Direct electrochemistry and bioelectrocatalysis of H2O2 reduction of recombinant tobacco peroxidase on graphite. Effect of peroxidase single-point mutation on Ca2+-modulated catalytic activity

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Abstract

Direct electron transfer (DET) reactions and bio(electro)catalytic reduction of H2O2 catalysed by native and recombinant forms of tobacco peroxidase (nTOP and rTOP) were studied in homogeneous-phase catalysis and when TOPs were adsorbed on graphite electrodes. Non-glycosylated wild type and Glu141 → Phe mutant forms of rTOP were produced using an Escherichia coli expression system. Mutation was introduced to explore the mechanisms for modulation of the catalytic activity of TOP by Ca2+ ions. At the pH optimum of 5.0, direct electrochemical Fe3+/2+ transformation of the peroxidase heme was characterised by potentials of −208 mV (nTOP) and −239 mV vs. Ag/AgCl (rTOP), and 0.9 ± 0.1 and 1.1 ± 0.4 pmoles of adsorbed nTOP and rTOP, correspondingly, were in DET contact with graphite. Kinetic analysis of amperometric (at +50 mV) data on H2O2 reduction at TOP-modified electrodes, placed in a wall-jet flow-through electrochemical cell, yielded 82% (nTOP) and 88% (rTOP) of adsorbed TOP molecules active in the DET reaction. The efficiency of DET (and bioelectrocatalysis) increased 3.5-fold when changing from glycosylated nTOP to rTOP. The Glu141 → Phe mutation in the heme-binding pocket of rTOP enabled to achieve a Ca2+-tolerance of TOP in the reaction with H2O2, which is characteristic of other plant peroxidases, and to a large extent in heterogeneous DET and reaction with a second substrate catechol. The results promote further applications of TOP for biosensor- and solid-phase biocatalysts development.

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1. Introduction

There is a constant and considerable interest in application of enzymes as mild, efficient and highly selective catalysts in different biotechnological processes, also including the development of enzyme electrodes for a specific detection/conversion of relevant species in food, drink and paper industries [1–3], in clinical practice [4], and in agricultural [5] and environmental analysis [6,7]. Amongst other enzymes, peroxidases are undoubtedly the most studied enzymes in enzyme-electrode systems for the detection of hydroperoxides and a variety of organic compounds [7–20]; along with a number of ligninolytic enzymes, e.g., from white rot fungi, peroxidases are intensively probed in the enzymatic delignification processes and for detoxification of high-redox-potential xenobiotics [3,21–24]. The main problem in application of peroxidases for these purposes is their general instability and sensitivity to elevated temperatures and concentrations of H2O2.
A heme-containing anionic tobacco peroxidase (TOP, pl 3.5 [25]) is unique among all other known peroxidases due to its attractive stability, substrate specificity, and environmentally modulated catalytic and spectral properties [26–28]. Like all members of the plant peroxidase superfamily, TOP catalyses the oxidation of a wide variety of organic and inorganic substrates by hydroperoxides, through the formation of oxidised peroxidase states, Compound I and Compound II [29]:

\[
\begin{align*}
E(\text{Fe}^{3+}) + \text{H}_2\text{O}_2 & \xrightarrow{k_i} E(\text{Fe}^{4+} = \text{O}, P^+) + \text{H}_2\text{O} \quad (1) \\
E1(\text{Fe}^{4+} = \text{O}, P^+) + e^- (S) + H^+ & \xrightarrow{k_2} E2(\text{Fe}^{4+} = \text{O}) + S^+ \quad (2) \\
E2(\text{Fe}^{4+} = \text{O}) + e^- (S) + H^+ & \xrightarrow{k_3} E(\text{Fe}^{3+}) + S^+ + \text{H}_2\text{O} \quad (3)
\end{align*}
\]

This cycle represents a two-electron oxidation of the ferriheme prosthetic group of the peroxidase (E designates the native or resting ferric state of the enzyme) by \( \text{H}_2\text{O}_2 \) to form Compound I (E1), containing an oxyferryl iron (\( \text{Fe}^{4+} = \text{O} \)) and a porphyrin cation radical \( P^+ \). The two-electron reduction of E1 to the initial ferriperoxidase occurs through the intermediate formation of Compound II (E2) by a sequential one-electron transfer from an electron donor S, which is then one-electron oxidised to the radical product \( S^+ \). S may be also a proton donor, as in the case of phenolic compounds, or may display only electron donor properties.

Anionic TOP is surprisingly stable with respect to \( \text{H}_2\text{O}_2 \), also at a very low pH and in the presence of \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \) cations, which is a unique property of this enzyme distinguishing it among all other known peroxidases [26]. Anionic TOP also exhibits a unique catalytic activity with high-redox-potential substrates at low pHs, thus resembling the biological function of fungal lignin peroxidase (LiP) [25–27]. Certain structural peculiarities of TOP may account for this LiP-like catalytic behaviour: contrary to other plant peroxidases, TOP has a negatively charged Glu residue at the entrance of the heme-binding pocket (Glu141) like LiP does (Glu146), and in the presence of \( \text{Mg}^{2+} \) or \( \text{Ca}^{2+} \) TOP is believed to change the conformation of the heme active site in a way resembling LiP [26,27,30]. This protects the enzyme against inactivation at low pHs and provides E2 of TOP with a redox potential sufficient to catalyse the oxidation of lignin-model-compounds such as veratryl alcohol, at a pH as low as 1.8 [26]. Thus, the presence of a low-affinity site for bivalent metal binding (presumably, Glu141, as one of ligands), in addition to the two constitutive \( \text{Ca}^{2+} \)-sites present in all plant peroxidases, may account for the improved catalytic and stability properties of TOP at low pHs [26,27] and a reversible \( \text{Ca}^{2+} \)-induced inhibition of the catalytic activity of TOP at moderate pHs [30]. Therewith the high stability of E1 and the high reactivity of E2 of TOP, and the surprising modulation of the enzymatic activity of TOP by the bivalent cations at extremely low pHs attract much attention both from fundamental science and biotechnological points of view. Namely, the unique stability of TOP with respect to \( \text{H}_2\text{O}_2 \) gives an opportunity to create a LiP-minicking enzymatic system, stable towards inactivation by \( \text{H}_2\text{O}_2 \) and at elevated temperatures, with promising catalytic properties for biodegradation and detoxification of high-redox-potential natural aromatic compounds and xenobiotics.

