Review

Direct Electron Transfer Between Ligninolytic Redox Enzymes and Electrodes

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Abstract
The electrochemistry of the ligninolytic redox enzymes, which include lignin peroxidase, manganese peroxidase and laccase and possibly also cellobiose dehydrogenase, is reviewed and discussed in conjunction with their basic biochemical characteristics. It is shown that long-range electron transfer between these enzymes and electrodes can be established and their ability to degrade lignin through a direct electron transfer mechanism is discussed.

Keywords: Laccase, Lignin Peroxidase, Manganese Peroxidase, Cellobiose Dehydrogenase, Graphite Electrode, Gold Electrode, Thiol Modified Electrodes

1. Introduction

This review focuses on one of the most intriguing phenomenon that has been intensively studied over the last 25 years. Understanding "Bioelectrocatalysis" at atomic resolution is not only an intellectual challenge but has already contributed greatly to improving the quality of life since it is the basis of a range of biosensors, of which the personal blood sugar monitor is perhaps the most successful. Extending this technology will depend on determining the parameters that modulate direct electron transfer (DET) between an electrode material and redox active proteins or protein clusters [1]. The very first reports on DET with a redox active protein were published in 1977 when Eddowes and Hill [2] and Yeh and Kuwana [3] independently showed that cytochrome *c* on bipyridyl modified gold and tin doped indium oxide electrodes, respectively, showed virtually reversible electrochemistry as revealed by cyclic voltammetry. Cytochrome *c* is a small redox protein, which is active in biological electron transfer (ET) chains but has no intrinsic catalytic activity [4]. These first publications were soon followed in 1978/79 by reports from Russian scientists that provided indirect evidence that DET was also possible for larger redox proteins with enzyme activity (oxidoreductases or 'redox enzymes'). They showed that, laccase [5, 6] and peroxidase [7] modified carbon electrodes exhibited DET in the presence of the substrates dioxygen and hydrogen peroxide, respectively. These findings were reported some 10 years after the first papers were published on combining redox enzymes and electrodes (enzyme based amperometric biosensors or "enzyme electrodes" [8]). The electronic coupling between redox enzymes and electrodes for the construction of devices for practical applications (enzyme electrodes, biofuel cells, bioelectroorganic synthesis) has, however, in most cases, not been based on DET but rather on the electroactivity of the enzyme primary or secondary substrate or product (*first generation biosensors*) or through the use of nonphysiological redox mediators (*second generation biosensors*), most typically illustrated by numerous biosensors based on glucose oxidase [9].

A high percentage of all enzymes are redox active revealing their great importance for all living organisms. Although most of them are intracellular enzymes, there are also numerous extracellular redox enzymes. Efficient DET reactions with electrodes have been shown for many of the high and low molecular weight redox proteins, which have no intrinsic catalytic activity but act as electron transfer components in biochemical pathways (e.g., ferredoxins, flavodoxins, cytochrome *c*) [10–13]. In contrast efficient DET reactions with electrodes have only been reported for a small number of redox enzymes [10, 11, 14–24]. There are in principle two experimental approaches to establishing

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whether DET is occurring between redox enzymes and electrodes:

1. Indirect evidence from observing a catalytic response current in the presence of the enzyme substrate.
2. Direct evidence from observation of independent electrochemical activity of the redox cofactor comprising the active site in the absence of substrate.

The vast majority of redox enzymes with known DET properties contain redox active metallocenters in their active site, e.g., heme, iron-sulfur clusters, and copper [18]. The number of redox enzymes with DET properties lacking metallocenters and having only an organic cofactor (e.g., a flavin or a quinone) is small [18]. Many of the redox enzymes with DET properties with electrodes are intracellular enzymes located in membranes, where they participate in biological electron transfer pathways. This requires complementary docking sites on each redox partner that often minimize the electron transfer resistance-distance between the two redox active metal or organic cofactor centers. However, there are also extracellular redox enzymes exhibiting efficient DET reactions with electrodes. The in vivo functional significance of this phenomenon, if any, is uncertain. However, it is interesting to note that the redox enzymes secreted by the ligninolytic white-rot fungi, *viz*. lignin peroxidase (LiP) [25, 26], manganese peroxidase (MnP) [25], laccase [5, 6, 27 – 29], and cellobiose dehydrogenase [18, 30, 31], all show efficient DET. This contrasts with extracellular redox enzymes produced by other fungi, e.g., glucose and pyranose oxidase, which do not exhibit DET. Therefore it is interesting to further study the bioelectrochemistry of these enzymes in order to investigate whether there exists any correlation between their ability for DET with common electrode materials (bare and surface modified carbon, gold, platinum, etc.) with their 3D-structure and their interaction with lignin. This should also lead to a better understanding of the chemistry of enzymatic lignin degradation. Oxidative degradation of natural polymers such as lignin by soluble enzymes involves heterogeneous catalysis effected either by a direct interaction between the enzyme and the polymer or by small molecules, redox active mediators such as veratryl alcohol (VA, 3,4-dimethoxybenzyl alcohol).

Research on DET based reactions between redox enzymes and electrodes has been undertaken in our laboratory at Lund University since our first report on DET between graphite electrodes modified with adsorbed horseradish peroxidase (HRP) [32]. It is now our central research theme from both fundamental and applied perspectives [18, 21, 27, 33 – 38]. There follows below a review of our previous and current ongoing research in this area focused on the ligninolytic redox enzymes.

2. Lignin

Lignin, a highly branched, irregular three-dimensional organic polymer, is the most abundant biopolymer in nature next to cellulose [39]. Lignin provides the protective matrix surrounding the cellulose microfibrils of plant cell walls. White-rot fungi are the only known microorganisms capable of efficient depolymerization and mineralization of lignin. Thus they play a crucial role in the carbon cycle of the biosphere [40 – 43]. In culture, white-rot fungi secrete besides several cellulose and hemicellulose hydrolyzing enzymes, an array of ligninolytic redox enzymes [42, 44 – 46]. It is today generally accepted that two peroxidases, i.e., lignin peroxidase (LiP) and manganese peroxidase (MnP), and laccases are directly involved in the breakdown of lignin. These enzymes act nonspecifically via the generation of lignin free radicals, which undergo spontaneous cleavage reactions [47]. This is the reason that these extracellular proteins of basidiomycetes origin are denoted "enzymes of the ligninolytic complex". White-rot fungi, however, also secrete cellobiose dehydrogenase (CDH), the physiological role of this enzyme has not yet been elucidated, but it is suspected to be involved in both cellulose and lignin degradation [48 – 59].

3. Lignin and Manganese Peroxidase

Among the other ligninolytic enzymes, white-rot fungi secrete two types of extracellular heme-containing peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), which are believed to be the main components of the bio-degradation system of lignin. They also degrade a wide variety of environmental pollutants that ultimately yield carbon dioxide. These ligninolytic peroxidases follow a classical peroxidase catalytic cycle [44]:

$$E(Fe^{3+}) + H_2O_2 \xrightarrow{k_1} E1(Fe^{4+}{=}O,\ P^{\bullet+}) + H_2O \quad (1)$$

$$E1(Fe^{4+}{=}O,\ P^{\bullet+}) + e^- (S) + H^+ \xrightarrow{k_2} E2(Fe^{4+}{=}O) + S^{\bullet} \quad (2)$$

$$E2(Fe^{4+}{=}O) + e^- (S) + H^+ \xrightarrow{k_3} E(Fe^{3+}) + S^{\bullet} + H_2O \quad (3)$$

This cycle represents a two-electron oxidation of the ferriheme prosthetic group of the peroxidase by H_2O_2 to form compound I (E1), containing an oxyferryl iron ($Fe^{4+}{=}O$) and a porphyrin π cation radical $P^{\bullet+}$. The two-electron reduction of E1 back to the initial ferriperoxidase occurs through the intermediate formation of compound II (E2) by two sequential one-electron transfers from an electron donor S (the reducing substrate). A key step in the catalytic cycle is the heterolytic cleavage of bound peroxide to yield compound I and water. Mutation of the distal pocket histidine (H42) and arginine (R38) of horseradish peroxidase (HRP), not only supports the original mechanism for peroxide binding and cleavage proposed by Poulos and Kraut [60] but has also allowed the identification of a ferric-peroxyanion (Compound 0) intermediate [61]. Crystal structures of high-valency redox intermediates have recently been obtained using X-ray driven catalytic reduction of a bound oxygen species of HRP [62]. The H42Leu mutant of HRP catalyzes a significant amount of homolytic cleavage

of bound peroxide that yields a hydroxyl radical that can react with the protein matrix to yield a free radical located more than 10 Å from the heme iron [61]. A related observation is that the X-ray structure of LiP shows that a surface Trp(W171) is hydroxylated at the C_β position and that this posttranslational modification is autocatalytic [63, 64]. It is also proposed that Trp 171 is part of the site that binds and oxidizes the small molecule mediator VA that is involved in lignin degradation. These observations are also significant in the context of defining electron transfer pathways through the protein and suggest that protein radicals could be generated electrochemically and contribute to both catalytic function and better understanding of protein inactivation/degradation processes.

Despite the structural similarities and common catalytic cycle, MnP and LiP exhibit differences in their substrate specificity [42]. MnP is unique among peroxidases in that its principal substrate S is Mn^{2+}, which is oxidized to Mn^{3+} [65]. The physiological substrate for LiP is thought to be VA, which is oxidized by a one-electron mechanism to yield the veratryl alcohol cation radical [66]. Both cosubstrates, Mn^{2+} and VA, are secondary metabolites naturally produced (Mn^{2+}) or de novo synthesized (VA) by white-rot fungi, which accumulate in the extracellular medium of fungal cultures and induce elements of the ligninolytic system. Therewith LiP and MnP generate powerful diffusible oxidants capable of oxidizing secondary substrates such as phenolic compounds, dyes, thiols, and in the case of polymers such as lignin, initiating their free-radical depolymerization, see Figure 1. In addition, LiP has been shown to directly oxidize lignin which was confirmed using a resonant mirror biosensor [67] and lignin model compounds [68]. Furthermore, a direct 1 e^--transfer from a lignin model compound to Compound I as well as to Compound II was shown using stopped flow technique [67]. These findings indicate that long-range ET must occur also at the surface of the enzyme in the absence of any soluble mediator.

The main distinction between LiP/MnP and plant peroxidases (e.g., HRP) is that the ligninolytic peroxidases have much higher redox potentials for the transformation between the ferric state and Compound I/II [44]. LiP and MnP can catalyze the oxidation of substrates with oxidation potentials higher than 1.3 V (vs. NHE) [44]. Thus, despite some similarities with other peroxidases, ligninolytic peroxidases display unique redox characteristics in the oxidation of aromatic macromolecules. Specifically, they exhibit high redox potentials for the reduction of compounds E1 and E2 and have a low pH optimum for activity, of pH 3 – 4 [47, 69, 70]. At pH 9.0 lignin peroxidase loses two structurally defined calcium ions, the heme becomes 6-coordinate with a second histidine ligand and catalytic activity is lost. However, the inactivation is reversible since on lowering the pH to 6.0 in the presence of calcium ions, the heme returns to five-coordinate with a single histidine ligand, the two structural calcium ions are reincorporated and full activity is recovered [71].

