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Direct and Mediated Electron Transfer Catalyzed by Anionic Tobacco Peroxidase

Effect of Calcium Ions

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Abstract

The properties of anionic tobacco peroxidase (TOP) adsorbed on graphite electrode have been studied in direct and mediated electron transfer in a wall-jet flow injection system. The percentage of tobacco peroxidase molecules active in direct electron transfer is about 83%, which is higher than that for horseradish peroxidase (40–50%). This observation is explained in terms of the lower degree of glycosylation of TOP compared with horseradish peroxidase and, therefore, a reduced interference from the oligosaccharide chains with direct electron transfer. Calcium ions cause an 11% drop in the reaction rate constant toward hydrogen peroxide. The detection limit of calcium chloride has been estimated as 5 m*M*. The results obtained by means of bioelectrochemistry, stopped-flow kinetics, and structural modeling provide evidence for the interaction between calcium cations and negatively charged residues at the distal domain (*Glu-141*, heme propionates, *Asp-79*, *Asp-80*) blocking the active site. The observation that both soluble and immobilized enzyme undergo conformational changes resulting in the blockade of the active site indicates that the immobilized enzyme preserves conformational flexibility. An even stronger suppressing effect of calcium ions on the rate constant for mediated electron transfer was observed. In the case of direct electron transfer, this could mean that there is no direct contact between

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the electrode and the active site of TOP. The electrons are shuttled from the active site to the surface of the electrode through electron transfer pathways in the protein globule that are sensitive to protein conformational changes.

Index Entries: Direct electron transfer; mediated electron transfer; orientation effect; conformational changes; model structure; tobacco peroxidase; biosensor; calcium effect; rate constant.

Introduction

Heme-containing peroxidases are widely available in Nature and play important roles in defense mechanisms of mammals and plants *(1)*. The enzymes are of both fundamental and practical interest. Horseradish peroxidase (HRP) is the most popular enzyme among plant heme-containing peroxidases due to its applications for various analytical procedures. Besides common spectrophotometric and luminescent assays, the enzyme is widely used in biosensors *(2)*. The basic reaction includes the enzyme oxidation by hydrogen peroxide and then its subsequent reduction by electrons provided by an electrode (Fig. 1, top):

$$E + H_2O_2 \rightarrow EI + H_2O \qquad (k_1) \qquad (1)$$

$$EI + 2e^- + 2H^+ \rightarrow E + H_2O \qquad (k_s) \qquad (2)$$

This process is usually referred to as direct electron transfer (ET) when an electrode substitutes the donor substrates in a common peroxidase cycle. The latter proceeds via two steps and in the case of re-reduction of the electron donor by the electrode it is considered as mediated ET (Fig. 1, bottom):

$$EI + AH_2 \rightarrow EII + AH^\bullet \qquad (k_2) \qquad (3)$$

$$EII + AH_2 \rightarrow E + AH^\bullet + H_2O \qquad (k_3) \qquad (4)$$

$$AH^\bullet + e^- + H^+ \rightarrow AH_2 \qquad (k_{s,m}) \qquad (5)$$

where E, EI, and EII are the ferric enzyme and its Compounds I and II, respectively, AH_2 and $AH^\bullet$ are a donor substrate and a radical product of its one-electron oxidation.

The reaction sequence Eqs. 1, 3–5 known in electrochemistry as mediated ET is much more efficient compared to direct ET *(3)*. The higher efficiency of bioelectrocatalytic reduction of hydrogen peroxide on peroxidase-modified carbon electrodes compared with metal surfaces was ascribed to phenolic and quinoidal groups mimicking the structure of peroxidase donor substrates *(4–6)*. The molecular mechanism of direct ET between an electrode and the protein active centers remains a challenging problem since its discovery in 1978 *(7)*.

