$ are steady-state parameters. The activity with ferro-cyt c as substrate was determined with 16 μM ferro-cyt c, 120 μM H2O2 in 10 mM sodium succinate pH 5.

<table>
<thead>
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<th></th>
<th>$k_1 \times 10^5$ M$^{-1}$s$^{-1}$</th>
<th>$k_2 \times 10^5$ s$^{-1}$</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>activity with cyt c (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native LiP465</td>
<td>6.9 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>0.12 ± 0.01</td>
<td>2.7 ± 0.14</td>
</tr>
<tr>
<td>NBS-LiP465</td>
<td>6.8 ± 0.3</td>
<td>0.20 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>2.0 ± 0.15</td>
</tr>
</tbody>
</table>

Since the rate-limiting step in the turnover of LiP is compound II reduction, these rates can be approximated from the steady-state kinetic parameter $k_{cat}$ (Wariishi and Gold, 1989; Chung and Aust, 1995). The plot of the initial turnover rates against VA concentration show saturation behaviour and the steady-state parameters $k_{cat}$ and $K_M$ were estimated by fitting the data points to a hyperbolic function (Figure 2.9, right). Table 2.1 lists the steady-state parameters for native and NBS-treated LiP465. Whereas the $K_M$ values differ only insignificantly, the turnover number of NBS-LiP is about 6-fold smaller than that of the native treatment does not perturb the distal heme pocket where the catalytic processes leading to compound I take place.
enzyme suggesting an effect of NBS on the electron transfer properties of LiP. The monomolecular step at saturating VA concentration has been attributed to electron transfer from the substrate to the oxo-ferryl heme of compound II (Khindaria et al., 1995b).

The enzyme concentration is a critical quantity for the determination of turnover numbers. Therefore it had to be ruled out that the reduced activity observed with NBS-LiP was the result of an unspecific destruction of a fraction of the enzyme by NBS and that another fraction was still fully active. For this purpose, a substrate was sought whose oxidation would not depend on NBS-treatment of LiP, e.g. because a mechanism not involving Trp171 would be employed. Cytochrome c (cyt c) was chosen which had been introduced as a substrate for LiP by Wariishi et al. (1994). Because of its low redox potential of 0.2 V one could expect that a mechanism distinct from that of VA oxidation would be employed. In particular, electron transfer over quite long distances will be feasible with cyt c due to a large driving force. The rates of ferro-cyt c oxidation from Table 2.1 differ only by a factor of 1.4 for native and NBS-treated LiP indicating that the 6-fold decrease observed with VA cannot be explained by a total inactivation of a fraction of the enzyme caused by NBS.

In conclusion, the chemical modification of LiP465 with NBS, a reagent rather specific for tryptophan, provided evidence that Trp171 is important for both the reduction of LiP compound I and compound II. From these data it could be argued that Trp171 is not essential for the oxidation of VA because the catalytic activity of the enzyme is not entirely lost upon NBS treatment. The reason for this behaviour is not known. The possibility exists that the oxindole derivative of Trp171 is partially able to carry out the function of Trp171. However, more compelling results regarding the functionality of Trp171 in the catalytic cycle of LiP are presented in the next section from mutant data.

2.2.5 Kinetic analysis of recombinant enzyme variants

Lifetime of compound I.

Whereas compound I of most heme peroxidases is fairly stable the absorbance spectrum of LiP compound I transforms within seconds to a typical compound II-like spectrum (Tien et al., 1986; Harvey et al., 1989). The source of the electron which brings about this reduction of the heme has remained unknown. Our investigation of the Trp171 modification led us to assume an electron transfer from Trp171 to the oxidised heme as the process setting off the sequence of reactions leading to hydroxylation (see discussion in chapter 4.1). This would implicate Trp171 as a possible electron donor which could also be
Figure 2.10 Spontaneous compound I decay of LiPH8* variants at pH 4.5 in the stopped-flow. Compound I was rapidly formed by mixing resting state enzyme with 200 μM H2O2 and compound II formation was followed at 426 nm. The residuals of exponential fits are also shown (experimental data minus fit).

A) pristine LiPH8*. The trace was fitted biexponentially.

B) W171S mutant of LiPH8*, fitted single exponentially.

C) LiPH8* pretreated with 3 equiv. of H2O2, biexponential fit.

responsible for the short lifetime of compound I. With the recombinant enzyme and a Trp171 mutant at our disposal the hypothesis of Trp171 being an intramolecular reductant for LiPI became checkable.

Compound I was quickly created by mixing resting state enzyme with a large excess of 200 μM H2O2 (all concentrations in stopped-flow experiments are after mixing) at pH 4.5 and its spontaneous conversion to compound II was followed using the stopped-flow technique. Figure 2.10 A presents the time course observed in a typical experiment using pristine LiPH8* followed at 426 nm, an isosbestic point of compound I and ferric enzyme (Koduri et al., 1996). From a biexponential fit first order rates of 5.3 and 0.91 s⁻¹ (Table 2.2) were obtained for the fast and the slow phase, respectively. The fast one accounts for ≈60% of the absorbance change. The residuals between the observed trace and the fit function are also displayed in Figure 2.10. The traces are somewhat noisy but systematic deviations are not
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<table>
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<tr>
<th></th>
<th>compound I decay (s⁻¹)</th>
<th>turnover number (s⁻¹)</th>
<th>K_M (µM)</th>
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<tbody>
<tr>
<td>pristine LiPH8⁺</td>
<td>k_f = 5.3 ± 0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>k_s = 0.91 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LiPH8⁺(H₂O₂)</td>
<td>k_f = 3.7 ± 0.5</td>
<td>25.3 ± 1.5</td>
<td>182 ± 15</td>
</tr>
<tr>
<td></td>
<td>k_s = 0.63 ± 0.03</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>W171S LiPH8⁺</td>
<td>k = 0.40 ± 0.04</td>
<td>&lt; 0.02</td>
<td>—</td>
</tr>
<tr>
<td>LiP465</td>
<td>not determined</td>
<td>27 ± 1.5</td>
<td>152 ± 15</td>
</tr>
</tbody>
</table>

Table 2.2 Rates of spontaneous compound I decay (in the absence of reducing substrates) and steady-state parameters of LiP variants at 25 °C. k_f denotes the fast phase and k_s the slow one. For pristine LiPH8⁺ no steady-state parameters exist because the enzyme is hydroxylated during the first few turnover cycles of an activity assay (compare chapter 2.2.3). Rates of compound I decay were determined at pH 4.5 in 5 mM citrate, 5 mM phosphate buffer of ionic strength 50 mM. Steady-state data of LiPH8⁺ forms were obtained in the same buffer but at pH 3.0. LiP465 data are from 100 mM sodium tartrate buffer pH 3.0.

recognisable. Compound II formed in this reaction is much more stable than compound I. Hence, the 426 nm traces are not biased by a potential backformation of the resting state enzyme as was confirmed by a 397 nm trace of this reaction (trace not shown but compare the results of the next section and Figure 2.12). Then the same experiment was carried out with the W171S mutant (Figure 2.10 B). In this case the course of LiPI decay allowed a single-exponential fit with a rate of 0.4 s⁻¹. The residuals reveal slight systematic deviations but these are not significantly above the overall noise level. Thus replacement of the redox active tryptophan by an inert serine leads to a thirteen-fold longer half-life of compound I providing strong evidence that Trp171 can act as an endogeneous reductant for LiP compound I. Comparable rates which are roughly half of those reported here were obtained at pH 4.0 (Doyle et al., 1998).

Analogous experiments were also done with LiPH8⁺ pretreated with three equiv. H₂O₂ which leads to an enzyme variant hydroxylated at Trp171. The trace of Figure 2.10 C had to be fit biexponentially with rates of 3.7 and 0.63 s⁻¹ and the fast phase accounted for ~40% of the absorbance change. Both rates are smaller than those obtained with the pristine enzyme but only about 30%. It had been anticipated that the presence of the Trp171 Cβ hydroxy group would lead to a somewhat increased redox potential of the indole system which would be reflected by a slower electron transfer rate from Trp171 to the heme of compound I.
Figure 2.11 Compound I reduction of W171S LiPH8* by VA at pH 4.5 monitored at 417 nm. One syringe of the stopped-flow contained the enzyme and the other one 400 μM H₂O₂ and 20, 80 or 160 μM VA. See Table 2.3 for the rates obtained from single exponential fits. Traces are offset for display purposes.

Table 2.3 Rates of compound I reduction of W171S LiPH8* by different concentrations of VA at pH 4.5 and 25 °C. See legend of Figure 2.11 for experimental details.

<table>
<thead>
<tr>
<th>[VA] (mM)</th>
<th>rate (s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>0.01</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>0.04</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>0.08</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.51 ± 0.02</td>
</tr>
</tbody>
</table>

the compound I lifetimes of pristine and processed LiP differ with respect to the experimental error limits of the experiments, the effect is not pronounced enough to call for a functional interpretation. kcat of LiPH8* under the same conditions is 4.5 s⁻¹ (Doyle et al., 1998). The spontaneous compound I decay rate of 3.7 s⁻¹ is therefore slightly too small to be catalytically competent. At pH 4.0 the discrepancy is even more pronounced (Doyle et al., 1998). It follows that during turnover with VA a substrate-induced rate acceleration of the electron transfer to the heme must take place.

Reactivity of the W171S variant of LiPH8*

Initial velocities of VAld formation by LiPH8* with increasing VA concentrations exhibit saturation behaviour as observed with fungal enzymes and the steady-state parameters kcat and KM(VA) were determined to be 25 s⁻¹ and 182 μM at pH 3.0. These values compare well with those of the fungal enzyme LiP465 of 27 s⁻¹ and 152 μM (Table 2.2), respectively, at the same pH but different buffer conditions. When the same experiments were carried out with the W171S mutant of LiPH8* the activity was below the detection limit of about 0.02 s⁻¹ even at 4 mM VA (Table 2.2). Although an effect of the Trp171 mutation on the VA oxidase activity had been expected from the chemical modification experiments these striking results showing an at least 1000 fold reduced activity were surprising. Remarkably, the activity with the two non-physiological negatively-charged dye substrates ABTS and DFAD (see Figure 3.16 for structures) was only slightly affected by this mutation (see the values listed in Table 3.6 and Doyle et al., 1998). This finding established unambiguously the essential requirement
for a tryptophan at position 171 for the oxidation of the natural substrate VA and suggests this residue to be a substrate interaction site.

The question came up whether both high oxidation states of W171S LiP, compound I and II are inactive against VA or only one of them. Compound I reactivity was again determined using a stopped-flow instrument. In the single-mix mode ferric W171S LiP was reacted with a mixture of 200 μM H₂O₂ and various concentrations of VA and the reaction was followed at 417 nm, a wavelength reported to be an isosbestic of ferric enzyme and compound II (Khindaria et al., 1995b). The rates obtained from single-exponential fits of the traces from Figure 2.11 are compiled in Table 2.2 and some reaction traces are shown (Figure 2.11). Irrespective of the VA concentration they are all virtually the same and match the spontaneous compound I decay rate of this mutant in the absence of a reducing substrate. Without doubt, compound I of the W171S mutant is completely inert against VA. At the high VA concentration of 2 mM the decay rate of LiPI increases slightly to 0.5 s⁻¹. This small effect can be regarded as insignificant and is more readily explained by a tiny fraction of reductive impurity of VA than by the action of VA itself (Koduri and Tien, 1994). For comparison, one can calculate the rates of LiPI reduction in the wild-type enzyme: using the second order rate of 2.8·10⁵ M⁻¹s⁻¹ determined in chapter 2.4 with LiP465 at pH 4.5 and 20 °C rates of 2.8, 11 and 22 s⁻¹ are obtained for the three VA concentrations of Figure 2.11.

To check the reactivity of compound II of W171S LiP with VA, the enzyme was reacted with a mixture of 10 equiv. H₂O₂ and 2 mM VA in the stopped-flow. Figure 2.12 compares the traces at 397 and 426 nm with those in the absence of VA. Under these conditions, compound I formation was completed within about one second as demonstrated by the 397 nm trace. The trace at the isosbestic point of compound I and II (397 nm) implies that, once compound I is formed, no ferric enzyme is received back from one of the high oxidation states within the time frame of Figure 2.12. Therefore, the traces at 426 nm are a direct measure of the ratio of LiPI to LiPII. Both in the absence and in the presence of VA accumulation of compound II was observed although in the latter case a little faster. This must be due to the above mentioned impurity. If compound II reacted with VA at a rate
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comparable to compound I decay, the enzyme would enter a second turnover cycle and an accumulation or a higher fraction of compound I would be expected. Because the enzyme fraction in the compound II state is always higher in the presence of VA, compound II must be inactive against VA or at least react slowly as compared to the rate of spontaneous compound I decay. The experiments of Figure 2.12 must therefore be true single turnover ones. As a more quantitative supplement to this somewhat qualitative result it is recalled that the activity of W171S LiPH8* was below the detection limit of 0.02 s⁻¹. Since compound II reduction is the rate-limiting step in turnover one can compare the latter number with the turnover number of the wild-type enzyme at pH 4.5 of about 4.5 s⁻¹ (Doyle et al., 1998) to get an at least 200-fold decreased rate of compound II reduction in the mutant. If compound II was fully active and compound I not at all one could expect a turnover number in the range of 0.4 s⁻¹, which is the rate of the spontaneous compound I decay.

