Electrochemical Characterization of Purified *Rhus vernicifera* Laccase: Voltammetric Evidence for a Sequential Four-Electron Transfer†

Daniel L. Johnson, Janene L. Thompson, Sandra M. Brinkmann, Kathryn A. Schuller, and Lisandra L. Martin*

Schools of Biological Sciences and Chemistry, Physics, and Earth Sciences, Faculty of Science and Engineering, Flinders University, GPO Box 2100, Adelaide 5001, South Australia, Australia

Received February 17, 2003; Revised Manuscript Received June 12, 2003

ABSTRACT: *Rhus vernicifera* (Rv) laccase was purified to electrophoretic homogeneity by hydrophobic interaction chromatography. A comprehensive study of the direct electrochemistry of Rv laccase covalently immobilized at a gold electrode using alkanethiol monolayers was undertaken. The observed midpoint potential was 410 mV versus the normal hydrogen electrode (NHE), consistent with reduction potentials obtained by potentiometric titration for the T1 copper site. Evidence is presented for a concerted 4-electron reversible process at slow scan rates (υ) on the basis of peak current ratios (i_{pa}/i_{pc}). Catalytic currents were observed in the presence of the biological substrate oxygen, indicating that laccase activity is retained throughout the immobilization process. Electrochemical characteristics of the immobilized laccase were essentially invariant across the pH range 5.5−8.5 and the temperature range 5−35 °C. The purified enzyme displayed a pH optimum of 9.0, when assayed spectrophotometrically with syringaldazine as a substrate. Inhibition of the laccase activity with azide or fluoride showed an $I_{50}(NaN_3)$ of 2.5 mM and an I_{50}(NaF) of 18.5 mM. Electrochemistry in the presence of azide reduces the anodic current by ca. one-half, consistent with the 4-electron process decreasing to a 2-electron process. However, fluoride has no effect on anaerobic electrochemistry. These electrochemical results suggest that the pH dependence of laccase activity is related to the effects of pH on the structure or binding of the substrate.

Multi-copper oxidases have realized a renaissance in the past few years with applications in biofuel cells (*1*−*3*), bioelectrocatalysis (*4*, *5*), biological and oxygen sensing (*5*−*9*), pulp delignification (*10*, *11*), and bioremediation (*12*−*15*). The simplest members of the multi-copper oxidase family are laccases, capable of catalyzing the 4-electron reduction of oxygen to water with concomitant oxidation of a broad range of substrates (*13*).

The monomeric laccase contains four copper atoms that are spectroscopically classified as an EPR[1]-active type 1 (T1), an EPR-active type 2 (T2), and the EPR-inactive type 3 (T3) consisting of a binuclear pair of copper atoms. The T1 copper is characterized by an intense ($\epsilon \sim 3000\ M^{-1}\ cm^{-1}$) absorption band in the visible region at ∼600 nm. The accepted mechanism for laccase activity suggests that the electron entry point is the T1 copper which rapidly transfers the electron to the triangular active site, consisting of the antiferromagnetically coupled T3 copper atoms and the T2 copper atom (*7*, *16*−*18*). The in vivo electron donor can be substituted by an artificial electrode, thereby enabling estimates for the reduction potentials, electron transfer rate constants, and thermal and pH stability, in addition to the screening of effective inhibitors (*3*, *12*, *17*, *19*, *20*).

Early electrochemical studies of *Rhus vernicifera* (Rv) laccase using potentiometric titration with spectrophotometry indicated three reduction potentials at +420 mV (T1), +390 mV (T2), and +460 mV (T3), which were assigned to each distinct type of copper atom (*7*, *21*). Considering that all laccases are thought to perform the same basic function in vivo, it is surprising that a considerable difference in reduction potential is observed between species (*18*). For example, the T1 reduction potential of *Polyporus versicolor* is +785 mV (*22*) whereas Rv is +420 mV. However, the majority of electrochemical studies of laccases reported to date show a single, poorly defined redox response (*6*, *17*, *20*). Although Armstrong et al. (*23*) identified two distinct redox processes at 10 °C, other workers have since been unable to reproduce this behavior (*17*). The more typical, single redox process observed for Rv laccase has been explained either by the coincidental redox waves of the four copper atoms or by the response arising from a single copper atom, most probably the T1 site (*6*, *17*, *20*). Only a few studies have achieved direct electrochemistry of large proteins, presumably limited because the active site often tends to be "buried" within the protein matrix. In the case

† L.L.M. acknowledges support from the Grape and Wine Research and Development Corp. (GWRDC).

* Address correspondence to this author at the School of Chemistry, Monash University, Clayton 3800, Victoria, Australia. Phone: (+613) 99051338. Fax: (+613) 99054597. E-mail: Lisa.Martin@sci.monash.edu.au.

[1] Abbreviations: EPR, electron paramagnetic resonance; Rv, *Rhus vernicifera*; T1, T2, and T3, type 1, 2, and 3 copper atoms, respectively; E_{mid}, midpoint (reduction) potential; ΔE, peak separation; k_s, electron transfer rate constant; I_{50}, the inhibitor concentration required to reduce initial laccase activity by half; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MPA, 3-mercaptopropionic acid; SAM, self-assembled monolayer; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; EDTA, ethylenediaminetetraacetic acid; AcOH, acetic acid; NHE, normal hydrogen electrode.

10.1021/bi034268p CCC: $25.00
Published on Web 08/09/2003

of laccases, this problem has been exacerbated by the apparent experimental limitation to a narrow range of pH values, temperatures, and scan rates, thereby preventing significant conclusions (*6*, *7*, *17*, *20*). Over the past decade, some of these problems have been overcome by covalently attaching the protein to an electrode through a self-assembled monolayer, thereby preventing diffusion of the protein away from the electrode (*24*−*26*).

Careful fabrication of stable protein films on electrodes for voltammetric analysis can assist in the elucidation of reaction mechanisms and the correlation of structure with function (*27*, *28*). Protein purity is crucial in this pursuit, especially when considering low surface coverage where a monolayer of protein often results in small current responses. Purification of Rv laccase was first reported in 1970 using a mixture of cation- and anion-exchange chromatography (*29*), and subsequent workers have almost exclusively followed this purification protocol. We have improved this method of purification using hydrophobic interaction chromatography. In the present study, laccase purified by our procedure was used to study its biochemical and electrochemical behavior in solution and immobilized on a gold electrode, respectively. Our specific aims were (i) to investigate the activity of Rv laccase over a range of pH values, (ii) to electrochemically identify the types of copper atoms involved in the electron transfer response, and (iii) to use electrochemistry to monitor the interaction of laccase with known inhibitors, thus gaining an insight into the catalytic process and enzyme function of Rv laccase.

