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Do Carbohydrates Play a Role in the Lignin Peroxidase Cycle? Redox Catalysis in the Endergonic Region of the Driving Force#

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Abstract—The redox cycle of lignin peroxidase (LiP) is discussed in terms of the Marcus theory of electron transfer. The difference in kinetic behaviour of the two redox couples LiP-Compound I/LiP-Compound II (LiPI/LiPII), respectively LiPII/LiP, in the oxidation of veratryl alcohol is attributed to an estimated increase in reorganization energy of about 0.5 eV for the conversion of LiPII to native enzyme compared to the reduction of LiPI to LiPII. Whereas LiPI/LiPII involves a transition from a low-spin oxyferryl porphyrin radical cation to a low-spin oxyferryl porphyrin system, the conversion of LiPII to native enzyme involves a change in spin-state to high-spin ferric, accompanied by a conformational change of the protein. In addition, a molecule of water is formed after protonation of the oxyferryl porphyrin system by the distal His-47 and Arg-43. Furthermore, the reduction of LiPI to LiPII is observed as an irreversible process. Since the oxidation of veratryl alcohol by oxidized LiP will occur in the endergonic region of the driving force, it is postulated that the thermodynamic unfavourable formation of veratryl alcohol radical cation is facilitated by reaction of a nucleophile with the incipient radical cation. It is further postulated that the ordered carbohydrate residues found near the entrance to the active site channel in the LiP crystal structure play a role in this process.

Introduction

Lignin peroxidase (LiP), a heme-containing glycoprotein isolated from ligninolytic cultures of white-rot fungi like *Phanerochaete chrysosporium*,[1,2] will oxidize non-phenolic electron-rich aromatic compounds to form radical cations.[3,4] LiP also catalyzes the depolymerization of the lignin polymer.[5] However, this depolymerization only occurs in the presence of 3,4-dimethoxybenzyl (veratryl) alcohol,[6] a secondary metabolite of *P. chrysosporium* and other white-rot fungi. As can be inferred from the LiP crystal structure, no direct interaction between the heme and the lignin polymer is possible.[7-9] It has been suggested that veratryl alcohol functions as a mediator between the oxidized forms of the enzyme and the lignin polymer.[10-14] At first sight rather contradictory, veratryl alcohol is also the preferred substrate for LiP and is oxidized to veratraldehyde at the expense of one equivalent of hydrogen peroxide.[15-19] As side-products quinones and muconic acid derivatives can be isolated, indicating that the oxidation to the aldehyde is not a simple hydrogen atom abstraction process, but involves electron transfer from the aromatic ring.[13,14]

In this paper we will discuss the LiP redox-cycle semi-quantitatively in terms of the Marcus theory of electron transfer.[20-22] We will compare the one-electron oxidation of veratryl alcohol and dimeric lignin model compounds with fundamental studies on methylarene oxidations by tris(phenanthroline)iron(III) complexes.[23] The latter oxidations occur in the endergonic region of the driving force.

Substantial work by many groups has focused on determining the parameters that control protein electron transfer rates, such as distance of electron transfer, driving force, medium and orientational effects, and specific electron transfer pathways.[24-31] Although successful separation of the variables involved is difficult, these studies have found some agreement between experiment and Marcus theory. Here, we will especially focus on the reorganizational energy accompanying spin-state changes.[32-34] In addition, the possibility that LiP-Compound II is reduced by hydroxy-substituted benzylic radicals via long-range electron transfer is discussed.

Finally, in one of the LiP crystal structures solved,[7] the occurrence of ordered carbohydrate residues near the entrance to the active site channel could be established. We postulate that these carbohydrate residues, originating from an O-glycosylation site at Ser-334, may play a crucial role in the LiP redox-cycle.[35]

#Dedicated to Professor J. Bryan Jones, in honour of his 60th birthday.

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			second-order rate constant [$M^{-1}s^{-1}$]
$Fe^{III}(phen)_3 + ArCH_3$	$\overset{k_1}{\underset{k_1}{\rightleftharpoons}}$	$Fe^{II}(phen)_3 + [ArCH_3]^{+\cdot}$	$k_1 = 14$ $k_{-1} = 1.3 \times 10^8$
$[ArCH_3]^{+\cdot} + B$	$\overset{k_2}{\rightarrow}$	$BH^+ + ArCH_2^{\cdot}$	$k_2 = 1.5 \times 10^5$
$Fe^{III}(phen)_3 + ArCH_2^{\cdot}$	$\overset{k_3}{\rightarrow}$	$Fe^{II}(phen)_3 + [ArCH_2]^+$	$k_3 = >10^6$

Scheme I.

Literature Data

Aromatic oxidative substitutions via electron transfer

Veratryl alcohol and dimeric lignin model compounds are oxidized by a tris(phenanthroline)iron(III) complex (E_{Fe}^0 = 1.09 V vs SCE, the Saturated Calomel Electrode) in a manner mimicking the one-electron oxidation of these compounds by LiP.[36] Redox potentials of these model compounds are about 1.3–1.4 V vs SCE,[37–40] indicating that the reactions occur in the endergonic region of the driving force. This is analogous to the oxidation of methylarenes by tris(phenanthroline)iron(III) complexes studied by Kochi and coworkers,[23,41–43] as depicted in Scheme I.

In this scheme the second-order rate constants for the oxidation of hexamethylbenzene (E_{Ar}^0 = 1.62 V vs SCE) by the tris(phenanthroline)iron(III) complex are given. Although the first oxidation step is endergonic, the reaction proceeds to completion via a fast and irreversible proton abstraction to form a benzylic radical. Subsequently, the benzylic radical is rapidly oxidized by a second tris(phenanthroline)iron(III) complex. The latter reaction is exergonic (and thus very fast) since the redox potential of the benzylic radical is less than 0.73 V vs SCE.[44] It has been shown that a catalytic version of Scheme I is also possible, *viz* in the electrochemical oxidation of methylbenzenes mediated by ruthenium(III)polypyridine complexes as catalysts.[45]

Kochi and coworkers[23] have shown that there is excellent agreement with the theoretical free energy relationship developed by Marcus. The total reorganization energy obtained as the sum of the inner- and outer-sphere contributions was estimated[23] as 24 kcal mol^{-1}, which is approximately 1 eV. In this reaction the Gibbs free energy change ΔG^0 is about + 0.5 eV (1.62 V–1.09 V), indicating that even with such an unfavourable driving force, reactions may readily proceed.