In our previous work on kinetic models of HRP action on a graphite electrode *(3,8)* it has been shown that between 40 and 50% of the adsorbed

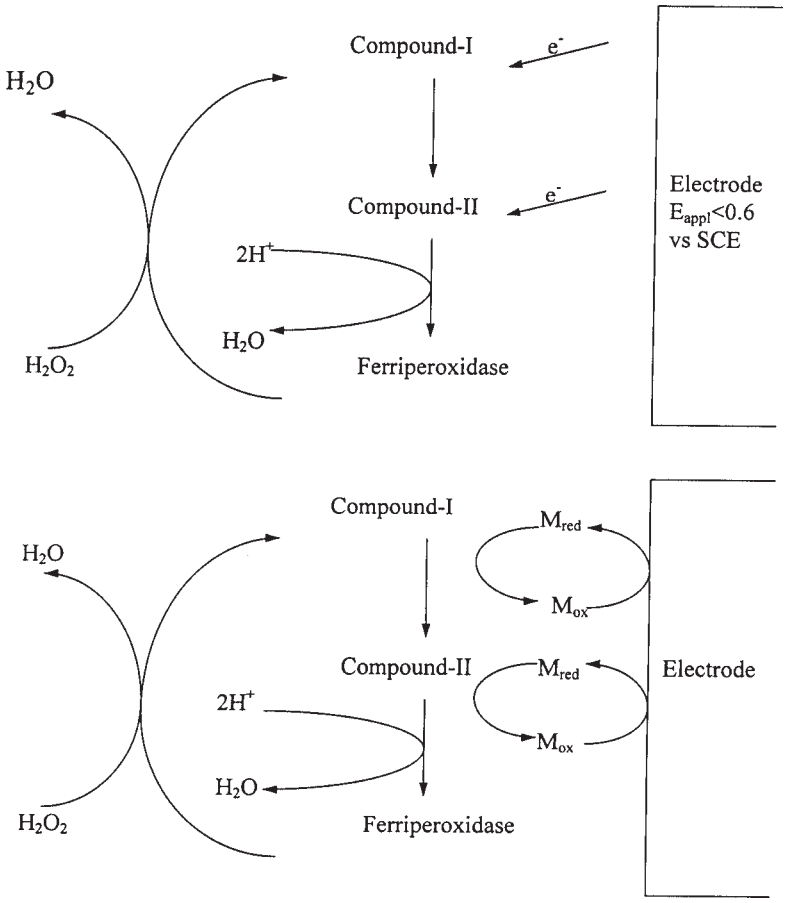

Fig. 1. Schemes for direct (top) and mediated (bottom) electron transfer mechanisms. M, mediator (electron donor substrate).

HRP molecules are able to participate in direct ET. The access channel to the HRP active center is known to be narrow *(9)*, and this again raises the question about the molecular mechanism of ET between graphite and the peroxidase active center. The percentage of "silent" molecules is only between 50 and 60%, which suggests that the protein mediates an ET between heme and graphite and that there is no direct contact between the active site and surface C–O functionalities.

The availability of new peroxidases with unusual modulation of the catalytic properties allowed us to gain new insights into the problem of direct ET. A newly isolated and described anionic tobacco peroxidase (TOP) *(10)* was demonstrated to undergo conformational changes in the active site structure in the presence of calcium ions at low pH yielding an enzyme resembling lignin peroxidase *(11)*. Further studies on the enzyme stability and activity toward hydrogen peroxide at the extremely low pH allowed us to propose a blockade of the active site by calcium cations *(12)*.

The aim of this article is to study the effect of the active site accessibility on direct ET catalyzed by TOP. The results obtained by means of bioelectrochemistry, stopped-flow kinetics, and structural modeling provide evidence for a reversible and rapid interaction between calcium cations and the negatively charged residues at the distal domain (*Glu-141*, heme propionates, *Asp-79*, *Asp-80*). The proposed blockade and conformational changes in the active site decrease the rate constant for peroxidase catalyzed direct and mediated electron transfer reactions at the surface of electrode. This supports the conclusion that there is no direct electrical contact between the active site of TOP and the electrode and that ET is mediated by electron transfer pathways in the protein backbone.