EXPERIMENTAL PROCEDURES

Materials. 2-(*N*-Morpholino)ethanesulfonic acid (MES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), [[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid (TAPS), EDTA, NaCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), NaF, KCl (Sigma); $KMnO_4$, $K_3[Fe(CN)_6]$, NaOH (Ajax chemicals); NaN_3, H_2SO_4, AcOH, H_2O_2, KOH (Merck); 3-mercaptopropionic acid (MPA), *N*-hydroxysucinimide (NHS) (Aldrich); and 24-carat gold sheets (0.30 mm thickness) and gold wire (0.25 mm diameter, 99.999%) (Peter W. Beck) were used as purchased without further purification.

Laccase Activity Assay. Laccase activity was assayed spectrophotometrically by monitoring the oxidation of syringaldazine at 530 nm ($\epsilon = 65000\ M^{-1}\ cm^{-1}$) using a Beckmann DU650 spectrophotometer (Beckmann Instruments). The assay mixture consisted of 0.3 mL of 28 μM syringaldazine (dissolved in absolute ethanol), 0.7 mL of mixed buffer (pH 8.5), and an appropriate volume of the enzyme-containing solution in a final volume of 1.5 mL. The mixed buffer consisted of 25 mM MES, HEPES, TAPS, and acetic acid. The pH was adjusted to a value of 8.5 with NaOH. All assays were performed at 25 °C. The reaction was started by the addition of the enzyme-containing solution. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of syringaldazine per minute.

Purification of Laccase. Laccase was purified from a commercially available acetone precipitate (Saito and Co. Ltd., Tokyo, Japan) of the lacquer from the Japanese lacquer tree, *Rhus vernicifera*. In the first step of the purification protocol, 4 g of the acetone precipitate was stirred with 100 mL of 0.1 M potassium phosphate buffer (pH 6.0) at room temperature for 16−20 h. Any undissolved material was then removed by filtration through a series of MF-Millipore filters (Millipore Corp., Bedford, MA) with decreasing pore sizes of 0.80, 0.45, and 0.2 μm. In the second step, $(NH_4)_2SO_4$ was added to the filtered extract at a ratio of 30 g to 100 mL of extract, and after the $(NH_4)_2SO_4$ had dissolved, the resulting solution was centrifuged at 15000*g* for 20 min to remove any insoluble material. In the final step, the supernatant from step 2 was applied at a flow rate of 3 mL min^{-1} to a column (1.5 cm diameter × 14.0 cm height) of phenyl-Sepharose (Amersham Pharmacia Biotech) equilibrated with 2.67 M $(NH_4)_2SO_4$ in 0.01 M potassium phosphate buffer (pH 6.0). The column was then washed sequentially with 2.67, 1.34, and 0.27 M $(NH_4)_2SO_4$ in 0.01 M potassium phosphate buffer (pH 6.0).

Electrochemistry. Electrochemical experiments were performed using a Bioanalytical Systems (BAS) 100B potentiostat controlled by BAS 100W Windows control software version 2.3. Measurements were performed at least three times on separate gold sheets. A bright platinum wire was used as the auxiliary electrode. A Ag/AgCl (saturated KCl) reference electrode purchased from BAS was used throughout the experiments and was routinely referenced to the calibrant $K_3[Fe(CN)_6]$ under standard conditions of 1 M KNO_3 (*22*, *30*). The solution volume in the electrochemical cell was 5 mL. Unless otherwise specifically defined, all electrochemical experiments were anaerobic, and solutions were rigorously purged with N_2 for 1 h or more before being used. For aerobic experiments, compressed air was bubbled through the solution for at least 10 min prior to application of a potential difference, and a blanket of compressed air was maintained throughout the experiment. Reduction (midpoint) potentials (E_{mid}) were determined from the average of anodic (E_{pa}) and cathodic (E_{pc}) peak potentials and corrected to the NHE by adding 197 mV. Peak separations (ΔE) were evaluated as the difference between the E_{pa} and E_{pc} peak potentials.

Electrochemical Cell. A modified electrochemical cell (*31*) was used for all experiments, either with or without a water jacket enabling thermal control. The electrode area was always 0.071 cm^2, defined by an opening in the base of the cell to the working electrode.

Working Electrode Surface Preparation. Gold sheets were rigorously cleaned before chemical functionalization using the following procedures. Previously immobilized thiol monolayers were first removed by reductive desorption in 0.5 M KOH. The surface was then sequentially polished to a mirrorlike finish using 1, 0.3, and 0.05 μM Al_2O_3 powders on polishing cloths. Excess Al_2O_3 was removed by sonication twice in water. The surface was then soaked in a 0.1 M $KMnO_4$ solution containing 0.5 M NaOH solution overnight. After extensive washing with water, the gold surface was placed into a "pirhana solution" for 5 min, comprising a 1:3 v/v mixture of 30% H_2O_2 and concentrated H_2SO_4. This was followed by exhaustive washing with water and then ethanol. The cleanliness of the surface was verified by cycling between −0.5 and 1.7 V in 0.5 M H_2SO_4 for approximately 30 min (*32*). The electrode was washed with water followed by ethanol, then dried under a stream of N_2, and immediately placed in a 5 mM solution (75:25 v/v ethanol/water) of MPA

Table 1: Purification of Laccase from *Rhus vernicifera*

fraction	volume (mL)	activity (units)[a]	protein (mg)	specific activity (units/mg)	purification (*x*-fold)	yield (%)
1. filtered extract	86	53.5	34.4	1.56	1.00	100
2. filtered extract plus $(NH_4)_2SO_4$	93	39.1	34.4	1.14	0.73	73
3. PS−HIC chromatography						
peak 1	6.5	22.9	12.0	1.91	1.23	43
peak 2	6.5	1.1	2.7	0.42	0.27	2

[a] A unit is defined as the amount of enzyme required to oxidize 1 μmol of syringaldazine min^{-1} at 25 °C.

and left overnight to allow self-assembly of the thiol. While there have been reports of successful self-assembly within several minutes (*33*), it is generally believed that a long deposition period facilitates a monolayer with a high degree of order by enabling surface rearrangement. This is particularly important for short chain alkanethiols such as MPA, since intermolecular van der Waals forces do not significantly contribute to the final monolayer (see, for example, refs *33* and *34*). The surface was then washed with 95% ethanol to remove unbound MPA and dried under a stream of N_2. The dried surface was placed into an aqueous solution containing 75 mM EDC and 25 mM NHS to activate the terminal acid functionality. After being washed with water and dried under a stream of N_2, the gold surface was placed into a solution of Rv laccase (0.013 mg of protein/mL in 10 mM phosphate buffer, pH 6.0) overnight at 4 °C to complete covalent immobilization. The gold electrode was then immersed in a solution containing 10 mM phosphate buffer (pH 6.0) to remove excess or loosely bound laccase, then dried under a gentle stream of N_2, and immediately used for electrochemical experiments.