The unique redox properties of ligninolytic peroxidases actually invite electrochemical investigation, both for the development of highly efficient catalysts for bioelectrochemical oxidation/degradation of polyaromatic compounds with high oxidation potentials and for fundamental investigations of the relevance of long-range ET between these peroxidases and their natural substrate lignin. Optimally, direct electronic communication (in the absence of any mediator) between the electrode and the peroxidase active site may provide not only direct electrochemical data on redox potentials but also kinetic parameters for the bioelectrocatalytic reduction of H_2O_2 catalyzed by peroxidases. Our initial bioelectrochemical study of the ligninolytic peroxidases [25] was also a logical extension of our previous bioelectrochemical studies on plant peroxidases, in which we investigated how the DET properties of HRP are influenced by factors such as: deglycosylation [72], "inner" [72] and "outer" mutants [73 – 76], electrode material [77], pH [77, 78]. We also characterized the DET properties of several other plant peroxidases adsorbed onto graphite electrodes [79 – 81].

The most intensively studied of all ligninolytic peroxidases are LiP and MnP secreted by *Phanerochaete chrysosporium*. They share 43% amino acid sequence identity

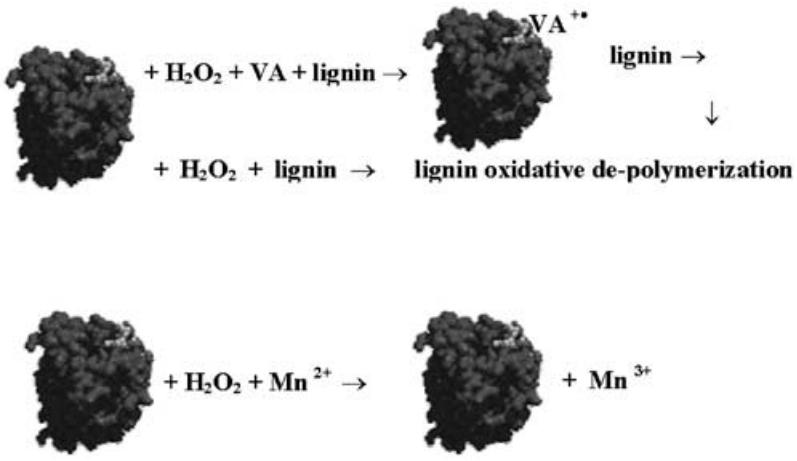

Fig. 1. Schematic representation of the action of ligninolytic peroxidases and lignin.

and have very similar heme environments to those of other plant peroxidases (the distal H_2O_2-binding pocket contains a catalytically active histidine and an arginine residue essential for efficient heterolytic cleavage of bound peroxide to yield compound I) [42, 44]. Both LiP and MnP are anionic at physiological pH, have similar molecular weights and extent of glycosylation [44, 82]. The difference in function of these two homologous peroxidases is attributed to different substrate-binding sites. In MnP, the Mn^{2+}-binding site is believed to be located at the γ-meso edge of the porphyrin [83]. In contrast to MnP, the substrate binding site(s) in LiP is (are) the subject of ongoing research. Modeling studies suggested that various aromatic substances could be oxidized at the surface tryptophan residues (W17 or W171) [84], whereas other researchers have proposed substrate oxidation at the surface residue H239 [67] and at the heme access channel near the heme δ-meso edge [85]. The VA oxidation site near W171 was later confirmed by mutational analyses [64, 86] and through the use of chemically modified LiP [87].

The 1 e^--redox potentials for the ferrous/ferric transitions in a number of LiP isozymes determined by potentiometric titration with mediators are in the range of −142 to −127 mV, and for MnP in the range of −88 to −93 mV (vs. NHE), pH 7.0 [88] compared to −278 mV determined for HRP [89, 90]. Cyclic voltammetry of LiP and MnP at pyrolytic graphite electrodes demonstrated similar values for the redox potentials of the ferrous/ferric transitions in LiP, MnP, and HRP, −126, −122, and −136 mV (vs. NHE), pH 7.0, respectively [26, 91], see Fig 2. The overall 1 e^- reaction was coupled to the transfer of one proton to the ferric enzyme, in acidic media, which was interpreted as the presence of a heme-linked ionization of an amino acid in the reduced forms of the LiP and MnP similar to that of other plant peroxidases [26, 88]. The results of studies of the ferri/ferrous redox states are consistent with a more electron deficient heme environment of ligninolytic peroxidases compared to that of HRP. This could explain the large differences in the redox potentials of E1/E2 transformation for LiP and MnP compared to HRP. In contrast the 2 e^--2 H^+ redox reaction for the ferric/CompoundI/II transition has been reported with electrochemical techniques and in the absence of H_2O_2 only for cytochrome *c* peroxidase [92, 93].

The direct bioelectrocatalytic reduction of H_2O_2 catalyzed by ligninolytic peroxidases from *Phanerochaete chrysosporium* adsorbed on graphite electrodes has been studied in detail through rotating disk electrode experiments performed at 0 V (vs. SCE), pH 7.0 [25]. The direct bioelectrocatalytic reduction of H_2O_2 at peroxidase modified electrodes can be followed at applied potentials more negative than the $E^{\circ\prime}$ of the ferric state/Compound I/II [32, 94–97]. In previous investigations [34] it was shown that this process is, however, very much dependent on the applied potential and reaches a maximum value around 0 mV (vs. SCE) ($\approx$+250 mV vs. NHE). The reason why the highest efficiency is exhibited several hundred millivolts away from the $E^{\circ\prime}$ of the ferric state/Compound I/II is not known, but

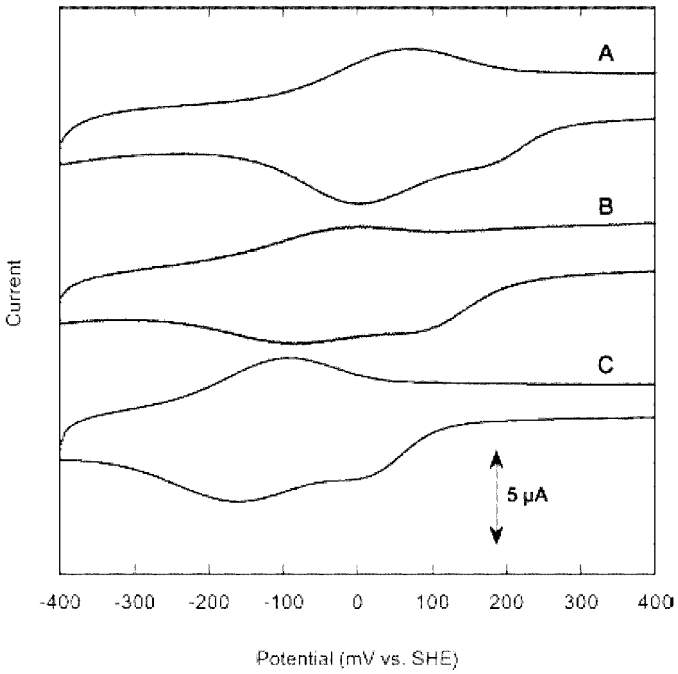

Fig. 2. Cyclic voltammograms of tributylmethyl phosphonium chloride polymer (TBMPC) entrapped LiP at a pyrolytic graphite electrode in the potential range between 400 and −400 mV (vs. NHE) at a scan rate of 80 mV s^{-1}. Cyclic voltammograms were run at a room temperature in: A) 100 mM succinate buffer, pH 3.5; B) 100 mM malonate buffer, pH 5.0; C) 100 mM phosphate buffer, pH 7.0. Potential was corrected vs. NHE from vs. Ag | AgCl. Reproduced from [26] with permission from Elsevier.

may indicate that the number of adsorbed enzyme molecules in DET contact with the electrode is dependent on the applied potential, i.e., the thermodynamic driving force [98]. Analyzing the rate constants at around +250 mV (vs. NHE) is thus within a potential range that is not compromised by risking the reduction of the catalytically active ferri-state of the enzyme into its noncatalytic ferrous state.

It was demonstrated that LiP and the various forms of MnPs, in the absence of their specific substrates, i.e., VA and Mn^{2+} exhibit similar behavior for H_2O_2 reduction to that observed previously for HRP (Fig. 3) [18, 33–37]. The bioelectrocatalytic response to H_2O_2 of the LiP-modified graphite electrodes was stable and pronounced but lower than those of the corresponding MnP- and HRP-modified electrodes. These results demonstrated that LiP can establish a direct electronic communication with the electrode surface (and also possibly with lignin) and supports the suggestion of Johjima et al. [67] that long-range electron transfer occurs between LiP and lignin. The rate constants for heterogeneous ET, k_s, that characterize the electronic communication between graphite and the ligninolytic peroxidases were close to 1.6 s^{-1}, pH 7.0, which is close to the value of 2.3 s^{-1} reported for HRP [34, 37, 99] (Table 1). The very similar heterogeneous ET rates for HRP and the ligninolytic peroxidases at an electrode surface contrast with the large differences in their homogeneous catalytic activity towards ABTS (i.e., 1400 U mg^{-1} for HRP and

ranging between 0.02 and 8 U mg^{-1} for the ligninolytic peroxidases [25]). The reason for this is not known but point to the fact that these peroxidases exhibit high substrate specificity.

The similar values for the k_s may simply reflect similar ET pathways from the electrode surface to the active sites of the three studied peroxidases. However, the percentage of the peroxidase molecules, active in DET reactions with the graphite electrode, was significantly lower for LiP, than that for HRP. This most likely reflects differences in the surface topology and structural features of the peroxidases studied (Table 1). The relative efficiency of proton-coupled DET between these peroxidases and a graphite electrode can be determined from the variation of k_s with pH (Table 2) [25, 78, 99, 100]. LiP from *Phanerochaete chrysosporium* exhibited a four-fold increase in k_s on decreasing the pH from 7.4 to 6.0 while LiP from *Trametes versicolor* exhibited only a two-fold increase (work in progress). This compares to a 10-fold increase in the ET rate observed with HRP [99, 100]. The amount of peroxidase molecules undergoing DET with the electrode remained unchanged when the pH was varied. Thus, within the pH range studied, although ligninolytic peroxidases and HRP appear to use similar routes for DET on graphite, the coupling of DET to proton transfer is clearly different.