Alternatively, due to the substrate specificity different from other plant peroxidases, TOP was also shown to be highly promising for the development of enzyme electrodes for detection of aromatic phenols and amines [31]. In the bioelectrocatalytic reduction of \( \text{H}_2\text{O}_2 \) mediated by electron/proton donors S (e.g., phenols), the oxidised donor \( S^+ \) produced in step (2) and (3) is then electrochemically reduced at the electrode:

\[
S^+ + e^- + \text{H}^+ \xrightarrow{k_m} S
\]

and can thereafter participate again in the bioelectrocatalytic cycle (1)-(3) and provide catalytic amplification of the electrode response in the presence of \( \text{H}_2\text{O}_2 \). In the absence of the second substrate S the oxidised peroxidase can be directly reduced by the electrode to the initial ferriperoxidase state (so-called direct electron transfer (DET) reaction):

\[
E1(\text{Fe}^{4+} = \text{O}, P^+) + 2e^- + 2\text{H}^+ \xrightarrow{k_s} E(\text{Fe}^{3+}) + \text{H}_2\text{O}
\]

The DET between the peroxidase heme active site and electrodes is commonly known to be less efficient than the mediated one due to the value of \( k_s \) is lower than that of \( k_{s,m} \) [16,32–34]. Nevertheless, the case of the efficient DET enables one to develop highly effective biosensor systems for the detection of \( \text{H}_2\text{O}_2 \) and organic hydroperoxides based on DET reaction (see for review [7,35,36] and references therein). Both removal of the oligosaccharide overcoat (recombinant forms of the enzymes) and the use of metal electrodes as alternative to carbonaceous ones were shown to increase the efficiency of the heterogeneous DET reaction [36–42]. In many cases a detailed kinetic analysis of the DET reactions enabled to correlate the natural function of the peroxidases with their bioelectrocatalytic function and provide deeper knowledge on the pathways and ways of regulation of the catalytic mechanisms [30,43–45].

In general, DET of native anionic TOP (nTOP) on graphite has been reported not to be as efficient as that of another plant peroxidase, cationic horseradish peroxidase (HRP). DET rate constants ranged between 0.5 and 2.6 s\(^{-1}\) at the pH optimum of TOP [19,30], compared to 11 s\(^{-1}\) for HRP [32]. In the present work we achieved efficient DET-based bioelectrocatalysis of \( \text{H}_2\text{O}_2 \) reduction by using non-glycosylated recombinant forms of TOP (rTOP) for modification of graphite electrodes: absence of the oligosaccharide overcoat improved adsorption/orientation of TOP onto graphite and resulted in higher DET rates and sensitivities for \( \text{H}_2\text{O}_2 \) comparable with those observed for HRP. Wild-type rTOP and Glu141 → Phe141 mutant of rTOP, with the negative charge removed from the entrance to the heme-binding pocket, were studied and compared with respect to \( \text{Ca}^{2+} \)-induced inhibition of the catalytic
function of TOPs at moderate pHs, to clarify the mechanisms of the bivalent-cation-induced modulation of the catalytic activity of TOP.

2. Materials and methods

2.1. Reagents

Native anionic TOP (nTOP, hydrogen peroxide oxidoreductase, EC 1.11.1.7, MW 36 kDa, 0.2 mg ml\(^{-1}\), RZ = 3.4, activity 130 U mg\(^{-1}\)) was purified from transgenic Nicotiana sylvestris plants as described elsewhere [46]. Recombinant TOP (rTOP, MW 32 kDa, 2 mg ml\(^{-1}\), RZ = 3.0, activity 410 U mg\(^{-1}\)) has been produced in Escherichia coli strain BL21(DE3)CdPlus, using the gene of anionic TOP kindly provided by Prof. L.M. Lagrimini, Syngenta BioTech, NC, USA. The construction of the expression vector was done in accordance with a protocol developed earlier for a wild-type TOP (rTOP, MW 32 kDa, 2 mg ml\(^{-1}\), RZ = 1.5, activity 330 U mg\(^{-1}\)) was produced from Escherichia coli strain BL21(DE3)CdPlus, using the gene of anionic TOP kindly provided by Prof. L.M. Lagrimini, Syngenta BioTech, NC, USA. The construction of the expression vector and the procedure of rTOP expression, refolding and purification are covered in detail elsewhere [47]. rTOP with a Glu141 replaced for Phe point mutation at the entrance of the heme-binding pocket (mutant rTOP, 2 mg ml\(^{-1}\), RZ = 1.5, activity 330 U mg\(^{-1}\)) was produced as follows. Glu141Phe mutation was introduced with the primers E141F_fwd: 5′-CCT AGC CCC TTT TTC ACA CTT GCT GTA –3′ and E141F_rev: 5′-TAC AGC AAG Glu141Phe mutation was introduced with the primers E141F_fwd: 5′-CCT AGC CCC TTT TTC ACA CTT GCT GTA –3′ and E141F_rev: 5′-TAC AGC AAG TGT GAA AAA GGG GCT AGG –3′ in accordance with the protocol and using the Quik-Change kit from Stratagene (USA). Mutation efficiency was 100% as judged from plasmid sequencing from 3 individual clones. The plasmid with the mutation was used to transform E. coli BL21(DE3)CdPlus cells, and the subsequent steps, e.g., cultivation, induction, inclusion bodies isolation, refolding and purification of mutant rTOP, were performed in accordance with a protocol developed earlier for a wild-type rTOP [47]. The typical yield of the homogenous mutant enzyme with a specific activity of 900 U/mg in ABTS oxidation was ca. 10 mg per 1 L of culture.