Gold Surface Characterization. The electrochemical roughness of the gold electrodes (electrochemically available area divided by the geometric area) was determined to be 1.5 ± 0.1 using the gold oxide reduction method (*35*). All measurements of surface coverage (Γ) were adjusted accordingly. The surface coverage of MPA was 0.83 nM cm^{-2} as determined by reductive desorption at the gold−thiol bond. The surface coverage of electroactive laccase was then determined by integrating the area under the anodic peak, dividing this by the scan rate, and converting to moles per unit area on the surface by dividing by *nFA*, where *F* is Faraday's constant and *A* is the actual electrode area. This gives $\Gamma = \sim 2.8 \times 10^{-12}$ mol cm^{-2} assuming $n = 4$ and $\sim 0.7 \times 10^{-12}$ mol cm^{-2} assuming $n = 1$ (*36*).

RESULTS

Purification and Biochemical Characterization of Laccase. (A) Purification of Laccase. Laccase was purified to a single band on an SDS−PAGE gel using a combination of $(NH_4)_2SO_4$ fractionation and phenyl-Sepharose−hydrophobic interaction chromatography (PS−HIC) (Table 1, Figures 1 and 2). Two major fractions eluted from the PS−HIC column showing laccase activity. Peak 1, eluted with 1.34 M $(NH_4)_2SO_4$, contained the majority (95%) of the laccase activity and no other proteins whereas peak 2, eluted using 0.27 M $(NH_4)_2SO_4$, contained a mixture of proteins (Figure 2) including one at 113 kDa, which is the accepted molecular mass of laccase (*29*). Peak 1 contained 95% of the laccase activity remaining at the end of the purification protocol.

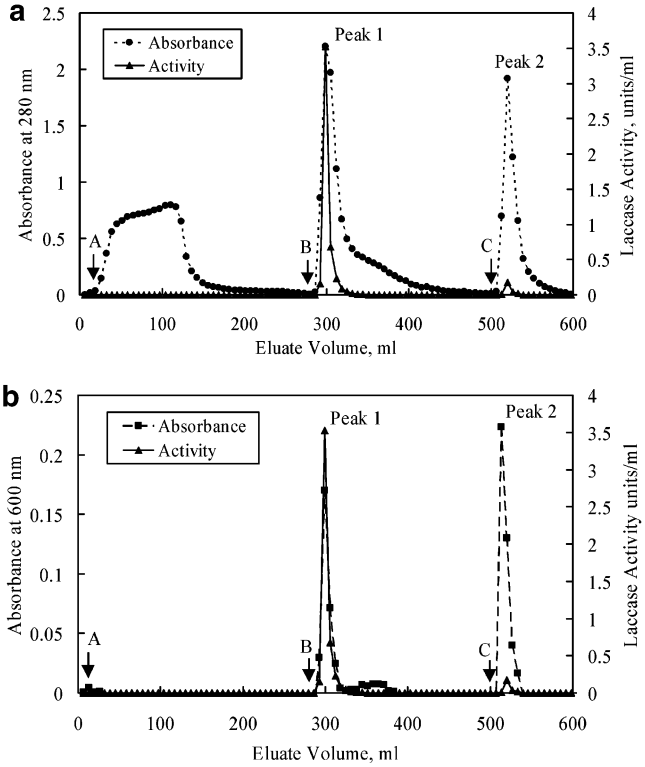

FIGURE 1: PS−HIC chromatography of the filtered extract plus $(NH_4)_2SO_4$ derived from the resuspended acetone precipitate of Rv laccase (refer to Table 1). The column was washed sequentially with 2.67, 1.34, and 0.27 M $(NH_4)_2SO_4$, commencing at the points labeled A, B, and C, respectively. Panel a illustrates the elution of laccase and the elution of the other proteins applied to the column, monitored at 280 nm. Panel b illustrates the elution of laccase, monitoring absorption at 600 nm due to the presence of a T1 copper site.

The major laccase peak corresponded with the major protein peak as indicated by the peak in absorbance at 280 nm (Figure 1a). The enrichment of laccase in peak 1 was only 1.23-fold, indicating that laccase was the most abundant protein in the original acetone precipitate. Both peaks from the PS−HIC column had high absorbance at 600 nm (Figure 1b). This is attributed to the T1 copper site. This suggests that peak 2 contains an inactive form of laccase and/or another protein(s) containing a T1 copper. On the basis of the work of Reinhammer (*29*) and SDS−PAGE (Figure 2), the other protein is probably stellacyanin. Thus, although peak 2 had low laccase activity, it exhibited high absorbance at 600 nm, indicating that either an inactive form of laccase or another protein containing a T1-type copper was also present.

When analyzed by SDS−PAGE, peak 1 contained only one protein band (113 kDa) whereas peak 2 displayed several

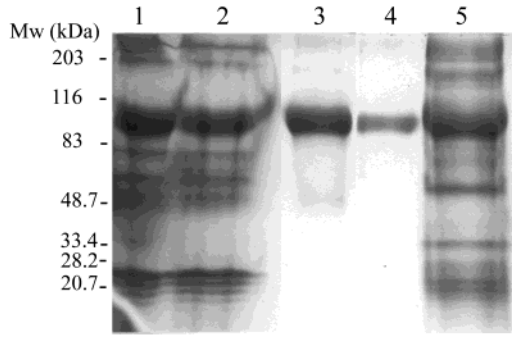

FIGURE 2: Silver-stained SDS−PAGE gel of the key fractions from the purification of laccase. The contents of the lanes were as follows: lane 1, the filtered extract (2.95 μg of protein); lane 2, the filtered extract plus $(NH_4)_2SO_4$ (2.86 μg of protein); lane 3, peak 1 from the PS−HIC column (2.95 μg of protein); lane 4, peak 1 from the PS−HIC column (0.295 μg of protein); and lane 5, peak 2 from the PS−HIC column (3.08 μg of protein).

bands (Figure 2). Peak 1 contained pure laccase close to the reported molecular mass of laccase (*27*) whereas peak 2 contained a mixture of proteins, one of which is probably either a distinct isoform of laccase or a degradation product of the laccase found in peak 1. Rv laccase is a glycoprotein with up to 80% glycosylation by weight, and when glycosylation is blocked by tunicamycin, laccase activity is lost (*37*). Thus, if the form of laccase found in peak 2 were partially deglycosylated, this would explain both its low level of activity and its increased hydrophobicity relative to the form of laccase found in peak 1.

(B) Biochemical Characterization of Laccase. Using the standard biochemical assay with syringaldazine as substrate, the two peaks from the PS−HIC column were assayed for the effect of pH on laccase activity. Peak 1 exhibited a pH optimum of 9.0 whereas peak 2 was unresponsive to pH. For peak 1, activity declined sharply below pH 9.0 with virtually no activity at pH 8.0 and below. NaN_3 and NaF, known inhibitors of laccase (*32*), were also tested for their effects on the laccase activity of peak 1 from the PS−HIC column. The $I_{50}(NaN_3)$ was 2.5 mM whereas the I_{50}(NaF) was 18.5 mM, demonstrating the effectiveness of azide as an inhibitor of laccase activity.