Materials and Methods

Chemicals

Catechol and *p*-cresol were purchased from Sigma, hydrogen peroxide, calcium chloride, and other salts were from BDH Merck. All solutions were prepared with Milli-Q water. The concentration of H_2O_2 was determined spectrophotometrically on a Ultraspec II instrument (LKB, Sweden) using a molar absorption coefficient of 72.7 M^{-1} cm^{-1} at 230 nm *(13)*. The concentration of TOP, RZ 3.4, an acid isozyme, pI 3.5 (for detailed description of the enzyme preparation see ref. *10*) was determined using a molar absorption coefficient of 108, 000 M^{-1} cm^{-1} at 403 nm *(10)*. HRP was purchased from Boehringer Mannheim GmbH, Mannheim, Germany (cat. no. 814407, RZ 3.2–3.3, 1000 U/mg [ABTS]).

Electrode Preparation

Solid graphite electrodes were prepared by the following procedure. Rods of solid spectroscopic graphite (SGL Carbon AG, Werk Ringsdorff, Bonn, Germany, type RW001, 3.05 mm diameter) were cut and were polished on wet fine emery paper (Tufbak Durite, P1200) and additionally polished on white paper, carefully rinsed with deionized water, and allowed to dry at room temperature. An 8-µL aliqout of TOP solution (5 mg/mL) was added to the polished ends of the graphite rods and the electrodes were then placed at 4°C for 20 h in a glass beaker covered with a sealing film, to prevent rapid evaporation of the droplet of enzyme solution. The enzyme electrodes were thoroughly rinsed with 0.1 *M* Na-acetate buffer at pH 5.0 and were stored in the buffer at 4°C prior to experiments.

Electrochemical Experiments

The enzyme electrodes were fitted into a Teflon holder and inserted into a flow-through wall-jet amperometric cell *(8,14)*. The enzyme electrode was used as the working electrode, an Ag/AgCl (0.1 *M* KCl) electrode

as the reference electrode, and a platinum wire serves as the auxiliary electrode. The electrodes were connected to a three-electrode potentiostat (Zäta Elektronik, Lund, Sweden) and the current was recorded. The measurements were performed at an applied potential of –50 mV vs Ag/AgCl *(3)*. The flow cell was connected to a two-channel flow-injection system with a peristaltic pump (Gilson, France) *(15)*. In one line, the $CaCl_2$ samples were injected using a six-port valve (injection loop: 30 μL). The eluent was 0.1 *M* Na-acetate buffer, pH 5.0, containing freshly prepared hydrogen peroxide. Increasing concentrations of $CaCl_2$ were injected into the system and the changes in the steady-state current achieved for 100 μ*M* H_2O_2 at a flow rate of 0.5 mL/min were recorded. The value of the inverted (negative) peaks in the steady-state current was plotted versus the concentration of calcium chloride.

To follow the enzymatic kinetics of direct and mediated ET, a wall-jet flow-through electrochemical detection system can be used under certain defined conditions *(8,16)*. Under these conditions plots of $1/I$ vs $1/V^{3/4}$ (where I is the registered steady-state current and V is the volumetric flow rate of the flow entering the flow cell) for a wall-jet system are equivalent to the corresponding Koutecky–Levich plots for a rotating-disk electrode *(8)*. The dependence of the kinetically limited current (obtained from the intercepts of the $1/I$ vs $1/V^{3/4}$ plots) on hydrogen peroxide concentration for direct and mediated was studied with two mediators (100 μ*M* catechol or *p*-cresol) in the presence and in the absence of 50 m*M* of $CaCl_2$. For comparison equivalent experiments were run with electrodes modified with HRP.

Transient Kinetics

Single-wavelength transient kinetics were monitored using a stopped-flow spectrophotometer (model SF-51, Hi-Tech Scientific, Salisbury, UK). Data were recorded through an RS232 interface on a microcomputer and analyzed by fitting absorbance-time curves to exponential functions using a least-squares minimization program supplied by High-Tech Scientific. The rate constant for hydrogen peroxide interaction with the ferric enzyme (k_1) was determined from the traces followed at 400 nm (the isosbestic point between TOP Compound I and II at pH 5.0) with 1 μ*M* enzyme shot against rising concentrations of hydrogen peroxide in the range of 0.6–10 μ*M* at pH 5.0 in 0.1 m*M* Na-acetate buffer in the presence and the absence of 50 m*M* $CaCl_2$. The kinetic studies were performed thermostatted at 25°C using Techne C-400 circulating water bath with an integral heater–cooler system.