Substrates and buffer components were from Merck, Darmstadt, Germany. Diammonium 2,2'-azino-bis(3-ethyl-benzothiosaline-6-sulfonate) (ABTS) was from Sigma (St. Louis, MO, USA). All reagents were of analytical grade or of ultra-high purity and used as received. All solutions were prepared with de-ionised Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Activity assay

Concentrations of TOP and H\(_2\)O\(_2\) were determined spectrophotometrically. A molar absorption coefficient of 108 nM\(^{-1}\) cm\(^{-1}\) at 403 nm was used in the TOP assay [46] and 43.6 M\(^{-1}\) cm\(^{-1}\) at 240 nm – in H\(_2\)O\(_2\) determination [48]. Peroxidase activity for ABTS was measured by monitoring the increase of the absorbance at 405 nm using an Ultrospec II spectrophotometer (LKB, Bromma, Sweden) at 25 °C. For ABTS oxidation 0.1–5 nM TOP was added to a solution containing 0.4 mM ABTS and 1 mM H\(_2\)O\(_2\) in 0.05 M sodium-acetate buffer, pH 5.0. A molar absorp-

2.3. Instrumentation

Cyclic voltammetric (CV), differential pulse voltammetric (DPV), and chronoamperometric (CA) measurements with graphite disk electrodes (rods of solid spectroscopic graphite, SGL Carbon AG, Werk Ringsdorf, Bonn, Germany, type RW001, 3.05 mm diameter, fitted in Teflon holders) were performed at room temperature (24 ± 1 °C) either in a standard three-electrode cell connected to a potentiosstat AUTOLAB (PGSTAT 30, Eco Chemie, Utrecht, the Netherlands) equipped with GPES 4.9 software (Eco Chemie), or in a three-electrode wall-jet electrochemical flow-through cell [33] connected to a three-electrode potentiostat (Zäta Elektronik, Lund, Sweden). An Ag|AgCl (0.1 M KCl) electrode and a platinum wire were used as the reference and auxiliary electrodes, respectively. In the flow-through cell, the distance between the nozzle and the working electrode was 0.8 mm. The flow of the solutions was maintained by a peristaltic pump MINI-PULS 2 (Gilion, Villiers-le-Bel, France). Pump pulsations were attenuated by a 0.5 m length Teflon tubing (0.61 mm diameter) placed between the cell and the pump.

2.4. Electrode modification and measurements

Prior to the electrochemical studies, the surface of the graphite disk electrodes was renewed on fine emery paper (Tufbak Durite, P800). After a short rinse with water, the electrodes were immersed either in a 0.2 mg ml\(^{-1}\) (nTOP) or in 1 mg ml\(^{-1}\) solution of rTOPs in 0.05 M phosphate buffer solution containing 0.15 M NaCl (PBS), pH 5.0, for 2 h. After thorough washing with a buffer solution, the TOP-modified electrodes were mounted in the electrochemical cell and CV, DPV, or alternatively CA measurements were performed either in 0.05 M PBS, in 0.05 M Tris or 0.05 M Na-acetate buffer solution, containing 0.15 M NaCl (Na-AcBS), of a corresponding pH. In CV and DPV, the working solutions were thoroughly de-aerated with nitrogen during 1 h prior to the measurements, if not stated otherwise. In CA in the wall-jet flow-through cell, steady-state currents were measured at an applied potential of +50 mV. For the measurement of the amperometric response of the rTOP-modified electrodes to H\(_2\)O\(_2\) the flow-carrier buffer solution containing different concen-
trations of H₂O₂ was used. The flow rates were 1.0, 0.9, 0.7, 0.55, 0.4 and 0.35 ml min⁻¹. The response signal difference between the background current and the steady state current in the presence of the substrate was measured to assess the signal corresponding to the bioelectrocatalytic reduction of H₂O₂ on the modified graphite electrodes. The reproducibility of the data was measured with at least five equivalently prepared electrodes. The sensitivity of the enzyme electrodes was calculated from the slopes of calibration curves with respect to the geometric surface area of the electrodes used.

3. Results and discussion

The direct electrochemistry and the bioelectrocatalytic function of native and recombinant forms of TOP adsorbed onto graphite electrodes were studied in the absence and in the presence of H₂O₂, correspondingly.