Electrochemistry of Purified Laccase. (A) Cyclic Voltammetry. No Faradaic current was observed in any of the electrode preparative stages including bare Au, Au-MPA [gold modified with an MPA self-assembled monolayer (SAM)], or Au-MPA-NHS (Au-MPA activated with EDC/NHS). After laccase immobilization, a single, well-defined redox wave was observed with a midpoint potential of +410 mV, consistent with the T1 copper reduction potential as determined by potentiometric titration (*21*, *22*). Only one redox couple was observed for the active laccase samples throughout the voltammetric experiments. Midpoint (reduction) potentials and peak separations are provided in Table 2.

(B) Electrode Characterization. The observed surface coverage of electroactive laccase is consistent with those of other proteins immobilized on metal surfaces. Our values for Γ range from $\sim 0.7 \times 10^{-12}$ to 2.8×10^{-12} mol cm^{-2} and are comparable with those obtained for azurin electrostatically adsorbed onto a gold electrode, $\sim 2 \times 10^{-12}$ mol cm^{-2} (*24*), and for glucose oxidase immobilized on an MPA SAM, $\sim 1.9 \times 10^{-12}$ mol cm^{-2} (*38*). Glucose oxidase (*Aspergillus niger*, ~200 kDa) is roughly double the molecular mass of laccase (113 kDa), so our calculated surface coverage of laccase is consistent with this report.

Table 2: Experimental Data for Rv Laccase Immobilized on an MPA SAM[a]

pH	scan rate (mV/s)	E_{mid} (mV)	ΔE (mV)	i_{pa}/i_{pc}
5.5	0.5	418	28	1.2
	1	419	30	1.5
	2	421	41	1.9
	5	419	50	2.1
	10	420	53	2.3
	20	420	60	2.6
	50	419	63	3.0
	100	419	71	3.4
	200	421	82	3.7
	500	427	108	3.8
7	0.5	409	30	1.2
	1	409	30	1.7
	2	409	38	2.1
	5	411	48	2.1
	10	409	51	2.2
	20	410	56	2.4
	50	409	63	2.9
	100	411	67	3.3
	200	412	78	4.0
	500	415	107	4.0
8.5	0.5	403	28	1.3
	1	402	26	1.5
	2	403	37	1.8
	5	402	48	2.0
	10	403	53	2.2
	20	402	61	2.5
	50	402	63	2.8
	100	403	71	3.2
	200	403	80	3.8
	500	411	105	4.0

[a] Potentials versus NHE; results are averaged over five data sets and the error lies within 1 SD of the mean.

(C) Stability of the Modified Electrode. Over the temperature range considered (5−30 °C), the laccase-modified gold electrode provided consistent results for up to 8 h. However, leaving the electrode in the electrochemical cell containing buffer and electrolyte overnight at 20 °C led to a considerable reduction in peak current at +410 mV and the introduction of a second, reversible process at +150 mV. This peak was sharp and narrow, unlike the broad peaks attributed to laccase redox chemistry. Accordingly, this new wave is most probably due to free copper ions at the gold electrode. This was verified by examining the voltammetry of free copper ions at an MPA-modified gold electrode, which resulted in a wave at similar potentials. Thus it was possible to directly examine the in situ demetalation of laccase under a variety of conditions. This process was complete within 1 h after addition of azide or fluoride ions at 20 °C, indicating that these inhibitors of laccase activity do not just bind irreversibly at the active site but they also contribute to copper depletion when provided with an electron flux. Under ideal conditions, this depletion could be significantly slowed by storing the electrode at 4 °C, with an acceptable loss in peak current over several weeks (50% over 3 weeks), correlating with biochemical assays.

(D) Thermal, pH, and Kinetic Behavior. Contrary to other reports, temperature did not affect E_{mid} over the range 5−30 °C (*17*). Electroanalytical experiments were undertaken at pH 5.5, 7.0, and 8.5, where laccase is biochemically inactive,

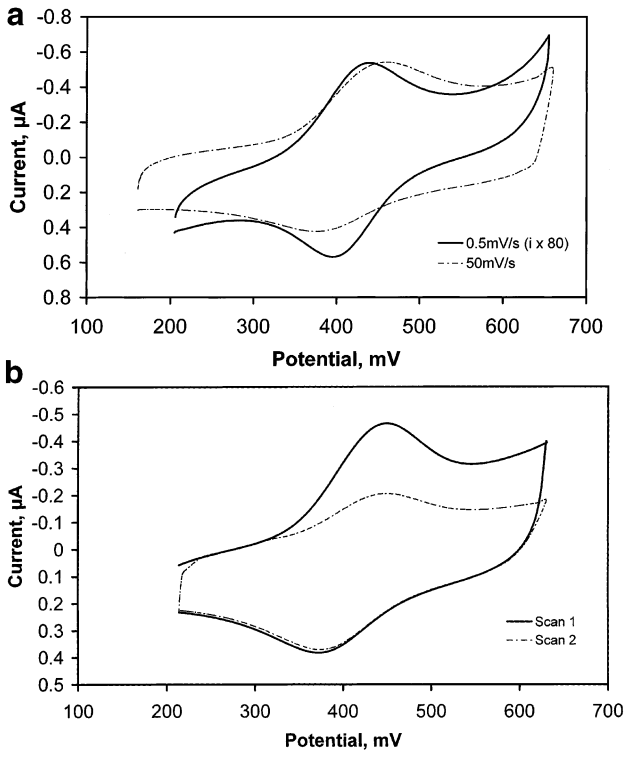

FIGURE 3: Representative cyclic voltammograms of Rv laccase immobilized on an MPA SAM. (a) Single scans at rates of 0.5 and 50 mV/s. (b) Successive scans at 50 mV/s. The electrochemical cell contained mixed buffer at pH 7.0, with 0.1 M NaCl as supporting electrolyte. Experiments were performed in the absence of oxygen at room temperature (21 ± 1 °C).

marginally active, and approaching optimal activity, respectively. The electrochemical response was essentially invariant across this pH range, suggesting that surface-confined laccase may exhibit a different pH profile compared to that of laccase in the solution phase. Alternatively, the pH-dependent behavior of laccase in the biochemical assay may be related to the effect of pH on binding of the reduced substrate (in our case syringaldazine). The T1 copper in laccases is believed to be a traditional "blue copper" electron transfer center, and such centers have been shown to display a pH-dependent reduction potential (*18*, *27*). Laccase reduction potentials remained fairly consistent with scan rate (υ), but ΔE values increased proportionally with υ above 10 mV/s. The apparent electron transfer rate constant, k_s, determined using the method of Laviron (*39*) was 8−30 s^{-1}, depending on the number of electrons transferred ($n = 1-4$) over the pH range 5.5−8.5. Two representative voltammograms at pH 7.0, 50 mV/s and 0.5 mV/s, are shown in Figure 3a, highlighting the slight increase in ΔE with increasing scan rate even though E_{mid} remained constant. Figure 3b illustrates two successive voltammograms at 50 mV/s showing the loss of current between the initial and subsequent anodic scans.