TOP Structural Modeling

TOP structure modeling was performed using *Insight II* (version 95.0) program (Biosym/MSI, San Diego, CA). The peanut peroxidase crystal structure *(17)* was used as a basis for the homology modeling.

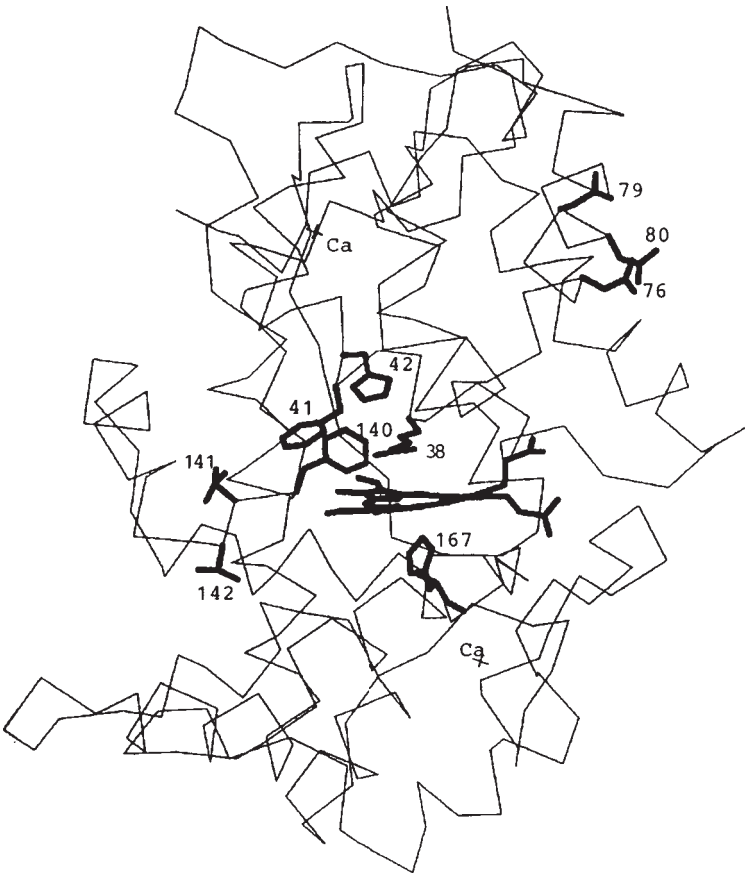

Fig. 2. Tobacco peroxidase model: a general view (left) and the active site (right, on opposite page).

Results and Discussion

There are two possible variants to imagine how direct ET occurs between the enzyme active centers and an electrode surface. A first is to consider that the active center of the enzyme participating in direct ET has a real contact with the surface functionalities mimicking the substrate structure. And a second is to suggest that ET between the active site and the electrode surface proceeds via ET channels through the protein.

In the case of peroxidases there is some evidence for the second mechanism, e.g., the crystal structure for cytochrome *c* peroxidase–cytochrome *c* complex shows the existence of an electron transfer channel through the protein molecules between two spatially separated hemes *(18)*. The enzyme is able to rearrange the ET transport chain: a single-point mutant lost the activity, whereas a double-point mutant restored it *(19)*. The percentage of silent molecules of HRP determined earlier is comparable with those active in direct ET (48 and 52% *[8]*, respectively). This could mean that the orientation of HRP on the electrode surface is not crucial for direct ET and