3.1. Direct (non-catalytic) electrochemistry of TOP on graphite electrodes

Non-catalytic DPV in de-aerated solutions enabled to detect a redox process associated with a direct electrochemical conversion of the heme active site of TOPs, switching between its Fe³⁺/²⁺ states (Fig. 1, inset):

\[
E(\text{Fe}^{3+}) + e^- + H^+ \rightarrow E(\text{Fe}^{2+})
\]

At pH 5.0, the heme Fe³⁺/²⁺ couple of TOP was characterised by the redox potentials of −208 ± 4 mV (nTOP) and −239 ± 15 mV (rTOP). For both nTOP and rTOP similar values for the heme redox potential were expected, however, the electrode surface environment can differently affect the adsorbed state of peroxidases, e.g., the conformation and thus the microenvironment of the heme redox cofactor. A different conformational stability at the electrodes of these two forms of TOP, one of which is not glycosylated, could be the reason for the shift in the redox potential for nTOP compared to rTOP.

These potentials are also somehow different from −143 mV obtained for rTOP adsorbed on gold [50]. Generally, adsorption on electrodes may provide certain partial denaturation of peroxidases thus shifting the redox potentials of the heme active site to more positive values [36]. Thus, this difference in the redox potential of the heme in rTOP can be ascribed to more pronounced conformational changes of non-glycosylated (and thus more hydrophobic) rTOP on the hydrophobic gold electrode surface than on the essentially hydrophilic graphite. Surface interactions of rTOP with gold then result in conformational changes in the active centre as well as possible partial unfolding of the enzyme, shifting the potential in a more positive direction.

Keeping this in mind, we consider the more negative potential values obtained for the heme of TOP on graphite as being closer to the inherent redox potential of TOP in solution. The exact values for the “solution” redox potentials for TOP are unknown, but, for comparison, potentiometric titration of other peroxidases with mediators, at pH 5.0, gave around −360 mV for horseradish peroxidase (HRP) [51] and −219 mV for LiP (data obtained at pH 7.0 were recalculated by taking into account a −118 mV shift per two pH units characteristic of 1e⁻/1 H⁺ reaction (6)) [52]. Thus, the redox potentials of TOP on graphite seem to be closer to the redox potentials of LiP, which reasonably correlates with similarities in the reactivity of both peroxidases towards high-redox-potential aromatic compounds [26].

The average amount of nTOP and rTOP in DET contact with graphite electrodes was calculated from the DPVs [53] and was found to be close for both forms, 0.91 ± 0.12 pmoles for nTOP and 1.1 ± 0.4 pmoles for rTOP. Evidently, not all adsorbed TOP molecules were active in DET, but only a part representing some percentage of the total amount of adsorbed TOPs. The total amount of adsorbed catalytically active TOP calculated from the CA data on DET-based and catechol-mediated bioelectrocatalysis of H₂O₂ reduction (part 3.2) was 1.1 and 1.24 pmoles for nTOP and rTOP, correspondingly.

CV and DPV failed to detect any signals from direct electrochemical oxidation of ferric TOP to E1/E2 by the electrode at potentials lower than 0.8–0.9 V. Intensive oxidation of the graphite surface itself excluded studies of the electrochemistry of TOP at higher potentials. We can just suppose the redox potentials for E1/E2 of TOP to be higher than 0.9 V and thus closer to the redox potentials of LiP (similarly to their heme Fe³⁺/²⁺ redox potentials), which are higher than 1.2 V [25–27]. Of all electrochemically studied peroxidases so far, yeast cytochrome c peroxidase (CcP) was the only one shown to exhibit a unique redox activity when adsorbed on edge-oriented pyrolytic graphite: it gave distinct redox signals corresponding both
to direct electrochemical (at 0.55 V) and bioelectrocatalytic (upon addition of \( \text{H}_2\text{O}_2 \)) oxidations to E1 and its further re-reduction back to the native enzyme [43,44].

### 3.2. Direct bioelectrocatalysis of \( \text{H}_2\text{O}_2 \) reduction at TOP-modified graphite electrodes

CVs of TOP-modified graphite electrodes in air-saturated solutions are presented in Fig. 1. In the presence of oxygen, its bioelectrocatalytic reduction by adsorbed TOP (catalysis by the heme Fe\(^{3+/2+}\) couple [36]) occurred at potentials 100–150 mV more positive than the reduction of \( \text{O}_2 \) at bare graphite electrodes (Fig. 1).

In de-aerated solutions, in the absence and in the presence of varying concentration of \( \text{H}_2\text{O}_2 \), the bioelectrocatalytic reduction of \( \text{H}_2\text{O}_2 \) at the TOP-modified electrode was followed over the 0.7 to –0.2 V potential range (Fig. 2, data presented for rTOP, potential range from 0.5 to –0.2 V). At potentials higher than 0.7–0.8 V the intensive oxidation of the graphite surface impeded the studies. Thus, the redox potential for E1/E2 of TOP could be assumed to be higher than 0.7 V. Both nTOP and rTOP electrodes demonstrated a substantial current response to \( \text{H}_2\text{O}_2 \) due to its bioelectrocatalytic reduction based on DET between graphite and the active site of TOP. It is worth to stress that the reduction of \( \text{H}_2\text{O}_2 \) at bare graphite starts from sufficiently less positive potentials and the concentrations of \( \text{H}_2\text{O}_2 \) providing detectable response are in the mM range (Fig. 2, inset).

To analyse and compare the kinetics of bioelectrocatalysis of nTOP and rTOP, CA experiments with TOP-modified electrodes were performed at +50 mV, since at lower potentials an increasing current due to the contribution from the reactions of \( \text{O}_2 \) reduction (Fig. 1) and \( \text{H}_2\text{O}_2 \) reduction (Fig. 2) catalysed by the Fe\(^{3+/2+}\) couple of peroxidase [36] could be observed.