Lignin peroxidase redox-cycle

In its most general form the LiP redox-cycle can be depicted[16,17] as in Scheme II.

$Enz\text{–}Fe^{III}P + H_2O_2$	$\rightarrow$	$H_2O + Enz\text{–}Fe^{IV}{=}O\ P^{+\cdot}$	(LiPI)
$Enz\text{–}Fe^{IV}{=}O\ P^{+\cdot} + S$	$\rightarrow$	$S^{+\cdot} + Enz\text{–}Fe^{IV}{=}O\ P$	(LiPII)
$Enz\text{–}Fe^{IV}{=}O\ P + S + 2H^+$	$\rightarrow$	$S^{+\cdot} + H_2O + Enz\text{–}Fe^{III}P$	(LiP)

Scheme II.

LiP is oxidized first by H_2O_2 to form LiP Compound-I (LiPI) with a second-order rate constant[17,46,47] of 4.2–6.5 $\times 10^5$ $M^{-1}s^{-1}$. In the oxidation of veratryl alcohol (Scheme II, S = Valc), the rate constant for the conversion of LiPI to LiPII is 2.6×10^6 $M^{-1}s^{-1}$ at pH = 3, the optimum pH for the reaction. The reaction of LiPI with veratryl alcohol strictly obeys second-order kinetics and the reduction of LiPI to LiPII is irreversible.[16] In contrast, the rate-limiting reduction of LiPII by veratryl alcohol shows completely different characteristics. Starting from LiPII—prepared via metachloroperbenzoic acid oxidation of LiP, followed by one-electron reduction with ferrocyanide—saturation kinetics were observed upon reduction with veratryl alcohol. A first-order rate constant of about 30 s^{-1} was reported.[16] If LiPII were prepared by oxidation of LiP with H_2O_2, followed by a slow spontaneous conversion of LiPI to LiPII, results were difficult to interpret. LiPII reduction rates appeared to be independent of veratryl alcohol concentration[18,19] and were in the order of 2–6 s^{-1}.

In contrast, for the conversion of LiPI to LiPII with veratryl alcohol as the reductant, saturation behaviour was not observed[16] in the range of veratryl alcohol concentrations used (up to 0.5 mM). However, in principle saturation behaviour might be observed at higher concentrations of veratryl alcohol, but the reaction rates would be too fast to be measured. These results are rather puzzling for a number of reasons.

First, from a comparison of the redox potentials of the substrate and the oxidized enzyme it is difficult to understand why the reduction of LiPI is observed as an irreversible reaction. Although the redox-potentials of the oxidized forms of LiP are not known, they can be estimated by comparison with the known redox-potentials[48] for the corresponding redox couples of horse radish peroxidase (HRP). Near neutral pH the HRPI–HRPII couple has a redox potential of 0.92 V vs NHE, the Normal Hydrogen Electrode. The redox potential for the HRPII–HRP couple is 0.94 V vs NHE.[32] Like HRP, LiP will act like a bromoperoxidase, but not as a chloroperoxidase,[49,50] indicating that the redox potentials of the LiP redox couples are well below 1.35 V vs NHE[50] at pH = 3. Like LiP, at low pH values HRP will oxidize non-phenolic aromatic compounds with redox potentials up to 1.36 V vs NHE,[51] indicating that it is not required for oxidized HRP (and by the same token for oxidized LiP) to have a redox potential higher than its substrate. LiP will oxidize aromatic compounds with redox potentials up to 1.6 V vs NHE.[51] In comparison, the reduction potential of

the ferric/ferrous couple is higher for LiP (–0.14 V vs NHE) than for HRP (–0.28 V vs NHE).[52] However, this does not necessarily mean that the redox potentials of the LiP redox couples have to be considerably higher than the corresponding HRP couples.[50] From the discussion above it can be inferred that the redox potentials for the LiPI–LiPII couple and the LiPII–LiP couple may be only slightly higher than the corresponding couples in HRP.[50] This rationale implies that the redox potential of veratryl alcohol, which is 1.45 V vs NHE at pH = 3 in aqueous buffer,[37] is higher than the redox potential of the LiPI–LiPII couple. This being true, the reduction of LiPI to LiPII by veratryl alcohol as depicted in Scheme II, should not have been observed as an irreversible reaction.[16] Therefore, the mechanism of formation of substrate radical cations is more complicated than suggested in Scheme II.

Second, from a comparison of the redox potentials of the LiPI–LiPII couple and the LiPII–LiP couple—which in analogy to HRP may be expected to be quite similar—one should expect rather similar behaviour in the oxidation of veratryl alcohol by both LiPI and LiPII. However, in the slow reduction of LiPII by veratryl alcohol saturation kinetics is observed, whereas the fast conversion of LiPI–LiPII strictly obeys second-order kinetics.[16]

A rationale for these puzzling observations is given in the sequel.

Active site modeling studies

Starting from the crystal structure of LiP-2—determined from crystals obtained[8] by microseeding in hanging drops using polyethylene glycol as the precipitant at pH 4.5—veratryl alcohol has been modeled into the active site channel. The substrate is approaching the heme to within 6–7 Å. In this study,[9] the major contacts between the protein and the substrate are with Ile-85, Val-184, Gln-222, Phe-148 and His-82.