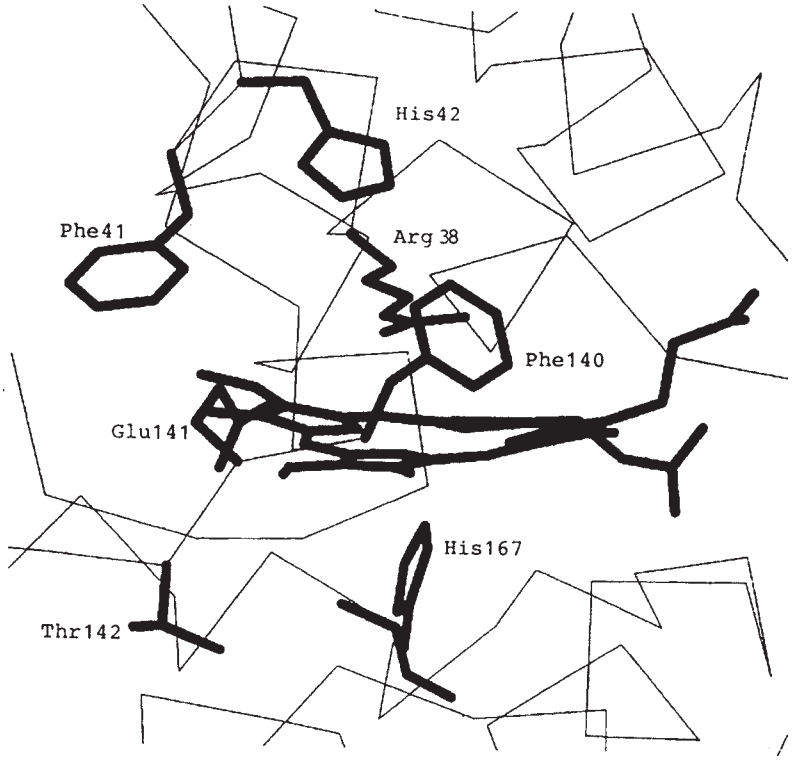

Fig. 2. *(right)*

that several ET channels are possible/probable between the active site of the peroxidase and the electrode.

This work presents a new peroxidase, TOP, in direct and mediated ET. The enzyme was shown to undergo changes in the active site structure in the presence of calcium ions *(11)*.

The structure of TOP created by homology modeling using the crystal structure of peanut peroxidase *(17)* is presented in Fig. 2. The active site of TOP has no principal differences compared to those of peanut and HRP *(9,17)*. However, it does contain the negatively charged glutamic acid residue, whose presence at the entrance to the active site may account for the absence of any catalytic activity toward iodide *(10)*, which is known to be oxidized directly at the edge of the porphyrin ring *(20)*.

The active site of TOP in the model structure is influenced by the electrostatic repulsion of *Glu141*, heme propionates and the triad of *Asp76*, *79*, *80*. This results in an increase of the distances between the above residues (19–23 Å, see Fig. 3, top) compared to the analogous distances in peanut peroxidase (12–15 Å, see Fig. 3, bottom). The entrance to the TOP active site is screened by both *Glu141* and *Phe140* (corresponding to *Phe143* and *Phe142* in peanut and horseradish peroxidases), and the distal histidine is shifted from the heme iron toward the negatively charged *Glu141*.