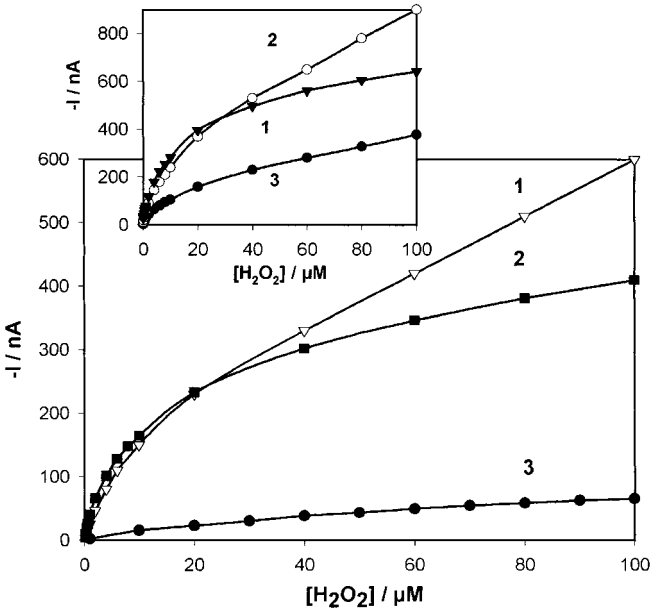

Fig. 3. Dependence of the steady-state current on the H_2O_2 concentration determined with graphite rotating disk electrodes modified with 1) HRP; 2) MnP; 3) LiP in the case of direct ET and in the insert in the presence of 5×10^{-4} M catechol (mediated ET) in 0.1 M PBS, pH 7.0. Applied potential was 0 mV (vs. SCE), rotation speed 959 rpm. Reproduced from [25] with permission from Wiley-VCH.

From the data discussed above, it follows that despite the clear differences in substrate specificity and lower pH optimum for homogeneous catalytic activity, ligninolytic peroxidases from white-rot fungi exhibit bioelectro-catalytic activity similar to that of plant peroxidases. This has been demonstrated under conditions of heterogeneous ET between the protein and a graphite electrode, in the absence of their specific reducing substrates at physiological pH values. In order to improve the efficiency of direct ET during bioelectrocatalysis with ligninolytic peroxidases, a bioengineering approach has been adopted. The most bioelectrocatalytically active of all the native and variant ligninolytic peroxidases studied to date are the recombinant nonglycosylated forms of MnP, specifically, with an engineered veratryl alcohol oxidizing activity, rMnP(S168W) [25]. This variant exhibited both MnP and LiP type activities [86]. Thus rMnP(S168W) is able to oxidize a wide range of substrates, including those that are readily oxidized by the native forms of LiP and MnP. The recombinant forms of MnP gave a higher percentage of peroxidase molecules active in DET between the graphite electrode and the enzyme (around 30 and 60% compared with 30% for native MnP, Table 1 and [25]). However, the highest kinetic parameters for compound I formation, the largest bioelec-

Table 1. Enzymatic and bioelectrocatalytic characteristics of HRP and ligninolytic peroxidases. Bioelectrochemical rate constants were determined for peroxidases on graphite (0.1 M PBS, pH 7.0, applied potential 0 mV vs. SCE) [25]

Peroxidase	Activity (ABTS) (U/mg)	% direct ET	k_s (s^{-1})	k_1 ($10^{-6}M^{-1}s^{-1}$)
wild type HRP	1400	45 ± 3	2.3 ± 0.6	0.20 ± 0.05
wild type LiP	8	9.4 ± 0.6	1.6 ± 0.5	0.025 ± 0.03
wild type MnP	0.02	27.4 ± 4.2	1.67 ± 0.33	0.35 ± 0.05
recombinant MnP	not detectable	50 ± 7	0.71 ± 0.07	0.21 ± 0.02
recombinant MnP (S168W)	0.91 ± 0.22	57.4 ± 9.6	1.51 ± 0.38	0.28 ± 0.02
recombinant MnP (A48C/A63C)	0.14	37.2 ± 5.6	1.67 ± 0.36	0.37 ± 0.05
recombinant MnP (A48C/A63C/S168W)	0.74	33.8 ± 12.6	1.67 ± 0.21	0.30 ± 0.07

Table 2. Bioelectrocatalytic properties of the ligninolytic peroxidases at different pHs. Those of HRP are included for comparison

Peroxidase	% direct ET	k_s (s^{-1}), pH 7.4	k_s (s^{-1}), pH 6.0
HRP [100]	48	1.2	11.9
LiP (*Phanerochaete chrysosporium*)	10	0.7	3.0
LiP (*Trametes versicolor*)	31	0.9	2.1

trocatalytic currents for reduction of H_2O_2, and the highest percentage of enzyme molecules in DET were obtained for the rMnP(S168W) mutant (Table 1). Thus, the rMnP(S168W) variant with an additional, unique LiP activity, is a possible candidate for the development of a solid-phase biocatalyst for lignin bioelectro-oxidation with clear potential for the commercial development of biosensors for polymeric phenol or lignin.

4. Laccase

The blue copper phenol oxidases, also known as laccases, represent one enzyme activity implicated in lignin degradation [101]. Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyzes the oxidation of *ortho*- and *para*-diphenols, aminophenols, aryl diamines, polyphenols, polyamines, and lignin, as well as some inorganic ions coupled to the reduction of molecular dioxygen to water [102, 103]. The enzyme exhibits a broad substrate specificity, which can be enhanced by addition of redox mediators. The efficiency of the latter has been demonstrated in a number of publications [103, 104]. Laccases are classified into two groups depending on their source, i.e., plant and fungal. However, diphenol oxidases (laccases) have been also identified in eubacteria [105] and insects [106]. Laccase is a copper protein and contains 4 metal ions classified into 3 types, i.e., T1, T2, T3 [102, 103, 107]. It was shown that laccases have a minimum of one mononuclear copper site containing one T1 Cu, and a trinuclear copper cluster containing one T2 Cu and two T3 Cu [102]. The single T1 copper is responsible for the blue color of the enzyme and has a characteristic absorbance around 610 nm. The single T2 copper cannot be detected spectrophotometrically in the UV-vis. range, however, it generates a characteristic EPR-signal ($g_{\|} \approx 2.3$, $A_{\|} 160-210 \times 10^{-4}$ cm^{-1}) [108–111]. The bi-nuclear T3 copper is diamagnetic. It has an absorbance shoulder in the region of 330 nm and a characteristic fluorescence spectrum [112, 113].

The X-ray structures of laccase isolated from several organisms have been determined to date only for a limited number of enzymes, e.g., *Coprinus cinereus* [114], *Trametes versicolor* [115, 116], *Pycnoporus cinnabarinus* [116] and *Melanocarpus albomyces* [117] (Fig. 4). The laccase structure is a monomer, organized in three sequentially arranged domains and has dimensions of about $65 \times 55 \times 45$ Å [115]. Each of the three domains has a similar β-barrel type architecture, related to the small blue copper proteins such as azurin or plastocyanin. Although local structural differences are apparent (e.g., in the loops organizing and forming the substrate-binding pocket), all of the laccase structures show a significant degree of overall structural homology. It should also be noted that only structures for laccases from basidiomycetes or ascomycetes have been determined to date. The low potential laccase from the lacquer tree *Rhus vernicifera* has quite distinct biochemical and physicochemical properties, therefore a unique structure can reasonably be anticipated.

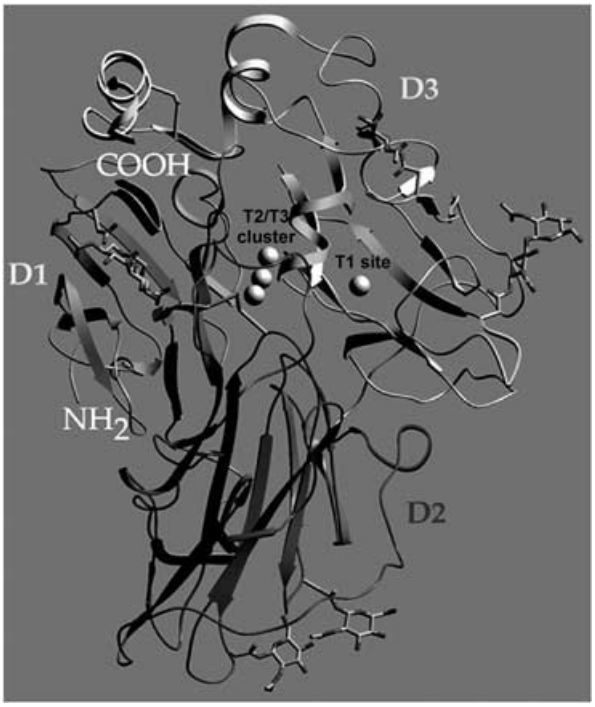

Fig. 4. Crystal structure of *Trametes versicolor* laccase (ribbon diagram) according to [115]. Reproduced with permission from The American Society for Biochemistry and Molecular Biology.

The electrostatic surface potential distribution of laccase (e.g., for *T. versicolor* laccase) reveals a dominance of negative charges, which is in accordance with the acidic pI of 3.5. From the crystal structure of an enzyme/substrate complex, it is known that the electron donor substrate binds in a small negatively charged cavity near the copper T1 site [115], which lies embedded in domain 3, about 7 Å below the surface of the enzyme. The T1 copper is the primary electron acceptor site and it is connected to the trinuclear cluster by a His-Cys-His tripeptide, which is highly conserved among blue multicopper oxidases. Oxidation of simple organic substrates occurs via a ping-pong type mechanism [102, 103, 118, 119]. Substrates are oxidized near the mononuclear site, and the electrons are transferred to the trinuclear site, where molecular oxygen is reduced. Neither the electron transfer nor the oxygen reduction to water mechanisms are fully understood. However, a number of mechanistic schemes have been proposed, see Figure 5, which are consistent with the kinetic and structural data currently available [102, 120].