In a parallel study,[35] veratryl alcohol has been modeled in the active site channel of LiP415. Crystals of LiP415 were obtained from saturated ammonium sulfate buffered to pH 4.0 with sodium citrate, using the hanging drop method with macroseeding.[7] Interestingly, in this crystal structure ordered carbohydrate residues were found at the entrance to the active site channel. In the 2.5 Å resolution structure, the terminal carbohydrate residue at the entrance was tentatively assigned a mannose structure, further refinement of the structure however, indicates that this carbohydrate residue is a glucose unit.[35] Straightforward model building has been performed to accommodate the substrate veratryl alcohol into the active site channel. No unfavourable contacts, which would have made it necessary to move protein atoms, were found. The major hydrophobic contacts of the modeled veratryl alcohol molecule with the enzyme are with Ile-85, Val-184, Pro-147, Phe-148, Asp-183 and Gln-222, forming at the same time a hydrogen bond with His-82 at the entrance to the active site channel. The 4-methoxy-group of the substrate veratryl alcohol approaches the 8-Me-substituent and the propionate group of the heme within Van der Waals distances. Surprisingly, it was found that the glucose residue at the entrance to the active site channel is positioned with the C4-hydroxy-group at a distance of about 3.5 Å from the aromatic C1-atom of the veratryl alcohol.

From the modeling studies it was also clear that in the proposed binding mode, a putative *meta*-methoxy-substituent in the 5-position would have unfavourable interactions with Ile-85 and Asp-183. Thus, 3,4,5-trimethoxybenzyl alcohol is predicted to be a poor substrate for LiP, as observed experimentally.[53]

Although similar in many aspects, the two modeling studies are not identical. The veratryl alcohol modeled in the LiP415 structure is closer to the heme, without the necessity to move protein atoms.

Marcus theory and electron transfer in proteins

We will discuss the LiP redox cycle in terms of Marcus theory of electron transfer.[22] The application of this theory to the process of electron transfer in proteins has been studied extensively and several excellent reviews have appeared on the subject.[28,30,31]

In the present discussion, we will use the following formulae:

$$\Delta G^{\#} = (\Delta G^0 + \lambda)^2/4\lambda \quad (1)$$

$$k_{ET} = A \exp [-\Delta G^{\#}/k_B T] \quad (2)$$

In equation (1), $\Delta G^{\#}$ (in eV) is the intrinsic barrier for electron transfer, ΔG^0 (in eV) is the Gibbs free energy change (driving force) and λ (in eV) is the overall reorganization energy. For the rate of electron transfer k_{ET} between donor and acceptor in close contact, we use equation (2) with $A = ca\ 10^{13}\ s^{-1}$.

For k_{ET} between weakly coupled donor and acceptor sites[54] we use the 'golden rule' expression (3):

$$k_{ET} = [4\pi^2/h]\ FC_{DA}\ H_{DA}^2 \quad (3)$$

in which H_{DA} is the electronic interaction integral (in cm^{-1}) and FC_{DA} is the Franck–Condon weighted density of states, which can be approximated for weak coupling (small values for H_{DA}) in a high temperature limit by equation (4), as has been demonstrated by Marcus and Sutin:[22]

$$FC_{DA} = [1/\sqrt{4\pi\lambda k_B T}] \exp[-(\Delta G^0 + \lambda)^2/4\lambda k_B T]. \quad (4)$$

In practice, it is impossible to determine the electronic interaction integral in proteins accurately. However, k_{ET} in equation (3) can be approximated in a simple exponential decay model, equation (5), in which the protein is considered as a uniform organic glass:[22,29]

$$k_{ET} = A \exp[-\beta R].\exp[-(\Delta G^0 + \lambda)^2/4\lambda k_B T]. \quad (5)$$

In (5), the damping factor β depends upon the properties of the intervening medium and R is the distance defined by

the center of the edge atom of the donor to the center of the edge atom of the acceptor.

Alternatively, the electronic interaction integral H_{DA} can be approximated using the experimentally derived equation (6), in which n is the number of bonds in a through-bond process in Å:[55]

$$H_{DA} \text{ (in cm}^{-1}) = 2566 \exp(-0.51\ n). \quad (6)$$

Equation (6) represents the first direct measurement of H_{DA} as a function of distance. However, it should be noted that (6) was derived in model studies using a series of conformationally rigid, all-*trans* carbon–carbon systems. The long-range electron-transfer rates measured by Oevering *et al.*[55] are the fastest ever determined experimentally. Therefore, values obtained using (6) for a through-bond process in proteins should be considered as upper-limits. Changes in conformation and orbital symmetry may lower the rate significantly.[54]

Estimation of Parameters Relevant for Marcus Theory

Essential features of the Marcus theory include the driving force, the reorganization energy, distance between redox centers and the electronic coupling matrix. Since none of these parameters is accurately known, we have to make some estimations based on analogous systems described in the literature. Please note that in order to simplify the arithmetics some values are given in two digit numbers, suggesting an accuracy that is without any physical basis.

Furthermore, in the sequel it is tacitly assumed that the first step of the reaction is the formation of an enzyme–substrate complex. Thus, the calculated first order rate constants should be considered as intra-complex electron transfer rates.

Driving force

The redox potential for veratryl alcohol is about 1.45 V vs NHE.[37] We assume that the redox potential for non-phenolic lignin (the inner-part of the polymer) has a similar value. We estimate the redox potential for both the LiPI–LiPII and the LiPII–LiP couple to be 1.05–1.15 V vs NHE at pH = 3.5. The resulting Gibbs free energy change ΔG^0 = 0.3–0.4 eV for the reaction with veratryl alcohol or with non-phenolic lignin, indicating a process in the endergonic region of the driving force.

The oxidation of veratryl alcohol by LiP has been investigated extensively.[13] Veratraldehyde is the major product, but depending on reaction conditions up to 30 % of quinones and ring-opened products can be isolated.[56] The first step is the formation of the radical cation, proton loss from the initially formed radical cation leads to the formation of a hydroxy-substituted benzylic radical, which is further oxidized by the enzyme or by oxygen to form veratraldehyde (see also Figure 1). The redox potential of this radical is not known, but is estimated to be less than 0.55 V vs NHE. This estimate is based upon the known value for the benzyl radical, 0.96 V vs NHE.[44] Introduction of two methoxy substituents in the aromatic ring is expected to lower the redox potential significantly. In addition, the presence of a Cα hydroxy-substituent will lower the redox potential considerably, as can be judged from the known redox potential for the $MeOCH_2$·-radical, which is *ca* 0 V vs NHE.[44] This means that reduction of LiPII by the veratryl alcohol radical has a driving force of at least ΔG^0 = –0.5 eV– –0.6 eV, a strongly exergonic reaction. In Figure 1 the endergonic and exergonic nature, respectively of the two different oxidation steps are schematically depicted.