Calibration curves were constructed using the data of amperometric detection of \( \text{H}_2\text{O}_2 \) with nTOP- and rTOP-modified graphite electrodes at different pHs (Fig. 3). Over the entire studied pH-range the bioelectrocatalytic DET-based responses of rTOP-modified electrodes were sufficiently higher than those of nTOP-modified electrodes. For both forms of TOP the maximal current responses were obtained at pH 5.0 (Fig. 3), which correlates with the maximum of the catalytic activity of TOP in solution [27]. The following CA experiments were done at the pH optimum of 5. The stability of the CA response (correlating with the stability of the adsorption of TOP on graphite) was sufficient to perform kinetic studies: 10% decrease in the response of the electrode was observed during 2 h of continuous steady-state measurements at different flow rates.

Calibration curves, at pH 5.0, both in the absence and in the presence of a soluble mediator (catechol), are presented in Fig. 4. The sensitivity of nTOP- and rTOP-modified electrodes to \( \text{H}_2\text{O}_2 \) in the DET-mode was 0.38 and 0.71 A M\(^{-1}\) cm\(^{-2}\), correspondingly, and 0.5 and 0.82 A M\(^{-1}\) cm\(^{-2}\) in the mediated ET-mode. The detection limit was 10 nM of \( \text{H}_2\text{O}_2 \) for both direct and mediated ET reactions. Upon addition of a mediator (5 \( \times \) 10\(^{-4}\) M catechol), the current response moderately increased revealing certain kinetic restrictions and/or implying that not all adsorbed catalytically active rTOP molecules are active in DET reaction. The registered bioelectrocatalytic currents were thus determined both by the diffusion of \( \text{H}_2\text{O}_2 \) to the electrode surface and by the kinetics of the direct and mediated ET reactions, making it possible to perform a kinetic analysis of the amperometric data.
The substrate was 0.9 ml min\(^{-1}\) / C\(_0\) (0.5 mM catechol acting as a mediator, (d) PBS with 0.15 M NaCl, pH 5.0. (d) is the kinematic viscosity of water (0.01 cm\(^2\) s\(^{-1}\)). Concentration determined with nTOP-modified (Fig. 4. Dependence of the steady-state current density on the H\(_2\)O\(_2\) concentration determined with nTOP-modified (\(\bullet, \bigcirc\)) and rTOP-modified (\(\nabla, \bigtriangledown\)) graphite disk electrodes in a wall-jet flow-through cell; flow rate of the substrate was 0.9 ml min\(^{-1}\). Applied potential was +50 mV, 0.05 M PBS with 0.15 M NaCl, pH 5.0. (\(\bigcirc, \bigtriangledown\)) Response in the presence of 0.5 mM catechol acting as a mediator, (\(\bullet, \nabla\)) DET reaction.

To separate the kinetic and diffusion parts of the measured current, amperometric measurements were performed at different flow rates and the obtained data were processed in accordance with the Koutecky–Levich (KL) approach adapted for an electrode in a wall-jet cell [33] in combination with the kinetic model of the bioelectrocatalytic action of HRP at electrodes [16]. The basic rate constants of the peroxyxidase bioelectrocatalytic cycle, represented by reactions (1)–(4), specifically, the rate constant for the enzymatic reduction of H\(_2\)O\(_2\), \(k_1\), the heterogeneous DET rate constant, \(k_s\), and the rate constant \(k_3\) of mediated ET (an assumption is made that the enzymatic reaction (3), i.e., the oxidation of a mediator S by E\(_2\) is the limiting step in the overall mediated ET process (2) and (3) in accordance with the fact that 10 \(k_3\approx k_2\), [29]) were estimated with Eqs. (7) and (8):

\[
1/I = 1/I_k + 1/I_{lim} = 1/nFEDET(1/(k_1 CH\(_2\)O\(_2\)) + 1/k_3) + 1/0.898nFCH\(_2\)O\(_2\)D\(^{2/3}\)A\(^{1/3}\)V\(^{-5/12}\)P\(^{3/4}\)a\(^{-1/2}\) \quad (7)
\]

\[
1/I = 1/I_k + 1/I_{lim} = 1/2n_FEMET(1/k_1 CH\(_2\)O\(_2\) + 1/k_3[S]) + 1/0.898nFCH\(_2\)O\(_2\)D\(^{2/3}\)A\(^{1/3}\)V\(^{-5/12}\)P\(^{3/4}\)a\(^{-1/2}\) \quad (8)
\]

Here, \(I\) is the measured current of the bioelectrocatalytic reduction of H\(_2\)O\(_2\) on the TOP-modified electrodes, \(I_k\) is the kinetically limited current of the enzymatic reaction and \(I_{lim}\) is the diffusion current limited by the mass-transfer of H\(_2\)O\(_2\) to the electrode; \(n\) is the number of electrons transferred in the reaction; \(n_1\) is the number of electrons transferred per mediator molecule and equals 0.5\(n\) for catechol; \(F\) is the Faraday constant; \(C_{H\(_2\)O\(_2\)}\) is the bulk concentration of H\(_2\)O\(_2\); \(D\) is the H\(_2\)O\(_2\) diffusion coefficient (1.6 \times 10\(^{-5}\) cm\(^2\) s\(^{-1}\)); \(A\) is the geometrical electrode area; \(v\) is the kinematic viscosity of water (0.01 cm\(^2\) s\(^{-1}\)); \(V\) is the volume flow rate; \(a\) is the radius of the capillary nozzle (0.025 cm); \(E_{DET}\) is the amount of the enzyme (in moles) participating in direct ET; \(E_{MET}\) is the total amount of the active enzyme (in moles) on the electrode surface involved in the electrode reaction; [S] is the mediator concentration.