To sum it up, the activity of the W171S mutant of LiPH8* is inactive against VA and both rates of compound I and II reduction are negligible. The fact that the rate of compound I formation is unchanged and that the activity versus artificial substrates is only slightly affected indicates that catalytically important moieties other than Trp171 are basically unperturbed in the Trp171 mutant (Doyle et al., 1998).

2.2.6 Detection of a transient radical on Trp171

The results of the previous paragraph have provided evidence that an electron abstracted from Trp171 is responsible for the short lifetime of LiPI. A Trp171 radical cation is also postulated to be the first intermediate in the sequence of reactions leading to the hydroxylation at its Cβ (see chapter 4.1). In addition, it is well conceivable that the indole moiety of Trp171 is involved also in the general redox/radical chemistry of catalysis. An independent observation of a Trp171 radical would therefore reinforce the aforementioned points.

ESR spectroscopy is the only analytical technique which allows the direct observation of radicals. Since the late sixties it has been complemented by spin-trapping, which makes use of a longer lifetime of the secondary radical obtained by reaction of the primary radical with a trapping agent (see Janzen, 1980 for a review). More recently, with the availability of soft ionisation methods like ESI and MALDI, the use of mass spectroscopy as a very sensitive tool to analyse spin-trapped protein fragments has been introduced, which has the ability to replace, at least in part, ESR spectroscopy (see e. g. Fenwick and English, 1996; Deterding et al., 1998).

In the present study, yet another approach has been taken that is based on the uv/vis absorption properties of the spin-adduct or a resulting stable reaction product. LiP465 was
incubated with the spin-trap methyl nitroso propane (MNP) and H₂O₂ and then tryptically digested and analysed by HPLC similar to what had been done for the detection of the Cβ hydroxylation of Trp171. In initial experiments no changes in the peptide maps could be detected. By observing the absorbance of the enzyme during MNP/H₂O₂ incubation it was found that MNP supports the turnover of LiP465 by acting as a moderate reducing substrate (data not shown). Since the redox potential of the MNP⁺/MNP couple is about 2 V (vs NHE, McIntire et al., 1980) this reductive effect is probably not due to the MNP monomer in the ground state. Possible alternatives are traces of the MNP dimer which is hardly soluble in water and the MNP excited state which is much more redox active (Eberson, 1998). Conditions were therefore devised that would render the desired trapping reaction more important and at the same time suppress deactivation of the enzyme due to a too high excess of H₂O₂. About 5 equiv. of peroxide were simply added 5 to 10 times to the LiP/MNP mixture in 5 minute intervals.

Peptide maps from this enzyme preparation revealed peaks with absorbance characteristics different from those obtained when either H₂O₂ or MNP were left out indicating that a trapping reaction had occurred. Figure 2.13 demonstrates that the peak with the most intense 333 nm absorbance from the native protein (marked with 1) is weakened upon MNP/H₂O₂ treatment and that instead another peak eluting at 72.6 min (marked with 2) is formed whose absorbance extends into the yellow region of the spectrum (Figure 2.14). The fraction of this peak was collected and partial sequencing identified it as the Trp171 containing tryptic peptide. Since Trp171 is the most redox active amino acid of this peptide, it was presumed to be the trapped residue. Support for this assignment comes from the following observation: a compound with an absorbance spectrum strikingly similar to that of the spin-trapped Trp171 containing tryptic peptide can be generated by treating the tryptophan model compound N-acetyl tryptophanamide with MNP and a one-electron oxidant consisting of LiP, VA and H₂O₂ (Figure 2.14). This compound, whose formation was absolutely
dependent on the presence of N-acetyl tryptophanamide and MNP, could be purified on a very low scale from the reaction mixtures of these experiments by column chromatography and a uv/vis spectrum of the pure compound was obtained (spectrum 3 in Figure 2.14, see Materials and Methods).

Unfortunately however, not enough of this spin-adduct model could be prepared by the used method to allow a chemical structure elucidation. The structure of the chromophore resulting from MNP spin-trapping with tryptophyl radicals is therefore still unknown and, apparently, such a study has not been published yet. However, from the similarities between the absorbances from the trapping reaction with LiP and with the tryptophan model it is very likely that the two products share the same or a very similar chromophore. This result suggests that Trp171 indeed harbours a transient radical after the oxidation of LiP with H₂O₂. Since the model compound N-acetyl tryptophanamide is not hydroxylated at its Cβ, the hydroxy group of Trp171 is probably not a major determinant of the absorbance characteristics of the Trp171-spin trap adduct, but this issue requires further investigation.

An attempt was undertaken to detect the spin-trapped Trp171 in a protein crystal structure, referred to as LiP415MNP. After the trapping reaction LiP415 was crystallised and diffraction data were collected up to 1.9 Å (see Materials and Methods for details). After some refinement cycles using phases from the original LiP415 structure, electron density maps in the Trp171 vicinity were inspected. The 3Fo-2Fc map at Trp171 shown in Figure 2.15 is of good quality. In a difference map contoured at 3.5 σ a relatively weak spherical density close to the 6-position of the indole ring was found within the indole plane.
Figure 2.15 Electron density at Trp171 in the crystal structure of MNP/H\textsubscript{2}O\textsubscript{2}-treated LiP415 superimposed on β-hydroxy-Trp171. The thin-lined density is a 3Fo-2Fc map contoured at 3 σ. The thick-lined one is a difference map contoured at 3.5 σ. When this density was modelled by a nitrogen atom and refined, the distance to the indole C6 refined to 1.5 Å and the B-factor to =40 Å\textsuperscript{2}.

Since this particular density is not present in maps from untreated LiP415 (data not shown) it was supposed to be due to the spin trap. MNP is thought to trap radicals by bond formation with its nitroso nitrogen (Lion et al., 1982). Therefore a nitrogen atom was modelled into the density and refined. The B-factor of the N atom rose to about 40 Å\textsuperscript{2} indicating an occupancy of roughly 30-40% and the distance refined to 1.51 Å. The remaining parts of MNP appear to be disordered which, in combination with only partial occupancy, makes it impossible to identify the whole group attached to the 6 position of Trp171. It should, however, be pointed out that this is the first time to our knowledge that a spin-trapping reaction product could be detected by protein crystallography.
The C3 of the indolyl radical cation is generally believed to be the prime target of a spin-trap like MNP or dioxygen (Gunther et al., 1995; DeGray et al., 1997), since this is the site of the highest spin density in trytophan radicals and their cations both experimentally (Huyett et al., 1996) and theoretically (Jensen et al., 1996; Himo and Eriksson, 1997). In LiP the positions of the pyrrole moiety of the Trp171 indole ring are hardly accessible from the solvent. In contrast, those of the benzene moiety are (Figure 1.6) and here the highest spin-densities are calculated at the 4 and 6 positions. Figure 2.16 gives the numbering of the indole atoms and the spin densities of the 3-methyl indolyl radical cation (taken from Jensen et al., 1996). Evidently, the density in the LiP415MNP structure is found at a site that combines good accessibilty for a reactant with high spin-density of the Trp171 radical cation.

The first step of the trapping reaction is supposed to be coupling of MNP to the indole C6 of Trp171 radical cation 1 in Scheme 2.4 to form the aminoxyl radical 2 which has a tetrahedral indole C6 atom. As nitric oxide derivatives aminoxyl radicals are relatively stable and that is exactly why they were introduced as trapping agents. In general, however, they are not stable enough to allow chromatographic purifications to be carried out (Makino et al., 1985; Fenwick and English, 1996). In particular, aminoxyl radicals are much more redox active than the parent nitroso compound due to the unpaired electron and under oxidising conditions as in the presence of LiP/H₂O₂ they will be oxidised to the corresponding nitrosonium ions. As an example, the redox potential of the di-²butynitroxyl/di-²butynitrosonium couple is 0.81 V (vs NHE; Eberson, 1998) which is about 1.2 V less than that for the oxidation of MNP.

Therefore it is assumed that the described yellow colour (Figure 2.14) is not due to the initially formed aminoxyl radical spin-adduct but to a stable consecutive reaction product. At present, one can only speculate on these consecutive reactions leading to the observed

![Scheme 2.4 Proposed mechanism of spin-trapping of a tryptophyl radical cation by MNP and subsequent formation of possible stable end products.](image-url)
chromophore. However, as discussed above, the aminoxyl radical is likely to be oxidised to the nitrosonium ion 3. Another stabilisation reaction could be the deprotonation and rearomatisation of 3 to give 4 which could explain the observation that the electron density at C6 is in the plane of the indole ring. Quaternary nitrogens are good leaving groups in nucleophilic substitutions. Therefore, water could substitute the nitrosonium moiety at the central butyl carbon to form 4-butyl alcohol and 6-nitroso tryptophan 5. 5 or the 6-nitro derivative 6 could be stable end products. Obviously, these considerations should be substantiated by further work. Helpful experiments would be the mass-spectroscopical determination of the weight of the spin-trapped Trp171-containing peptide and the chemical structure elucidation of the trapped tryptophan model compound.
3. Crystal structures of recombinant LiP variants

3.1 Materials and Methods

3.1.1 Crystallisation and data collection

The hanging drop vapour diffusion method was used throughout for crystallisation with plastic tissue culture plates (Linbro) whose wells were sealed with siliconised cover slips and grease. 0.5 -1 ml of precipitant solution was equilibrated against a 1:1 mixture of precipitant and protein solution, each 3-5 µl in volume.

Recombinant LiP enzyme forms were provided by Dr. A. T. Smith at a concentration of 8 mg/ml in 10 mM sodium succinate pH 6. Neither any of the Hampton Research crystal screen I or II conditions nor excessive and systematic variation of ammonium sulfate conditions which are successful with LiP415 yielded crystals of LiP*. The first LiP* crystals were obtained after changing the protein buffer to 5 mM sodium tartrate pH 4.5 and with 10 % PEG 1000, 10 % PEG 8000. All subsequent native LiP* crystals used for data collection were obtained with protein in the initial succinate buffer and with 17 % PEG 6000 (Microselect quality from Fluka) at 20 °C. These crystals grew readily de novo and reached their full size, well-suited for data collection, within about four days. Considerable amounts of precipitated protein always formed initially but redissolved while the crystals were growing. In 23% PEG 6000 the crystals could be washed. About half of them were twinned (see Figure 3.1). Four weeks old crystals stored in hanging drops progressively deteriorated by shrinking and growing pale. Therefore, only up to two weeks old crystals were used for data collection. Mounted in sealed capillaries, they were stable for several months.

The [H82P:P83A:E146G] triple mutant LiP*-tm was crystallised with a precipitant that was obtained by mixing 50% PEG 6000, 35 mM sodium succinate pH 4.5 and water to final concentrations of 17% PEG, 20 mM sodium succinate to give a final pH of 4.0 (see below). The drops became very turbid and small crystals were visible after about one week. These conditions were difficult to reproduce and only 2 crystals of this LiP variant were obtained in total. The precipitant used for the wild-type enzyme was unsuccessful.

A peculiarity of the PEG 6000 (Fluka, 50% aqueous solution) used should be mentioned: in contrast to what is stated in the catalogue of this company, the pH was not neutral but acidic, e.g. a 17 % aqueous solution had a pHe of 3.1.

All data were collected at 20 °C with synchrotron radiation on 30 cm MAR Research image plates. Data were processed with DENZO and scaled and merged with SCALEPACK (Otwinowski, 1993).
3.1.2 Determination of the LiP*-H₂O₂ structure

Most calculations were done on Indigo 2 workstations (Silicon Graphics) using programs from the CCP4 package (1994) unless stated otherwise. The program CHAIN (Sack, 1988) was used for model building and visualising molecular structures.

Solution of the phase problem with molecular replacement

The structure determination was started with the data from the LiP*-H₂O₂ crystals. LiP415 was taken as a starting model for LiP* because of the high sequence identity of these isozymes of 91%. Sugars were removed from the LiP415 model and 21 side chains which are different in the two proteins were chopped down to alanines. The program AMORE was used for molecular replacement calculations. Two molecules were expected in the asymmetric unit corresponding to a V_M value of about 2.6 Å³/Da and, accordingly, two maxima of similar height of the cross rotation function. Indeed, two peaks were found in the resolution range 12 - 4 Å by the rating routine of AMORE with correlation coefficients of 11.1. The next best solution had a correlation coefficient of 9.1 and this discrimination could be improved by extending the resolution range. The ten highest peaks of the rotation function were then subjected to a translation search in a resolution range of 12 - 4 Å. Correlation coefficients of 27.1 (R=0.478) and 32.1 (R=0.467) were obtained for the two most promising peaks of the translation function compared to values of 17.7 (R=0.507) and 20.2 (R=0.506) for the next best translation functions, respectively. Again, the discrimination was good suggesting that the molecular replacement problem had been solved correctly. Fixing one of the translation solutions and searching for the other one confirmed the result and yielded correlation coefficients of 58.2 (R=0.369) and 62.2 (R=0.354) up to 4.5 Å resolution. No major overlaps between the two molecules in the asymmetric unit and between these molecules and crystallographically related ones were found. A final rigid body refinement in AMORE up to 3.8 Å improved the correlation of the model with the data to 72.6 % (R=0.299).