The key characteristic of laccase is the standard redox potentials of its redox centers; the T1, T2, and T3 sites. The value of the redox potential of the T1 Cu-site has been determined using potentiometric titrations with redox mediators for a great number of different laccases and varies between 420 and 790 mV (vs. NHE) [104, 121–125]. It has been shown for some laccases that T1 is the primary center at which electrons are accepted from reducing substrates [102, 103, 121]. Moreover, the catalytic efficiency (k_{cat}/K_M) for some reducing substrates depends on the redox

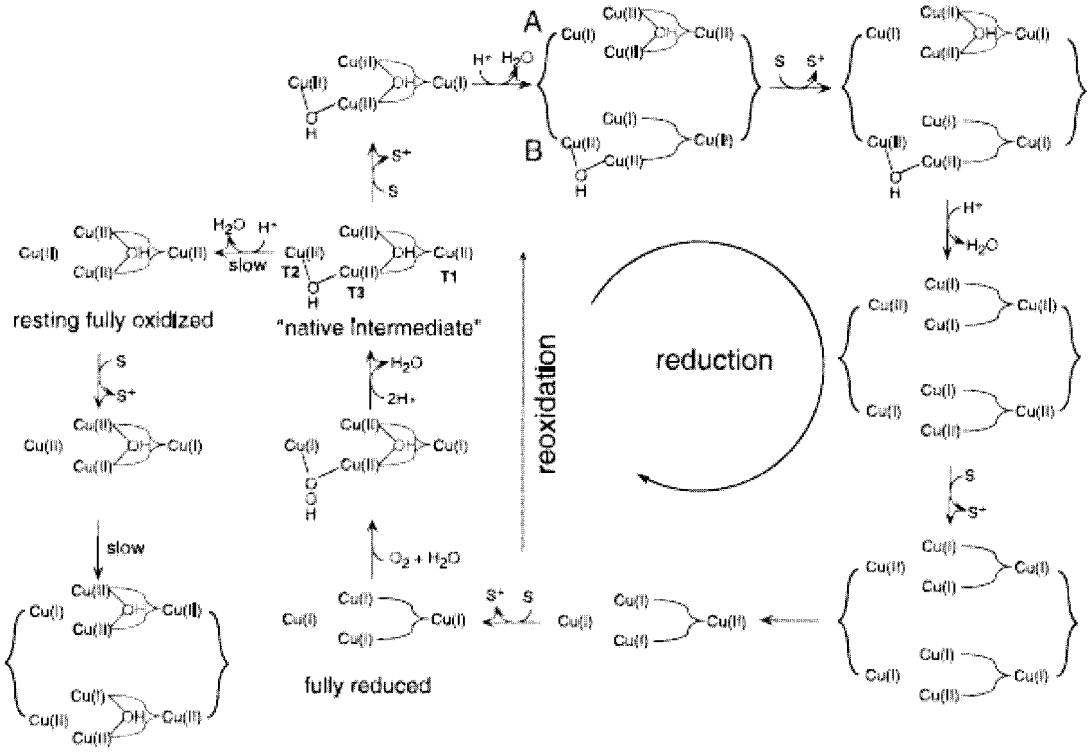

Fig. 5. Proposed catalytic cycle of laccase according to [102] showing the mechanism for reduction and oxidation of the copper sites. Reproduced with permission from The American Chemical Society.

potential of the T1 copper [121, 126]. This is the reason why laccases with a high redox potential T1 site are of special interest in biotechnology, e.g., for different bleaching [127–130] and bioremediation processes [131, 132]. This dependence of the catalytic efficiency on the redox potential of the T1 site suggests that the rate limiting step (or at least partially limiting step) for some reducing substrates in the catalytic cycle is the 1 e^- transfer from the substrate to the protein, specifically the T1 site of the laccase [126]. In case the electron donation from the reducing substrate is rapid Solomon et al. [102, 120] point to the fact that the rate limiting step be found in the reaction sequence in the molecular mechanism of the 4 e^- reduction of oxygen to water by laccase. The enzyme binds one oxygen and one water molecule prior to the formation of a bound peroxide intermediate. The reduction of this intermediate involves two two-electron steps (Fig. 5). The first is rate-determining and the second is fast. Such a reaction path is thermodynamically more profitable compared with a sequential 1 e^- transfer reaction from the T3 site to the bound intermediate.

From an electrochemical point of view all laccases can be divided into three groups depending on the potential of the T1 site: low, middle and high potential laccases. The low potential group includes laccases from trees, e.g., *Rhus vernicifera* with a potential of the T1 site of about 420 mV (vs. NHE) [122, 133]. The middle group includes laccases from basidiomycetes like *Myceliophthora thermophila* [121], basidiomycete C30 [134], *Rhizoctonia solany* [121], and *Coprinus cinereus* [124]. The enzymes have a potential of the T1 site ranging from 470 to 710 mV (vs. NHE). The high potential laccases (e.g., those from *Trametes (Polyporus. Coriolus) hirsuta (hirsutus)*, *T. versicolor*, *T. villosa*) all have a potential of the T1 site of about 780 mV (vs. NHE) [121, 122, 125].

Redox potential data for the T2 and T3 sites were published in 1970s by Reinhammar et al. [122, 133]. A redox potential of 390 mV was determined for the T2 site of the low potential laccase from *Rhus vernicifera* [122, 133]. This Cu site has the lowest potential reported for laccases. The redox potential of the T3 site was measured for two different laccases, *viz.* the low potential enzyme from *Rhus vernicifera* (450 mV) and the high potential enzyme from *T. versicolor* (785 mV) [133].

The first publication on DET for a large redox protein with enzymatic activity was a high potential laccase from the basidiomycetes *T. versicolor* [5, 6]. The authors of this paper showed that in the presence of molecular oxygen, laccase modified carbon electrodes exhibited DET. They found that addition of laccase into an electrochemical cell shifts the equilibrium potential of the working electrode from 30 to 380 mV. The process was dependent on the electrode material, its method of preparation and the partial pressure of oxygen in the system. Moreover, in the presence of molecular oxygen a reduction current was recorded at the laccase modified carbon electrode due to DET between the electrode and the adsorbed laccase. A second paper about the electroreduction of oxygen at laccase modified electrodes was published in 1984 [135]. The electroreduction of oxygen using a highly ordered pyrolytic graphite electrode coated with laccase from *Trametes* (*Polyporous) versicolor*

(high potential fungal laccase) was shown. The potential at which the laccase begins to catalyze the electroreduction of oxygen (about 735 mV vs. NHE) is in the vicinity of the redox potential of the T1 site (780 mV vs. NHE). However, under anaerobic conditions the laccase modified graphite electrode exhibited cyclic voltammograms that were indistinguishable from the background voltammograms obtained in the absence of enzyme. In the presence of an electrochemically inactive promoter, 2,9-dimethylphenanthroline, an electrochemical response was, however, observed with an average of the peak potentials of 645 mV (vs. NHE). Kinetic parameters for the electroreduction for molecular oxygen at carbonaceous electrode materials with adsorbed laccase have been reported subsequently [135–137].

The characteristics of the electroreduction of oxygen at laccase modified graphite/carbon electrodes depend on the origin and also on the amount of enzyme on the electrode surface [27, 29, 138]. Laccases from different sources (from *Rhus vernicifera* and *Trametes hirsuta*) catalyze the heterogeneous electroreduction of oxygen in very different potential regions. The pH dependence of the heterogeneous electrocatalytic currents for both enzymes adsorbed on electrodes was found to be very similar to that obtained for the corresponding enzyme reactions in homogeneous media [27]. The difference in the redox potential of the T1 site of the two laccases is most likely the major determinant of this behavior with a similar mechanism operating for both homogeneous catalysis in solution and heterogeneous electrocatalysis at an electrode. A DET process has been reported on the basis of small cyclic voltammetric peaks observed for the high potential laccase from *T. versicolor* under anaerobic conditions, see Fig. 6 [28]. The process was quasi-reversible with the E_m close to the redox potential of the T1 site of this laccase (*ca.* 780 mV vs. NHE). Under aerobic conditions a clear catalytic wave was shown commencing close to the potential of the T1 site. A mechanism for the bioelectrocatalytic reduction of oxygen by laccase adsorbed on carbon electrode materials has been proposed by Tarasevich et al. [29]. At potentials close to the steady-state potential, the rate-determining step is proposed to be a concerted transfer of two electrons to the oxygen molecule. In the region of polarization, where the current is essentially potential independent, the process is limited by the formation of a "peroxide" intermediate. Further support for this was recently obtained for laccases from basidiomycetes [138]. Three high potential basidiomycetes laccases (having differences in the redox potential of the T1 site of only 50 mV) had the same electrocatalytic activity on graphite. In addition the potential of electroreduction of oxygen correlated with the values of the redox potential of their T1 sites. This supports the idea that carbon electrodes act as the primary electron donor to the T1 site of the enzyme by DET. This would also explain the similar catalytic behavior of laccases adsorbed on graphite electrodes with that in homogeneous solution in the presence of soluble electron donors.

Only in 1997 was independent DET shown for *Rhus vernicifera* laccase (low potential) under anaerobic conditions. The laccase was adsorbed onto a β-mercaptopropionic acid modified gold electrode and under anaerobic conditions well-pronounced reversible cyclic voltammetric peaks were obtained [139] with a difference in potential between the anodic and cathodic peaks (ΔE_p) of around 60 mV and a midpoint potential (E_m) of about 330 mV (vs. NHE). A subsequent paper on gold electrodes modified with laccase embedded in a polymeric film of an anion exchange resin [140] not only confirmed these results but also reported a reversible and diffusion-controlled electrochemical process with an ΔE_p of around 30 mV and an E_m of about 410 mV, which is very close to the potential of the T1 site of the enzyme (420 mV vs. NHE). The pH-dependence of the electrochemical process and the electrochemical activity of the T2 depleted enzyme were also studied. The holoprotein and the T2 depleted laccase had the same electrochemical characteristics, whereas the apoprotein was electrochemically inactive. Moreover, the pH-dependent redox potential was similar to that of the free protein. The authors concluded that the T1 site is the primary electron acceptor during electrochemical activity of this low potential laccase on their gold electrode. In the last few years, a few abstracts (see, for example, [141]) have been published in which DET between laccases and electrodes has been claimed, however, little or no experimental evidence has been published in peer reviewed journals.

In depth studies of the mechanism of DET between high potential laccases and electrodes, have recently been carried out in our laboratories and some initial results have been published [138]. Cyclic voltammograms using graphite electrodes modified with adsorbed *T. hirsuta* laccase were recorded in order to provide unambiguous experimental evidence for direct heterogeneous ET of this laccase under anaerobic conditions. Osteryoung square-wave voltammograms recorded using edge plane highly ordered pyrolytic graphite (HOPG) with and without *T. hirsuta* laccase entrapped between the electrode and a dialysis membrane in 0.1 M phosphate buffer solution at pH 6.5 show a single wave only in the presence of the enzyme (Fig. 7), centered around + 800 mV (vs. NHE). The recorded faradaic process is very close to the potential of the T1 site and probably only the relatively high background noise of carbon electrodes (high capacitive currents) most likely prevents the observation of the same process by more conventional cyclic voltammetry measurements. When equivalent experiments were run with the less well defined carbon electrode material spectroscopic graphite the voltammograms did not reveal any redox transformation of the enzyme in the potential range between + 1 100 and − 100 mV (vs. NHE) at sweep rates varying from 1 to 1 000 mV s^{-1}. Varying the pH between 3 to 7 did not lead to the appearance of any clearly identifiable faradaic currents on the recorded voltammograms. It was, however, possible to observe indirect DET for the *T. hirsuta* laccase under aerobic conditions as reflected in Fig. 8. All our experiments with carbon electrodes modified with laccase from *T. hirsuta* and other high potential fungal laccases [142] confirm that the T1 site is the primary electron acceptor site from carbon electrodes