(E) Peak Current Ratio. Analysis of the ratio of cathodic and anodic wave peak currents reveals an interesting correlation. The peak current ratio (i_{pa}/i_{pc}) varied from 1:1 at very slow υ (<1 mV/s) to 4:1 at fast υ (>200 mV/s). This effect is illustrated in Figure 4 for each pH value studied. Although this dependence appears at first glance to be sigmoidal, there could also be three distinct phases. The first phase involves a rapid increase from a 1:1 ratio at 0.2 mV/s to a 2:1 ratio at 5 mV/s. Then, the peak current ratio increases

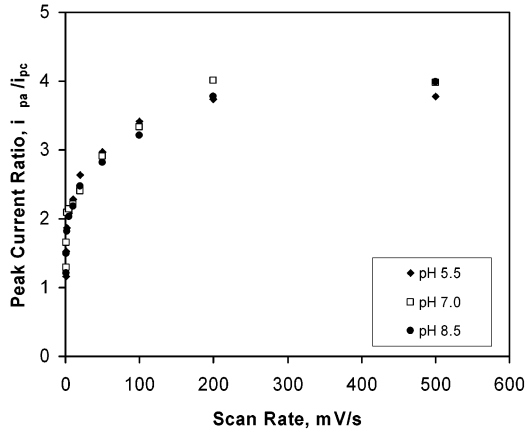

FIGURE 4: Influence of scan rate on the peak current ratio at a variety of pH values. Peak current ratios were determined by dividing the anodic peak current by the cathodic peak current (only Faradaic component used). The electrochemical cell contained mixed buffer adjusted to the required pH, as well as 0.1 M NaCl as the supporting electrolyte. Experiments were performed in the absence of oxygen at room temperature (21 ± 1 °C).

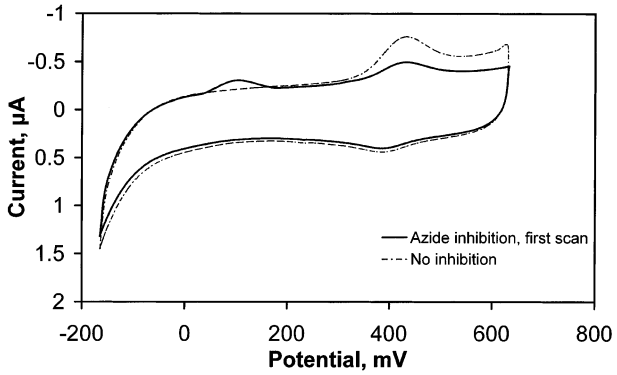

FIGURE 5: Cyclic voltammograms of the laccase-modified gold electrode immediately before and after the addition of sodium azide. The aqueous azide solution was deaerated separately before addition to the cell to give a final concentration of 6.6 mM. The electrochemical cell contained mixed buffer at pH 7.0, with 0.1 M NaCl as supporting electrolyte. The scan rate was 50 mV/s. Experiments were performed in the absence of oxygen at room temperature (21 ± 1 °C).

gradually from 2:1 to 4:1 as υ is increased from 5 mV/s to 200 mV/s. Above 200 mV/s, the ratio remains constant at 4:1, irrespective of scan rate, up to 2 V/s. At faster scan rates, such as at 50 mV/s shown in Figure 3b, the first scan in the anodic direction gave a peak current that was approximately three times greater than that observed for subsequent scans. This suggested that the electron transfer was limited by the reductive step at fast scan rates. Cyclic voltammograms measured over all scan rates showed no change in either peak position or current over the pH range 5.5−8.5.

(F) Electrochemical Inhibition with Azide and Fluoride. The most frequently reported inhibitors of laccase activity, N_3^- and F^- (*40*), were studied in order to assess their influence on the redox response of laccase. Figure 5 displays an overlay of a voltammogram taken before and after incubation with 6.6 mM NaN_3. After incubation with azide, two prominent effects were noticed. First, the anodic current of the original laccase redox process was roughly half that of the preincubation current. Second, a new peak at considerably lower potential (+100 mV) was observed, with peak

current roughly equivalent to that observed for the inhibited laccase redox process. The combined magnitude of the postincubation peak currents was equivalent to that of the native laccase prior to inhibition. The new peak (at +100 mV) is irreversible, and rapid loss of signal occurred after repeated cycling of the potentials between the upper and lower potential limits. This instability of the azide-inhibited form of laccase limited a more thorough examination of the redox mechanism; however, the results obtained are consistent with two copper atoms dissociating from the protein matrix following binding by azide anion(s).

It appears that when a potential difference is applied, the protein denatures, leading to loss of a $Cu-N_3$ species, which then diffuses to the electrode surface via defects (*33*) in the SAM, giving rise to the second wave. In the presence of N_3^-, the redox signal of laccase reverted from an anodic peak current of 0.27 to 0.14 μA. Repeated cycling of the potential between −100 and 600 mV led to total loss of laccase current, probably due to protein denaturation following demetalation. While this conclusion is difficult to prove, the loss of current for the initial anodic process is approximately half of its original magnitude, and the presence of a new wave at a lower potential with the same intensity provides a reasonably strong indication.

Similar experiments using NaF resulted in no change to the laccase voltammograms. This result was unexpected, because several groups have shown F^- to be an effective inhibitor, while other workers have presented convincing evidence for the presence of a Cu−F bond in the fluoride-inhibited form (*41*−*43*). It is possible that, in the case of surface-confined laccase, F^- inhibits in either a loosely coordinated or "blocking" manner, preventing oxygen from gaining access to the active site. The fluoride inhibition of the catalytic behavior of laccase supports this interpretation.

(G) Catalytic Behavior. Observation of the catalytic activity of laccase confirmed that the in vivo function was retained throughout the immobilization process. Conveniently, dioxygen is the electron acceptor of laccase so an assessment was readily obtained by saturating the electrochemical cell with compressed air. Cyclic voltammograms obtained in the presence of dioxygen displayed a significant increase in Faradaic current, where the difference between the anaerobic Faradaic current and the aerobic Faradaic current is attributed to catalytic behavior. Other groups have shown that this catalytic behavior follows the typical Michaelis−Menten profile (*7*). Inhibition of laccase was directly observed by addition of gradually increasing concentrations of NaF (curves 1−9 in Figure 6) and NaN_3 (data not shown). It was thus possible to confirm the inhibitory action of F^-, albeit at somewhat higher concentrations than for azide (total inhibition with N_3^- at 6.6 mM, compared with F^- at 52.8 mM).