Density modification

In order to avoid model bias from the LiP415 structure it was decided to carry out two-fold averaging before the first model building. The program DM from the CCP4 suite was employed for this purpose and NCSMASK to place a mask around the protein. The automatically created mask was extended manually around the N-terminus where the prosequence of LiP* was expected to be. Averaging was done in combination with histogram matching and solvent flattening. Phases were extended in 20 cycles from 2.93 to 2.0 Å. During the run, the R_free dropped from 0.41 to 0.3 and the non-crystallographic symmetry (NCS) correlation increased from 0.79 to 0.89. In addition to a reflection file which contains
Chapter 3

modified phases one obtains a refined non-crystallographic symmetry operator which can be used in NCS-constrained refinement.

Model building and refinement

The density modified 2Fo-Fc map could be used to model most of the residues which are different in LiP* compared to LiP415 and the first two residues of the prosequence. Only a small number of surface residue side chains, the remaining part of the prosequence and of the three C-terminal residues could not be modelled at this stage. The density of the Trp171 Cβ hydroxy group was visible at a 6 σ contour level in a difference map. Apart from the protein, the model contained 2 calcium ions and the heme. 5% of the reflection data were left aside for cross validation. The refinement was started with a simulated annealing run using X-PLOR 3.8.5.1 (Brünger, 1992) and the slow-cooling protocol applying NCS constraints. The standard Engh-Huber parameters (Engh and Huber, 1991) were employed. For the proximal His the parameters from the param19x.heme file were used to define the iron-His bond and to prevent the iron from escaping into the distal pocket due to van der Waals repulsion. Apart from the electrostatic energy terms which were switched off, it was made use of the default energy functions. No σ cutoff was applied. An R-factor of 0.26 (Rfree= 0.31) was obtained for 8 to 2.5 Å resolution data. Then the NCS constraints were suspended and the model alternatively refined with individual B-factor, positional refinement and manual rebuilding up to 2.0 Å resolution (R=0.258, Rfree=0.293). In order to make efficient use of ARP to build in water molecules automatically it was switched to PROTIN/REFMAC for further refinement, since these programs can be combined conveniently and can be carried out loopwise with a shell script. A maximum of 50 waters was added in each cycle of ARP. The σ threshold was determined by ARP. Waters were accepted if they were between 2.2 and 3.3 Å distant from an already existing electronegative atom. The real space refinement routine was used. The threshold for removing waters was 1 σ. In REFMAC restrained refinement with a σ weighting of x-ray and geometric terms against a maximum-likelihood residual was done in combination with isotropic B-factor refinement and bulk solvent scaling. The Trp171 hydroxy groups were introduced as waters and their distance to the Trp171 Cβ atoms restrained to 1.43 Å. After 511 waters had been built the model was extensively checked on the graphics, corrected when necessary and some more water molecules were added manually. They were removed if their B-factor exceeded 60 Å². The non-protein electron densities in the distal heme cavity and the heme channel were all interpreted as water molecules. Problems of the model as detected by PROCHECK (Laskowski et al., 1993) were inspected on the graphics and corrected. Subsequently, the weighting factor was varied from 0.3 to 0.8 around the default of 0.5 in various cycles in order to get an optimal combination of good stereochemistry and an optimal fit of the model to the data. Moreover, the effect of the weighting on the heme geometry and the iron ligation was studied. During the final
Chapter 3

refinement cycles the distance restraints of the Trp171 hydroxy groups was removed and the cut-off radius for van der Waals repulsion set to 0.1 in PROTIN in order to get unrestrained distances between the Trp171 Cβ and the hydroxy groups as well as of those between the iron and its axial ligands. For the final refinement cycles a σ weighting of 0.55 was used.

3.1.3 Structure determination of LiP*-nat

For the structure determination of the pristine enzyme LiP*-nat the complete LiP*·H2O2 model with waters but excluding the Trp171 hydroxy groups served as a model. Since the cell constants of these two crystal forms are slightly different, a rigid body refinement using the fitting routine of AMORE was carried out initially. Refinement with REFMAC was started with Trp171 omitted to reduce bias from the LiP*·H2O2 model on its conformation but no significant differences to it were found. ARP was used to remove bad waters and to add new ones following the same criteria as above. After the refinement had converged, waters were added in the rest electron densities at the Cβ of Trp171. Their occupancies were refined with X-PLOR (see below) and with REFMAC by systematically varying their occupancy until their B-factors coincided with those of the Trp171 Cβ atoms. The same procedure was done with the two water molecules which hydrogen bond to the hydroxy groups in both subunits. The final refinement conditions were the same as those applied to LiP*·H2O2.

Occupancy refinement was done with X-PLOR. A new residue type was introduced for β-hydroxy tryptophan to define the interactions of the hydroxy group suitably. In the topology file, the X-PLOR atom type OH1 was assigned to the hydroxy oxygen and that of the Cβ was changed from CH2E to CH1E. Thus for the bond between these two atoms the same parameters as in threonine were used. Some entries in the parameter file had to be added to fit to the new atom type of the Cβ atom leaving the energy constants unchanged. For the OH1-CH1E-C5W angle (the latter atom corresponds to the Cγ of Trp) a value of 109° and an energy constant of 200 was used. Since electrostatic energy terms were not included in the refinement charge assignments were of no importance. Positional refinement in X-PLOR carried out on the model refined with REFMAC and after individual B-factor refinement in X-PLOR did not improve the R-factors any further. B-factors from X-PLOR refinement were found to be in general some Å² lower than those from REFMAC.

Starting from the final models of LiP*·H2O2 and LiP* (refined with REFMAC) individual B-factor refinement was carried out firstly in X-PLOR until convergence was achieved. Occupancy refinement for selected atoms was done for five cycles leaving all B-factors constant and after setting the B-factors of the selected atoms to the desired value.
3.1.4 Structure determination of LiP*-orc

This crystal variant was obtained by the inclusion of 4 mM orcinol (3,5-dihydroxy toluene) in the precipitant solution of the pristine recombinant enzyme. The refinement was as described for the LiP*-nat structure with the exception that there was no need for occupancy refinement.

3.1.5 Structure determination of the triple mutant LiP*-tm

For the [H82P:P83A:E146G] mutant the refined LiP*-orc structure was used as a starting model including all waters but with omitted side chains of the mutated residues. Because the resolution of these data was only 2.51 Å a more conservative refinement protocol was carried out to avoid overfitting. Medium main chain and medium side chain non-crystallographic symmetry (NCS) restraints (according to the definitions in PROTIN/REFMAC) for positional shifts and medium thermal restraints (1.5 Å for main chain atoms and 2.5 Å for side chains) were applied throughout to reduce the effective number of parameters. First some refinement cycles were done with strong positional shift magnitude restraints with the mutated residues omitted and waters whose B-factors rose over 60 Å² were removed. Then the mutations were carried out on the graphics and the side chain of Pro83 was modelled into the electron density of an omit map. Further refinement used again the default positional shift magnitude restraints of REFMAC and individual isotropic B-factors with restraints for bonded, 1-3, and planar 1-4 bonding relations which allowed only half of the shifts which the default values do. Manual modelling was basically restricted to the adjustments of the water model.
3.2 Results and Discussion

3.2.1 Crystallisation, data collection, and structure determination of recombinant LiP variants

Recombinant LiPH8* (LiP*) consists of 344 amino acids of the mature isozyme H8 and a 7-residue prosequence. It crystallised from 17 % PEG 6000 under conditions of low ionic strength. The crystals were of irregular morphology and those used for data collection had the size shown in Figure 3.1. Figure 3.2 shows an oscillation photograph from which it is evident that the crystal must have at least one relatively long unit cell axis. The images from data collection were indexed in a C-centered orthorhombic lattice type which leaves only two possible space groups, namely C222 and C2221. When the data were merged in space group C2221 no significant violations of the special extinction rule $l=2n$ were found which provides strong evidence for the presence of a screw axis parallel to the c-axis.

The cell parameters of the crystals rendered data collection, especially attaining complete data sets, rather complicated. The crystals diffracted up to 1.5 Å but only for a limited time in the beam. After about 15 frames they deteriorated considerably. Their irregular shape prevented the correlation of the lattice orientation with the macroscopic morphology. For this reason it was not possible to mount the crystals with the long c-axis aligned with the spindle axis and it was very difficult to continue data collection with a new crystal in the orientation where the previous one had given up.

Four data sets from recombinant enzyme crystals have been collected:
(i) the LiP*-H2O2 data are from LiP* that was treated with 3 equiv. H2O2 prior to crystallisation,
(ii) the LiP*-nat data from pristine LiP* (enzyme that has never come in contact to H2O2),
(iii) pristine LiP* cocrystallised with 4 mM orcinol (1,3-dihydroxy-5-methyl benzene) gave the LiP*-orc data set,
(iv) the LiP*-tm data from the [H82P:P83A:E146G] triple mutant of LiP*.

Table 3.1 lists data processing statistics and the cell constants from postrefinement.

The data set from H2O2 treated LiP* (LiP*-H2O2) was the first one that was collected and the determination of the recombinant enzyme structure was started off with it. The phase problem was solved with the structure of the fungal enzyme LiP415 which shares a sequence identity of 91% with LiPH8. Details of the molecular replacement, model building and refinement are given in chapter 3.1. The other enzyme variants crystallised all in the same space group with virtually the same unit cell dimensions. They were refined with the
the LiP*-H₂O₂ structure as a starting model and only relatively little model building had to be carried out. There are two protein molecules in the asymmetric unit denoted as subunit A and B, each with a heme and two calcium ions, corresponding to a Matthews coefficient of about 2.6 Å³/Da and a solvent content of 53%. Refinement statistics are compiled in Table 3.2.

Crystals resulting from cocrystallisation with orcinol showed a rather interesting change in their behaviour compared to those without that phenol. They were considerably more stable in the beam so that a complete data set could be collected from one single crystal. The same behaviour was found during data collection from a triple mutant crystal which has
Table 3.1 Data collection statistics of the recombinant LiPH8* structures determined in this study. Values in brackets give statistical values from the outmost of 10 resolution shells of those data collected in Grenoble and of 20 shells of the data from Hamburg. All data were processed and merged with DENZO and SCALEPACK. The statistics are from the output of the latter program. The space group in all crystal forms is C222₁. The unit cell constants are from postrefinement in SCALEPACK.

<table>
<thead>
<tr>
<th>x-ray source</th>
<th>LiP*-H₂O₂</th>
<th>LiP*-nat</th>
<th>LiP*-orc</th>
<th>LiP*-tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNBL, ESRF</td>
<td>SNBL, ESRF</td>
<td>BW7B, EMBL</td>
<td>BW7B, EMBL</td>
<td></td>
</tr>
<tr>
<td>Grenoble</td>
<td>Grenoble</td>
<td>Hamburg</td>
<td>Hamburg</td>
<td></td>
</tr>
<tr>
<td>wavelength (Å)</td>
<td>0.873</td>
<td>0.873</td>
<td>0.8345</td>
<td>0.8345</td>
</tr>
<tr>
<td>No. of crystals</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>1</td>
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<tr>
<td>No. of observations</td>
<td>391636</td>
<td>223168</td>
<td>245690</td>
<td>82594</td>
</tr>
<tr>
<td>No. of unique refl.</td>
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<td>54256</td>
<td>73943</td>
<td>24232</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.93</td>
<td>4.11</td>
<td>3.32</td>
<td>3.41</td>
</tr>
<tr>
<td>Completeness</td>
<td>94.4 (91.1)</td>
<td>85.5 (85.9)</td>
<td>92.7 (68)</td>
<td>88.2 (84.1)</td>
</tr>
<tr>
<td>Resolution</td>
<td>20 - 1.73 (1.79-1.73)</td>
<td>20-1.9 (1.97-1.9)</td>
<td>40-1.75 (1.78-1.75)</td>
<td>14 -2.51 (2.55-2.51)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>12.2 (4.4)</td>
<td>8.0 (4.0)</td>
<td>19.7 (4.7)</td>
<td>10.2 (6.0)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.078 (0.27)</td>
<td>0.085 (0.27)</td>
<td>0.042 (0.21)</td>
<td>0.069 (0.13)</td>
</tr>
<tr>
<td>unit cell (Å)</td>
<td>a</td>
<td>73.48</td>
<td>73.46</td>
<td>73.26</td>
</tr>
<tr>
<td>b</td>
<td>94.93</td>
<td>94.73</td>
<td>94.42</td>
<td>94.15</td>
</tr>
<tr>
<td>c</td>
<td>230.13</td>
<td>229.23</td>
<td>228.05</td>
<td>227.54</td>
</tr>
</tbody>
</table>

also been grown in the presence of orcinol. An advantageous consequence is that these data
merge with a considerably better $R_{merge}$ than those collected from several crystals (Table 3.1).
The effect of orcinol and very probably of other phenols to mitigate radiation damage during
x-ray data collection could be useful also for other proteins to increase the lifetime of crystals
in the beam at non-cryo temperatures.

### 3.2.2 Validation and quality of the final models

All quality indicators and the electron densities of the LiP*-H₂O₂ and LiP*-orc
Table 3.2 Refinement statistics of the four crystal structures. Refinement was carried out with REFMAC (see Materials and Methods for details). The rms coordinate error was calculated with the program SIGMAA.