Reorganization energy

Typical values for the reorganization energy of proteins are in the order of 0.7–1.5 eV.[29,30] Even values of about 2 eV have been reported for Zn^{II}/Fe^{III} hybrid hemoglobins.[57] In the oxidation of methylarenes by tris(phenanthroline)-iron(III) complexes the total reorganization energy was estimated to be 1 eV.[23] For the reduction of LiPI by veratryl alcohol we estimate a total reorganization energy λ_1 of 1.2 eV. In contrast, the reduction of LiPII by veratryl alcohol is accompanied by a spin change and a

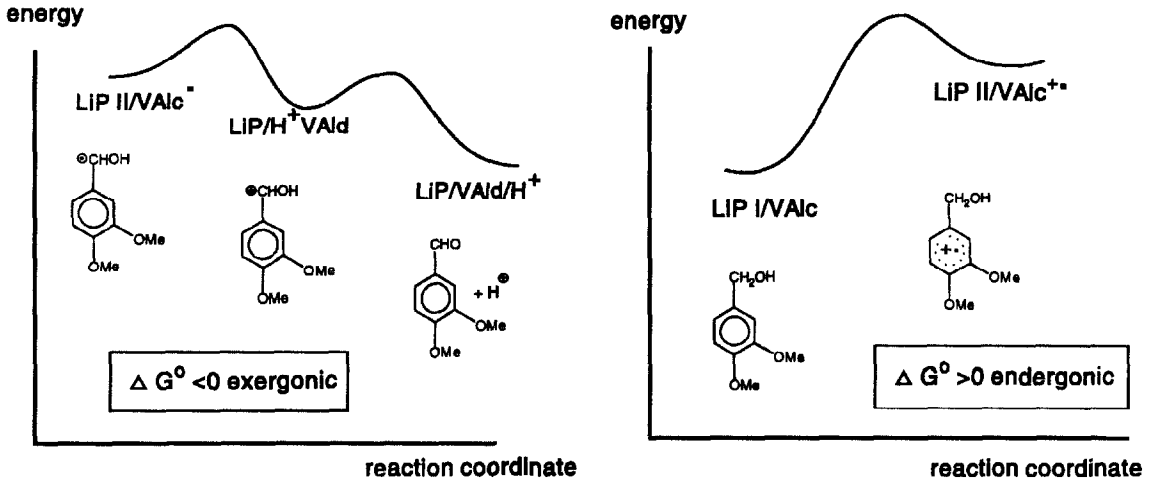

Figure 1. Redox catalysis in the endergonic region; oxidation of veratryl alcohol by LiP.

conformational change in the protein.[32,58] Moreover, in the reduction of LiPII a molecule of water is formed upon protonation of the oxyferryl system. The distal His-47 and Arg-43 are the most likely proton donors (*cf* Figure 2). Such changes may easily account for an extra contribution to the reorganization energy of 0.5 eV.[33,34] Thus, we estimate the total reorganization energy λ_2 for the conversion of LiPII to native enzyme to be 1.7 eV. The crystal structure of LiP[7-9] is quite similar to the crystal structure of cytochrome *c* peroxidase (CcP).[59] It is to be expected that the crystal structure of oxidized LiP also resembles oxidized CcP—of which the crystal structure is known[60]—irrespective of the fact that the second oxidizing equivalent in the two oxidized enzymes resides on different residues. There is a significant conformational difference between oxidized CcP and native CcP, manifested both in the structure of the heme and in the metal ligand distances. Also, literature data suggest that the reorganization energy of the reduction of oxidized CcP with cytochrome c is about 1.5 eV.[31,58] This value is comparable to the value of 1.7 eV estimated here for the reduction of LiPII by veratryl alcohol.

Distance

In LiP, the distance R of the heme edge to the surface of the protein (e.g. to His-82) is about 10 Å.[7-9] We use a value of 1.4 $Å^{-1}$ for the damping factor β in equation (5).[29]

The LiP Redox Cycle in Terms of Marcus Theory

Long-range electron transfer in LiP?

From the LiP crystal structure it is obvious that there is no direct interaction between the heme and the lignin polymer.[7-9] Thus, it has been suggested that both LiPI and LiPII are reduced by non-phenolic lignin via long-range electron transfer.[61,62] However, in view of the redox potential of the polymer compared to the redox potentials of the redox-states of the oxidized enzyme, such a long-range electron transfer process in the endergonic region of the driving force seems rather unlikely. In principle this suggestion can be probed with the Marcus theory. Using the simple exponential decay model[29] of equation (5) with $A = 10^{13}$ s^{-1}, R = 10 Å, $\beta = 1.4$ $Å^{-1}$, $\Delta G^0 = 0.4$ eV and $\lambda_1 = 1.2$ eV for the reduction of LiPI by veratryl alcohol or the lignin polymer via long-range electron transfer, yields $k_{ETlr} = 4.5 \times 10^{-3}$ s^{-1}. This indicates that such long-range electron transfer in the endergonic region of the driving force is catalytically non-competent. The corresponding value for $H_{DA} = 0.03$ cm^{-1} as can be inferred from equations (3) and (4). Even if we substitute $\Delta G^0 = 0.2$ eV (assuming an unrealistically high redox potential of 1.25 V for the LiPI–LiPII couple) and a low estimate for the reorganization energy of $\lambda_1 = 1.0$ eV in (5), then a long-range electron transfer rate of 4.6 s^{-1} is calculated for the reduction of LiPI, again too low for catalysis as compared with the actual experimental data for the reduction of LiPI.[16]

Although this simple exponential decay model has been used rather satisfactorily for a fair number of enzymes,[22,29] it has recently been suggested that in certain proteins specific through-bond pathways exist which would allow for increased electron transfer rates.[24-26] Although not all possibilities were excessively probed, a more detailed study of the LiP crystal structure allowed the identification of a potentially highly efficient through-bond pathway, depicted in Figure 2.