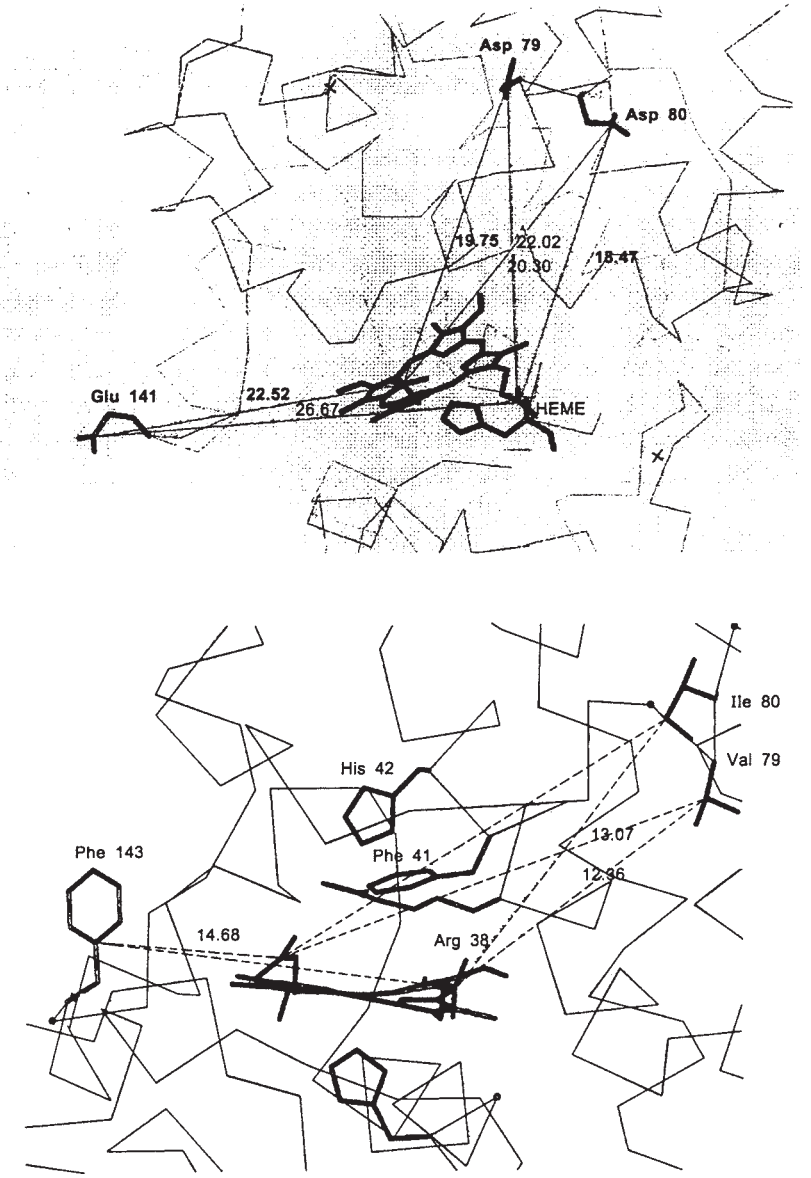

Fig. 3. View of the active sites of tobacco (top) and peanut (bottom) peroxidases from the side of heme propionates.

The extensive protonation of the enzyme in the presence of calcium cations should decrease the repulsion and, moreover, allow an extra calcium cation to be bound and block the access to the active site. Taking into account the stabilization of the TOP holoenzyme at extremely low pH in the presence of calcium cations *(11,12)*, we propose that binding of the calcium ion blocks and stabilizes the heme in the protein matrix.

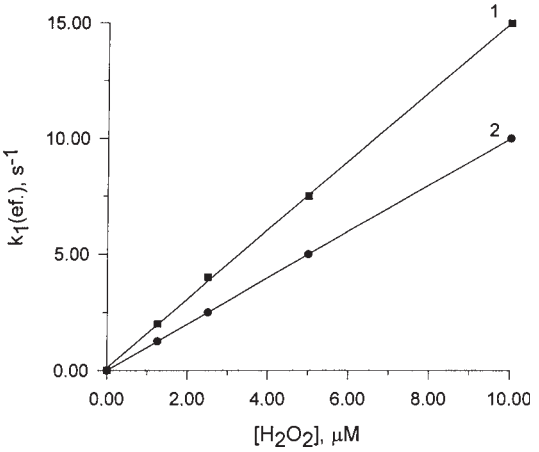

Fig. 4. Determination of the rate constants toward hydrogen peroxide (1) in the absence and (2) in the presence of 50 mM calcium chloride.

At a pH close to neutral (5.0) calcium ions still cause conformational changes affecting the rate constant toward hydrogen peroxide. The measurements of the rate constants performed by stopped-flow spectrophotometric techniques show that the rate constant toward hydrogen peroxide k_1 in the presence of calcium ions is decreased by 25 ± 5% and becomes equal to $(1.10 \pm 0.05) \times 10^5\ M^{-1}\ s^{-1}$ (Fig. 4). We suppose that the decrease in k_1 is due to the blockade of the active site by calcium cations.

Figure 5A presents the effect of calcium ions on the steady state current obtained in a wall-jet flow-injection electrode system. The inverted peaks observed on the saturation curve could be considered as a result of lowering the efficiency of direct ET in the presence of calcium ions. The detection limit for calcium ions is about 5 m*M* (Fig. 5B), which is in agreement with the previously reported minimal concentration of calcium cations affecting the rate of veratryl alcohol oxidation catalyzed by TOP (1 m*M*) *(11)*. When equivalent experiments were performed with HRP-modified electrodes, no such effects were observed at all reflecting that the effect of calcium ions is solely due to the presence of TOP. To discriminate between the effects of calcium ions on the rate constants for hydrogen peroxide reduction and electron transfer for TOP-modified electrodes, plots of $1/I_{KIN}$ vs $1/[H_2O_2]$ were examined (Fig. 6).