From the intercepts of the KL plots (Fig. 5) with the Y-axis the 1/I\(_k\) values were obtained for direct and mediated ET reductions and plotted versus the reciprocal H\(_2\)O\(_2\) concentrations (Fig. 6). The 1/I\(_k\) versus 1/c\(_{H\(_2\)O\(_2\)}\) plots showed a linear dependence, and from the slope of these plots \(k_1\) was determined; from the intercepts with the Y-axis the value of \(k_1\) was evaluated in the case of direct ET (Eq. (7)) and \(k_3\) in the case of mediated ET (Eq. (8)). From the experiments when only the direct (no mediator in solution) or only the mediated (saturating concentration of mediator in solution) ET takes place, the fraction of the enzyme molecules active in direct ET was calculated from the ratio of the 1/I\(_k\) versus 1/c\(_{H\(_2\)O\(_2\)}\) slopes in the presence and in the absence of the mediator [16] (Fig. 6). Therewith it was assumed that in the presence of a saturating concentration of the mediator (5 \times 10\(^{-4}\) M catechol [33,39]) all adsorbed catalytically active peroxidase molecules participate in mediated ET, whereas in the absence of the mediator only a fraction is available for DET [16]. Here, with saturation is meant a concentration of the mediator, enabling mediated ET with a rate so that reactions (2)–(4) proceed much faster than reaction (5). This ensures that the total amount of active enzyme adsorbed at the electrode surface is involved in the bioelectrocatalytic cycle. Catechol, a classical substrate in peroxidase catalysis [29] and a good substrate for TOP [31], was chosen as the mediator since its reaction product, S\(^+\) in reactions (2) and (3), is reduced at the electrode surface (reaction (4)) with no kinetic restrictions in a potential range of around +100 to −50 mV vs. Ag|AgCl [33].

The ratio of the slopes in the presence and in the absence of a mediator gave the ratio between \(E_{DET}\) and \(E_{MET}\) equal to 82% (nTOP) and 89% (rTOP), which implies in average 1.1 pmoles (nTOP) and 1.24 pmoles (rTOP) of the total amount of the adsorbed catalytically active enzyme, recalculated from the non-catalytic DPV data for both TOPs on graphite. These values are lower than 3 pmoles of peroxidase molecules adsorbed on graphite, which is commonly assumed (corresponding to a surface coverage of 40 pmoles cm\(^{-2}\), if referred to the geometric surface area of the used graphite electrodes) [16,30,32,33]. Thus, all kinetic parameters in reactions (1)–(5) were calculated using the experimental values of the surface coverage.

The percentage of DET-wired TOP molecules and the catalytic rate constants that characterise the direct and catechol-mediated bioelectrocatalytic reduction of H\(_2\)O\(_2\) are summarised in Table 1. The efficiency of DET of nTOP was characterised by a moderate \(k_1\) equal to 12 s\(^{-1}\), which approached the \(k_1\) values for cationic HRP on graphite at the pH-optimum of 6.0 [32]. The obtained catalytic rate constant \(k_1\) (reaction (1)) was 10 times lower than that reported for nTOP in solution, 1.5 \times 10\(^8\) M\(^{-1}\) s\(^{-1}\) [27]. That is a typical case observed with plant peroxidases immobilised at electrodes [32,39,45,54]. When changing from
nTOP to rTOP, the efficiency of the DET reaction increased and was characterised by a $k_s$ of 35 s$^{-1}$. Thus, the absence of the oligosaccharide overcoat in rTOP enabled adsorption of rTOP with a surface orientation providing more favourable ET pathways through the protein medium, with increased ET rates. The rate constant $k_3$ for the reaction with catechol (reaction (3)) increased when changing from nTOP to the rTOP form as well, which can also be connected with adsorption of rTOP on graphite more favourable for the catalytic reaction. The obtained $k_3$ suited well the range of values previously reported for bioelectrocatalysis of some other plant peroxidases adsorbed on graphite, with catechol [55] and structurally related $p$-cresol [19] working as mediators. However, $k_1$ again appeared to be 10 times lower than that reported for rTOP in solution, $4 \times 10^5$ versus $3.6 \times 10^6$ M$^{-1}$ s$^{-1}$ [47]. Nevertheless, in general, the kinetic parameters of both DET-based and catechol-mediated bioelectrocatalysis demonstrated advantageous bioelectrocatalytic properties of the recombinant form of anionic TOP adsorbed on graphite electrodes, for further biosensor development.