<table>
<thead>
<tr>
<th></th>
<th>LiP*-H₂O₂</th>
<th>LiP*-nat</th>
<th>LiP*-orc</th>
<th>LiP*-tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolution (Å)</td>
<td>20 - 1.73</td>
<td>20 - 1.9</td>
<td>30 - 1.75</td>
<td>14 - 2.51</td>
</tr>
<tr>
<td>outmost shell</td>
<td>1.81 - 1.73</td>
<td>1.99 - 1.9</td>
<td>1.83 - 1.75</td>
<td>2.62 - 2.51</td>
</tr>
<tr>
<td>R-factor (%)</td>
<td>18.7</td>
<td>19.6</td>
<td>18.9</td>
<td>19.6</td>
</tr>
<tr>
<td>outmost shell</td>
<td>26.0</td>
<td>22.7</td>
<td>21.8</td>
<td>20.2</td>
</tr>
<tr>
<td>free R-factor (%)</td>
<td>22.3</td>
<td>24.3</td>
<td>22.6</td>
<td>24.7</td>
</tr>
<tr>
<td>outmost shell</td>
<td>31.6</td>
<td>28.8</td>
<td>25.9</td>
<td>32.4</td>
</tr>
<tr>
<td>total No. of atoms</td>
<td>5831</td>
<td>5796</td>
<td>5834</td>
<td>5636</td>
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<tr>
<td>No. of waters</td>
<td>531</td>
<td>496</td>
<td>536</td>
<td>338</td>
</tr>
<tr>
<td>rms coordinate error (Å)</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.18</td>
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<tr>
<td>B-factors (Å²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>overall</td>
<td>22.15</td>
<td>25.8</td>
<td>25.7</td>
<td>31.6</td>
</tr>
<tr>
<td>main chain (subunit A)</td>
<td>20.7</td>
<td>24.9</td>
<td>24.6</td>
<td>32.9</td>
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<tr>
<td>main chain (subunit B)</td>
<td>18.7</td>
<td>23.2</td>
<td>22.3</td>
<td>29.9</td>
</tr>
<tr>
<td>water only</td>
<td>36.2</td>
<td>38.5</td>
<td>39.5</td>
<td>39.0</td>
</tr>
</tbody>
</table>

structures are of good quality. For these structures the ratio of the No. of observations/No. of parameters is 3.4 and 3.2, respectively, assuming individually refined B-factors. The rms coordinate error as determined by SIGMAA is about 0.15 Å. The quality of the LiP*-nat data and structure is lower and is not considered here in detail since its only importance is to demonstrate the ease of oxidative modification. Apart from a few surface residues like Glu57, Glu59, Lys61, Gln137 in subunit A and Gln99, Arg211 and Glu319 in subunit B whose side chains are disordered the electron density could be interpreted unambiguously. The program PROCHECK (Laskowski et al., 1993) was used to analyse geometric properties of the refined models. 92 % of the residues are in the most favoured regions of the Ramachandran plot (Figure 3.3 ) and 8 % in additionally allowed regions. No non-glycine residues are found in generously allowed or disallowed regions. No cis peptide bond is present and no van-der-Waals clashes were detected. The only big protein void, the distal heme cavity, is occupied by well-ordered water molecules. Statistics of overall geometric deviations from ideality are given in Table 3.3. As a representative example, the R-factor dependence on the resolution is given in Figure 3.4 for the LiP*-orc structure. The R-factors increase only moderately when
Table 3.3 Statistics of overall geometric rms deviations from ideality. Data taken from the output of REFMAC.

<table>
<thead>
<tr>
<th></th>
<th>LiP*-H₂O₂</th>
<th>LiP*-nat</th>
<th>LiP*-orc</th>
<th>LiP*-tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ω angle (°)</td>
<td>4.2</td>
<td>3.1</td>
<td>3.6</td>
<td>2.3</td>
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<tr>
<td>chiral volume (Å³)</td>
<td>0.13</td>
<td>0.11</td>
<td>0.13</td>
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<tr>
<td><strong>distance information</strong></td>
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<tr>
<td>bond rms (Å)</td>
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<td>0.01</td>
<td>0.015</td>
<td>0.005</td>
</tr>
<tr>
<td>angle distances (Å)</td>
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<td>0.023</td>
<td>0.027</td>
<td>0.019</td>
</tr>
<tr>
<td>planar 1-4 distances (Å)</td>
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<td>0.027</td>
<td>0.034</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>plane information</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peptide bonds (Å)</td>
<td>0.022</td>
<td>0.018</td>
<td>0.021</td>
<td>0.013</td>
</tr>
<tr>
<td>aromatics (Å)</td>
<td>0.0088</td>
<td>0.0062</td>
<td>0.0079</td>
<td>0.0032</td>
</tr>
<tr>
<td><strong>torsion angles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>planar (°)</td>
<td>3.9</td>
<td>3.1</td>
<td>4.0</td>
<td>2.3</td>
</tr>
<tr>
<td>staggered (°)</td>
<td>14.8</td>
<td>15.0</td>
<td>14.0</td>
<td>17.2</td>
</tr>
<tr>
<td>orthonormal (°)</td>
<td>34</td>
<td>34</td>
<td>34</td>
<td>34</td>
</tr>
</tbody>
</table>

Plot statistics

- Residues in most favoured regions [A,B,L] 518 92.2%
- Residues in additional allowed regions [a,b,l,p] 44 7.8%
- Residues in generously allowed regions [-a,-b,-l,-p] 0 0.0%
- Residues in disallowed regions 0 0.0%
- Number of non-glycine and non-proline residues 562 100.0%
- Number of end-residues (excl. Gly and Pro) 4
- Number of glycine residues (shown as triangles) 64
- Number of proline residues 62
- Total number of residues 692

Figure 3.3 Ramachandran plot of the LiP*-orc structure produced by PROCHECK.
approaching the resolution limit indicating that the diffraction limit of the crystals is at an even higher resolution.

The data set of the [H82P:P83A:E146G] triple mutant LiP*-tm is of less resolution and completeness than those of the wild-type structures. Refining individual B-factors would have given a ratio No. of observations/No. of parameters of only 1.4. Consequently, non-crystallographic symmetry restraints were applied and elevated B-factor restraints which results in a situation similar to grouped B-factor refinement (see Materials and Methods for details). The electron density allowed the unambiguous identification of the mutated residues which is especially important for residue 82 and rather trivial for residue 146. As all other prolines the newly introduced Pro83 has also a trans-configurated peptide bond.

3.2.3 General description of the recombinant LiP structures

As expected from the high sequence identity of LiPH8 and LiP415 the two structures are very similar. Therefore, the overall description that has been given for the structures of the fungal enzymes (Choinowski, 1996) apply also to the recombinant enzyme and are not repeated here. Only selected features of the LiP* structures which are considered to be of special importance and a brief comparison to LiP415 are presented. The three wild-type recombinant structures are basically the same with the exception of the Trp171 hydroxylation and one of these is used exemplary.

The main chain rms displacements (rmsd) of LiP415 superimposed on LiP*-H2O2 subunit A and B are 0.64 and 0.68, respectively. Apart from the C-terminal four or five residues which are substantially different (see below) all other major deviations (data not shown) can be ascribed to differences of the crystal packing which affects a small number of
Figure 3.5 Crystal packing in the LiPH8* structures viewed along the crystal a axis (left), along the b axis (middle) and along the c axis (right). The protein molecules are shown as Cα traces and the boxes represent the unit cell.

溶剂暴露的残基。重要的是，最大 RMS 移动和两个同工酶中不同的残基的位置之间没有相关性。

这七个残基的前肽序列只能部分建模。前肽序列的第一残基 Arg0 在亚基 A 中定义良好，在亚基 B 中定义较弱。模型还包含链 A 中的 Lys-1 和 Glu-2，其 B 值因子在 30 和 40 Å² 之间仍然合理。前肽序列的剩余部分在两个亚基中都未建模，无法建模。它很可能不会对酶产生功能性影响。

重组酶中糖基化的缺失似乎与真菌酶 LiP415 和 LiP465 的结构相比没有明显的结构后果。甚至与糖基化残基的结合方式相比，真菌酶的这些结构非常相似。

非晶洛洛格的对称性和晶体堆积
在空间群 C2221 中，单胞中包含八个不对称单位，每个单位中包含两个蛋白质分子。分子 B 可以通过围绕 c 轴旋转 -75.2° 来获得分子 A，这对应一个 a = 0°，b = 180°，γ = -75.2° 的操作，然后沿 (-0.241; 0.341; -0.257) 旋转。因此，旋转轴与晶体学对称操作不重合。

晶体中的蛋白质分子的堆积如图 3.5 所示。沿 c 轴堆积最密，溶剂体积最小。沿 b 轴堆积最少，溶剂体积最大。在第 3.2.4 章中推测，这些堆积差异导致了晶体的各向异性，并引发了各向异性重原子波动。
Comparison of the two molecules in the asymmetric unit

Figure 3.6 shows the rms displacements of the main chain atoms of the two protein molecules superimposed (overall rmsd 0.24 Å) and the average main chain temperature factors of both chains. The maxima in the rmsd plot coincide with those of the B-factor plot in at least one of the chains. Elevated B-factors are generally due to increased mobility of surface residues which are less well-fixed by surrounding ones. In fact, the major rms displacements occur on surface parts of the protein and can be attributed to crystal packing effects as can be verified by inspection of the superimposed subunits:

- in the range of the first ten residues relatively high B-factors and rms displacements are present. This is a relatively loose range of residues with only a small number of protein-protein interactions which are different in the two subunits. In molecule B a van-der-Waals interaction exists between Asn5 and Ile77 of a crystallographically related subunit B which is not present in subunit A.
- the second most striking rmsd is that of the loop region around residue 60 which coincides with elevated B-factors in both chains. The fact that the highest main chain B-factors in chain B are much less than those in chain A can be explained by crystal contacts which are present in subunit B (H-bond between the carbonyl oxygen of Gly60 and the side chain of His101 of a related subunit B) but not in subunit A.

- the large rmsd at residue 220 can be rationalised similarly. In subunit A Gly220 is firmly hold in place by van-der-Waals interactions with Gly142 of a related B chain. This interaction does not exist in subunit B resulting in higher B-factors.

The C-terminus

Substantial differences between LiP415 and the LiP* structures exist at the C-terminus onwards from residue 341 which is not conserved among LiP isozymes and lacks secondary structure. Apart from having an additional residue, Ala344, LiPH8 has different residues at positions 341, 342 and 343 leading to the unusual feature of four successive prolines at positions 339 to 342. This results in an altered α trace which changes its direction compared to that of LiP415 and approaches Asp183. Thereby, the side chain of Glu89 is forced to a different conformation than it has been modelled in LiP415 (not shown). The terminal carboxy group of Ala344 hydrogen bonds to the side chain of Asp183 which in turn binds to the heme propionate of pyrrole ring A forming the unusual constellation of a hydrogen bonded array of three carboxy groups in close proximity to the active site channel (Figure 3.7, also Figure 3.11). Asp183 is an interesting residue in that it is conserved among LiP isozymes but replaced by non-ionisable residues in other class II as well as in class I and III peroxidases whose pH optima are much higher. It is therefore one of the promising targets for mutagenesis studies aimed at pinning down structural features which may have evolved in connection with the low pH requirement of LiP. From the close proximity of these three carboxy groups it can be predicted that they together will not carry more than one negative elementary charge. If this was the case they would repel one another and the flexible C-terminus would move away from the propionate-Asp183 couple. In the high resolution structures of this study and those of LiP415 and LiP465 the hydrogen bonds between Asp183 and the propionate are all unusually short and therefore strong with distances between 2.45 of 2.5 Å. Those between Asp183 and the C-terminal carboxy group refined also to short distances (2.5-2.63 Å) but their accuracy will be less since the positions of the C-terminal atoms are less well defined (B-factors between 34 and 38 Å²). These high B-factors indicate that more open conformations may also exist in which the C-terminus is not in contact to the protein but stretches into bulk solvent. These conformations will be favoured at pH values higher than that of the present.
crystal structures (pH=3.5) and, as already pointed out earlier (Poulos et al., 1993), it is rather unlikely that the heme propionate is still hydrogen bonded to Asp183 at neutral pH.

The functional role of different C-terminal arrangements of LiP isozymes is hard to estimate from the structure alone. In general, functional significance is only assigned to conserved residues. On the other hand, the fact that the different LiP isozymes of *P. chrysosporium* are encoded by different genes (Stewart et al., 1992) suggests that a certain functional variability is needed. Although the above mentioned triad of carboxy groups will carry only marginal negative charge at the pH optimum of 3, the C-terminus in this conformation may contribute in determining the pH dependence of activity towards charged substrates which are oxidised at the active site channel.
3.2.4 The heme and its axial coordination

At less than atomic resolution, which is usually the case in protein crystallography, prior geometrical knowledge has to be used as restraints during the refinement since the number of unique reflections does not fully determine the protein structure. These restraints are based on geometric properties of small organic molecules whose structures are known with high precision. Unfortunately, estimating correct weights for the restraints is a major problem (Dodson et al., 1998). E.g. weighting the restraints too heavy leads to a too tight clustering of geometric properties around the expected mean values, but may not represent the reality of the protein structure. On the other extreme, considerable distortions from planarity of groups which are supposed to be planar, like aromatics, peptide bonds or carboxyl groups, are indications for too weakly weighted geometric restraints. The choice of the weighting factor can exert a considerable effect on the result of the refinement. Therefore, refinement cycles with various weightings of the restraints were carried out in order to study the effects on critical parts of the structure. The situation is even more tricky with a cofactor like heme since model compounds cannot mimic the effect of a protein environment.

