Biotransistors

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Transistor-Like Behavior of a Fungal Laccase**

Sergey Shleev and Tautgirdas Ruzgas*

Herein, we describe a mechanism by which the activity of an enzyme is modulated by means of an electrical potential. Specifically, when *Trametes hirsuta* laccase (Lc) is adsorbed on the surface of a gold electrode, a potential applied to the metal site (the gold electrode) modulates the chemical structure of the three-nuclear copper cluster of the enzyme (Figure 1). The structure of the cluster affects the intramolecular electron transfer (IET) steps in the surface-confined Lc and, thus, determines the catalytic activity of the enzyme. We refer to the mechanism as a transistor-like behavior of the enzyme following a report on diode-like behavior of succinate dehydrogenase, published more than a decade ago,[1a] and the hypothesis of a possible occurrence of transistor-like behavior in redox enzymes.[1b]

Lc (EC 1.10.3.2) is a copper protein that contains four metal ions classified into three types, referred to as T1, T2, and T3 (Figure 1). The T2 and T3 sites form the trinuclear cluster, which binds O_2 and reduces it to H_2O.[2] The catalytic cycle starts with the abstraction of electrons from electron-donor molecules (for example, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) in Figure 1) at the T1 site (the first electron acceptor) followed by subsequent IET to the T2/T3 cluster. It has recently been confirmed that, when Lc is absorbed on the surface of a bare gold electrode,[3] the T2/T3 copper cluster becomes the first electron acceptor upon electrochemical reduction of the enzyme (Figure 1).

Based on our previous work,[3a] we hypothesized that one of the copper ions in the cluster, most likely the T2 copper ion, plays the same role as a control terminal in a transistor structure. The key point is that the electronic coupling of the T2 copper ion enables electrochemical control over the redox states of the T2/T3 cluster. Thus, depending on the applied potential, it would be possible to produce different intermediates of the enzyme with different structures of the cluster. Several intermediates of Lc that have previously been characterized structurally and kinetically are, for example, the peroxy and native intermediates (IET rate of $>1000\ s^{-1}$ between the T1 ion and T2/T3 cluster), the resting fully oxidized enzyme (IET rate $\approx 1\ s^{-1}$), and the fully reduced enzyme (no IET).[2] Moreover, many other intermediates with different structures of the cluster were predicted by hybrid