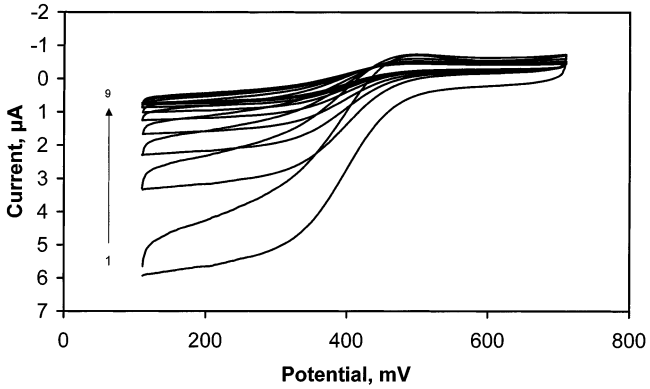

FIGURE 6: Inhibition of catalytic voltammograms with flouride ions. The solution was aerated, and catalytic behavior is obvious in the absence of any inhibitors (1). Addition of sodium flouride to create 6.6, 13.2, 19.8, 26.4, 33.0, 39.6, 46.2, and 52.8 mM [F^-], respectively, results in a corresponding drop in catalytic current (2 → 9). Conditions: pH 7.0, temperature 21 ± 1 °C, and scan rate 20 mV/s.

DISCUSSION

Studies of the 4-electron reduction of Rv laccase have been undertaken over several decades using redox titrations monitoring spectrophotometric changes (*21*, *44*−*49*). Our investigation confirms some of these findings but also offers new insights into the electron transfer process, as we have explored the redox waves under kinetic control rather than equilibrium studies. Significantly, we have used highly purified protein as demonstrated by SDS−PAGE. No other studies of Rv laccase have illustrated the purity of their Rv laccase, and certainly the original purification reported by Reinhammer (*29*) offered no data to support the method of purification although this is widely reported as the standard method for laccase purification since 1970. Our purification has enabled us to confidently undertake the direct (unmediated) electrochemistry of Rv laccase to obtain a reproducible redox "signature" using various samples from different purifications.

We observed cyclic voltammograms for laccase immobilized on a gold electrode that are consistent with 4-electron transfer events occurring via the T1 center, supporting the currently accepted mechanistic model (*16*). In addition, laccase activity is limited by electron transfer kinetics in the absence of substrate. The observed redox process(es) is (are) pH and temperature independent. Known inhibitors of laccase, N_3^- and F^-, appear to act by entirely different mechanisms consistent with results of other workers (*41*, *50*). The cyclic voltammograms achieved by immobilization of Rv laccase onto a self-assembled monolayer of a thiol-modified gold electrode enabled very good signal to noise ratios to be achieved. Although we were aware that this method of enzyme immobilization results in heterogeneous orientations of laccase molecules, the advantages of a diffusionless system outweighed this disadvantage. In this way we have been able to access extremely slow scan rates, allowing analysis of the kinetic features of electron transfer events for Rv laccase. The peak current ratio is proportional to the amount of charge transferred in the redox step. The plot of peak current ratio (anodic:cathodic) as a function of increasing scan rate indicated that a minimum value (approaching 1:1) occurred at slow scan rates. As the scan rate increased, the current ratio reached a maximum value of 4:1. These results suggest that the rate of laccase oxidation (k_{off}) is considerably faster than the rate of reduction (k_{on}). Thus over the entire range examined here, 0.5 mV/s to 2 V/s, the oxidation process involving four 1-electron steps was always observed; however, the reduction process was scan rate dependent. The oxidation process is consistent with rapid electron transfer via the T1 site until all four copper atoms are oxidized. However, while at the fast scan rate limit

(>∼200 mV/s) it appears that a 4-electron oxidation occurs, the associated reduction has just 1 electron equivalent, i.e., a 4:1 current ratio. As the scan rate is lowered to 0.5 mVs^{-1}, the ratio of peak currents approaches 1:1. Normalizing the Faradaic current (dividing i_p by υ) confirmed that, at slow scan rates, the peak current ratio was actually 4:4: four distinct oxidative processes and four distinct reductive processes. Additional evidence supporting this mechanism is obtained by monitoring successive scans at fast scan rates. As illustrated in Figure 3b at 50 mV/s, the initial anodic scan gives a current that is approximately four times greater than the subsequent scan. These data demonstrate that all four electrons are removed through the initial oxidation scan, one electron is returned via the reduction scan, and the subsequent reoxidation scan is only able to oxidize the 1-electron-reduced center, presumably the T1 copper. If we are correct, and the oxidation wave is always 4 electron equivalents, then the 4:4 limit could be rationalized as either only one copper atom (T1) being reduced four times, i.e., 4 × 1:1 ratio, or four copper atoms being reduced, i.e., 4:4 ratio. This kinetic phenomenon is consistent with in vivo requirements of laccase, where, despite a rapid turnover rate, the rate-limiting step for catalysis is the intramolecular electron transfer from the T1 atom to the trinuclear active site. While it is not thought to be necessary in vivo for electrons to be passed from the T2/T3 site to the T1 for an oxidation, this process is considerably faster than the reductive transfer from the T1 to the triangular active site. Thus the redox process at 410 mV is consistent with electron transfer occurring through the T1 copper "gate". The T1 atom sequentially accepts electrons from the donor and subsequently reduces the T2 and T3 copper atoms; that is, the electron transfer involves four distinct steps through the T1 copper. In fact, the 4-electron transfer process is quite contrary to earlier reports where redox titrations indicated 3.5−3.7 electron equivalence. These discrepancies could well have arisen from impurities or experimental errors in measurement of the very small changes in absorption in the UV (*48*, *49*). Also, these authors suggest that at least two distinct reduction potentials have been observed by chemical titration methods; however, as mentioned above, we saw no indication of a higher potential process. Our data suggest that only one copper ion (T1) is redox observable under our experimental conditions.

The T1 site has long been considered the electron entry point for electrons, and this is consistent with recent spectroscopic data (*7*, *16*, *17*). Interestingly, the superior currents observed for the anodic compared with the cathodic waves have also been observed for ascorbate oxidase using cyclic voltammetry, although the authors attributed this feature to the enzyme existing in a partially reduced form (*51*).

The cyclic voltammograms of Rv laccase displayed a single cathodic and anodic wave well discriminated from the baseline and reproducible with samples from different purification preparations. The temperature dependence of this redox process was assessed and shown to have no effect on either the position or nature of the signal over the temperature range 5−30 °C, nor was there any significant effect of pH over the range 5.5−8.5. The observed values fall within ±5 mV of those reported by Santucci et al.: 410 mV at pH 5.5 and 400 mV at pH 7.0 (*17*). The peak separation varied with scan rate, the smallest value of ca. $\Delta E = 30$ mV was observed at 0.5 mV/s, indicating electrochemical reversibility. The insensitivity of the redox process to pH was intriguing as the optimum activity for laccase using syringaldazine as the electron donor was pH 9.0. This is consistent with the results obtained by Xu et al. (*52*), who compared Rv laccase with several fungal laccases and found that, in general, Rv laccase had a more basic pH optimum than any of the fungal laccases. Thus the effect of pH appears to be on the structure or binding of the electron-donating substrate (in this case syringaldazine) and not on the components of the active site (T1, T2, or T3) that give rise to the redox waves. Although protonation close to metal clusters has been shown to give rise to pH dependence of redox processes in ferredoxins (*53*), for laccase the pH effect may only occur in the presence of the electron acceptor, oxygen.