Distortions of the heme from planarity

Non-planar distortions of tetrapyrroles are prevalent in the hemes of hemoproteins (Shelnutt et al., 1998). This is corroborated by atomic resolution x-ray structures of cytochromes c6 determined to 1.3 Å (Frazão et al., 1995) and by the yeast cytochrome c structure at 1.23 Å (Louie and Brayer, 1990). In c-type cytochromes the most important factor leading to distortions of the heme is the strain imposed by the two thioether linkages to the protein with its Cys-X-X-Cys motif (Ma et al., 1998). In larger heme proteins the resolution of available crystal structures is generally much less than those of the above mentioned cytochromes as well as their reliability with respect to critical parts of a macromolecular structure like a heme. There are, however, studies which indicate that different types of heme distortions, which are energetically unfavourable (Anderson et al., 1993), are conserved for proteins with the same function (Jentzen et al., 1998). Whether a certain type of functionality requires a certain type of heme distortion or whether the similarity of these proteins is responsible for the heme distortions remains, after all, unclear.

As in the fungal LiP structures, the heme is saddle-shaped in the LiP* models of this study (compare Figure 3.8). The hemes in the two subunits as well as those of LiP415 and LiP465 can be superimposed nicely, i.e. the deviations from planarity go in the same directions indicating that this type of distortion is real and caused in a conserved way by the protein matrix. The degree of distortion from planarity depends hardly on the weight of the geometric restraints. In order to rule out the possibility that the heme distortion type of the recombinant LiP structures originate from the molecular replacement model LiP415 rather
Table 3.4 Distances between opposing pyrrole nitrogens in LiP structures. For the models refined in this work some refinement conditions are also given. w is the overall σ weight from REFMAC and vdw is the van-der-Waals cut-off radius from PROTEIN which sets an upper limit for atom distances for which van-der-Waals repulsion is taken into account.

<table>
<thead>
<tr>
<th></th>
<th>D(NA-Nc) (Å)</th>
<th>D(NB-Nd) (Å)</th>
<th>Refinement cond.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiP*-H2O2 A</td>
<td>4.16</td>
<td>3.8</td>
<td>w=0.4, vdw=0.1</td>
</tr>
<tr>
<td>LiP*-H2O2 B</td>
<td>3.95</td>
<td>4.09</td>
<td>&quot;</td>
</tr>
<tr>
<td>LiP*-H2O2 A</td>
<td>4.08</td>
<td>3.94</td>
<td>w=0.7, vdw=0.1</td>
</tr>
<tr>
<td>LiP*-H2O2 B</td>
<td>4.0</td>
<td>4.05</td>
<td>&quot;</td>
</tr>
<tr>
<td>LiP*-H2O2 A</td>
<td>4.11</td>
<td>3.90</td>
<td>w=0.55, vdw=0.1</td>
</tr>
<tr>
<td>LiP*-H2O2 B</td>
<td>3.99</td>
<td>4.07</td>
<td>&quot;</td>
</tr>
<tr>
<td>LiP*-orc A</td>
<td>4.10</td>
<td>3.85</td>
<td>w=0.55, vdw=0.1</td>
</tr>
<tr>
<td>LiP*-orc B</td>
<td>3.93</td>
<td>4.07</td>
<td>&quot;</td>
</tr>
<tr>
<td>LiP415</td>
<td>4.16</td>
<td>4.06</td>
<td></td>
</tr>
<tr>
<td>LiP465 A</td>
<td>3.80</td>
<td>4.31</td>
<td></td>
</tr>
<tr>
<td>LiP465 B</td>
<td>3.91</td>
<td>4.03</td>
<td></td>
</tr>
</tbody>
</table>

Heme symmetry

A perpendicular view onto the heme plane revealed a difference between heme A (in subunit A) and B (in subunit B) in the LiP* models. Whereas heme B is almost symmetrical with respect to the Fe-N(pyrrole) distances heme A is distorted in that the distances between opposing pyrrole nitrogens are different. The degree of the distortion depends on the weight of the restraints during refinement with stronger restraints leading to higher symmetry. Table 3.4 lists some distances between pyrrole nitrogens in both subunits of all wild-type LiPH8* models and those of the fungal enzymes for comparison. The heme of LiP415 is very symmetrical but in LiP465 the situation is similar to the recombinant enzyme structures in
Figure 3.8 Stereo plots of the heme in subunit A (a) and subunit B (b) of the LiP*-orc structure and difference maps of the refined structure contoured at 5 σ. These pronounced residual densities are discussed to derive from crystal anisotropicity. The distal and proximal histidines are also shown.

that there is one symmetrical and one substantially distorted heme present. The direction of the distortion in LiP465, however, is reverse to that in LiP*-H_2O_2. All LiPH8 wild-type structures behave similarly but not LiP*-tm which has a considerably lower resolution of 2.51 Å.

Difference electron density maps of the final LiPH8* models revealed strong residual electron densities between the iron and pyrrole nitrogens N_B and N_D of heme A (Figure 3.8). Compared to heme A, these densities are less intense in heme B and are located between the iron and pyrrole nitrogens N_A and N_C but somehow below and above the heme plane (Figure 3.8). In fact, these densities are by far the most intense rest densities of the final LiP* models. Apparently, the pyrrole nitrogens must have been pulled towards these densities during the refinement giving rise to the disturbed symmetry. No significant negative electron density is present in the heme vicinity.

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Initially, no explanation for this puzzling behaviour apart from the well-known phenomenon of heavy atom ripples could be thought of and no similar observation was known from the literature. It was however very likely that the observed differences between the two molecules in the asymmetric unit are of crystallographic origin and most probably due to a crystal anisotropy. This interpretation was corroborated by the observation that imaginary lines drawn through the centers of these rest densities are almost parallel to the crystal b-axis and that similar, albeit weaker, rest densities exist for the calcium ions. An analysis of the crystal packing revealed that the molecules are in fact less densely packed along the unit cell b-axis compared to the a- and c-axis which could result in a slight degree of more mobility along b. The fact that these electron densities do still appear in difference maps at σ contour levels as high as 7 is simply due to the higher number of electrons of iron and calcium compared to the elements of the second row of the periodic table which dominate the scattering power of the unit cell and determine the σ value of an electron density map. Anisotropic electron densities of these light atoms could not be observed. In summary, these rest densities at the iron may be described best as anisotropic heavy atom ripples.

A number of other structures of heme proteins were checked with respect to their heme (α-)symmetries. Most revealed only slight distortions of about the same order of magnitude as in the heme of subunit B which is certainly within the error limit. However, no detailed information on the applied restraints are in general available. In a talk given by Carlos Frazão on his 1.3 Å structure of cytochrome c6 a 2Fo-Fc map of the heme was presented that showed the same characteristics as such a map of our LiP* structures, namely connection of the electron density between one set of opposing pyrrole nitrogens but not between the iron and the pyrrole nitrogens of the other set. In spite of that anisotropic electron density the heme symmetry was almost perfect (pdb entry code 1ctj). Interestingly, it then turned out that the heme had been restrained to be symmetric (Frazão et al., 1995), which is an illustrative example for the importance of restraints in protein structure refinement even at near atomic resolution.

**Heme vinyl groups**

The vinyl stretching frequencies are common markers for RR spectroscopy. They have been correlated with the degree of overlap of the vinyl π system and that of the heme macrocycle, i.e. the torsion angle τ, and a linear correlation has been found (Smulevich et al., 1997). The higher the frequency of the vinyl stretch, the less conjugated is its double bond with the porphyrin resulting in a blue shift of the pp* transition of the heme (the Soret band) in the electronic absorption spectrum (Neri et al., 1997).

The results from the different LiP* models and the two molecules in the asymmetric unit can be summarised as follows: The vinyl group of pyrrole ring A is almost coplanar with
the porphyrin having an angle that deviates by 10° to 25° from optimal overlap of the π orbitals. It is twisted towards the proximal domain and is oriented towards the α meso atom of the heme. However, the electron density of the external vinyl carbon is such that considerable probability must also exist for conformations twisted towards the distal side about the same angles. The vinyl group of pyrrole ring B is best modelled in two different conformations with equal occupancy. One is essentially coplanar with the porphyrin macrocycle and points towards the α meso atom of the heme. The other one points towards the distal domain and the β meso atom with deviations from coplanarity of 25° - 40°. That this type of modelling is appropriate is supported by the resulting B-factors which are nearly identical for both the outermost vinyl carbons in the two conformations and those of the two preceding carbons.

In the fungal structures LiP415 and LiP465 the situation was also ambiguous and the vinyl groups were modelled in different conformations. It can be concluded that the heme vinyl groups of LiP are not forced into one particular conformation by the surrounding protein and that their conformation is probably not crucial for the function of ligninases. Conformations with more than about 50° deviation from coplanarity of the vinyl groups and the porphyrin do not appear to be present.

**Proximal heme coordination**

Whereas the equatorial iron coordination in heme proteins is very similar, the axial coordination determines to a great extent the properties of a particular protein, e.g. oxygenases like cytochrome P-450 and nitric oxide synthase have a cysteine as proximal ligand. Most heme proteins like globins, peroxidases and c-type cytochromes share a histidine as the proximal ligand (His176 in LiP) and heme peroxidases also share an aspartate which hydrogen bonds to the Nε2 atom of the proximal histidine with its carboxy group. Since the bonding distance of the proximal His to the iron has been correlated with the redox potential of the Fe3+/Fe2+ couple among heme peroxidases (Piontek et al., 1993; Choinowski, 1996; Banci, 1997) it was of interest to see whether the values found in the fungal LiP structures would be confirmed by those of the recombinant enzyme. Table 3.5 compares the distances found in this study with those of the fungal enzymes. When the refinement conditions of LiP*-H2O2 were varied only slight changes within the error limits were encountered (data not shown). Removal of the van-der-Waals repulsion restraint had the expected effect of shortening the distance but also in a marginal way. Thus, the relatively long Fe-Nε2(His176) distance found in LiP415 is reproduced nicely by the structures of the recombinant enzyme.
Table 3.5 Axial heme coordination in some refined LiP models.

<table>
<thead>
<tr>
<th></th>
<th>LiP*-H_2O_2 A</th>
<th>LiP*-H_2O_2 B</th>
<th>LiP*-orc A</th>
<th>LiP*-orc B</th>
<th>LiP415</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-distal H_2O</td>
<td>2.16</td>
<td>2.12</td>
<td>2.23</td>
<td>2.19</td>
<td>2.27</td>
</tr>
<tr>
<td>dist H_2O-H_47(Ne2)</td>
<td>2.99</td>
<td>3.00</td>
<td>2.86</td>
<td>2.85</td>
<td>2.82</td>
</tr>
<tr>
<td>Fe-H_47(Ne2)</td>
<td>5.14</td>
<td>5.11</td>
<td>5.09</td>
<td>5.04</td>
<td>5.07</td>
</tr>
<tr>
<td>Fe-H_176(Ne2)</td>
<td>2.15</td>
<td>2.14</td>
<td>2.17</td>
<td>2.13</td>
<td>2.15</td>
</tr>
</tbody>
</table>

From a comparison of the redox potentials of various heme peroxidases with the distances between the iron and the proximal histidine it follows that this distance cannot be the main determinant of the redox potential of these enzymes. In horseradish peroxidase (HRP) the distance is identical within experimental error to that found in LiP although the redox potentials of the Fe^{3+}/Fe^{2+} couples differ by about 150 mV (Millis et al., 1989; Makino, 1976). In the HRP model from the pdb entry 2ATJ a distance of 2.21 Å is found. Upon refinement with REFMAC and the same settings as those used for the LiP* models, distances around 2.12 - 2.15 Å were obtained.

The larger distance in LiP compared to that of 1.96 Å found in CcP was attributed to the presence of Ser177 in LiP enabling hydrogen bonds and coordination to the proximal calcium thereby moving the proximal helix away from the heme (Piontek et al., 1993). Since then, some more crystal structures of members of all classes of the plant peroxidase superfamily have been solved and this interpretation does still appear to be valid. Apart from CcP which has an alanine at the position corresponding to Ser177 of LiP and no cation site in the proximal domain, the other members of the plant peroxidase superfamily have either a serine or a threonine following the proximal histidine and a cation nearby of which the serine/threonine is a ligand. In all cases for which crystal structures have been solved the iron-His distances are in the order of 2.15 Å.