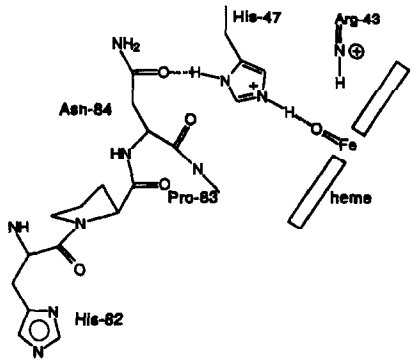

Figure 2. Proposed electron transfer pathway.

His-82 is located at the entrance to the active site channel and—as can be inferred from two different modeling studies (*vide supra*)[9,35]—is hydrogen bonded to the substrate veratryl alcohol. His-82 is connected to the distal His-47 via Pro-83 and Asn-84, Asn-84 is connected to His-47 via a hydrogen bond. A second hydrogen bond connects the protonated His-47 and the oxyferryl system. In this pathway both the distal His-47 and His-82 are considered to be part of the through-bond system, because of the hydrogen bond between the distal histidine and the oxyferryl system. Using equations (3), (4) and (6) with n = 19 (corresponding to $H_{DA} = 0.15$ cm^{-1}), $\Delta G^0 = 0.4$ eV and $\lambda_1 = 1.2$ eV yields a $k_{ETlr} = 8.9 \times 10^{-2}$ s^{-1}. This rate is a factor of 20 higher than the rate calculated with the simple exponential decay model. Even then, the rate is still too low to be catalytically competent. As discussed above, it should be stressed that equation (6) was determined with an ideally designed all-carbon system.[55] In the protein the rates are expected to be significantly lower. This can be inferred from the fact that a single change in the all-carbon system from a *trans* to a *cis* configuration already lowered the rate at least one order of magnitude.[54] In general, for long-range electron-transfer rates over distances of 10–12 Å, H_{DA} values in the range 0.01–0.25 cm^{-1} have been reported for a number of different enzymes.[28,63-66] Thus, both the simple exponential decay model (by definition) and equation (6) used in the estimation of the long-range electron transfer rates in LiP yielded reasonable values for these types of systems.

At this point it is appropriate to comment on the magnitude of the calculated value for H_{DA} using equation (6). The electron transfer pathway proposed here may be compared with a recently published pathway in cytochrome b_5 of approximately similar size.[66] In this pathway, the authors designated the distal histidine as part of the heme

system due to the direct link of the Fe with the distal ligand. This is in contrast with the oxyferryl system discussed here, which is connected to the distal His-47 via a hydrogen bond, thereby elongating the through-bond pathway. The cytochrome b_5 distal histidine was connected via 12 covalent bonds to the other redox center. Using equation (6), a value of $H_{DA} = 5.6\ cm^{-1}$ (upper-limit) can be calculated for this 12 bond system. The experimentally determined value was $H_{DA} = 0.25\ cm^{-1}$, more than a factor of 20 less. The authors also estimated H_{DA} with the simple exponential decay model[29] and the pathway model.[24–26] Using these models, values for $H_{DA} = 0.21$–$0.23\ cm^{-1}$ were obtained.

These two examples show that the calculated value for H_{DA} using equation (6) may be a factor of 5–25 higher than the value obtained with the exponential decay model and—most probably—the experimental value. The electron transfer rate depends on H_{DA}^2, thus the rate may be overestimated by a factor of more than 500 using equation (6). However, in the present discussion a difference of 2–3 orders of magnitude in the calculated value for the intracomplex k_{ET} does not significantly effect the qualitative argument. An optimistic (too high) value for the calculated rate indicates that long-range electron transfer in the endergonic region of the driving force would be even more improbable (*vide supra*). In the exergonic region of the driving force a 500 fold decrease in the calculated rate for a long-range electron transfer process with a large driving force (e.g. $\Delta G^0 = -0.5$eV) would still be catalytically relevant (*vide infra*).

For the present case the values obtained with the simple exponential decay model form a good starting point for further discussion, hence a more accurate estimate of H_{DA} is not necessary. Analogously to the reduction of cytochrome *c* peroxidase by cytochrome *c*,[22] in the present crude analysis there is no need for invoking a specific electron pathway, although of course such a pathway may very well exist.

The reduction of LiPII by veratryl alcohol

Having established that substrates with high redox potentials should be oxidized in close contact with the heme and assuming that veratryl alcohol can approach the heme sufficiently close for efficient electron transfer—as supported[9,35] by modeling studies (*vide supra*)—we can now start to analyze the LiP redox cycle in terms of the Marcus theory using the estimated set of values for the different parameters described above.

First we will investigate the reduction of LiPII by veratryl alcohol. Substituting $A = 10^{13}\ s^{-1}$, $\Delta G^0 = 0.4$ eV and $\lambda_2 = 1.7$ eV, in equations (1) and (2) gives a first order rate constant of $k_{ET} = 54\ s^{-1}$ for a LiPII–veratryl alcohol complex. This value should be compared to the reported values[16,18,19] of 2–$70\ s^{-1}$. This simple back of an envelope calculation indicates that electron transfer may be the rate-limiting step in the conversion of LiPII to native enzyme. Thus, upon formation of a LiPII–veratryl alcohol complex, saturation kinetics may be observed.[22] Substituting $\Delta G^0 = 0.4$ eV and $\lambda_1 = 1.2$ eV in (1) and (2) affords $k_{ET} = 5.4 \times 10^3\ s^{-1}$, indicating that electron transfer probably is not the rate-limiting step in the reduction of LiPI. Evidently, if we substitute $\Delta G^0 = 0.3$ eV (assuming a redox potential of 1.15 V vs NHE for the LiPII–LiP couple) and $\lambda_2 = 1.7$ eV in (1) and (2), we get a first-order rate constant of $604\ s^{-1}$ for the reduction of LiPII by veratryl alcohol, showing the sensitivity of these types of calculations to small changes in the estimated values. It should be remembered, however, that we have used the maximum value $A = 10^{13}\ s^{-1}$ in the calculation. A value of $A = 10^{12}\ s^{-1}$ again would yield a potential rate-limiting electron transfer step.