The activity of laccase, as monitored by the oxidation of syringaldazine, was inhibited by NaN_3 and NaF. NaN_3 was found to be a more potent inhibitor than NaF gauged by the different I_{50} values. This may be related to differences between these two inhibitors in how they affect or access the active site. Spectroscopic studies, in particular EPR, support the binding of azide to the T2/T3 active site although the nature of this interaction has not been unequivocally assigned (*41*, *42*, *54*, *55*). Fluoride has also been implicated in binding to both the T2 (*43*) and T3 (*42*) copper atoms, with the number of bound molecules influenced by concentration. Research contributions from the 1980s also examined the role of F^- binding, by comparison of the native with the T2-depleted laccase indicating binding was occurring at the T2 site (*45*−*47*, *56*, *57*). Thus, in this study, these inhibitors were introduced into the electrochemical cell containing laccase under both anaerobic and aerobic conditions. Earlier studies have observed that the mode of inhibition appears to be different for the two inhibitors, azide and fluoride, although there remain discrepancies in the literature as to which copper centers are involved in these interactions. Xu et al. suggest that N_3^- binds to the T2/T3 and F^- binds to the T2 alone (*50*) whereas Spira-Solomon et al. suggest that the F^- can also bind to the T2/T3 although this occurs when the enzyme is fully oxidized (*41*).

Incubation of immobilized laccase with N_3^- under anaerobic conditions resulted in loss of current at 410 mV and appearance of a second wave at lower potential. The first lies at the same potential as the initial redox process and equivalent to two 1-electron redox processes. The second wave at ∼100 mV was roughly consistent with the potential expected for a copper−azide complex external to the protein matrix. Rapid loss of current followed, rendering only the first scan suitable for analysis and preventing a more thorough analysis of this effect. The combined magnitude of the postincubation oxidative currents is equivalent to the preincubation current, implying that all copper atoms contribute to the voltammogram. Thus, the effect of N_3^- on the laccase protein under electrochemical conditions results in a change from a protein capable of a 4-electron transfer to one capable of only a 2-electron transfer. This is presumably due to the absence of two of the four original copper atoms. One possible explanation as to why these data appear to contradict the results of other workers is that our results were obtained *after* a potential difference was applied; thus the metal ions are probably more labile under these conditions.

However, these results do support N_3^- binding to the triangular active site, since the electron flow through the T1 gate is not disturbed after N_3^- addition, suggesting that any combination of the T2 and T3 copper atoms may be coordinated to N_3^-.

We were surprised to see no comparable effect for the fluoride-inhibited laccase under similar conditions. Incubation of immobilized laccase with 6.6 mM F^- resulted in no change in redox properties under anaerobic conditions, even after several successive scans (data not shown). The binding affinity of F^- to the active site has been previously reported to be somewhat lower for the reduced T2 copper than the oxidized T2 copper (*41*). In the potential range considered (−170 to +650 mV), the laccase molecule is in the reduced state for much of the experiment and only oxidized at potentials ≥410 mV. Thus, the F^- ion may be binding weakly to the copper atom(s) for much of the experiment, resulting in a reduced effect on the laccase redox chemistry.

The effect of inhibitors on the ability of laccase to catalyze the reduction of the natural substrate oxygen was also investigated. The laccase sample was incubated with 6.6 mM N_3^- prior to cyclic voltammetric analysis; however, no catalytic current was observed. Similar experiments with increasing concentrations of F^- illustrate that 52.8 mM inhibitor was required to totally inhibit catalysis. These data are approximately consistent with the biochemical I_{50} values [$I_{50}(F^-) = 18.5$, $I_{50}(N_3^-) = 2.5$], suggesting that azide is seven times more effective as an inhibitor than fluoride.

Earlier reductive/oxidative titration data elegantly illustrated that the electrons are transferred from the T1 to the T3 centers (*44*). However, in that study the oxidative reaction used small molecules such as H_2O_2, known to bind irreversibly to the T3 site. These data are difficult to compare with direct electrochemistry as the influence of chemical mediators is not easily assessed. We believe direct electrochemistry enables us to obtain a better redox-only picture of the enzyme's electronic requirements. It must also be stressed that the use of a metal electrode as the electron donor and receptor provides electrons at a much greater rate than feasible in vivo, where the rate is typically controlled by diffusion and then later by intramolecular transfer. Therefore, while we have seen evidence for fast oxidation and comparatively slower reduction, and for loss of laccase activity with azide binding, these conclusions must be considered in that context. However, evidence for all four copper atoms participating in the in vitro activity of Rv laccase remains a significant finding and should be applicable irrespective of the electron source.

Finally, the feasibility of long-term use of laccase on an MPA SAM was considered for relevance in practical biosensing. At room temperature, laccase activity is lost presumably due to copper depletion within 24 h, thereby limiting practical use to several hours, but this could be improved to several weeks by storing the prepared electrode at 4 °C.

ACKNOWLEDGMENT

The authors thank Dr. D. Losic for assistance in electrochemical experiments and Dr. J. J. Gooding for discussions on glucose oxidase.