**Distal heme coordination**

Those members of the plant peroxidase superfamily for which structural information exists have a water molecule as the closest neighbour of the iron on the distal side of the heme. In the resting state the iron of LiP is in the ferric high spin (HS) state. The exact determination of the coordination state is not an easy task, although methods like crystallography and RR spectroscopy should in principle be well-suited. One problem which has been encountered is the apparent discrepancy between results obtained from different methods. Manganese peroxidase, for example, whose crystal structure shows a 5-c heme with
a water molecule 2.8 Å distant from the heme iron (Sundaramoorthy et al., 1994) is a mixture of 5-c and predominant 6-c HS heme in solution according to results from RR spectroscopy (Kishi et al., 1996). We have calculated a difference electron density map using the MnP model and the structure factors deposited with the protein data bank by Sundaramoorthy et al. Therein, a residual electron density not mentioned by the authors at a 4σ contour level next to the distal water and 2.5 Å distant from the iron was found (data not shown). This density could be due to a partially occupied water and could explain the mixture of coordination states found by RR spectroscopy. MnP may not be the only case with a 5-c/6-c equilibrium of the heme in the resting state of the enzyme.

Another problem connected to the determination of the coordination state of the heme seems to be the definition of a maximum distance at which a potential ligand can still be considered to be coordinated to the iron. It appears that the distance range of 1.89-2.15 (± 0.08) Å given in the International Tables of Crystallography for 6-c Fe³⁺-O distances of inorganic compounds may be too small for heme proteins. Metal ligands should be considered to be coordinated if the distance between the metal and the ligating atom is clearly less than the sum of their van-der-Waals radii. Taking 1.2 Å for the radius of iron from mean iron-iron distances in different metallic phases and 1.4 Å for the van der Waals radius of oxygen (Handbook of Chemistry and Physics, 1977) we conclude that substantial attractive forces must be operating if the iron to water distance is lower than about 2.5 Å. Otherwise the water is expected to be pushed further away. Naturally, no fix number generally valid to differentiate 5-c from 6-c cases can be given.

From the foregoing discussion it should come as no surprise that contradictory results regarding the distal iron coordination exist also for LiP. In the crystal structure at 1.7 Å resolution of LiP415 a distance of 2.27 Å was refined between the iron and the closest water molecule and interpreted to be weakly bound to the iron (Choinowski, 1996). Poulos et al. (1993) reported corresponding distances between 2.43 Å and 2.73 Å, dependent on the refinement restraints, in the structure of the same isozyme. The latter values were interpreted by these authors as to reflect a 5-c heme.

Figure 3.9 shows a difference omit map of the distal water of the final LiP*-orc model and Table 3.5 lists the distances found in the LiP*-H₂O₂ and LiP*-orc models in both subunits. Within the accuracy of these structures they can be regarded as to be the same with a mean of 2.18 Å, a value still in the distance range given by the International Tables. Since this distance is also much shorter than the minimal van der Waals distance of about 2.5 Å and since the B-factors of these waters refined to low values of 16.5 ± 1.5 Å² we conclude that the iron is 6-c in LiP. The distances of Table 3.5 were only insignificantly dependent on the refinement restraints. Moreover, perturbation experiments were carried out by shifting the distal waters to smaller or greater distances followed by a few refinement cycles. In all cases
Chapter 3

Figure 3.9 Stereoplot of the heme in subunit A of the LiP*-orc structure. The electron density is a difference omit map of the distal water contoured at 10 $\sigma$. The distance from the iron to the distal water is 2.23 Å in this case.

The distances of Table 3.5 were returned in good approximation. From the mean of the values of the LiPH8* structures of this study, the perturbation experiments, and the rms coordinate error it seems reasonable to give the iron-distal water distance as 2.18 ± 0.08 Å.

The correct determination of the coordination state of the heme has a practical impact on everyday labwork with heme proteins. Enzyme concentrations are conveniently determined by measuring the absorbance at the Soret band. As is known from related peroxidases whose distal coordination was manipulated experimentally, the coordination has a distinct influence on the uv/vis spectrum, particularly on the shape and intensity of the Soret band. 6-fold coordination leads to a narrow, sharp Soret with high intensity whereas 5-c heme has a less intense and broader Soret with a shoulder at 385 nm (Smulevich, 1998; Doyle et al., 1998; Rasmussen et al., 1998). Both the fungal and the recombinant enzymes used in this study show the spectral features of a 6-c heme in accordance with the crystallographic results. Most groups in the field as well as ours use the high extinction coefficient of 168 mM$^{-1}$cm$^{-1}$ at the Soret maximum as found by the hemochrome assay (Tien et al., 1986) to determine enzyme concentrations. Contrarily, Gold and coworkers use a much smaller one of 133 mM$^{-1}$cm$^{-1}$ which would fit to a 5-c heme as found in the their crystal structure solved in collaboration with Poulos' group and cited above. To complement the confusion, Aust's group uses the high extinction coefficient as we do but they state the heme in LiP to be 5-c although their absorbance spectra have the same shape as ours (see e.g. Nie and Aust, 1997). They
derive the spin-state of their enzyme preparations from EPR spectroscopy at 4 K and by referring to the RR study of Andersson et al. (1987). It should be pointed out that another report on a RR study from the same group (Mino et al., 1988) initially described also MnP to be 5-c. Later on however, a repetition of their RR experiments let them describe the coordination state of MnP to be a mixture of 5-c and 6-c heme species at room temperature (Kishi et al., 1996). The present study cannot fully resolve the contradictions which have accumulated during the years. From our present data, however, it appears safe to describe the heme in LiP to be 6-c at 20 °C. The typical changes in the uv/vis spectrum which are associated with a change of the coordination state (see e.g. Doyle et al., 1998) could not be observed between 8 and 30 °C.

3.2.5 Hydroxylation at Cβ of Trp171 from a structural point of view

The most intriguing finding in the crystal structures of the fungal enzymes was that of a covalent modification at the Cβ of Trp171 (Choinowski, 1996). The main goal of determining the crystal structures of pristine and H2O2-pretreated LiP was to sort out, by comparison, the structural consequences of the Trp171 hydroxylation.

LiP*-H2O2

The presence of the Trp171 hydroxy group in LiP*-H2O2 was evident already from maps calculated right after the molecular replacement solution had been found. In difference maps calculated with the final model and with omitted Trp171-hydroxy oxygens, their densities can still be seen at a 10 σ contour level. The B-factors of Trp171 are among the lowest in the whole structure indicating that it is part of a very rigid region. Those of the hydroxy oxygens are 13.8 and 13.1 Å² in subunit A and B, respectively, compared to those of the corresponding Trp171 Cβ atoms of 10.8 Å². The fungal enzymes are completely hydroxylated due to their long exposure to the H2O2-containing fungal cultures. In the structures of the latter enzymes, the B-factors of the Trp171 Cβ atoms and those of the hydroxy oxygens are the same within ± 1.2 Å². Consequently, since B-factors and occupancies are highly positively correlated, the occupancy of the hydroxy groups in LiP*-H2O2 must be slightly less than 1. The program X-PLOR was used to refine the occupancy of the hydroxy oxygens. Setting their B-factors to the values of the Cβ atoms, occupancies of 0.87 and 0.91 were calculated. Some control refinement cycles were carried out to explore the accuracy of the occupancy refinements using some well-defined protein atoms which must be
Figure 3.10 Environment of $\beta$-hydroxy-Trp171 in the LiPH8*-H2O2 model from two perspectives. A single water that occupies a little cavity is shown as a red ball. Nitrogens are in blue, oxygens in red and carbon atoms in gold. Hydrogen bonds are represented by black lines.
100 % occupied. Roughly speaking, the relative error of a refined occupancy is in the order of 10 %. It is therefore valid to treat the LiP*-H$_2$O$_2$ model as a completely hydroxylated enzyme form within the experimental error.

**LiP*-nat**

The refinement of the LiP*-nat data set was started from the complete LiP*-H$_2$O$_2$ model and only the Trp171 hydroxylation was left apart. After the R-factor had reached 0.2, electron density maps revealed clear signals next to the C$_\beta$ of Trp171. In initial maps calculated with phases from the Lip415 protein-only model and omitted Trp171 these densities had been present only very faintly and had not been considered to be significant. After refinement, however, their intensities were such that they vanished above a 4 $\sigma$ contour level in a difference map in subunit A and above 6 $\sigma$ in subunit B. When they were modelled as waters, their B-factors refined to 30 and 24 Å$^2$ in subunit A and B, respectively. The size of these values when compared to those of LiP*-H$_2$O$_2$ (13.7 and 13.0 Å$^2$) indicates a partial occupancy of the Trp171 OH groups in this enzyme form which was supposed to be pristine.

Two approaches were taken to assess the degree of Trp171 hydroxylation in LiP*-nat in a more quantitative way, both based on the assumption that the B-factors of the C$_\beta$ and that of the oxygen are very similar. The first one made use of X-PLOR as above. The B-factors of the hydroxy oxygens were again set to those of the Trp171 C$_\beta$ atoms and the occupancies refined to 0.44 in subunit A and 0.68 in subunit B. The B-factors of the Trp171 C$_\beta$ atoms were found to depend only marginally on whether the hydroxy groups were included in the refinement or not.

Secondly, the occupancies of the hydroxy group were systematically varied until similar B-factors for the C$_\beta$ and the OH oxygen were obtained after five cycles of refinement with REFMAC. This approach estimated 35 % hydroxylation of Trp171 in subunit A and 60 % in subunit B.

One of the hydrogen bonding partners of the Trp171 hydroxy group both in the fungal structures and in LiP*-H$_2$O$_2$ is a water molecule whose position is indicated in Figure 3.10. The interesting feature of this water molecule is that its temperature factor depends on the presence of the Trp171 hydroxy group. In LiP*-H$_2$O$_2$, its B-factors refined to 13.8 and 13.5 Å$^2$ in the two subunits compared to about 30 Å$^2$ in LiP*-nat. Their occupancies in the latter enzyme were again refined using X-PLOR and values of 0.5 and 0.75 were obtained. These values correlate well with those of the hydroxy groups in LiP*-nat. Therefore, these water molecules are only well-ordered if the Trp171 hydroxy groups are also present as hydrogen bonding partners.
LiP*-orc

In order to get a true pristine crystal structure of LiP it was chosen to add orcinol, a diphenolic reductant, to the crystallisation solutions of the pristine enzyme. The idea behind was that this compound would trap radicals that must have led to the partially hydroxylated Trp171 in the structure of LiP*-nat.

Interestingly, this approach not only turned out to be successful (Figure 3.11) but also increased largely the stability of the crystals presumably by preventing x-ray induced radiation damage. As a consequence, this data set is of higher quality than those collected earlier in the absence of orcinol. This function of orcinol will be exploited also in future projects when room temperature diffraction data are to be collected. However, the initial hope that orcinol would be visible in the electron density map and thereby identify a substrate binding site of LiP was disappointed.

Comparison of the hydroxylated and non-hydroxylated LiP structures

Figure 3.11 shows the Trp171 vicinity in the pristine and the oxidatively processed enzymes next to each other. When these structures were superimposed, no conformational differences of protein moieties near Trp171 nor anywhere else in the structures could be recognised. As an illustration an rmsd and a B-factor plot of subunit B of both variants is displayed in Figure 3.11.
Even the maxima in the rmsd plot (Figure 3.12a) are quite weak and rather distant from Trp171 in the three-dimensional structure. The most conspicuous difference affects the already mentioned water that occupies, together with the Trp171 hydroxy group, a little cavity. As depicted in Figure 3.10, it forms three hydrogen bonds in the hydroxylated enzyme, one of these to the Trp171 hydroxy group. Its B-factors are about 14 Å² which is very similar to those of the surrounding atoms but about 40 Å² in the pristine enzyme in which it lacks the Trp171 OH-group as a hydrogen bonding partner and has more space to move.
Chapter 3

3.2.6 Comparison of the triple mutant with LiP*-orc

The [H82P:P83A:E146G] triple mutant of LiPH8* (LiP*-tm) was made in order to engineer a LiP variant with a functionality similar to *Coprinus cinereus* peroxidase (CIP). Whereas LiP has a small channel-like opening to the heme, that of CIP is wide and broad which allows substrates to get closer to the heme. In LiP the side chains of His82 and Glu146 which form a hydrogen bond reduce the size of this opening and Ile85 and Asp183 divide it into a bigger part (shown in Figure 3.13, left) and a small one (not shown) that has been termed H2O2 channel due to its size (Choinowski, 1996). In CIP the corresponding residues to Ile85 and Asp183 are both glycines.

Figure 3.13 Heme access channel in the LiP*-orc structure (left) and of the triple mutant LiP*-tm (right). The upper pictures are stick models with amino acid labeling. The lower ones are space-filling CPK representations in which the mutated residues are coloured dark blue. The heme is in red.

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Figure 3.14 compares the rms distances and the B-factors of subunits B of LiP*-tm and LiP*-orc. The average main chain rms deviation between these structures is very low (0.18 Å) and there are no prominent maxima in the plot at the mutated positions. Thus, no structural rearrangements of the protein backbone have occurred as a consequence of the mutation. Whereas the overall B-factor in LiP*-tm is about 5 Å² higher than that of the LiP*-orc structure (Table 3.2), those of the residues at positions 82 and 83 are increased by 12 - 15 Å² and those of residue 146 by about 10 Å². Pro82 for example has an average B-factor of about 41 Å², which is associated with a moderate quality of the electron density map. Fortunately, as shown by the difference omit map of Pro82 and Ala83 in Figure 3.14, the electron density of these residues can still be recognised unambiguously. The B-factors, however, indicate substantially more flexibility in the mutated protein region. Among the interactions that get lost in the mutant are van-der-Waals interactions of the original Pro83.
and the hydrogen bond between His82 and Glu146 connecting protein moieties which are distant in the sequence. The substitution of the latter two residues makes the access channel to the heme from the outer medium, as intended, much more open (Figure 3.13).