It can be demonstrated (data not shown) that for realistic values for the pre-exponential factor $A = 10^{11}$–$10^{13}\ s^{-1}$ in equation (2) the intra-complex electron transfer step may become rate-limiting ($k_{ET} = 2$–$70\ s^{-1}$) in the reduction of LiPII to native enzyme for $1.4 < \lambda_2 < 1.8$ eV and $0.4 > \Delta G^0 > 0.2$ eV, corresponding to redox potentials for the LiPII–LiP couple in the range 1.05–1.25 V vs NHE. From the veratryl alcohol/LiP modeling studies[9,35] it can be inferred that the substrate will approach the heme in an edge to edge fashion. Consequently, the pre-exponential factor A may well be[23,67] in the range 10^{11}–$10^{12}\ s^{-1}$. Indeed, since the reduction of LiPI, respectively LiPII, occurs in the endergonic region of the driving force, the back electron transfer will be very rapid. An imperfect alignment of the reactants—manifested in a reduced value for the pre-exponential factor—will result in a slower maximum rate for the back electron transfer, thereby facilitating a secondary irreversible chemical reaction to drive the reaction to completion.[68] Similarly, reactions occurring in the endergonic region of the driving force, which are further driven by a secondary chemical process (*vide infra*), will benefit from a relatively high value for the reorganization energy λ. According to equations (1) and (2), this will limit the rate of the back-electron transfer reaction occurring in the exergonic region of the driving force (*vide infra*).

Thus, a difference of less than 0.5 eV in the reorganization energies for the reduction of LiPI and LiPII may explain the observed experimental results. Traylor *et al.*[32] have suggested a similar (qualitative) change in reorganization energies to rationalize the differences in reduction rates for HRPI and HRPII.

The reduction of LiPI by veratryl alcohol

As indicated above, the experimentally observed irreversible conversion of LiPI to LiPII by reduction with veratryl alcohol[16] is not in concert with the redox potentials of the two redox partners. Since the reaction occurs in the endergonic region of the driving force, an equilibrium of LiPI–veratryl alcohol and LiPII–veratryl alcohol radical cation is expected. In order for the reaction to go to completion, such a putative equilibrium should be displaced by a fast and irreversible secondary reaction, that should take place (almost) concomitantly with electron transfer.[68] In analogy with the oxidation of methylarenes with tris(phenanthroline)iron(III) complex, it may be postulated that in the oxidation of veratryl alcohol by LiPI, a (concomitant) proton abstraction would occur, yielding

LiPII and a veratryl alcohol radical. In this way, a novel redox cycle will result,[13,19] which is depicted in Scheme III. Noteworthy is the fact that this scheme is remarkably similar to Scheme I.

$LiP + H_2O_2$	→	H_2O + LiP-I
LiP-I + VAlc	→	$VAlc^{+\cdot}$ + LiP-II
$VAlc^{+\cdot}$ + B	→	VAlc· + BH^+
LiP-II + VAlc· + $2H^+$	→	H^+VAld + H_2O + LiP
H_2O + H^+Vald	→	H_3O^+ + Vald

H^+Vald = protonated veratraldehyde

Scheme III.

It may then further be postulated that the base B in Scheme III is His-82, located at the entrance to the active site. A similar deprotonation of a $NADPH^{+\cdot}$ radical cation by a histidine-residue has recently been proposed for bovine catalase.[69] The veratryl alcohol derived radical has an estimated redox potential of less than 0.55 V vs NHE (*vide supra*) and would immediately be oxidized to veratraldehyde in a highly exergonic reaction (again a reaction highly analogous to the oxidation of a NADPH· radical in the bovine catalase discussed above). Substituting $\Delta G^0 = -0.5$ eV and $\lambda_2 = 1.7$ eV in (1) and (2) with $A = 10^{13}$ s^{-1} would give a first-order rate constant of 2.1×10^9 s^{-1} for the reduction of LiPII by the veratryl alcohol radical. This hydroxy-substituted benzylic radical would be such a good reductant for LiPII that even long-range electron transfer would occur at a catalytically competent rate. Substituting $\Delta G^0 = -0.5$ eV, $\lambda_2 = 1.7$ eV, R = 10 Å and $\beta = 1.4$ $Å^{-1}$ in the simple exponential decay model (5) would give $k_{ETlr} = 1.7 \times 10^3$ s^{-1}. Alternatively, using (3), (4) and (6), with n = 19 a value of $k_{ETlr} = 2.9 \times 10^4$ s^{-1} can be calculated for a through-bond process.

In this array of reactions, the reduction of LiPI by veratryl alcohol would be irreversible, as observed experimentally. However, only veratraldehyde would be observed as the product at the expense of one equivalent of hydrogen peroxide. No veratryl alcohol radical cations would be available for charge-transfer with methoxymandelic acid or with polymeric lignin.[10–12,18] Still, polymeric lignin is only degraded in the presence of veratryl alcohol.[5,6] Also, in addition to veratraldehyde, quinones and ring opened products are formed in the LiP-catalyzed oxidation of veratryl alcohol. These products are derived from veratryl alcohol radical cations.[13] Scheme III, therefore, cannot be the only mechanism for the LiP redox cycle. In fact, in the veratryl alcohol stimulated oxidation of 4-methoxymandelic acid only anisaldehyde is found as the product, no veratraldehyde could be detected.[10,18] Thus, proton loss from veratryl alcohol radical cation appears to be a rather unlikely event in the LiP-catalyzed reaction. This notion is supported by the observation that also 1,4-dimethoxybenzene (no proton loss from this radical cation is observed) acts as a mediator in the LiP-catalyzed oxidation of 4-methoxymandelic acid.[10,14] Thus, in order to explain the irreversibility of the reduction of LiPI we are forced to conclude that step 2 in Scheme II is too simple a representation of the LiP redox cycle and that in the protein an additional or rather an alternative mechanism must also be operative. Experimental evidence for the occurrence of more than one redox-active species in the LiP redox cycle has been obtained in rapid-scan stopped-flow kinetic studies.[19]

Hypothesis

To rationalize the observations we propose that in the LiP redox cycle the unfavourable electron transfer equilibrium is displaced via reaction of the veratryl alcohol radical cation with a nucleophile in or near the active site (see Scheme IV). Thus:

LiP-I + ROH + Valc	→	LiP-II + Valc--OR + H^+
Valc--OR + H^+	→	$Valc^{+\cdot}$ + ROH
$2H^+$ + LiP-II + ROH + Valc	→	LiP + Valc--OR + H^+ + H_2O
Valc--OR + H^+	→	$Valc^{+\cdot}$ + ROH
$Valc^{+\cdot}$ + lignin	→	Valc + $lignin^{+\cdot}$ etc.