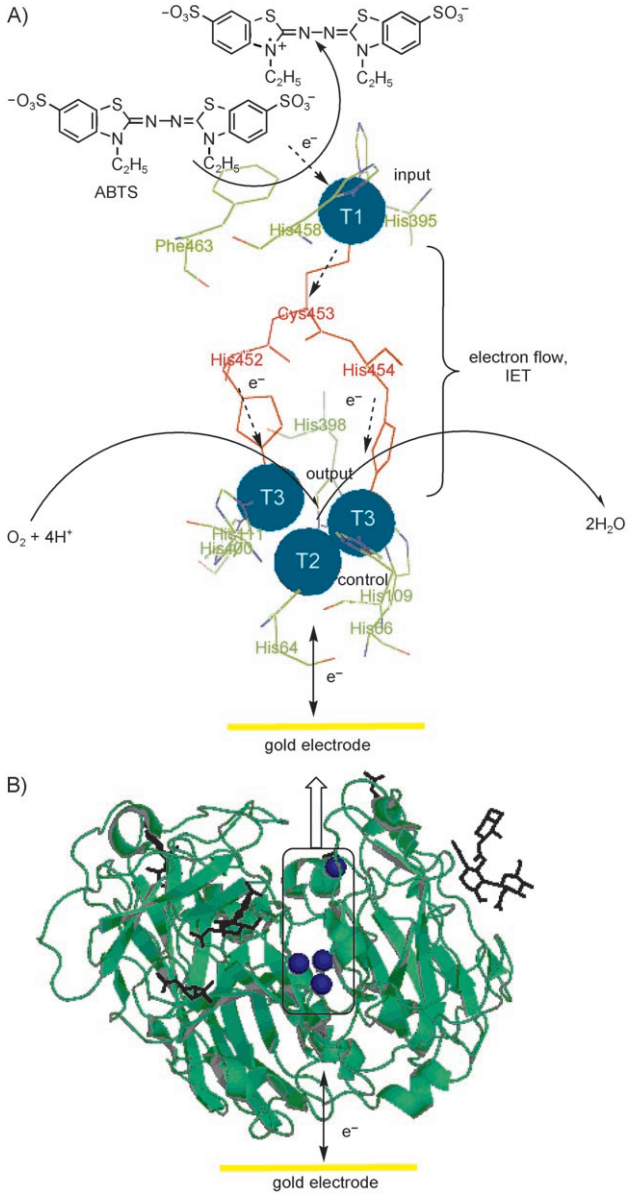

Figure 1. A) Electron-transfer pathways and copper centers in *Trametes* Lc on a gold electrode. The three-dimensional structure of the copper sites was modeled based on the structure of *Trametes versicolor* Lc (Protein Data Bank file: 1GYC) by using the PyMOL program (V. 0.99). B) Electron transfer between Lc and the gold electrode. Protein globule: green ribbons and strands; carbohydrates: black sticks; copper ions: blue spheres.

[*] Dr. S. Shleev, Prof. T. Ruzgas
Biomedical Science Laboratory
Faculty of Health and Society
Malmö University
Södra Förstadsgatan 101, 20506 Malmö (Sweden)
Fax: (+46) 40-665-8100
E-mail: sergey.shleev@mah.se
Homepage: http://www.mah.se/shleev
Dr. S. Shleev
Laboratory of Chemical Enzymology
A. N. Bach Institute of Biochemistry
Leninsky prospect 33, 119071 Moscow (Russia)

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quantum and molecular mechanics calculations.[4] The intermediates can probably be formed by applying different potentials. It is well known that the structure of the T2/T3 cluster strongly influences the IET rate between the T1 site and the cluster.[2]

From this, it follows that a potential applied to the Lc-modified gold electrode, where the enzyme is oriented with the T2 site ("control terminal") proximate to the electrode surface, would influence the rate of electron flow (current) between the T1 site ("input") and T3 copper site ("output") during the enzymatic oxidation of different substrates and reduction of O_2 (Figure 1). As shown in Figure 2, this

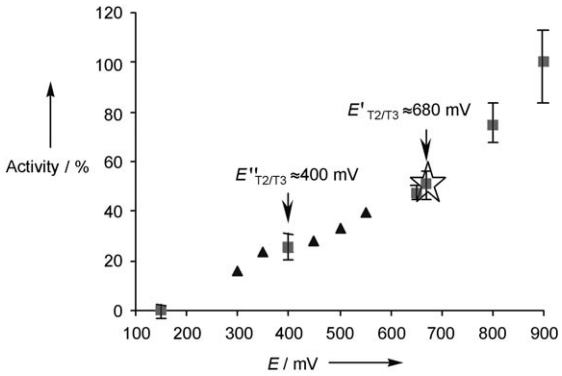

Figure 2. Activity of *Trametes* Lc adsorbed on bare gold electrodes versus the applied potential. Squares and triangles: normalized points from the spectroelectrochemical and amperometric experiments presented in Figures 3 and 4, respectively. (The activities of the enzyme at different potentials were first expressed in percent and then normalized versus the activity at 400 mV for both spectral and amperometric measurements.) Star: the activity of Lc adsorbed on a gold electrode without any potential applied.

behavior is borne out, and we conclude that Lc adsorbed on a bare gold electrode displays a "transistor-like" behavior for which the potential applied to the control terminal strongly influences the "input–output current", as represented by the catalytic turnover of the enzyme. The redox enzymes, while having neither the solid character nor precisely the same band-structure properties as semiconductors, nonetheless do allow for interactions of extended electronic orbitals. The semiconductor-like behavior of redox proteins is a consequence of nanometer-scale electron-transfer reactions along these extended orbitals within each individual protein molecule.[1b]