Some steady-state kinetic data from various heme access channel mutants have been measured with VA and the two artificial dye substrates DFAD and by W. Doyle and A. T. Smith (Doyle et al., 1998 and unpublished, personal communication) and are compiled in Table 3.6. The data from the E146G single mutant and the H82P:P83A double mutant allow partially to differentiate the effects seen with the triple mutant. The E146G mutation removes a large side chain and a potential negative charge. The double mutation will open up the heme access channel and remove the positive charge of His82.

As already mentioned in chapter 2.2.5, the weak dependence of the activity on these heme channel mutations with the natural substrate VA is not compatible with one of these residues being essential for the interaction with VA. For the artificial substrates ABTS and DFAD no specific interaction sites can exist on the enzyme which could be destroyed by a mutation and could lead to a drastic decrease of the activity. On the other hand, an improvement of the enzyme for these substrates cannot be expected to create an ideal interaction site leading to a dramatic increase of the activity. With the negatively charged ABTS neither the single nor the double mutant have an effect that would be worth mentioning. Only the triple mutant appears to give ABTS substantially better access to the heme increasing the activity three fold. For DFAD the situation is similar but Glu146 in the wild-type enzyme is a more seriously limiting factor of the activity than for ABTS. Triple
Table 3.6 Steady-state turnover numbers of LiPH8* and three mutants with VA and the two artificial negatively charged substrates ABTS and DFAD at 25 °C (all data by W. Doyle and A. T. Smith, Doyle et al., 1998). The ABTS assay contained 500 μM ABTS and 100 μM H₂O₂, the DFAD assay 50 μM DFAD and 100 μM H₂O₂, and that with VA 2 mM VA and 400 μM H₂O₂.

<table>
<thead>
<tr>
<th>LiPH8* variant</th>
<th>VA</th>
<th>ABTS</th>
<th>DFAD</th>
<th>pK</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiPH8*</td>
<td>29.7 ± 1.0</td>
<td>27.0 ± 0.9</td>
<td>31.3 ± 1.2</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>E146G</td>
<td>8.9 ± 1.3</td>
<td>35.8 ± 1.5</td>
<td>71.6 ± 8.2</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>H82P:P83A</td>
<td>24.3 ± 1.0</td>
<td>36.7 ± 0.7</td>
<td>28.1 ± 1.3</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>H82P:P83A:E146G</td>
<td>14.9 ± 0.6</td>
<td>80 ± 0.8</td>
<td>181 ± 8</td>
<td>n. d.</td>
</tr>
</tbody>
</table>

Figure 3.16 Structures of the artificial substrates ABTS and DFAD.

mutation achieves even a six fold increase of the turnover number. These kinetic data indicate that ABTS and DFAD are oxidised at the heme access channel in contrast to VA.
4. Discussion

4.1 Mechanism of Trp171 hydroxylation

The data of chapter 2.2 demonstrate that LiP catalyses its self-oxidation at Trp171 to form β-hydroxy-Trp171. From the finding that this reaction is accomplished by tiny amounts of $\text{H}_2\text{O}_2$, even one equiv. leads to about 50% hydroxylation, it is clear that the first step of the reaction sequence must be LiPI formation because the heme is the most reactive part of the enzyme towards $\text{H}_2\text{O}_2$. Trp171 can act as an intramolecular reductant for the heme in its high oxidation states as is suggested by the thirteen-fold longer lifetime of compound I when Trp171 is substituted by an inert serine or phenylalanine. The investigations on the lifetime of compound I and the spin-trapping experiments provided independent evidences for the formation of a transient radical on Trp171. This residue is located about 6 Å below the plane defined by the heme in the proximal domain of the LiP molecule (Figure 1.5) and approximately opposite to the narrow opening that gives access to the $\delta$-meso carbon of the heme. The shortest distance of an indole carbon of Trp171 to a heme pyrrole carbon is $\approx 11$ Å. It is thus two to three times as distant from the heme as a molecule of VA modelled into the heme edge channel (Poulos et al., 1993; Schoemaker et al., 1994). The one-electron redox potential of tryptophan free in solution is 1.19 V at pH 3 and lower at higher pH values (Jovanovic et al., 1991). It is thus at least 200 mV lower than that estimated for VA (Khindaria et al., 1996). Long-distance electron transfer from Trp171 to the oxidised heme, possibly via residue 172 which is in van der Waals contact to the heme, undoubtedly occurs resulting in a tryptophan radical cation. Scheme 4.1 proposes a mechanism leading to β-hydroxy-Trp171. The indolyl radical cation has a $pK_a$ of 4.3 (Jovanovic and Simic, 1985) and will be in equilibrium with its neutral radical depending on the pH. The latter could react with oxygen to form the 3-peroxyl derivative, a species that has been reported to occur in $\text{H}_2\text{O}_2$-treated metmyoglobin (DeGray et al., 1997). However, since the hydroxylation was found to be as effective in the absence of oxygen as in its presence another route is favoured which involves a second electron abstraction from the neutral Trp171 radical by the heme. The resulting cation could then deprotonate from its Cβ to give an indolenine structure that is highly electrophilic (Skiles and Yost, 1996) and will add a water molecule leading to the rearomatisation of Trp171. In both routes oxygen from water will be incorporated in the hydroxy group.
Scheme 4.1 Proposed reaction sequences for the autocatalytic Cβ hydroxylation at Trp171 of lignin peroxidase. As described in the text, the right hand sequence which does not require oxygen is more probable.

In principal, a similar reaction sequence to that in Scheme 4.1 could lead to the β-keto compound of Trp171. LiP415 and LiP465 have been exposed more intensively to \( \text{H}_2\text{O}_2 \) in the fungal cultures than the recombinant \( \text{H}_2\text{O}_2 \)-treated enzyme which was used for crystallisation. In the fungal LiP crystal structures the Cβ atoms of Trp171 are strictly tetrahedral and all atoms of Trp171 including the hydroxy oxygen have low temperature factors. Therefore, no other derivatives appear to be present. For LiPH8* no indication of the β-ketotryptophan absorbance (Noda et al., 1977) was found in tryptic peptides after turnover with hydrogen peroxide and VA for up to hundred cycles. Therefore, the further oxidation of Trp171 does not seem to be of relevance. Among the reasons for this behaviour may be the increased redox potential of the hydroxytryptophan (see below) and the increased steric hindrance introduced by the hydroxy group which restricts the movements which accompany the rehybridisations at the Cβ atom in the course of the oxidation (Scheme 4.1).
Stereochemistry of Trp171 Cβ-hydroxylation

In the crystal structures of all oxidatively processed LiP variants solved the Cβ is purely S-configurated which prompts to ask for the reason for this stereoselectivity. In chapter 3.2.5 a little cavity which is shielded from bulk solvent only by Asp264 has been described that contains a single water molecule and, if present, the Trp171 hydroxy group. As explained in the previous paragraph, the final step of the reaction sequence leading to hydroxylation is supposed to be the addition of a water molecule to the indolenine derivative of Trp171. The two sides of its molecular plane are prochiral. On the Re-side there are protein moieties in van der Waals distance that will prevent the attack of water on the Cβ atom. On the Si-side, however, the described water molecule in the cavity is ideally positioned to attack the Cβ atom leading to the stereochemistry that is observed in the crystal structures. Scheme 4.2 illustrates this reasoning.

LiP crystal structures are now available for three different isozymes. In all of them the single water cavity looks very much the same and the surrounding residues are conserved. A functional role of the right stereochemistry at the Cβ of Trp171 can therefore be surmised but not be understood for the time being.
Trp171 hydroxylation in the absence of added peroxide

The dependence of the autocatalytic hydroxylation of pristine LiPH8* on H2O2 and VA has been investigated in chapter 2.2.3. The crystallographic finding of processed enzyme that had not been treated with peroxide was therefore surprising. Whereas the protein chemical experiments have been carried out with freshly thawed enzyme stored at -80 °C there were about two weeks time at 20 °C between thawing the enzyme and the data collection from LiP*-nat crystals. Reactions involving the heme cofactor during this time period at ambient temperature could have brought about the partial hydroxylation of this enzyme form which was supposed to be pristine. More convincing, however, appears the possibility of x-ray induced radical damage during data collection. Radiation damage destroys every protein crystal in an x-ray beam sooner or later. As for metalloproteins, photoreduction of the metal has already been reported. Crystal structures of Cu,Zn-superoxide dismutases for example which have a CuII in the resting state of the enzyme have been obtained in the CuI form. If an analogous process happened in LiP the ferrous heme could reduce oxygen to superoxide which can disproportionate to give H2O2 that in turn could start the catalytic cycle. On the mechanistic details of reactions taking place in a crystal in an x-ray beam can only be speculated. What is important, however, is that this particular sort of radiation damage can be mitigated by adding the diphenolic antioxidant orcinol to the precipitant solution. This finding illustrates again the ease and specificity of the Trp171 hydroxylation.

Special reactivity of Trp171 of LiP as opposed to other tryptophans

Why is Trp171 prone to become hydroxylated? Tryptophans are present in many redox proteins and in many cases they are positioned close to the redox cofactor. They are the second most redox active natural amino acids after tyrosines (Stubbe and van der Donk, 1998). A definite participation in the redox chemistry of an enzyme, however, has only been established for Trp191 in CcP (Sivaraja et al., 1989). This residue is buried inside the protein, it is not solvent accessible and consequently cannot be hydroxylated according to the mechanism proposed above. The same reasoning applies to the buried Trp251 in LiP although some sort of redox activity cannot be excluded for this residue which is located only 9 Å from the heme. The third tryptophan in LiP, Trp17, is solvent exposed but rather distant from the heme presumably precluding electron transfer reactions. A peroxidase with a strikingly high number of tyrosines and tryptophans in the heme environment, both buried and solvent exposed, is mammalian myeloperoxidase whose redox potential is even higher than that of LiP. Although this peroxidase also has a very unstable compound I (Hurst, 1991), no tyrosine or tryptophan modifications were reported when its 2.2 Å resolution crystal structure came
out (Fenna et al., 1995; pdb entry code 1MHL). In conclusion, Trp171 in LiP appears to be a very unusual case, in fact at present it is the only one showing that peculiar feature. Other cases which may have escaped detection in the past will possibly be discovered using the relatively simple analytical approach developed in this thesis.

Redox activity and solvent accessibility are two necessary conditions which must be fulfilled for the LiP-type tryptophan hydroxylation but they are not sufficient alone. Experiments which were not described here have been carried out in the course of this work to see whether tryptophan-β-hydroxylation is an inevitable consequence of an one-electron oxidation of N-acetyl-tryptophanamide using CeIV salts or the LiP-system as oxidants. Under some conditions traces of the α,β-didehydro-tryptophan derivative could be identified but other oxidation products were far more abundant. The oxidative formation of indole-3-carbinol from indole-3-acetic acid (Candeias et al., 1994) or from 3-methyl indole in a cytochrome P-450 catalysed reaction has been described (Skiles and Yost, 1996) but many other products have also been found in peroxidase-catalysed or electrochemical oxidations of tryptophan (Nguyen et al., 1986). Further oxidation of initial oxidation products may be one reason among others for the great variety of products found in the latter study. One can conclude that the Trp171 modification in LiP is highly specific since neither other oxidation products nor further oxidation occurs. The special location of Trp171 in the protein must be the reason for this selective reactivity suggesting that the hydroxylation is not an accidental but a well-controlled event.

4.2 Reaction mechanism

4.2.1 Function of Trp171

A lot of kinetic and spectroscopic work done since the discovery of ligninases in 1983 was aimed at the understanding of the catalytic mechanism and quite some progress has been achieved; many other basic points have remained unclear. Before this work was started no research paper had ever addressed the possibility of Trp171 being a residue involved in catalysis, which is comprehensible in view that there was no particular reason to do so. Since the publications of the crystal structures in 1993 the heme access channel has been generally assumed to be the binding site of small substrates. It was the detection of the Trp171 hydroxylation that drew our attention to this residue and prompted us to look into that aspect in detail.

As initially indicated by chemical modification experiments with the fungal enzymes and was then unambiguously demonstrated by site-directed mutagenesis, Trp171 is an essential residue for the oxidation of the natural substrate VA but not for artificial substrates.
tested (Doyle et al. 1998; chapter 2.2.5). The rates of compound I reduction by VA of the W171S mutant matched the spontaneous compound I decay rate of this mutant in the absence of reducing substrate. Likewise, compound II formed by spontaneous W171S LiP decay did not reveal any significant reactivity towards VA. This clear-cut result is most easily explained by assuming a VA-interaction site at Trp171. As discussed in the following, Trp171 must be required both for VA binding and for the redox chemistry of its oxidation. In the W171S variant VA could take the place of the indole ring and thus get closer to the heme than in the wild-type protein. Nevertheless, this mutant is inactive with VA. The side chain of residue 171 in the W171F mutant is more similar to an indole ring in terms of steric requirements than a serine but yet it is completely inactive towards VA, too (Doyle et al., 1998). If VA is bound and oxidised at Trp171 it can be predicted that its indole system must also be involved in the redox chemistry of the enzyme. This follows from a comparison of the redox potentials of VA and tryptophan free in solution. As discussed above that of the VA+/VA couple is about 200 mV higher than that of the Trp+/Trp couple at pH 3 and it will, once generated in proximity to Trp171, inevitably oxidise the latter. Assuming equilibrium conditions, 200 mV corresponds to 19 kJ/mol energy difference and a 2500 fold higher probability to find an oxidised tryptophan than a VA+. Due to the pH dependence of the tryptophan redox potential, the ratio will approach 10000:1 at pH 4.5, further away from the pH optimum of LiP but closer to the physiological pH of the fungus.