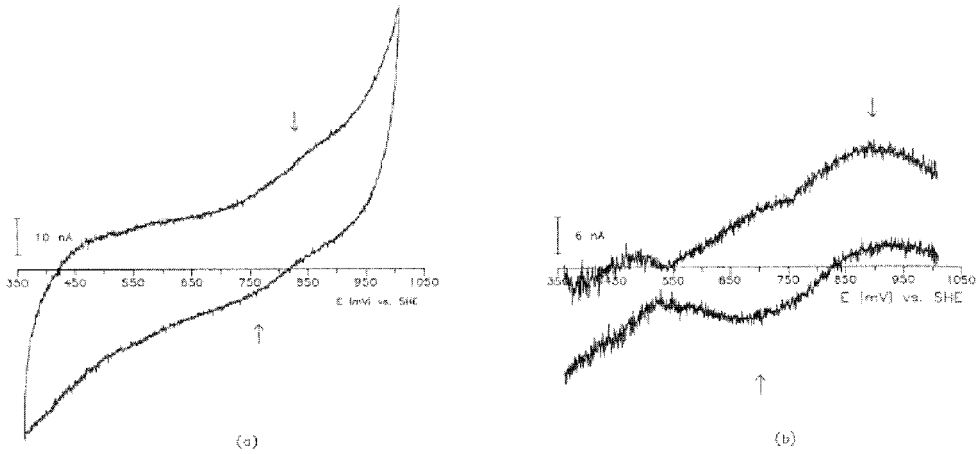

Fig. 6. a) Cyclic voltammetry (second scan) of 2.5 pmol *P. versicolor* (*T. versicolor*) laccase adsorbed onto a pyrolytic graphite electrode. Anaerobic conditions. 30 mM citrate, pH 3.31. Ionic strength 0.1 M ($NaClO_4$). Scan rate 1 mV s^{-1}. b) The background-corrected signal. Reproduced from [28] with permission from Acta Chemica Scandinavica.

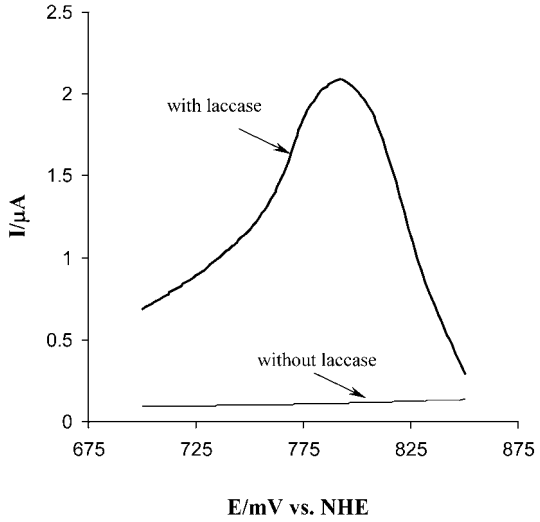

Fig. 7. Osteryoung square-wave voltammograms recorded with HOPG electrode with and without the *T. hirsuta* laccase entrapped under a dialysis membrane at the electrode surface. 0.1 M phosphate buffer pH 6.5; S. W. Amplitude – 10 mV; Frequency – 10 Hz.

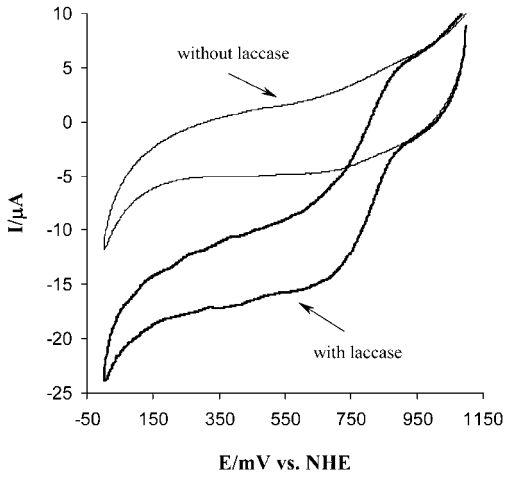

Fig. 8. Voltammograms recorded with bare and *Trametes hirsuta* laccase-modified spectroscopic graphite electrodes. 50 mM citrate-phosphate buffer with air-saturated oxygen containing 0.1 M $NaClO_4$. Scan rate 10 mVs^{-1}; start potential – 1 100 mV (vs NHE).

during heterogeneous reduction under both aerobic and anaerobic conditions.

To achieve and understand electrochemical reactions of redox proteins on gold electrodes are very important goals both for fundamental research and applications in biotechnology, since gold is often used as electrode material due to its inertness. There are only a few publications reporting on the electrochemical behavior of laccase on gold [139, 140, 143]. We know only two papers that describe electrochemical studies of high potential fungal laccases on gold [5, 144]. In 1978 it was shown for the first time that the presence of laccase shifted the steady-state potential of a bare gold electrode in oxygenated buffer to a value about 100 mV more positive than that without laccase [5]. In [144] Gelo-Pujic et al. published cyclic voltammograms showing an $E^{\circ\prime}$ of around 410 mV (vs. NHE) for recombinant *T. versicolor* laccase (not the native fungal laccase and only under anaerobic conditions) using a 4,4′-pyridine thiolate modified gold electrode. The authors suggested that the T1 site in the recombinant laccase is the primary electron acceptor from gold despite the considerable difference between the $E^{\circ\prime}$ measured by cyclic voltammetry (410 mV) and the redox potential of the T1 site (780 mV) determined by redox titration. Of course, it is well-known that the structural differences between wild type and recombinant protein, e.g., glycosylation, Cu-ligands, etc., can affect the surface interaction and the formal potential [72, 123, 145]. However, such a large shift of the $E^{\circ\prime}$ in case of laccase could be explained only if the enzyme acquires a dramatically changed (denatured) conformation at the gold surface or mis-folding has occurred during its production by recombinant technology. Although the data on DET processes

between laccases and gold are very limited, there is currently enormous interest in understanding and controlling the heterogeneous ET of laccases. This is exemplified by recent publications on the development of biofuel cells [129, 146–150].

In our laboratories we have recently obtained experimental evidence for DET for native high potential laccase at gold electrodes. As can be seen in Figure 9 cyclic voltammograms of *T. hirsuta* laccase at a gold electrode exhibit a quasi-reversible process with a ΔE_p of 170 mV and an E_m of 405 mV (vs. NHE), i.e., at a potential close to the value reported by Gelo-Pujic et al. [144] for recombinant *T. versicolor* laccase [144]. However, on gold it was not possible to obtain any electrochemical response close to the value of the redox potential of the T1 site (780 mV vs. NHE) as had previously been obtained on carbon electrodes for the same enzyme, see Figure 7 [27, 138]. Thus, the mechanism of DET between the laccase and the two different electrode materials may be totally different. At carbon electrodes DET is observed between the surface of the electrode and the T1 site, whereas in the case of gold the pathway of ET still remains unclear. It should be emphasized that to be able to elucidate the mechanism behind the electrochemical behavior of low potential laccases (like *Rhus vernicifera* laccase) and especially high potential enzymes (for instance, *T. hirsuta* or *T. versicolor* laccases) on gold electrodes under both aerobic and anaerobic conditions further investigations are indeed needed and are currently being carried out in our laboratories [142].

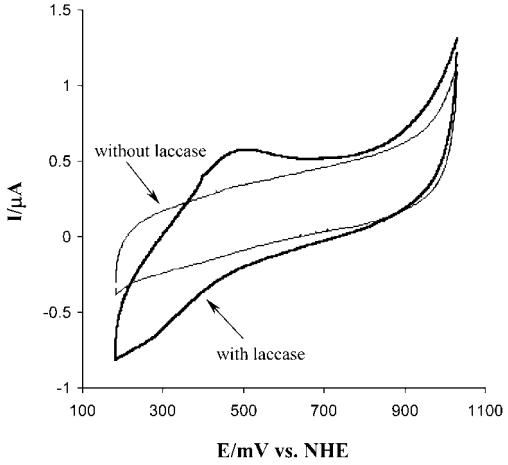

Fig. 9. Cyclic voltammograms of laccase in a bare gold capillary electrode. 0.1 M phosphate buffer pH 6.5; 10 mg/mL of *T. hirsuta* laccase solution; scan rate 10 mV/s; start potential – 1030 mV (vs NHE).

In conclusion, the mechanism of DET between electrodes and the various forms of laccases is not fully understood. The question is especially relevant for electrochemical investigations of "blue" high potential fungal laccases, since in many cases DET processes recorded by cyclic voltammetry do not coincide with the known $E^{\circ\prime}$ values for the different copper sites identified crystallographically in laccase structures. However, literature data and our most recent data (discussed above) are consistent with a mechanism for the DET process on carbon electrodes as shown in Figure 10.

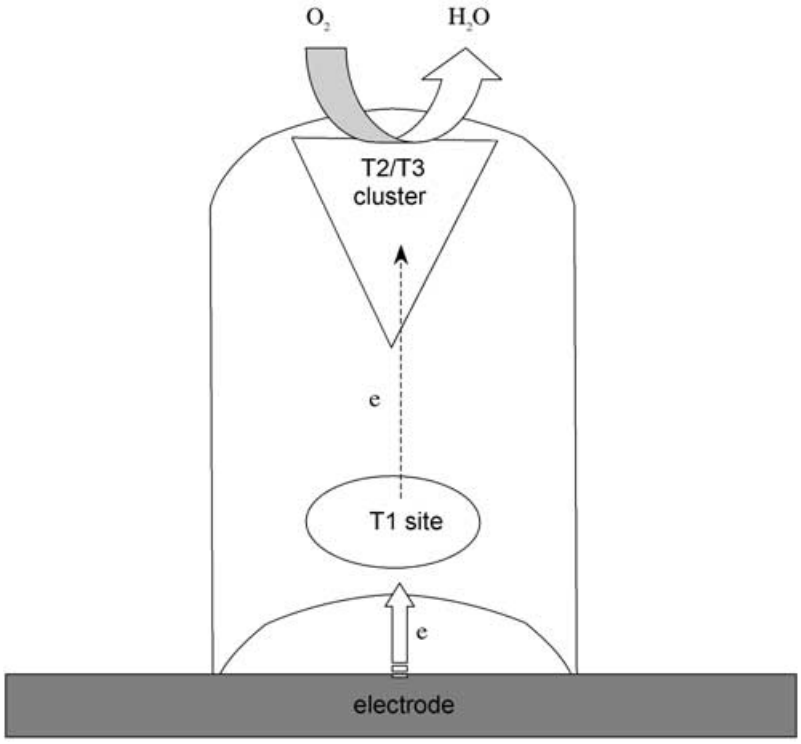

Fig. 10. Proposed scheme for electroreduction of oxygen at carbon electrodes with adsorbed laccase.