The slope of the plot in the absence of a donor substrate is equal to $1/k_1nFE_{DET}$ in accordance with the expression (Eq. 6) *(3,8)*:

$$1/I_{KIN} = 1/nFE_{DET}[(1/k_1[H_2O_2]) + (1/k_s)] \quad (6)$$

where I_{KIN} is the kinetically limited current, n is the number of electrons transferred, E_{DET} is the amount of the enzyme active in direct ET, and F is the Faraday constant.

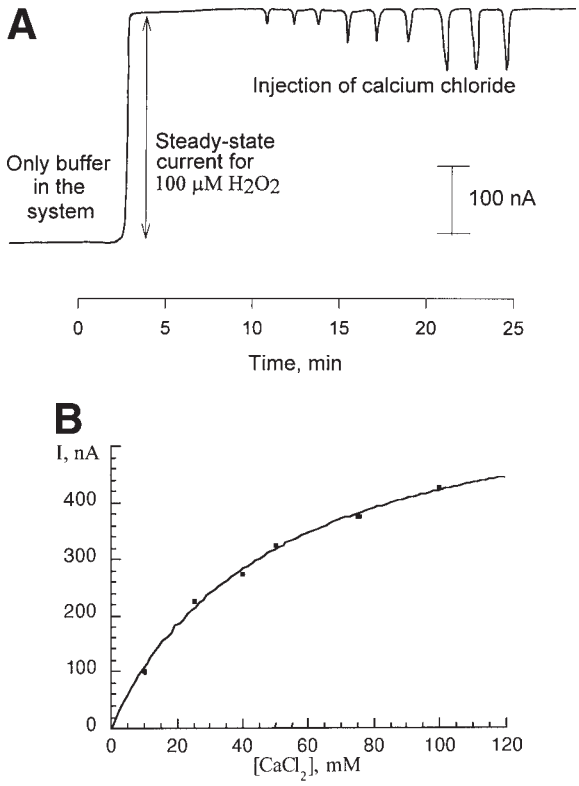

Fig. 5. **(A)** Effect of addition of calcium ions on the steady state current in the wall-jet system obtained with a hydrogen peroxide flow (100 μ*M*), and **(B)** dependence of the inverted peak maximum on the concentration of calcium chloride.

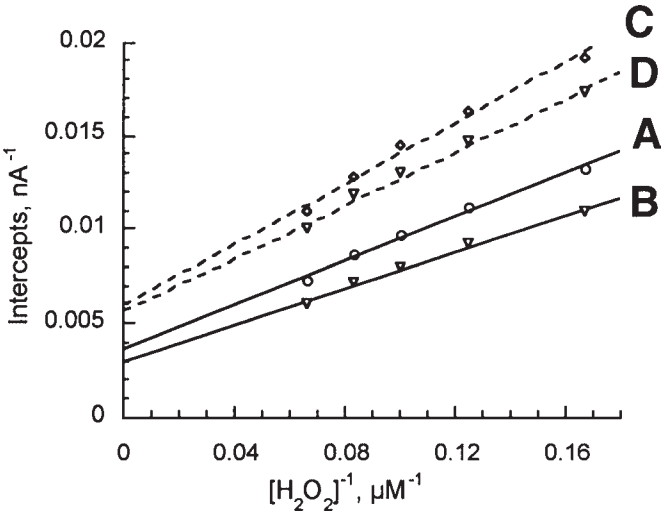

Fig. 6. Direct **(A,C)** and mediated **(B,D)** ET catalyzed by TOP in the absence of calcium ions (A,B) and in the presence of 50 m*M* calcium chloride (C,D). Catechol concentration in mediated ET is 100 μ*M*.