### Table 1

<table>
<thead>
<tr>
<th>TOP form</th>
<th>% DET</th>
<th>$k_s$ (s$^{-1}$)</th>
<th>$k_1$, 10$^{-5}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_3$, 10$^{-4}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTOP</td>
<td>82 ± 1</td>
<td>12.0 ± 1.1</td>
<td>1.45 ± 0.1</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Wild-type rTOP</td>
<td>89 ± 3</td>
<td>34.9 ± 0.8</td>
<td>3.95 ± 0.3</td>
<td>7.3 ± 0.5</td>
</tr>
</tbody>
</table>

3.3. Effect of Ca$^{2+}$ ions on the catalytic activity of rTOP and of the Glu141 → Phe mutant form of rTOP

Due to some variable structural domains, members of the plant peroxidase family show large variations in pl and extent of glycosylation, and substrate specificity, which appear to be dependent upon residues at the active site and
the substrate access channel. Thus that is the structural peculiarities of anionic TOP that predetermine its attractive substrate specificity and catalytic and stability properties at very low pHs [26, 27], making TOP one of the promising candidates for the development of efficient biocatalysts for delignification and detoxification of high-redox-potential aromatics. Both the catalytic and the spectral properties of TOP were shown to be essentially regulated by calcium ions [26, 28]. A peroxidase molecule itself contains 2 structurally important Ca\(^{2+}\) cations that participate in the enzyme folding and are tightly bound in the distal and proximal domains. In addition, nTOP apparently has a site with much lesser affinity for Ca\(^{2+}\): the maximum effect of Ca\(^{2+}\) on the enzyme activity and stability was observed at 20–100 mM Ca and low pH [26]. Computer modelling showed that extensive protonation or Ca\(^{2+}\) binding will reduce the repulsion between Glu141 and the triad of Asp residues 76, 79 and 80, providing the enzyme active centre with a more closed conformation [30]. Thus, the Ca\(^{2+}\)-dependent properties of TOP were ascribed to the presence of the negatively charged glutamic acid residue, Glu141, at the active centre entrance, capable of Ca\(^{2+}\) binding, while other plant peroxidases have Phe in this position [26, 28]. The replacement of Glu141 for Phe by site-directed mutagenesis was expected to remove the Ca\(^{2+}\)-effects on the enzyme activity thus providing TOP with tolerance towards Ca\(^{2+}\), which is characteristic for other plant peroxidases [30].

The effect of Ca\(^{2+}\) cations was studied on the catalytic activity of rTOP of the wild type and the mutant Glu141Phe rTOP in solution. As was shown earlier, nTOP is sensitive to Ca\(^{2+}\) ions and both \(k_1\) and \(k_3\) decreased in the presence of 50 mM CaCl\(_2\) [30]. Close results were obtained for homogeneous catalysis with rTOP of wild type (Table 2), which implies that it is the peculiarities of the polypeptide chain folding responsible for interactions with Ca\(^{2+}\) ions but not the glycoside moieties. However, unexpectedly, for both forms of rTOP in solution an inhibition of the catalytic activity by Ca\(^{2+}\) was observed in the catalytic reaction with ABTS. Kinetic analysis of the data on the homogeneous-phase catalysis demonstrated that the effect of Ca\(^{2+}\) on the activity of Glu141Phe rTOP was not removed, though a certain tendency for a decreasing Ca\(^{2+}\) influence could be followed (Table 2). Detailed studies of the catalysis over the pH range from pH 7 to pH 3 demonstrated a much lower stability of Glu141Phe rTOP compared to rTOP especially pronounced at acidic pHs and in diluted enzyme solutions used for the enzyme assay. Additionally, the pH optimum for the mutant rTOP widened and covered then the pH 5–6 range. These data imply conformational alterations in the active centre and its environment due to the introduced mutation, which affected the catalytic function of mutant rTOP as well. Thus, Glu141 appeared to control not only the heme access to the enzyme active centre, which might also be followed from the data on production of the Glu141Phe form. On the one hand, the yield of Glu141Phe rTOP during refolding was much higher (25%) than for the wild-type TOP (7% [47]) and was similar to that of recombinant HRP (20–25%, [56]). On the other hand, the mutant form appeared to be less stable upon purification and storage and showed a tendency to lose the heme (as judged by the decrease in RZ value). This instability might indirectly indicate not only the changes in the structure of the active site, but in the overall conformation of the enzyme as well. The question was to what extent could we separate the effects of Ca\(^{2+}\) and of Ca\(^{2+}\)-independent conformational changes on the catalytic activity of the mutant Glu141Phe rTOP?

The bioelectrocatalytic function of both rTOPs was studied within the pH-range from pH 7 to pH 3. Adsorption onto graphite somehow stabilised rTOPs, decreasing the inactivation of the mutant rTOP, which was especially pronounced at low pHs. Along with this, the general features of the pH-dependence of the catalytic activity and the pH optimum for both forms remained similar to those observed in homogeneous catalysis (Fig. 7). A reversible inhibition of the bioelectrocatalytic response to H\(_2\)O\(_2\) (apparent efficiency of bioelectrocatalysis) of both rTOPs and Glu141Phe-rTOP-modified graphite electrodes was achieved upon addition of Ca\(^{2+}\), the maximal effect being observed at pH 5 and with 0.1 M CaCl\(_2\) (Fig. 7). The apparent effect of Ca\(^{2+}\) was thus much less in the case of the mutant rTOP, over the whole pH-range studied.

Kinetic analysis of the data at pH 5, where the effects of Ca\(^{2+}\) and the stability of both forms were maximal, was performed using the KL-approach, and the calculated rate constants are summarised in Table 3. For both forms of TOP a decreasing percentage of DET-wired TOP molecules in the presence of Ca\(^{2+}\) was observed, giving evidence for structural rearrangements in the adsorbed peroxidase state upon interactions with Ca\(^{2+}\). This decrease was less in the case of the mutant form designating a decreasing effect of Ca\(^{2+}\). Therewith, a smaller percentage of mutant rTOP molecules in DET compared to the wild-type rTOP indirectly indicated a significant conformational change in the overall structure upon single-point mutation in this region. The bioelectrocatalytic function of wild-type rTOP allowed stabilising the enzyme against inactivation by Ca\(^{2+}\).