5. Cellobiose Dehydrogenase

Cellobiose dehydrogenase (CDH) is yet another type of enzyme that has been implicated in the degradation of lignin and lignocellulose, however, its exact in vivo function is not known at present even though the enzyme was first described almost 30 years ago [151, 152]. CDH activity was first discovered in 1974 by Westermark and Eriksson as a cellobiose dependent reduction of quinones by the white rot fungi *Trametes versicolor* and *Phanerochaete chrysosporium* [151, 152]. CDH is a hemoflavoprotein (EC 1.1.99.18; formerly cellobiose oxidase), in fact it is the only extracellular flavocytochrome known to date. It consists of a flavin domain containing one FAD (or alternatively one 6-hydroxy-FAD) per monomeric subunit, and the *N*-terminal heme domain with one heme *b*, which is noncovalently bound to the enzyme and hexacoordinate. These two domains are connected by a flexible linker region, which can be cleaved by proteases. In addition, CDH can carry a separate, C-terminal cellulose-binding domain [153], while it was suggested for some CDHs that the region responsible for the binding onto cellulose is part of the flavin domain [154, 155]. CDH was purified from a number of different organisms including basidiomycetes and ascomycetes [48, 49] including *P. chrysosporium* (*Sporotrichum pulverulentum*) [156], which in fact is the source for the best characterized cellobiose oxidoreductases, *Chrysonilia (Monilia) sitophila* [157], *Coniophora puteana* [158], *Heterobasidion annosum (Fomes annosus)* [159], *Humicola insolens* [160], *Irpex lacteus* [161], *Pycnoporus cinnabarinus* [162], *Schizophyllum commune* [163], *Sclerotium (Athelia) rolfsii* [164], *Thielavia heterothallica (Sporotrichum thermophile)* [153], and *Trametes versicolor* [165].

Typically, CDH is a monomeric, glycosylated protein with a molecular mass of roughly 100 kDa and an acidic pI of around 4. CDH oxidizes various sugars at the position C-1 to the corresponding lactones. These sugar substrates typically are β-1,4-linked di- or oligosaccharides and include cellobiose (glc-β-1,4-glc), which in fact is the preferred substrate of CDH [164, 166], cello-oligosaccharides, lactose (gal-β-1,4-glc), and even cellulose [167]. In contrast to this relatively high specificity of cellobiose dehydrogenase for β-1,4-linked di- or oligosaccharides it is highly nonspecific with respect to the electron acceptor and can reduce a wide range of quinones, redox dyes, complexed metal ions, organic radical species, and oxygen even though activity with this latter substrate is only low [48, 165, 168].

The catalytic mechanism of CDH and the role of the heme group are not well understood at present. CDH is unique in comprising two prosthetic groups, the flavin and the protoheme group. It transfers reducing equivalents from the electron donor (e.g., cellobiose) via its two redox centers to two different types of electron acceptors, two-electron proton acceptors such as benzoquinones and redox dyes, and one-electron no-proton acceptors such as ferric iron complexes, various radical molecules and cytochrome *c*. During these redox processes different functions have been proposed for the heme group. In the electron transfer chain model one-electron processes first require reduction of the heme and subsequent reduction of the acceptor directly by the heme, while two-electron events involve the interaction of reduced flavin and acceptor. In an alternative model, the electron sink model, it is suggested that both one-electron and two-electron acceptors are reduced by the flavin and the heme acts solely as a sink or parking place for the extra electron when one-electron acceptors react with the flavin [48, 169]. It was shown that the active flavin fragment can reduce both one- and two electron acceptors, however, the presence of the heme domain enhances the reduction of one-electron oxidants such as ferricyanide or phenoxy radicals, for which higher reaction rates were observed for the intact flavocytochrome compared to the flavin-only fragment [170]. Only intact CDH reduces cytochrome *c*. The natural electron acceptor of CDH is, however, not known at present.

To date the crystal structure of intact CDH has not been elucidated. Apparently crystallization of the complete flavocytochrome is hampered by the interdomain peptide linker. The group of Dr. Christina Divne in Stockholm (formerly Uppsala) has instead determined the crystal structures of the heme (Fig. 11) and the flavin domain of CDH from *Phanerochaete chrysosporium* separately [155, 171]. The heme domain was shown to have a sandwich fold of two antiparallel β sheets, referred to as the inner and the outer sheet. The heme pocket is formed on the inner β sheet close to the surface of the molecule. The heme iron is hexacoordinated with one Met and one His residue (Met65 and His163). This Met/His ligation is quite uncommon in *b*-type cytochromes, in fact, CDH is the first cyt *b*-containing enzyme with such a ligation. Furthermore, the heme propionate groups are surface exposed on the heme domain, which could indicate a role in the interdomain electron transfer as well as in the association between the heme and the flavin domain. The crystal structure of the flavin domain shows an overall PHBH (*p*-hydroxybenzoate hydroxylase) fold [172]. It can be partitioned into an FAD-binding subdomain and a substrate-binding subdomain. Based on docking studies of the separate heme and flavin domain it was proposed that the heme domain covers the active-site entrance of the flavin domain and that the resulting distance between the two prosthetic groups of less than 15 Å is within acceptable limits for interdomain electron transfer. Furthermore, it was suggested that this covering by the heme domain is only transient so as to allow substrate to enter the active site. This could probably indicate that the heme propionate groups are transiently exposed to the solvent even in the intact enzyme.

CDH has been proposed to be involved in both cellulose [173, 174] and lignin biodegradation [54]. Depending on what is considered the main reaction catalyzed by cellobiose dehydrogenases, i.e., the oxidative or the reductive half-reaction, several varying functions have been suggested. A participation of CDH in cellulose degradation is suggested from recent work performed in the group of Dr. Fred Archibald at the Pulp and Paper Research Institute of Canada. CDH-deficient strains of *T. versicolor* were unable

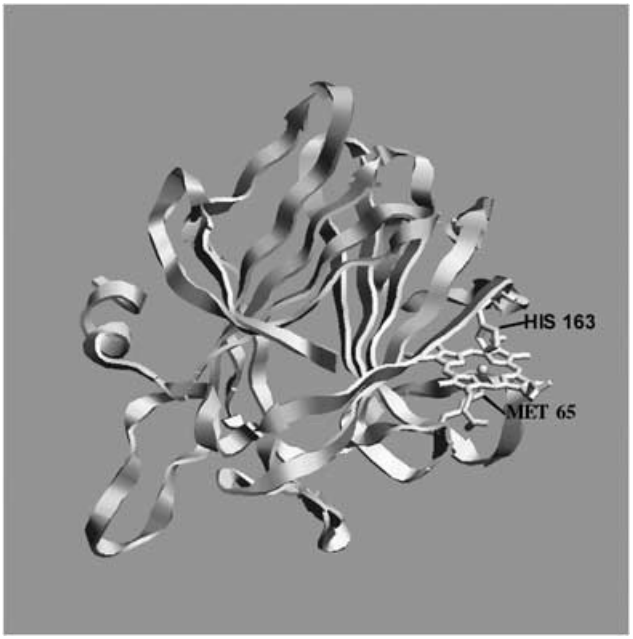

Fig. 11. Crystal structure of the heme domain of cellobiose dehydrogenase from *Phanerochaete chrysosporium* [171] (PDB 1D7C). Met65 and His 163 form the axial ligands of the heme iron and are marked.

to grow on agar plates containing highly crystalline cellulose as sole carbon source as well as on birch wood, while the reference strain producing CDH grew efficiently on these plates. In contrast, both the parent strain and the CDH-deficient strain were able to grow on amorphous cellulose (CM-cellulose). These data suggest that CDH plays an important role in the degradation of native cellulose and thus is essential for wood invasion and lignocellulose degradation by fungi [175]. Another proposed degradative function of CDH, which, however, is less specific, is via the production of Fenton's reagent. This is in agreement with the fact that CDH can continuously reduce Fe(III) to Fe(II) in the presence of cellobiose (which results from the degradation of cellulose by cellulases) and also possibly produces H_2O_2 – although hydrogen peroxide can also be formed by additional fungal oxidases or result from the reaction of Fe(II) with molecular oxygen [168, 174, 176]. The hydroxyl radicals thus formed can then degrade not only cellulose, but also xylan and lignin [50]. CDH has also been suggested to be part of the lignin degrading enzyme system of fungi. Its function in this context could be to prevent the repolymerization of aromatic radicals that are formed by the action of LiPs [52, 54]. CDH could also work in cooperation with MnP, making Mn(II) available by reducing $Mn(IV)O_2$, which is found during delignification as insoluble deposits in wood, and simultaneously forming suitable complexing agents, i.e., cellobionic acid, for the reactive Mn(III) ion [177]. However, it should also be pointed out that reduced CDH can additionally donate electrons to compound II of LiP, MnP and HRP [54] (the implication of this fact for lignin degradation remains unknown) and other heme proteins such as wild type cytochrome *c*, modified cytochrome *c* having its localized lysine residues at the surface close to the heme neutralized, myoglobin and hemoglobin [178], reflecting the ability of reduced CDH to act as an electron donor to various heme containing proteins with or without localized surface charges.

Because of its attractive and unique properties CDH has several technical applications, e.g., in biosensors for the sensitive and selective detection of toxic diphenols [179, 180], of cellodextrins and lactose [181 – 185], or as sensor in conjunction with flow immunoassays [186], in bioremediation for the degradation of recalcitrant pollutants including munitions and polyacrylate polymers [42, 56, 187], or in biocatalysis for the preparation of organic acids [187].

The coupling of CDH (from *Phanerochaete chrysosporium*) with electrodes was first done by Elmgren and coworkers [181, 182, 188], where they adopted the approach developed by Heller [189] of wiring redox enzymes with electrodes using a flexible polymeric network incorporating $Os^{2+/3+}$-functionalities acting as efficient redox mediators between the reduced enzyme and the electrode. However, earlier work by Ikeda et al. [190 – 192] and by Aizawa et al. [193, 194] inspired us to investigate whether CDH would also reveal DET properties with electrodes. Ikeda showed that there should be a great potential for DET between the heme domain and electrodes for enzymes similar to CDH in the respect that they have a two domain structure, one catalytic domain containing either a flavin or pyrroloquinoline quinone (PQQ) and a heme containing domain, where the heme would act as a "built-in mediator" [191]. Initially intact CDH from *Phanerochaete chrysosporium* was immobilized by simple adsorption onto graphite electrodes and in the presence of cellobiose (or lactose) a response current to cellobiose was registered providing indirect proofs for DET [195, 196]. After substrate oxidation and in the absence of external mediators, electrons from the reduced flavodomain are further transported to the heme domain through internal electron transfer and then further to the electrode, see Figure 12. When only the flavodomain (obtained from papain cleavage of the linker region) was adsorbed onto graphite no response to cellobiose was noticed, however, in the presence of a mediator a catalytic response to cellobiose was registered revealing the full catalytic activity of the sole flavodomain [195].