Table 1
Electrochemically Determined Rate Constants
for TOP Adsorbed on Graphite Electrodes (for futher details, *see* Eq. 6 and 7)[a]

	% direct ET	k_1 (M^{-1} s^{-1})	k_s (s^{-1})	Catechol, n = 5 k_3 (M^{-1} s^{-1})	*p*-Cresol, n = 3 k_3 (M^{-1} s^{-1})
Without Ca^{2+}	82.9 ± 3.2	$(4.7 \pm 1.1)10^4$	0.56±0.13	$(5.6 \pm 1.2)10^3$	$(4.2 \pm 2.1)10^3$
With Ca^{2+}	68.7 ± 6.1	$(4.2 \pm 0.95)10^4$	0.35 ± 0.1	$(2.5 \pm 1.5)10^3$	$(3.1 \pm 1.7)10^3$

[a]100 μ*M* catechol and *p*-cresol were used for determination of k_3. 95% confidence limits are given (n = 10 except for k_s, where n = 8 and, in case of k_3, n is specified.

In the presence of a saturating concentration of a donor substrate and assuming that all the adsorbed enzyme molecules are participating in mediated ET, the dependence of the kinetically limited current on hydrogen peroxide concentration follows from Eq. 7 *(8)*:

$$1/I_{KIN} = 1/(2n_1FE)\,[(1/k_1[H_2O_2]) + 1/k_3[AH_2]) \qquad (7)$$

where n_1 is the number of electrons participating in the rate limiting step (n_1 = 1), E is the total amount of active enzyme on the electrode surface. In this work as well as in previously published work from our group *(3,8)*, the surface coverage of peroxidase was assumed to be 40 pmol/cm^2, which is in accordance with values given by Paddock et al. (*21*). In a recent publication by Tatsuma et al. *(22)* a lower coverage (14 pmol/cm^2) was measured by using a quartz crystal microbalance. Keeping in mind that the porosity of the spectrographic graphite used in our work results in a 3–5 larger real surface than the geometric one, the assumption of a coverage of 40 pmol/cm^2 estimate is acceptable.

The ratio of the slopes obtained in the presence and in the absence of a donor substrate indicates the percentage of enzyme molecules active in direct ET *(3,8)*. The results obtained with TOP-modified electrodes are found in Table 1. It demonstrates that the percentage of TOP molecules active in direct ET is even higher than that for HRP and ranges from 69 to 83% both in the presence and in the absence of calcium cations, respectively. The higher percentage of TOP molecules in direct electrical contact with graphite can be explained by less interference (insulation) from the oligosaccharide chains. TOP is less glycosylated compared with HRP, e.g., oligosaccharides contribute to about 8% of the MW of TOP, whereas their content in HRP is as high as 22%.

Calcium ions affect k_1 determined in electrochemical experiments (see Table 1) resulting in an 11% drop of its value in the absence of calcium ions. The absolute value of this drop is, however, less compared with that estimated from the transient kinetic measurements. The observation that both the soluble and the immobilized forms of the enzyme experience the drop in the rate constant toward hydrogen peroxide, k_1, indicates that the immobilized enzyme preserves its conformational flexibility, however, in a slightly moderate fashion.

All kinetic characteristics describing the reduction reactions of oxidized TOP are, however, decreased more severely when compared with k_1. The percentage of TOP in direct ET contact is reduced by 17%, the direct ET rate constant, k_s, decreases by 38% and k_3 by 26% (for *p*-cresol) and 56% (for catechol). All these observations indicate that conformational changes or blockade of the active site reduce the probability for electrons to flow from the electrode or a soluble mediator to oxidized TOP (either the form of compound I or II). Taking into account that it has been proposed that phenolic compounds are oxidized by peroxidases at some distance from the heme *(17)* and that the decrease of k_3 and k_s are comparable in our case, it could be postulated that the heme of the active site of TOP does not have a direct contact with the electrode surface. In other words, the mechanism of direct ET from the electrode surface to the peroxidase active site is mediated by the protein itself, which is in agreement with the recent proposal on the mechanism of direct ET for cytochrome *c* peroxidase *(23)*. The question still remains open whether there are some preferential ET pathways in plant peroxidases for direct heterogeneous ET on an electrode surface.

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