### Table 2

<table>
<thead>
<tr>
<th>Rate constants, 10^{-8} (M^{-1} s^{-1})</th>
<th>Wild-type rTOP</th>
<th>Mutant Glu141Phe rTOP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Ca(^{2+})</td>
<td>25 mM Ca(^{2+})</td>
</tr>
<tr>
<td>(k_{\text{H}_2\text{O}_2}) ((k_1))</td>
<td>3.6 ± 0.6</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>(k_{\text{ABTS}}) ((k_3))</td>
<td>8.2 ± 0.9</td>
<td>7.0 ± 0.8</td>
</tr>
</tbody>
</table>
in the reaction with H₂O₂; only a 13% loss in k₁ was observed (Table 3) compared to a 2.5 times decrease of k₁ in the solution reaction, even at lower concentrations of Ca²⁺ (Table 2). Similar results were previously reported for the bioelectrocatalytic function of nTOP on graphite: the electrochemically determined k₁ decreased only 11% [30] compared to a 2.5–3 times decrease observed in homogeneous catalysis [27]. In parallel, a 2 times drop in k₂ and a 35–40% decrease in k₃ in the reaction with catechol was registered for rTOP (Table 3). In homogeneous catalysis, the Glu141Phe point mutation of rTOP enabled to achieve a 1.7 times decrease of k₁, while not affecting k₃ (Table 2). For mutant rTOP immobilised on graphite, the Glu141Phe mutation provided absolute Ca²⁺-tolerance in the reaction with H₂O₂ (k₁ being constant, Table 3) and only a 20% decrease in the rate constants with electron donors, either with the electrode (k₄) or with the mediator (k₅) (Table 3).

Thus, we can conclude that Glu141 is evidently involved in Ca²⁺-coordinative binding. However, the produced Glu141Phe point mutation in rTOP affected not only the Ca²⁺-induced change in the catalytic activity of rTOP, but also the overall conformational stability of the enzyme and possibly the structure of the active site. On the one hand, the negatively charged Glu141 evidently controls the access to the enzyme active centre providing its lower accessibility upon interaction with Ca²⁺, which is especially pronounced for the reaction with H₂O₂. On the other hand, this “controlling” role of Glu141 results in a lower stability (and thus rapid inactivation) of the mutant rTOP, in which Phe displaces this residue crucial for a stable catalytic function. These overlapping and in fact oppositely directed effects somehow interfere in a clear understanding of the mechanisms of Ca²⁺-induced modulation of TOP catalysis. The question still remains if there is only one residue, namely Glu141, which is involved in this type of the activity regulation? Further work on mutagenesis of the residues in the active site of TOP and kinetic studies of the mutant forms of rTOP at extremely low pHs will clarify the mechanism of the Ca²⁺-regulated activity and stability of TOP.

### 4. Conclusions

Direct immobilisation of native and recombinant (and thus non-glycosylated) forms of anionic TOP at graphite electrodes enabled studies of the bioelectrocatalytic behaviour of this anionic peroxidase, both in direct and catechol-mediated bioelectrocatalytic reduction of H₂O₂. The redox potential for the Fe³⁺/²⁺ couple of TOP, at pH 5.0, was –208 mV (nTOP) and –239 mV (rTOP), and 0.9 and 1.1 pmoles of adsorbed nTOP and rTOP, correspondingly, were in DET contact with graphite. The absence of the naturally occurring glycosylation provided adsorption/orientation of rTOP on graphite favourable for more efficient ET reactions, which resulted in DET rates and sensitivities of rTOP-modified graphite electrodes for H₂O₂ essentially higher than those observed for glycosylated (8%) nTOP adsorbed on graphite. The particular role of Glu41 in the mechanism of modulation of the catalytic activity of TOP by Ca²⁺ was demonstrated: the Glu141→Phe mutation in the heme-binding pocket of rTOP enabled to achieve a Ca²⁺-tolerance of TOP in the reaction with H₂O₂, and partially in the heterogeneous DET and in the reaction with a co-substrate, catechol. The obtained results are of particular interest for the development of LiP-mimicking enzymatic systems and enzyme electrodes for detection of aromatic phenols and amines, stable towards inactivation by H₂O₂, with promising catalytic properties for biodegradation and detoxification of high-redox potential natural aromatic compounds and xenobiotics.

### Table 3

<table>
<thead>
<tr>
<th>TOP form</th>
<th>% DET</th>
<th>k₄ (s⁻¹)</th>
<th>k₅₁, 10⁻⁵ (M⁻¹ s⁻¹)</th>
<th>k₅₃, 10⁻⁴ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type rTOP (no Ca²⁺)</td>
<td>81 ± 2</td>
<td>32.9 ± 0.7</td>
<td>3.2 ± 0.4</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>Wild-type rTOP (0.1 M Ca²⁺)</td>
<td>65 ± 2</td>
<td>17.2 ± 0.5</td>
<td>2.8 ± 0.3</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>Glu141Phe rTOP (no Ca²⁺)</td>
<td>66 ± 5</td>
<td>34.3 ± 0.8</td>
<td>3.4 ± 0.4</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Glu141Phe rTOP (0.1 M Ca²⁺)</td>
<td>61 ± 4</td>
<td>27.4 ± 1.1</td>
<td>3.5 ± 0.2</td>
<td>4.7 ± 0.5</td>
</tr>
</tbody>
</table>
Acknowledgements

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