4.2.2 Possible function of the Trp171 Cβ hydroxy group

From the above discussion it is clear that, if VA is oxidised at Trp171, there must exist a way of turning the reactivity from self-oxidation of the enzyme towards substrate oxidation. Whereas the concept of an irreversible reaction drawing the unfavourable VA+/Trp+ equilibrium to the right will be discussed below, the following paragraph will focus on the possible function of the hydroxy group.

As shown in chapter 3.2.5 no structural differences in the crystal structures between the pristine and the Trp171-hydroxylated enzyme forms could be detected other than the mere presence or absence of the OH group itself. Consequently, its role cannot be to bring about a certain side chain conformation of Trp171. It may well restrict the flexibility or torsional vibrations around the Cα-Cβ bond through additional H-bonding interactions of the introduced Cβ substituent. However, the requirement for such a function is not easy to see since the indole ring is already tightly fixed by three neighbouring acidic residues (see Figure 3.10). An involvement in VA binding is also not obvious because it is not accessible from the solvent without major structural reorganisations. It is therefore postulated, that the most important function of the hydroxy group will be to modulate the properties of the indole ring.
by electronic interaction. An OH-group at that position will exert a negative inductive effect on the ring system and thereby render it more electron deficient. Such an effect could potentially change the above mentioned unfavourable VA+/7Trp(+) ratio to a more productive one in terms of substrate oxidation. The assessment of this effect in a thermodynamically more quantitative way, however, is not an easy task since experimentally determined thermodynamic data on 3-hydroxymethylindole derivatives are apparently not available. In the toxicological literature a work was found that compared the antioxidant efficacies of various 3-indolyl congeners and detected a correlation with the reaction enthalpies of electron abstraction from these compounds (Shertzer et al., 1996). Although these enthalpies were calculated quantumchemically at a too low level of theory to allow them to be used as absolute measures of the redox potentials, 3-hydroxymethylindole was a poorer antioxidant than 3-methylindole. This fact supports the conjecture that the hydroxy substituent increases the redox potential of the indole ring.

In chapter 2.2.5 the rates of spontaneous compound I decay were measured and the results strongly suggested Trp171 to be the most effective reductant in this process. The rationale behind the comparison of the pristine and the oxidatively processed enzyme forms was to check whether the Trp171 hydroxy group would influence the electron transfer rate to the heme. A small, about 1.5-fold effect was in fact observed, again indicating an electron withdrawing effect to be operative. Unfortunately, however, it is difficult to derive quantitative information on the redox potentials from the rate constants unless more thermodynamic and kinetic data are measured.

One important piece of information present in the data from chapter 2.2.3 has not yet been mentioned so far. There it was shown that VA competed with the self-oxidation leading to Trp171 hydroxylation. This finding implicates that LiP can react with VA also in its pristine state. It follows that the activity of LiP with VA cannot be strictly dependent on the presence of the Trp171 hydroxylation. The hydroxy group may therefore be advantageous for the enzyme in the sense as described above but not absolutely required. It remains for future work to clarify the function of the Trp171 hydroxy group in more detail.

4.2.3 VA binding and its oxidation

The steady-state kinetic data of LiPH8* and some mutants cited in Table 3.6 show that all mutants affecting the heme access channel retain substantial activity with the natural substrate VA and only the Trp171 mutants are completely inactive towards this substrate. On the other hand these data indicate that the heme channel is the substrate interaction site for the two negatively charged artificial substrates tested. The most important conclusion that can be drawn is that none of the channel residues His82, Pro83 or Glu146 (see Figure 3.12) is
essential for the oxidation of VA. Contrarily, these data clearly show that Trp171 is essential for VA. Inspection of this residues' environment in the crystal structure does unfortunately not provide a hint on how VA could bind. No cavity or concave protein surface or what is generally associated with a substrate binding site of an enzyme is discernible in which VA could be modelled in. Perhaps a specific binding site for VA cannot even be expected since other dimethoxylated arenes like 1,4-dimethoxybenzene can replace VA e.g. in mediating the oxidation of MMA (Harvey et al., 1986). What stands out, however, is a remarkable number of acidic residues on the back side of LiP (Figures 3.10 and 4.1). Particularly, Trp171 is surrounded by Glu168, Asp264 and Glu250 which hydrogen bonds to the indole nitrogen. Although each of these residues will carry only partial negative charge at physiological pH they could provide the acidic microenvironment which has been postulated to be responsible for the stabilisation of VA** (Khindaria et al., 1996). Moreover, the negative electrostatic potential created by these negative charges will reduce the redox potential of Trp171 as well as of VA thereby rendering their oxidation by electron transfer to the heme thermodynamically more feasible. If a positive charge is created in the course of the reaction one of these acidic residues can simply drop a proton into bulk solvent or, in more general terms, the local cooperative ionisation equilibrium will be shifted somewhat towards a lower pK and thereby they may act as a charge buffer. There is only one positive charge near Trp171, that of Lys260 whose amino group is indicated in Figure 4.1 (right) as a blue spot below Trp171.

The redox potentials of LiPI and LiPII have been estimated to be in the range of 1.05-1.15 V (vs. NHE at pH 3.5; Schoemaker et al., 1994; see also discussion below). Recalling those of tryptophan and VA as cited above, the virtually irreversible reductions of LiPI and LiPII by VA cannot be understood without the concept of an effective stabilisation of the reaction products in the enzymatic system. Thus, after transfer of an electron to the heme of LiPI, VA and Trp171 could form a tight [VA-Trp171]** complex with a delocalised unpaired electron and positive charge. According to the relative redox potentials of VA and tryptophan the charge density will be higher on Trp171 and will be stabilised by the neighbouring acidic amino acids. At this point, special emphasis is put on the hydrogen bond between the Trp171 indole nitrogen and the carboxylate of Glu250: since the pK of Trp** (4.3) and glutamic acid are very similar this hydrogen bond is expected to become much stronger with comparable probability for the proton to reside on either residue once Trp171 is oxidised. This would result in a partial charge dissipation of the postulated [VA-Trp171]** complex on Glu250.

The LiPII-[VA-Trp171]** complex corresponds to the LiPII-VA** complex suggested by Khindaria et al. (1995b) with the decisive improvement that the site of VA interaction has now been identified with high probability. From here on, the mechanism of VA oxidation of these authors can be adapted: a second VA molecule can reduce the LiPII-[VA-Trp171]**
Figure 4.1 Grasp (Nicholls, 1993) surface plots of LiP415. Left: so-called front side of LiP with the heme access channel. Right: back side of LiP with Trp171 (green) as a surface residue. The heme is shown in yellow. Red indicates the presence of acidic surface residues and blue basic ones. The environment of Trp171 on the back side is strongly acidic. There is only one basic residue, Lys260, discernible as a blue spot below Trp171 in the right hand picture. The distal domain of the molecule is on the top and the proximal one on the bottom.

Figure 4.2 Backbone ribbon representation of LiP with the heme in yellow and Trp171 in green. The proximal domain is on the bottom and the distal side on the top. The arrow indicates the direction of an electrical dipole vector (pointing towards the positive pole) calculated with Grasp (Nicholls, 1993). For the calculation of the dipole a positive charge was assigned to all basic residues and to all histidines (apart from the proximal one) and negative charges to all acidic residues. Since this is a rather crude approximation the absolute size of the dipole is of no meaning. What is important, however, is that the direction of the dipole changed only slightly when the acidic residues were increasingly protonated to approach to more natural pH conditions.
complex to restore the ferric enzyme while giving rise to a second VA+*. This could deprotonate from the benzylic carbon and immediately reduce the [VA-Trp171]+* complex to form one VAld and regenerate one VA and Trp171. The deprotonation from the second VA represents the irreversible step which is needed to draw the overall reaction towards the product side. Regarding the interaction site of the second VA nothing reliable can be said. It could also be located at Trp171 or somewhere else on the enzyme, e.g. at the heme access channel. If it was at Trp171 the dismutation of one VA* and one VA+* would be easy to understand. In this case there would be no function that could be assigned to the heme channel at present.

Figure 4.2 shows a dipole vector calculated using the LiP415 crystal structure (see Figure legend for details). Its negative end points roughly in the direction of Trp171 and its positive one is near the heme channel. The orientation of this dipole vector initially let us recognise the acidic protein region near Trp171 as possible VA interaction site. Since then, various attempts to identify a substrate binding site in LiP crystal structures with VA and 1,2-dimethoxybenzene have been unsuccessful (this study, experiments not described). From the above discussion, one would expect that positively charged substrate analogues are more tightly bound at Trp171 than their neutral counterparts. In particular the binding site might be optimised for the intermediate VA+* rather than VA. In this respect, \(N-(3,4\)-dimethoxyphenyl\)-hydroxylamine which can be protonated at its nitrogen and which has a very similar structure to VA could mimick VA+*. It will be worth trying whether this compound produces an interpretable electron density in a crystal structure. It is also predicted that this hydroxylamine derivative will act as a competitive inhibitor of VA oxidation in its protonated form.

The finding from the crystal structures of LiP that large substrates like lignin cannot approach the heme has provoked much speculation and experimental work on the actual interaction between the enzyme and this substrate. The new finding that a catalytic site on the surface of the enzyme exists that is connected to the heme via an electron transfer pathway resolves much of that problem.

There are two recent reports on novel types of MnPs which have substantial VA oxidising activities. In one case, the amino acid sequence is known and a tryptophan was found at a position equivalent to Trp171 in LiPs (Heinfling et al., 1998) underlining the requirement of a tryptophan at this position for VA oxidation. In the other case (Mester and Field, 1998) the primary sequence has not come out yet and it will be exciting to see whether this hybrid enzyme from Bjerkandera has also a tryptophan at a comparable position where other MnPs have inert aliphatic residues.
4.2.4 Low pH-optimum and high redox potential of LiP

LiP differs from other peroxidases from the plant superfamily in that it has a higher redox potential and a lower pH optimum of \(\approx 2.5\). Both properties have attracted much attention but their structural origin have not been explained satisfactorily.

It has been proposed by several authors that the redox potential of the high oxidation states LiPI and LiPII will be higher at low pH. This can be illustrated considering the uncatalysed reaction

\[
2 H^+ + H_2O_2 + VA \rightarrow 2 VA^{+} + 2 H_2O
\]

Due to the two protons involved the equilibrium is shifted to the right and the redox potential of the \(H_2O_2/H_2O\) couple increases about 60 mV for each unit the pH is lowered. Thus, simply by lowering the pH from 7 to 3, the redox potential of \(H_2O_2\) increases by 240 mV. It will depend on the energetics of LiP compound I formation how much of this additional oxidative driving force can be transferred to compound I. However, if we add 240 mV to the redox potential of compound I and II of HRP of 920 and 940 mV at pH 7, respectively, we get 1160 and 1180 mV. If we further take into account that the redox potential of the ferric/ferrous couple of LiP is about 150 mV higher than that of HRP (the structural reason of which is not known), we can take \(\approx 1.2\) V as the lower limit of LiPI and LiPII at pH 3. While this value still does not explain exhaustively why LiPI reduction by VA is essentially irreversible it is quite a bit higher than that estimated elsewhere (Schoemaker et al., 1994). If an effective stabilisation of the primary reaction product \([VA-Trp171]^+\) is also taken into account as described above the oxidation of VA becomes well understandable from a thermodynamic point of view.

The LiP-type VA oxidation, especially the involvement of a tryptophan in the redox cycle also demands a low pH. If all the acidic residues in the environment of Trp171 get deprotonated the stabilisation of positively charged intermediates will be such that reactivity will be diminished. Particularly, if the proton from the indole nitrogen of Trp\(^+\) is lost then much of the oxidating ability of this residue will be lost too and the radical will be trapped at Trp171. In this case the oxidation of Trp171 represents an energetic sink in which the enzyme will get stuck. It is therefore proposed that the cooperative ionisation equilibrium of the charged residues at and including Trp171 is a major determinant of the pH dependence of enzymatic activity. Unless much better reductants than VA, e.g. phenolics, are available to restore the intact residue, the neutral Trp171 radical could decay by non-specific and presumably enzyme damaging reactions. The presence of reducing substrates is therefore absolutely required if peroxides are also present to prevent oxidative damage to LiP. VA is usually added to fungal cultures of \(P.\ chrysosporium\) to 'induce' LiP production. This inductive effect, however, is not due to an induction of \(lip\) gene expression (Cancel et al., 1993) but simply to prevent the deactivation of expressed LiP by \(H_2O_2\) produced by oxidases.
in the culture medium. Recently, it has been shown that tryptophan added to fungal cultures is even better suited to stabilise LiP and to increase peroxidatic activity (Collins et al., 1997).
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