Further work along this line on CDH from *Phanerochaete chrysosporium* adsorbed onto graphite was performed in order to register the independent electrochemistry of the enzyme, i.e., in the absence of substrate, and there were some indications of electrochemical activity revealed using square wave voltammetry, possibly exhibiting the heme as the $E^{\circ\prime}$-value was found to be between – 60 and – 20 mV (vs. Ag | AgCl) depending on the pH [196]. However, not until switching direction to the use of alkane thiol modified gold electrodes [197], clear independent evidence for DET was shown [18, 30, 31]. Initially cystamine, a rather short positively charged thiol derivative, was used, to form a positively charged self assembled monolayer (SAM) at the gold electrode surface. To increase the local concentration of CDH at the modified Au electrode-solution interface a

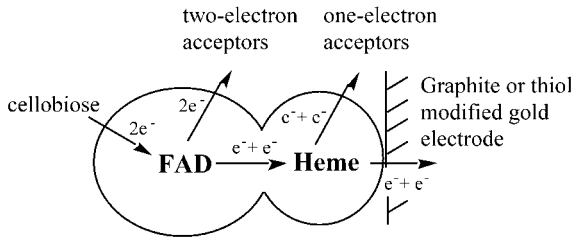

Fig. 12. Proposed reaction sequence at a CDH modified electrode.

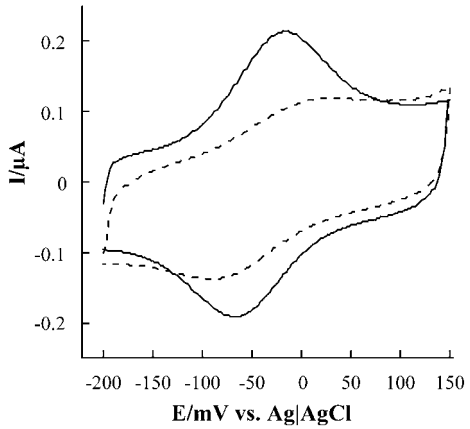

Fig. 13. Cyclic voltammograms obtained with CDH from *Phanerochaete chrysosporium* entrapped under a permselective membrane at a gold electrode modified with cystamine (solid line, $E^{\circ\prime}$; -42 mV, ΔE_p; 43 mV) and 3-mercaptoproprionic acid (broken line, $E^{\circ\prime}$; -40 mV, ΔE_p; 71 mV) in 50 mM acetate buffer, pH 5.1. Scan rate 50 mV s^{-1}. Reproduced from [31] with permission from Elsevier.

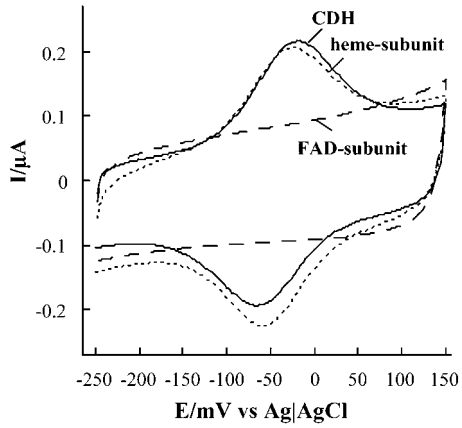

Fig. 14. Cyclic voltammetry of intact *P. chrysosporium* CDH ($E^{\circ\prime} = -42$ mV, $\Delta E = 44$ mV), heme-subunit ($E^{\circ\prime} = -42$ mV, $\Delta E_p = 28$ mV) and FAD-subunit trapped under a permselective membrane at a gold electrode (with cystamine). Experimental conditions: 50 mM Ac-buffer, pH 5.1, 50 mV s^{-1}. Reproduced from [31] with permission from Elsevier.

permselective membrane electrode [198] was used trapping the added enzyme solution between the electrode surface and a dialysis membrane. When investigated with cyclic voltammetry very clear voltammetric waves were registered, see Figure 13, facilitating the evaluation of the $E^{\circ\prime}$ of the redox wave [31]. When exchanging cystamine for 3-mercaptopropionic acid, thus forming a negatively charged SAM at moderate pHs, also clear voltammetric waves could be seen with cyclic voltammetry, however, not as pronounced as when using the cystamine SAM, see Fig. 13 [31]. The high content of primarily negatively but also positively charged amino acid residues on the surface of the heme domain [171] could explain that irrespective of the charge of the SAM clear voltammograms of CDH could be seen, however, as expected with a higher amplitude when using the positively charged SAM. When stripping the membrane and rerunning the electrode in pure buffer no traces of CDH electrochemistry was seen for either the cystamine or the 3-mercaptopropionic acid modified gold electrodes, revealing that CDH was not strongly bound to either type of electrode.

To confirm that what was seen with cyclic voltammetry was the electrochemistry of the heme, experiments were made with the intact CDH, the enzymatically fully functioning flavo domain as well as the heme domain and it could be concluded that it was the heme domain of the intact enzyme that caused the voltammetric wave as the voltammograms of the intact CDH and the heme domain were close to being similar, whereas the flavo domain within the investigated potential range did not reveal any electroactivity, see Figure 14. In the presence of cellobiose only for the intact enzyme a typical catalytic cyclic voltammogram could be seen, see Figure 15, supporting that the intact enzyme at the electrode/solution interface retains its catalytic properties and reflecting that the electron transfer occurs in a reaction sequence as follows: cellobiose → flavo domain → heme domain → electrode (Fig. 12). The intramolecular electron transfer rate between the reduced flavo domain and the oxidized heme domains is very much dependent on pH [48, 49]. This is reflected in Figure 15, showing cyclic voltammograms of CDH from *P. chrysosporium* on a cystamine modified gold electrode in the presence of 2 mM cellobiose at different pHs (3.5, 5, 6.3). At a pH of 3.5 the catalytic wave is very clear, however as pH is increased to 5, the catalytic wave is decreased and at pH 6.3 is virtually absent, reflecting efficient internal electron transfer at low pH and at around and above pH 6 it is blocked. Further support for the electroactivity of the heme domain of intact CDH was concluded with spectroelectrochemical investigations of CDH in an aldrithiol modified gold capillary electrode [38], see Figure 16.

The ability of the heme to act as a logic gate in the sense that depending on the redox state of the flavo domain, the heme domain can act both as an electron donor to $ABTS^+$ in the presence of cellobiose (flavo domain is in its reduced state) and as an electron acceptor to ABTS in the presence of hydrogen peroxide (flavo domain is in its oxidized state) [178]. With cyclic voltammetry it was also shown that the flow of electrons within CDH could be reversed [31]. Gold electrodes modified with adsorbed CDH initially in its fully

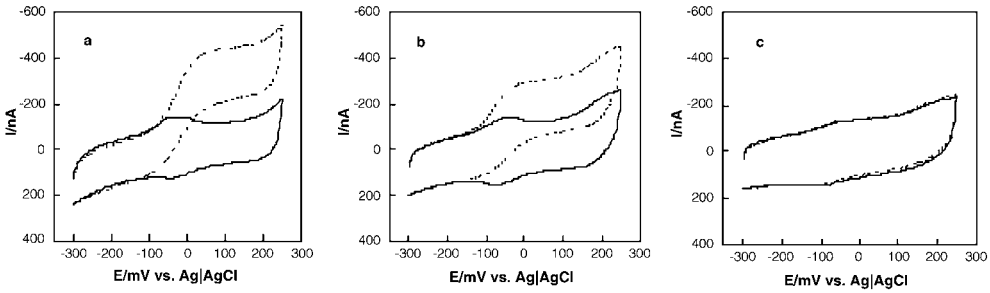

Fig. 15. Cyclic voltammograms of *Phanerochaete* CDH entrapped under a permselective membrane on a cystamine modified gold electrode in the absence (solid line) and in the presence (broken line) of 2 mM cellobiose at pH a) 3.5, b) 5, and c) 6.3. The same electrode was used for all pHs. Scan rate 50 mV s^{-1}. Reproduced from [30] with permission from Elsevier.

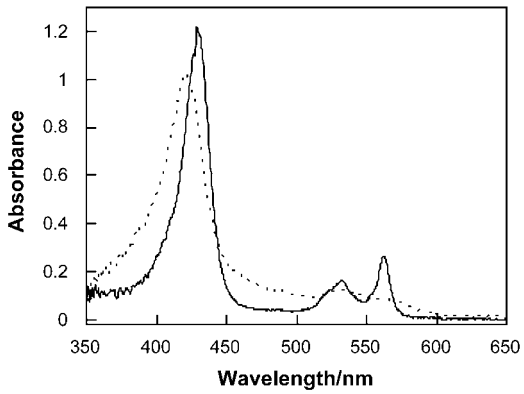

Fig. 16. The spectra of oxidized and reduced *P. chrysosporium* CDH at a concentration of approximately 1.7 g L^{-1} in 100 mM NaCl and 20 mM formic acid, pH 3.1, obtained in an aldrithiol modified gold capillary electrode. Reduced (solid line) and oxidized (broken line) spectra recorded at potentials of −250 mV and 150 mV (vs. $Ag\,|\,AgCl\,|\,KCl_{sat}$), respectively. Reproduced from [38] with permission from Elsevier.

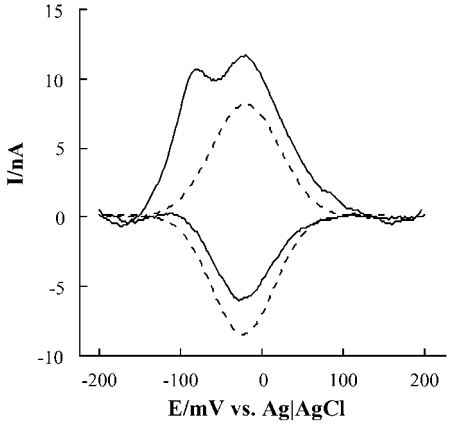

Fig. 17. Baseline-corrected cyclic voltammograms obtained with intact *P. chrysosporium* CDH (solid line) and heme-subunit (broken line) trapped under a permselective membrane at a gold electrode (modified with cystamine) in 50 mM Ac-buffer, pH 4.3. Start potential; +200 mV, scan rate; 10 mV s^{-1}. Reproduced from [31] with permission from Elsevier.

oxidized state (no cellobiose present, start potential; +200 mV) were subjected to a negative potential scan at a low sweep rate (10 mV s^{-1}) until −200 mV. Reversing the scan the resulting CV revealed an anodic prewave, due to that during the initial negative scan electrons from the electrode to the heme domain were further transported in an up-hill reaction to the flavo domain. When the scan was reversed as soon as the applied potential approaches the range, where heme can be reoxidized a very rapid reoxidation of the flavo domain occurs causing the anodic prewave (Fig. 17).