We now summarize experimental evidence demonstrating the reversible modulation of the activity of *T. hirsuta* Lc by the application of different potentials to the gold on which the enzyme is adsorbed. Spectrophotometric measurements were performed on the activity of Lc on gold after electrode equilibration at certain applied potentials (Figure 3) under air-saturated conditions. The dependence of the amount of enzymatically oxidized ABTS (directly proportional to Lc activity) on the applied potential showed a steeply rising, reversible, and typically "transistor-like" activity–potential characteristic (Figure 2, squares; Figure 3). Importantly, each point in the plot presented in Figure 2 is an average of data

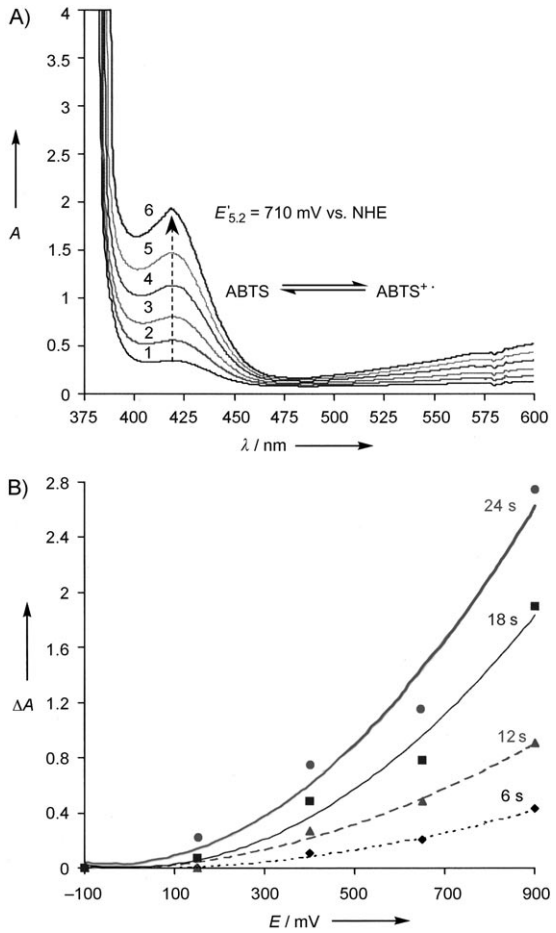

Figure 3. Spectroelectrochemical studies of ABTS oxidation by *Trametes* Lc adsorbed on the interior surface of a gold-capillary electrode (0.2 M phosphate buffer, pH 5.2). A) Spectrophotometric monitoring of ABTS oxidation by Lc adsorbed on the gold electrode. Curves 1–6: absorbance spectrum of the initially injected ABTS solution (0.6 mg mL^{-1}) and spectra after 6, 12, 18, 24, and 30 s, respectively. B) Dependence of ABTS oxidation by the Lc (expressed as absorbance changes at 419 nm after 6, 12, 18, and 24 s of enzymatic reaction) versus the potential of the Lc-modified gold electrode.

points acquired nonsequentially to verify the reversibility of the potential-modulated enzyme activity. Moreover, in the absence of Lc on the electrode surface, no significant electrochemical (that is, nonenzymatic) oxidation of ABTS was spectrophotometrically detected.

Additional amperometric results also confirmed that the activity of the enzyme at the surface of the gold electrode could be electrochemically modulated. In this case, a defined potential was applied at the Lc-modified electrode and, after injection of ABTS, a current response was measured under aerobic conditions (Figure 4 A). The redox potential of the ABTS/ABTS$^{+\cdot}$ couple is close to 700 mV versus the normal hydrogen electrode (NHE),[5] so no significant electrochemical oxidation of ABTS was observed in the potential range 150–550 mV in the absence of Lc. The dependence of the reduction current, originating from the electrochemical reduction of enzymatically oxidized ABTS, on the applied potential is presented in Figure 4 B. A strong background reduction current at applied potentials less than 300 mV was