Scheme IV.

Addition of nucleophiles to radical cations represents a mechanism which is well precedented in the literature[70–72] and is known to be competitive with proton loss, e.g. in the case of the radical cation of methoxy-toluene.[43] The nucleophilic addition should occur in the active site (almost) concomitantly with electron transfer, to rationalize the fast and irreversible reduction of LiPI.[68] The addition product of the nucleophile and veratryl alcohol radical cation subsequently diffuses out of the active site channel. Then, under the acidic reaction conditions, the veratryl alcohol radical cation is formed again. This radical cation subsequently will act as a mediator to oxidize the polymer. At the moment we do not know the nature of the nucleophile, it might be a protein residue or even water. However, a simple back of an envelope calculation indicates that the reaction of the nucleophile with the veratryl alcohol radical cation should be very fast to compete with the exergonic back electron transfer. Using equations (1) and (2) with $A = 10^{12}$ s^{-1} $\Delta G^0 = -0.2$ eV and $\lambda_1 = 1.2$ eV—conditions that would minimize the back electron transfer rate—will still yield a $k_{ETback} = 2.4 \times 10^8$ s^{-1}. Such a rate implies that the nucleophilic addition should be very fast. Pulse radiolysis experiments[11] indicate that the half-life of veratryl alcohol radical cations may be remarkably long (40 ms) in aqueous environment, thereby possibly excluding the solvent water as the nucleophile. Therefore, a fast nucleophilic addition to the radical cation has to occur in a pre-ordered (enzyme-like) configuration. From the modeling studies[35] (see also Figure 3) Asp-183 appears to be the only protein residue in the neighbourhood of veratryl alcohol to act as a nucleophile at the acidic pH of the reaction. The free carboxylate group of Asp-183, however, is hydrogen bonded to the heme-propionate group, an arrangement opening up the channel to the active site. Reaction of Asp-183 with the incipient veratryl

alcohol radical cation would have to involve cleavage of this hydrogen bond and rotation around the Cα–Cβ bond to bring the carboxylate group in the proximity of the veratryl alcohol radical cation. However, because of the restricted mobility of this Asp-183 residue, it is hard to imagine that the so formed Asp-183–veratryl alcohol radical cation adduct can move away from the heme. We therefore postulate that the carbohydrate residue observed near the entrance to the active site channel[7,35] will function as the nucleophile.

Discussion

Can carbohydrate residues be involved in the redox cycle?

Modeling of veratryl alcohol in the active site channel of LiP415 indicates that the 4-methoxy-substituent is in Van der Waals contact with the 8-Me-group of the heme and with the propionate substituent, whereas the alcohol function is hydrogen bonded to His-82 at the entrance to the active site channel.

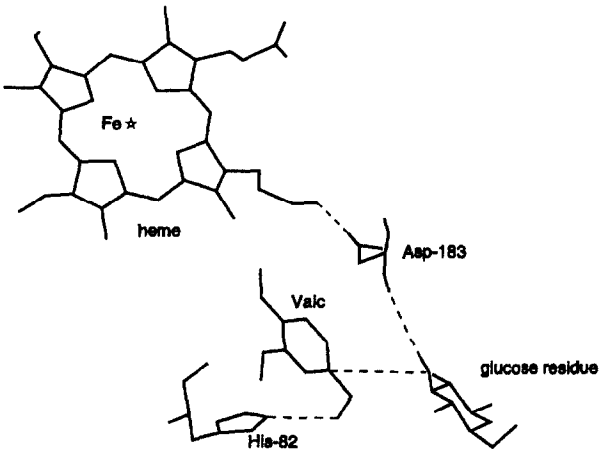

Figure 3. Schematic representation of veratryl alcohol modeled into the LiP active site. The possible interaction with the glucose residue is indicated. Asp-183 is hydrogen bonded to the heme propionate group.

Also, as depicted in Figure 3, the C4-hydroxy group of the glucose residue at the entrance, originating from the glycosylation site Ser-334, is 3.5 Å removed from the aromatic C1 atom of the aromatic ring. Furthermore, this C4-hydroxyl-group is hydrogen[35] bonded with the main-chain carbonyl oxygen of Asp-183. We now postulate that this is a physiologically and chemically relevant configuration for the reasons given above.

Electron transfer to LiPI will result in the development of a positive charge in the aromatic ring of veratryl alcohol (see Figure 4 for a schematic representation). Concomitantly, the hydroxy-group at C4 of the glucose-residue will react with the incipient radical cation, the C–O distance is shortened, whereas at the same time the hydrogen bond of the sugar residue with Asp-183 is broken with the assistance of solvent, resulting in deprotonation of the initially formed veratryl alcohol radical cation–glucose complex to form a glucose–cyclohexadienyl radical. Such a mechanism addresses the fundamental

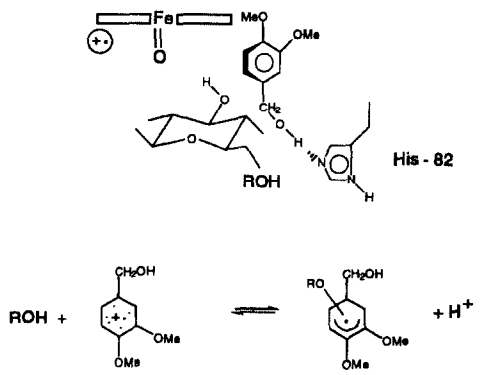

Figure 4. Reversible reaction of the glucose residue at the entrance of the active site channel with the incipient veratryl alcohol radical cation.

question how a reaction can occur in the endergonic region of the driving force. According to Perrin[68] electron transfer may be accompanied by nuclear motion. Subsequently, the glucose–cyclohexadienyl radical complex is 'forced' to move away from the heme, making the reduction of LiPI essentially irreversible. Outside the active site, protonation of the complex under the acidic reaction conditions (the optimum pH for veratryl alcohol oxidation by LiP is 3), regenerates a veratryl alcohol radical cation and the glucose residue.