Electrochemical investigations of other CDHs of soft-rot origin also reveal DET properties [30]. The pH optimum for CDHs from *Humicola insolens* and *Sclerotium rolfsii* differs and is acid for the one from *S. rolfsii H*. as for most other CDHs but CDH from *Humicola insolens* has a pH optimum at neutral values and is thus an exception [178]. When CDH from the two soft-rots *H. insolens* and *S. rolfsii* were adsorbed on graphite, catalytic currents were obtained in the presence of cellobiose and the difference in pH optima for the two enzymes was reflected as the highest catalytic current for cellobiose oxidation was obtained at 3.5 for *S. rolfsii* CDH and at 7 for *H. insolens* CDH [30]. Independent electrochemistry of the heme could, however, only be obtained for *H. insolens* CDH on thiol modified gold electrodes, whereas not for the CDH from *S. rolfsii.* The choice of thiol modifier to obtain efficient DET for *H. insolens* CDH was very critical. Neither of the two thiols used successfully as modifiers with *P. chrysosporium* CDH on gold electrodes resulted in electrodes exhibiting clear DET for *H. insolens* CDH. However, using a negatively charged thiol with a longer carbon chain, 11-mercaptoundecanoic acid, as modifier clear voltammograms could be shown, see Figure 18, however, in the presence of cellobiose no evident catalytic current could be seen. These results show the great need to carefully design the electrode surface for such two domain redox enzymes (and in future work for even more complex redox enzyme structures and whole membranes containing electron transfer pathways) so that not only the electrochemically active domain of the enzyme is properly oriented at the electrode surface for facile ET, but also allowing the two domains to productively interact with each other for the inter domain ET to proceed efficiently. Recently this was clearly shown for another

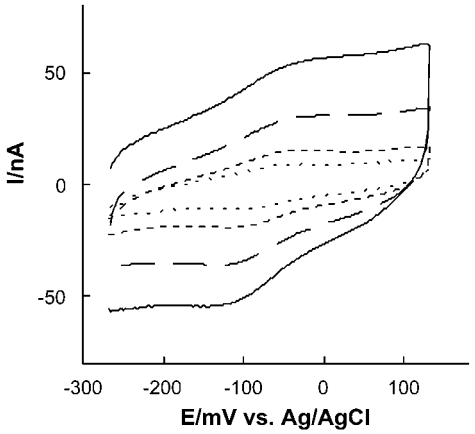

Fig. 18. Cyclic voltammograms of *H. insolens* CDH entrapped under a permselective membrane on a 1-mercaptoundecanoic acid modified gold electrode. Experimental conditions: The electrolyte used was phosphate/citrate buffer pH 5. Scan rate: 100, 50, 20, 10 mV s^{-1}. Reproduced from [30] with permission from Elsevier.

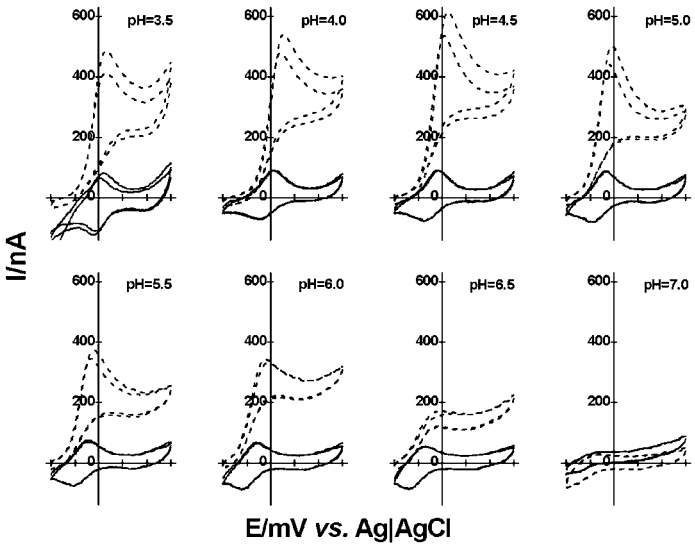

Fig. 19. First two consecutive cyclic voltammograms of CDH from *Trametes villosa* entrapped under a permselective membrane on an aldrithiol modified gold electrode registered in 20 mM acetate buffer at different pHs in the absence (solid curves) and in the presence (dashed curves) of 700 μM cellobiose. Experimental conditions are: scan rate 10 mV s^{-1}, scanning interval between −200 to +300 mV (vs. Ag | AgCl) (100 mV is the increment on the X-axes).

redox enzyme, sulfite oxidase, on thiol modified gold electrodes that both the chain length as well as the effective charge/hydrophilicity of the SAM was critical to obtain both efficient electrochemistry and efficient catalysis [21].

The current on-going work on the bioelectrochemistry of CDH is directed to investigating new CDHs from white rot fungi. Figure 19 reveals cyclic voltammograms of CDH from *Trametes villosa* on an aldrithiol modified gold electrode using the permselective electrode design. What is clear is that this white rot fungus CDH, similar to the one from *P. chrysosporium* exhibits facile DET. Additionally, in the presence of cellobiose very clear catalysis is seen, reflecting that the flavo and heme domains can freely communicate with each other allowing the internal electron transfer process to proceed. What is additionally shown in Figure 19 is as shown previously for *P. chrysosporium* CDH, the internal electron transfer process is more efficient at acidic pHs and at around pH 7 it drops off to zero.

In Figure 20 cyclic voltammograms are shown for another CDH from the white rot *Phanerochaete sordida* on various thiol modified gold electrodes. This figure further shows the great influence on the electrochemistry of the heme on the thiol used and moreover the influence on the communication between the flavo and heme domains. For some of the thiols, e.g., aldrithiol and 1-monothiolglycerol, pronounced electrochemistry is also coupled with high catalytic currents for cellobiose oxidation. For some other thiols, e.g., cystamine and 3-mercaptoethylamine, the electrochemistry of the heme domain is rather facile, whereas the catalytic current is low or hardly visible in the voltammograms. For some, e.g., 1-mercaptoundecanol, the electrochemistry of the heme is not so pronounced, yet in the presence of cellobiose clear catalytic currents are obtained. However, on virtually all the modified gold electrodes at least some traces of the electrochemistry of the CDH is exhibited and also catalysis in the presence of cellobiose.

Even though bioelectrochemical investigations of different CDHs from both white and soft rot origin are very limited, they might point to the conclusion that especially for the white rot CDHs facile DET reactions with both graphite and thiol modified gold electrodes are possible. In recent investigations (results not shown) of a series of other white rot CDHs, they all exhibit well-defined electrochemistry on thiol modified gold electrodes. In contrast for the soft rot CDHs investigated until now only for *H. insolens* CDH has independent DET been shown on gold, however, with an orientation of the enzyme on the electrode surface that does not promote either the catalytic function of the flavo domain or the intermolecular electron transfer from the flavo domain to the heme domain. For *S. rolfsii* CDH no electroactivity on thiol modified gold electrodes could be seen so far even though catalytic currents were exhibited when this CDH was adsorbed on graphite.

Future investigations on the bioelectrochemistry of various CDHs on different electrode materials may therefore reveal much more on the properties of this fascinating extracellular two domain redox enzyme. The influence of the composition of the SAM on gold (effect of chain length, hydrophobicity, hydrophilicity, charge, mixed monolayers) is currently at focus as well as other electrode materials and the use of other electrode modifiers.

6. Conclusions

In this paper we have summarized the current knowledge on the bioelectrochemistry between the redox enzymes secret-

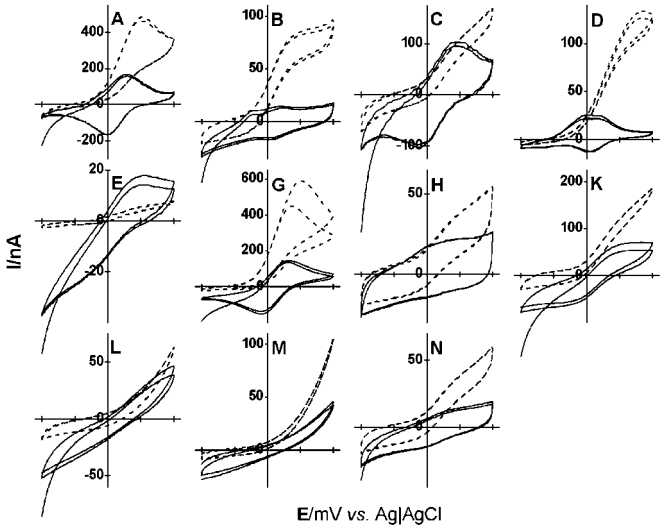

Fig. 20. First two consecutive cyclic voltammograms of CDH from *Phanerochaete sordida* in the absence (solid line) and in the presence of 1 mM cellobiose (dashed line) on various thiol modified electrodes. The used thiols are: A) 4,4′-aldrithiol, B) 3,3′-dithiodipropionic acid di(*N*-succinimidyl ester), C) cystamine dihydrochloride, D) 1-mercaptoundecanol, E) 3-mercaptoethylamine, G) 1-monothioglycerol, H) 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt, K) dithioheptanoic acid, L) dithioundecanamine hydrochloride, M) 1-mercaptoundecanoic acid and N) *N*-acetyl cysteine. Experiments were performed using a permselective membrane electrode in 20 mM acetate mM buffer at pH 3.5. Start potential, −200 mV (vs. Ag|AgCl), scan rate 10 mV s^{-1}, scanning interval between −200 to +200 mV (vs. Ag|AgCl) (100 mV is the increment on the X axes).

ed mostly by the ligninolytic white rot fungi (*Basidiomycetes*), lignin and manganese peroxidase, laccase, and cellobiose dehydrogenase. It is undoubtedly shown that long-range electron transfer between these redox enzymes and electrodes can be obtained. For all oxidoreductases, which are described in this review, direct as well as indirect proofs of DET were shown. For both LiP, laccase and especially for cellobiose dehydrogenase independent electrochemistry can be obtained.

In a recent publication it was shown that LiP from the basidiomycete *Phanerochaete chrysosporium* is capable of oxidizing synthetic lignin (dehydrogenated polymerizate) directly at the protein surface by a long-range electron transfer process, though laccase or MnP was not able to oxidize this polymer by the mechanism of DET [67]. Our unpublished data strongly suggest that laccase also can oxidize natural lignin by this mechanism. In our previous publication the suggestion for this ability was mentioned. Probably, there is a need for extracellular redox enzymes to communicate directly and electronically with a solid matrix in the course of lignin degradation at least at the initial phase of fungal attack on wood. The parallelism between direct bioelectrochemistry of ligninolytic enzymes and the mechanism of lignin degradation might require more stronger proof, however, it is definitely right to introduce the consideration of direct heterogeneous ET in the understanding of lignin biodegradation. Direct electron transfer of ligninolytic enzymes and the [199–201] recently discovered DET of whole living cells (e.g., *Geobacter sulfurreducens*) makes us believe that this principal of electron transfer (DET) is important in a wide range of processes in Nature, especially, when water insoluble materials are attacked by microorganisms, e.g., lignin and solid Fe_3O_4, acting as either electron donor or acceptor.

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