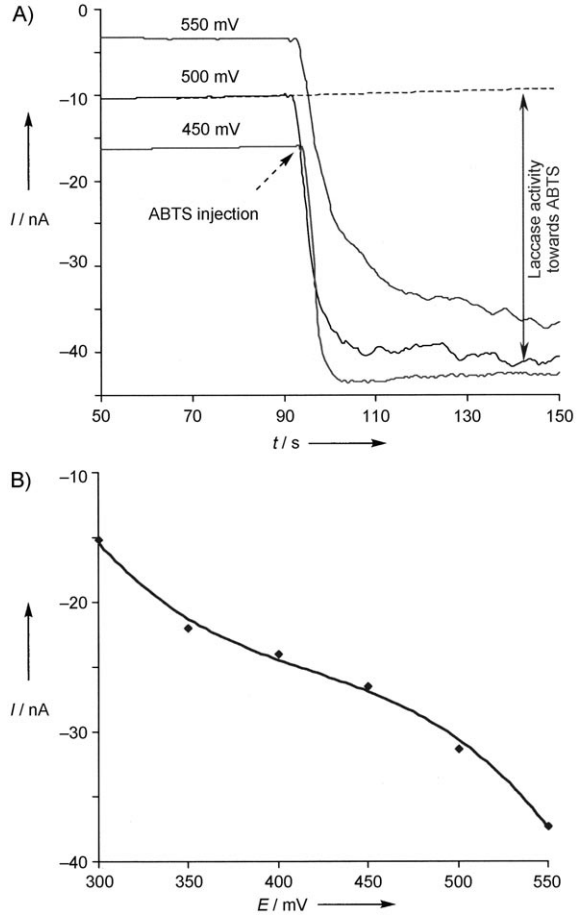

Figure 4. Amperometric study of ABTS oxidation by *Trametes* Lc adsorbed on a planar gold electrode (0.2 M phosphate buffer, pH 5.2). A) Typical chronoamperometric curves of the Lc-modified planar gold electrode under air-saturated conditions recorded at 550, 500, and 450 mV. The data recorded at 300, 350, and 400 mV are not shown. B) Dependence of the ABTS enzymatic oxidation rate (expressed as current output from the Lc-modified gold electrode) versus the potential applied to the electrode.

obtained; this corresponds to the mediatorless electroreduction of O_2 on the Lc-modified gold electrode.[3c,d] Thus, amperometric results from this potential range were not taken into account in the following interpretation of the mechanism of ABTS oxidation by Lc.

The results from two independent and absolutely different methods, that is, the spectrophotometric and amperometric determination of the activity of the Lc, are combined in Figure 2. A strong increase in Lc activity was observed with an increase in the applied potential at the Lc-modified gold electrode. This behavior is reversible and holds over a broad potential range of 150–900 mV.

At potentials below 150 mV, all of the copper ions of the enzyme are reduced,[3a] the IET rate is nullified, and Lc is catalytically inactive. Based on our previous work to describe the redox transformation of the T2/T3 copper cluster on gold,[3a] we suggest that one of the structures of the T2/T3 cluster that might be formed at approximately 400 mV is characterized by the loss of an O_2 bridge between the T2 and T3 copper ions, which leads to the formation of the resting form of the enzyme with low activity due to a very slow IET (turnover rate of approximately 1 s^{-1}).[2] Thus, according to the Nernstian law, half of the Lc molecules at this potential are essentially in an inactive state compared to the native enzyme, which has a turnover rate of approximately 50 s^{-1} under the same conditions. This situation results in an approximately halved activity of Lc at 400 mV compared to the activity of the adsorbed enzyme without any potential applied (Figure 2, star). Importantly, even at 900 mV, that is, 120 mV higher than the redox potential of the T1 site (E_{T1}) of *T. hirsuta* laccase,[3a,b] the maximum Lc activity is not achieved. This fact in particular, and the potential-modulated activity of the Lc in general, can be observed only if the IET rate (maximum 50 s^{-1} at pH 5.2) limits the enzymatic activity. This IET rate is at least five times lower than the maximal rate of ABTS oxidation at the T1 site of the Lc (260 s^{-1} at pH 3.2). Thus, in the potential window presented in Figure 2, the maximal IET rate and, consequently, the saturation of the activity–potential dependence was not approached. ABTS is oxidized by the enzyme through a cation-radical mechanism[5] and it belongs to the "electron no-proton donor" group of Lc substrates.[6] For these substrates, a decrease in laccase activity with an increase in the pH value is simply explained by conversion of the active enzyme into an inactive form due to structural changes in the T2/T3 cluster caused by OH^- binding to the T2 copper site, which plays the role of a pH-sensitive regulator of the enzyme activity.[7] The redox potential of the T2/T3 cluster ($E_{T2/T3}$) of Lc corresponding to the native intermediate of the enzyme[2] is 680 mV versus the NHE, as estimated from Figure 2 (star). Thus, at pH 5.2, the electron transfer from the T1 site (780 mV) to the T2/T3 cluster (680 mV) is an "uphill" reaction that is responsible for the relatively slow IET rate of 50 s^{-1}.