The fate of the resulting veratryl alcohol radical cation is complex.[13] In general, radical cation species are highly acidic[41,73] and will rapidly deprotonate to form a (hydroxy-substituted) benzyl radical. If this happens near the protein surface (e.g. near His-82), fast and irreversible long-range electron transfer may result in the reduction of LiPII and the formation of veratraldehyde. Alternatively, the benzylic radical may react with oxygen to form superoxide anion and veratraldehyde, a well documented process.[74–76] In these instances, after reduction of LiPI by veratryl alcohol, the enzyme is in the LiPII-redox state.

Although these two processes will efficiently lead to veratraldehyde formation, they are not productive from the point of view of lignin biodegradation. In addition, radical cations may also react with the solvent, water, and oxygen or perhydroxy-radical to form ring opened products and quinones.[56]

Alternatively, however, the radical cation may act as a powerful one-electron oxidant to oxidize aromatic structures like polymeric lignin. Thus, a lignin radical cation is formed, which subsequently might decompose via ether bond cleavage or Cα–Cβ bond cleavage, both depolymerizing reactions. This mechanism rationalizes the depolymerization of the lignin polymer in the presence of veratryl alcohol. LiPII may be reduced by the veratryl alcohol formed as a result of the charge transfer process. Thus, the mediator veratryl alcohol can be considered as a cofactor for LiP in the depolymerization of lignin.

Under the acidic reaction conditions the radical cation of veratryl alcohol has a short but distinct life-time. In pulse radiolysis experiments (measuring the lifetime in the reaction with solvent) a half-life of 40 ms has been

reported.[11] Still, in order to oxidize the lignin via charge transfer, the polymer should be in close contact with the protein, preferably near the entrance to the active site channel (e.g. His-82). At this point it is appropriate to note that the β-O-4-interunit linkages are the most prevalent substructures in lignin. Recently, the mechanism of Cα–Cβ cleavage of such lignin substructures and the consequences for the LiP redox cycle have been discussed.[19,77] Interestingly, this C–C bond cleavage proceeds with formation of a (polymer-bound) hydroxy-substituted benzylic radical at the Cα-position, and a $C\beta^+$-fragment.[77] Thus, polymeric lignin will only be oxidized in close proximity to the protein surface, due to the limited lifetime of the veratryl alcohol radical cation. The subsequent rapid Cα–Cβ cleavage results in the formation of a powerful reductant, the polymer-bound hydroxy-substituted benzylic radical, which has a redox potential of less than 0.55 V vs NHE, and is perfectly capable of reducing LiPII via long-range electron transfer (*vide supra*), a process arguably faster than the reduction of LiPII by veratryl alcohol. Evidently, in this hypothesis quite a number of assumptions have been made. The lignin polymer should bind to the protein and the resulting benzylic radical should not react with oxygen. Under *in vitro* conditions, this long-range electron transfer mechanism may be rather unlikely to occur. However, such a hypothetical mechanism might be envisaged under the physiological conditions of the lignin degradation process *in vivo*.[14]

Conclusion

The Marcus theory of electron transfer is a powerful tool in analyzing the redox cycle of LiP. The difference in reactivity of the two redox forms in the enzyme towards veratryl alcohol and other substrates is attributed to the difference in reorganization energy of the two different reduction steps. The conversion of LiPI to LiPII involves a low-spin to low-spin transition, whereas the conversion of LiPII to native enzyme involves a transition from low-spin to high-spin. The theory shows that long-range electron transfer is not possible in the endergonic region of the driving force and also indicates that the experimentally observed formation of radical cations outside the active site channel must be a secondary process. As an alternative to proton abstraction, reaction of veratryl alcohol radical cation with a nucleophile—more specifically the carbohydrate residue near the entrance of the active site channel—is considered to be a prerequisite to rationalize the, experimentally observed, irreversible nature of the reduction of LiPI to LiPII and to prevent, at least in part, the formation of veratryl alcohol radicals near His-82. Via a reversible reaction with the carbohydrate residue located at the entrance to the active site channel, veratryl alcohol radical cations are transported away from the 'gate-keeper' His-82 and are now able to oxidize the lignin polymer. Subsequently, Cα–Cβ bond cleavage leads to a polymer-linked hydroxy-substituted benzylic radical. The theory also predicts that long-range electron transfer in LiP is possible in the exergonic region of the driving force. During the redox cycle the enzyme may generate powerful reductants for LiPII, either veratryl alcohol radical as the result of proton abstraction, or polymeric hydroxy-substituted benzylic radicals formed after C–C bond cleavage in the polymer. The resulting driving force ΔG^0 is at least –0.5 eV and is high enough to overcome the large reorganization energy λ_2 for the conversion of LiPII to native enzyme via long-range electron transfer.

Although a specific but unidentified role for carbohydrate moieties in peanut peroxidase catalysis has been suggested,[78] to the best of our knowledge this is the first time that reaction with a carbohydrate residue near the active site is implicated in the mechanism of a peroxidase redox cycle. At this point, it should be stressed that in LiP catalyzed oxidation of veratryl alcohol or of dimeric lignin model compounds, the involvement of carbohydrate residues is *not* a prerequisite for veratraldehyde formation.[79] Proton loss or Cα–Cβ cleavage may provide the driving force for these reactions. At the moment we are actively pursuing further support for the postulated mechanisms. Also, we are investigating if the direct involvement of carbohydrate residues in enzyme catalysis is a more general phenomenon.

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