REFERENCES

1. Service, R. F. (2002) *Science 296*, 1223.
2. Palmore, G. T. R., and Kim, H.-H. (1999) *J. Electroanal. Chem. 464*, 110−117.
3. Chen, T., Barton, S. C., Binyamin, G., Gao, Z., Zhang, Y., Kim, H.-H., and Heller, A. (2001) *J. Am. Chem. Soc. 123*, 8630−8631.
4. Fernandez-Sanchez, C., Tzanov, T., Gubitz, G. M., and Cavaco-Paulo, A. (2002) *Bioelectrochemistry 58*, 149−156.
5. Lee, C.-W., Gray, H. B., Anson, F. C., and Malmstrom, B. G. (1984) *J. Electroanal. Chem. 172*, 289−300.
6. Hyung, K. H., Jun, K. Y., Hong, H.-G., Kim, H. S., and Shin, W. (1997) *Bull. Korean Chem. Soc. 18*, 564−566.
7. Thuesen, M. H., Farver, O., Reinhammer, B., and Ulstrup, J. (1998) *Acta Chem. Scand. 52*, 555−562.
8. Trudeau, F., Daigle, F., and Leech, D. (1997) *Anal. Chem. 69*, 882−886.
9. Leech, D., and Daigle, F. (1998) *Analyst 123*, 1971−1974.
10. Call, H. P., and Mucke, I. (1997) *J. Biotechnol. 53*, 163−202.
11. Li, K., Xu, F., and Eriksson, K.-E. L. (1999) *Appl. Environ. Microbiol. 65*, 2654−2660.
12. Duran, N., Rosa, M. A., D'Annibale, A., and Gianfreda, L. (2002) *Enzyme Microb. Technol. 31*, 907−931.
13. Yaropolov, A. I., Skorobogatko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. (1994) *Appl. Biochem. Biotechnol. 49*, 257−280.
14. Davis, S., and Burns, R. G. (1992) *Appl. Microbiol. Biotechnol. 37*, 474−479.
15. Hublik, G., and Schinner, F. (2000) *Enzyme Microb. Technol. 27*, 330−336.
16. Lee, S.-K., George, S. D., Antholine, W. E., Hedman, B., Hodgson, K. O., and Solomon, E. I. (2002) *J. Am. Chem. Soc. 124*, 6180−6193.
17. Santucci, R., Ferri, T., Morpurgo, L., Savini, I., and Avigliano, L. (1998) *Biochem. J. 332*, 611−615.
18. Xu, F. (1997) *J. Biol. Chem. 272*, 924−928.
19. Xu, F., Kulys, J. J., Duke, K., Li, K., Krikstopaitis, K., Deussen, H.-J. W., Abbate, E., Galinyte, V., and Schneider, P. (2000) *Appl. Environ. Microbiol. 66*, 2052−2056.
20. Scholz, F., Schadel, S., Schultz, A., and Schauer, F. (2000) *J. Electroanal. Chem. 480*, 241−248.
21. Reinhammar, B. B. M., and Vanngard, T. I. (1971) *Eur. J. Biochem. 18*, 463−468.
22. Reinhammar, B. R. M. (1972) *Biochim. Biophys. Acta 275*, 245−259.
23. Kroneck, P. M. H., Armstrong, F. A., Merkle, H., and Marchesini, A. (1982) *Adv. Chem. Ser.*, 223−248.
24. Chi, Q., Zhang, J., Andersen, J. E. T., and Ulstrup, J. (2001) *J. Phys. Chem. B 105*, 4669−4679.
25. Collinson, M., and Bowden, E. F. (1992) *Langmuir 8*, 1247−1250.
26. Song, S., Clark, R. A., Bowden, E. F., and Tarlov, M. J. (1993) *J. Phys. Chem. 97*, 6564−6572.
27. Johnson, D. L., Maxwell, C. J., Losic, D., Shapter, J. G., and Martin, L. L. (2002) *Bioelectrochemistry 58*, 137−147.
28. Johnson, D. L., Norman, S., Tuckey, R. C., and Martin, L. L. (2003) *Bioelectrochemistry 29*, 41−47.
29. Reinhammer, B. (1970) *Biochim. Biophys. Acta 205*, 35−47.
30. Bott, A. W., and B. P., J. (1996) *Curr. Sep. 15*, 25−30.
31. Wirtz, M., Klucik, J., and Rivera, M. (2000) *J. Am. Chem. Soc. 122*, 1047−1056.
32. Vitus, C. M., and Davenport, A. J. (1994) *J. Electrochem. Soc. 141*, 1291−1298.
33. Ulman, A. (1996) *Chem. Rev. 96*, 1533−1554.
34. Finklea, H. O. (1996) *Electroanal. Chem. 19*, 109−335.
35. Voicu, R., Ellis, T. H., Ju, H., and Leech, D. (1999) *Langmuir 15*, 8179−8177.
36. Ferrence, G. M., Henderson, J. I., Kurth, D. G., Morgenstern, D. A., Bein, T., and Clifford, P. K. (1996) *Langmuir 12*, 3075−3081.
37. Marbach, I., Harel, E., and Mayer, A. M. (1984) *Phytochemistry 23*, 2713−2717.
38. Losic, D., Shapter, J. G., and Gooding, J. J. (2002) *Langmuir 18*, 5422−5428.
39. Laviron, E. (1979) *J. Electroanal. Chem. 101*, 19−28.
40. Johannes, C., and Majcherczyk, A. (2000) *J. Biotechnol. 78*, 193−199.
41. Spira-Solomon, D. J., Allendorf, M. D., and Solomon, E. I. (1986) *J. Am. Chem. Soc. 108*, 5318−5328.

42. Severns, J. C., and McMillin, D. R. (1990) *Biochemistry 29*, 8592−8597.
43. Koudelka, G. B., and Ettinger, M. J. (1988) *J. Biol. Chem. 263*, 3698−3705.
44. Farver, O., Goldberg, M., Lancet, D., and Pecht, I. (1976) *Biochem. Biophys. Res. Commun. 73*, 494−500.
45. Morpurgo, L., Graziani, M. T., Finazzi-Agro, A., Rotilio, G., and Mondovi, B. (1980) *Biochem. J. 187*, 361−366.
46. Morpurgo, L., Graziani, M. T., Desideri, A., and Rotilio, G. (1980) *Biochem. J. 187*, 367−370.
47. Morpurgo, L., Desideri, A., and Rotilio, G. (1982) *Biochem. J. 207*, 625−627.
48. Fee, J. A., Malkin, R., Malmstrom, B. G., and Vanngard, T. (1969) *J. Biol. Chem. 244*, 4200−4207.
49. Malkin, R., Malmstrom, B. G., and Vanngard, T. (1969) *Eur. J. Biochem. 10*, 324−329.
50. Xu, F., Berka, R. M., Wahleithner, J. A., Nelson, B. A., Shuster, J. R., Brown, S. H., Palmer, A. E., and Solomon, E. I. (1998) *Biochem. J. 334*, 63−70.
51. Sakurai, T. (1996) *Chem. Lett.*, 481−482.
52. Xu, F. (1996) *Biochemistry 35*, 7608−7614.
53. Shen, B., Martin, L. L., Butt, J. N., Armstrong, F. A., Stout, C. D., Jensen, G. M., Stephens, P. J., La Mar, G. N., Gorst, C. M., and Burgess, B. K. (1993) *J. Biol. Chem. 268*, 25928−25939.
54. Gromov, I., Marchesini, A., Farver, O., Pecht, I., and Goldfarb, D. (1999) *Eur. J. Biochem. 266*, 820−830.
55. Hirota, S., Matsumoto, H., Huang, H.-W., Sakurai, T., Kitagawa, T., and Yamauchi, O. (1998) *Biochem. Biophys. Res. Commun. 243*, 435−437.
56. Frank, P., Farver, O., and Pecht, I. (1984) *Inorg. Chim. Acta 91*, 81−88.
57. Frank, P., and Pecht, I. (1986) *J. Phys. Chem. 90*, 3809−3814.

BI034268P