The above-proposed model of the enzyme function is also supported by the observation of Lc-activity modulation when genetic engineering is used to change the E_{T1} value. It has previously been shown that the rate of catalysis (k_{cat}) of different Lcs is strongly correlated with the difference in the thermodynamic driving force between the Lc substrates (for example, ABTS) and the T1 copper site.[6a,b] While a number of steps may contribute to the k_{cat} value, it was suggested that a redox-potential difference between the donor (substrate) and acceptor (E_{T1}) is one of the major components of the k_{cat} value. In spite of this widely held notion, an unexpected significant increase in the k_{cat} value of the T1-site-mutated *Trametes* sp. Lcs with lower E_{T1} values (for example, E_{T1} = 680 mV, $k_{cat} = 78\ s^{-1}$) was observed, relative to the high-redox-potential wild-type enzyme ($E_{T1} = 790$ mV, $k_{cat} = 45\ s^{-1}$), during the homogeneous oxidation of ABTS.[6b] These catalytic constants were measured at pH 5.5, when, in accordance with our model, the IET step limits the rate of enzymatic oxidation of ABTS. Thus, these kinetic data can rather be interpreted in terms of a decrease in the uphill Gibbs free-energy difference for the IET (electron transfer between the T1 and T2/T3 sites) after mutations. This strongly supports the proposed model of Lc function on gold, the essence of which is the potential control over the redox state of the T2/T3 cluster, which determines the IET rate, that is, the Lc activity.

The discussed transformations of the T2/T3 cluster imposed by an external electrical potential, resulting in modulation of the IET and ultimately in the possibility of reversibly modulating the enzymatic turnover of Lc, might also be of relevance in natural regulation of the enzyme activity. Besides enzyme regulation by the solution's pH value and inhibition by halide ions, interaction of the copper cluster of Lc with biological messengers, such as NO and H_2O_2, has also been previously shown.[8] A role for these interactions in the regulation of the enzyme activity was highlighted, in which the general mechanism of the activity regulation is the same as that invoked by the applied potential, that is, a mechanism involving changes of the chemical and electronic structure of the T2/T3 cluster with concomitant formation of different enzyme intermediates. It is interesting to note that OH^-, F^-, NO, and H_2O_2 bind to the T2 copper ion,[6–8] which is suggested in the present work to be the "control electrode". It should also be emphasized that the activity of the adsorbed Lc, when no potential is applied (Figure 2, star), is much lower than the maximum activity. This suggests that the physiological role of a "transistor-like" behavior of the enzyme in nature is probably realised through both activation and inhibition of Lc activity. Our finding thus provides insight into the enzyme function and regulation in nature by showing how both activation and inhibition of Lc activity can be realized and by shedding light on a structure–function relationship that might conceivably hold for many other complex redox enzymes.[1b]

Experimental Section

The basidiomycete *Trametes hirsuta*, strain *T. hirsuta* 56, was obtained from the laboratory collection of the Moscow State University of Engineering Ecology (Moscow, Russia). Accounts of the production of Lc on a preparative scale and detailed biochemical characterization of the enzyme have been previously published.[9] Homogeneous preparations of Lc were stored in 0.05 M phosphate buffer (pH 6.5) at −18 °C. The turnover numbers of the enzyme towards ABTS were measured spectrophotometrically by using a Uvikon 930 spectrophotometer (Kontron Instruments, Everett, MA, USA) and were found to be approximately 50 and 260 s^{-1} at pH 5.2 and 3.2, respectively.

Spectroelectrochemical studies were carried out by using previously described spectroelectrochemical equipment consisting of a gold capillary electrode with a total volume of 1 μL.[3a,10] The potential of the gold capillary of the cell was controlled by a three-electrode BAS CV-100W potentiostat (Bioanalytical Systems, West Lafayette, IN, USA). In these measurements, an Ag/AgCl/0.1 M KCl reference electrode and a platinum counter electrode were used. The gold-capillary working electrode was cleaned by washing the capillary with a peroxide/sulphuric acid mixture (Piranha solution) and subsequently rinsing with Millipore water. To adsorb the Lc at the bare gold electrode, a solution of Lc (18 mg L^{-1} in 50 mM phosphate buffer, pH 6.5) was injected inside the capillary and kept there for 1 h. After modification, the gold capillary was rinsed with 0.2 M phosphate buffer (pH 5.2) for 5 min and used for measurements. The Lc-modified capillary electrode was poised at different applied potentials for 300 s. After equilibration of the electrode at each potential, the gold working electrode was disconnected from the potentiostat. Immediately thereafter, reduced ABTS (0.6 mg L^{-1} in 0.2 M air-saturated phosphate buffer, pH 5.2) was injected into the cell and spectral changes were monitored with a step of 6 s between the recorded spectra. The activity of the adsorbed Lc was calculated as an absorbance increase at 419 nm (Figure 3), which is directly proportional to the amount of enzymatically oxidized ABTS. The error bars presented in Figure 2 reflect the results from two different Lc-modified gold electrodes, as well as the averaging of at least three data points acquired nonsequentially during several back-and-forth applied potential measurements continued over several hours. The acceptable operation stability of Lc-modified gold electrode under these conditions was confirmed; that is, the adsorbed Lc retained more than 75% of the initial activity after continuous measurements for 4 h.

Amperometric curves were recorded with a planar BAS gold electrode in a 20 mL electrochemical cell with constant stirring of the buffer solution in the cell. For these measurements, the three-electrode BAS CV-100W potentiostat, an $Hg/Hg_2Cl_2/KCl_{sat}$ reference electrode, and a platinum counter electrode were used. Prior to biomodification, the electrode surface of the working gold electrode was cleaned as described previously.[3] For physical adsorption of Lc on the bare gold electrode surface, the electrode was mounted with its surface facing up and the Lc solution (10 μL; 18 mg L^{-1} in 50 mM phosphate buffer, pH 6.5) was placed on the gold surface. To counteract evaporation, Millipore water was continuously added to the drop on top of the electrode. After modification, the electrode was carefully rinsed with 0.2 M phosphate buffer (pH 5.2) and was then placed into the electrochemical cell. The experiments were carried out under air-saturated conditions (approximately 260 μM dissolved O_2). After equilibration of the electrode at each potential, reduced ABTS solution was injected into the cell and the current outputs, corresponding to the activity of the Lc, were monitored. The final concentration of ABTS in the cell was always constant and equal to 0.06 mg L^{-1} (0.2 M phosphate buffer, pH 5.2). The activity of adsorbed Lc (in nA) was calculated as the difference between the currents before (mediatorless O_2 reduction) and after ABTS injection (Figure 4); this difference resulted from the electrochemical reduction of the enzymatically oxidized ABTS on the electrode surface. Excellent long-term stability of the Lc-modified gold electrode was observed; the adsorbed Lc retained more than 90% of the maximal activity during electrode storage in 0.2 M phosphate buffer (pH 5.2) for 4 h. As an independent assurance that the adsorbed Lc retained its catalytic activity, F^- ions, known to inhibit the enzyme, were added to the air-saturated solution at a final concentration of 10 mM or anaerobic conditions were created by bubbling argon through the electrochemical cell (trace O_2 concentration was measured to be approximately 2 μM). A dramatic decrease in the current was observed in both cases, which confirms without a doubt that the catalytic current was related to the activity of the adsorbed Lc.

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