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Bioelectrocatalytical Detection of H_2O_2 with Different Forms of Horseradish Peroxidase Directly Adsorbed at Polycrystalline Silver and Gold

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Abstract
The bioelectrocatalytical reduction of H_2O_2 based on direct electron transfer (ET) between polycrystalline silver and the heme containing active site of horseradish peroxidase (HRP) is studied and compared with that obtained at gold. Native HRP and recombinant wild type HRP, containing additionally a six-histidine tag at the N-terminus, N_{His}rHRP, have been used for adsorptive modification of the electrodes. The histidine sequences, introduced into the peroxidase structure by genetic engineering of recombinant HRP using an *E. coli* expression system, were supposed to affect adsorption/orientation of the enzyme at the electrode surface. The variations in direct ET efficiency when changing from gold to silver, as well as from native HRP to N_{His}rHRP are analyzed and discussed, specifically, the high ET rates obtained with N_{His}rHRP-modified preoxidized gold and silver electrodes, though accompanied by extremely rapid loss of the biolectrocatalytical activity of the latter (silver) in direct (but not mediated) ET. The advantages and drawbacks of the studied HRP-electrode systems for the electroanalytical detection of H_2O_2 are considered.

Keywords: Bioelectrocatalysis, Horseradish peroxidase, Histidine, Heterogeneous direct electron transfer, Polycrystalline silver, Polycrystalline gold

1. Introduction

Probably, the most important, though the least understood due to a limited or nonsystematized number of experimental data available, is the relation between the nature of the electrode and the efficiency of nonmediated bioelectrocatalysis for some electrode reactions with enzymes adsorbed at the electrode surface. Therewith, direct immobilization of redox active proteins could provide an increased efficiency of a bioelectrocatalytic event due to an enhanced electronic communication between the electrode and the redox active site of a biomolecule, thus offering necessary data for the analysis of the relationship between the electrode material – the bioelectrocatalysis for a justified choice of the optimal electrode support [1 – 4].

By now horseradish peroxidase (HRP), one of the most studied members of the family of heme enzymes [5], was shown to catalyze the reaction of nonmediated cathodic reduction of H_2O_2 resulting from direct (nonmediated) electron transfer (ET) from the electrode to the heme containing active site of HRP when immobilized at the electrode surface [6 – 9]. Due to numerous applications in electroanalysis of peroxides and of compounds yielding H_2O_2 in the presence of appropriate oxidases, this direct ET reaction has been extensively studied on a variety of electrode materials with different surface chemistry, e.g., carbon materials [6 – 13], gold [14 – 19], tin oxide [20] and some others. Nevertheless, the relation between the electrode support used for HRP immobilization and the efficiency of direct ET remains open since too many factors affect the heterogeneous ET rates when changing from one electrode material to another, even if the electronic structure of the interface is not taken into account: from the variations in ET pathways and enzyme orientation at the electrode surface to the variation in the surface coverage, possible denaturation/desorption of the enzyme etc. [3 – 4]. However, from the data available it becomes evident, that when changing from carbon/graphite surfaces [9 – 12] to metal electrodes, specifically gold [16 – 19], an increased ET efficiency from the electrode to the active site of HRP can be followed. The high ET rates, i.e., a high heterogeneous ET rate constant, k_s, as well as a high percentage of the adsorbed HRP molecules active in the ET reaction (up to 95 – 100%) attained with HRPs adsorbed directly at preoxidized polycrystalline gold surface [16 – 19] suggest that similar results might be expected with some other metal electrodes, under proper immobilization conditions of HRP and of the experimental procedure.

In the present work the bioelectrocatalytical reduction of H_2O_2 based on direct ET between polycrystalline silver/gold electrodes and the heme containing active site of HRP is studied and compared. Two forms of HRP, i.e., native glycosylated and recombinant nonglycosylated, were directly immobilized at the electrode surfaces through adsorption. The efficiency of direct ET between the electrode and the enzyme was analyzed with respect to the nature of

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the electrode material (gold and silver) trying to find any relationship between the bioelectrocatalytic activity of the adsorbed HRPs and the nature and pretreatment of the electrode material. The efficiency of the bioelectrocatalytic reduction reaction of H_2O_2 caused by the adsorbed peroxidase is estimated with regards to the stability, sensitivity and detection limit for H_2O_2.

2. Experimental

2.1. Materials

HRP from Boehringer Mannheim GmbH, Mannheim, Germany (nHRP, isoenzyme C, 1300 U mg^{-1} (ABTS)), and other reagents of analytical grade from Merck, Darmstadt, Germany, were used. Recombinant wild type HRP containing a 6 histidine tag at the N-terminus (N_{His}rHRP, 820 U mg^{-1} (ABTS)) was produced in *E. coli* strain BL21(DE3)pLysS transformed with the appropriate pET based expression vectors [15–17 and references therein] and kindly supplied by Dr. V. Grigorenko of the M.V. Lomonosov Moscow State University. All solutions were prepared with deionized Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Amperometric measurements with polycrystalline silver and gold disk electrodes (CH-Instruments, Austin, TX, USA, 0.031 cm^2) were performed at room temperature ($20 \pm 1\,^{\circ}C$) in a standard three-electrode flow-through wall-jet cell [10] connected to a potentiostat (μAUTOLAB, Eco Chemie B.V., Netherlands) equipped with GPES 4.8 software. The flow of the solutions was maintained by a peristaltic pump (MINIPULS 2, Gilson, Villiers-le-Bel, France). The cell contained an Ag | AgCl | 0.1 M KCl reference electrode; the auxiliary electrode was a Pt wire. The distance between the nozzle and the working electrode was about 0.8 mm. Amperometric measurements were performed at an applied potential of −50 mV (vs. Ag | AgCl). Cyclic voltammetry was performed with polycrystalline silver electrodes placed in a standard three-electrode electrochemical cell connected to a CV-50 W Voltammetric Analyzer (BAS, West Lafayette, IN, USA). SCE was the reference electrode and a platinum plate was the auxiliary one.

2.3. Immobilization and Measurement Procedure

The surface of the Au and Ag disk electrodes was polished on fine emery paper (Tufbak Durite, P2000), then to a mirror lustre on alumina slurry (0.1 μm, Stuers, Copenhagen, Denmark), rinsed with water, preoxidized in hot Piranha solution, i.e., in a mixture of 97% – 98% H_2SO_4 and 30% H_2O_2, in proportion 3 : 1, for 2 min, quickly rinsed with water, and immediately immersed in a 0.05 mg mL^{-1} HRP solution in 0.01 M phosphate buffer solution, containing 0.15 M NaCl, (PBS), pH 6.0, for 2 h. Ag electrodes were also modified with HRP as above after being polished but excluding any preoxidation step. After thorough washing with PBS, the modified electrodes were mounted in the electrochemical cell and cyclic voltammetric or amperometric studies were performed. PBS at pH 6.0 was used as the electrolyte. In the amperometric experiments steady state currents were measured at an applied potential of −50 mV [9, 16, 18]. For the measurement of the response of the HRP-modified electrode to peroxide the flow-carrier buffer solution containing H_2O_2 was used. The signal difference between the background current and the steady state current in the presence of H_2O_2 was taken as the response signal. For the experiments on mediated ET the flow-carrier buffer solution was used containing both H_2O_2 as well as 5×10^{-4} M catechol. In CV studies, the working solutions were deoxygenated by purging with argon gas for 30 min. The reproducibility of the data was verified by measurements with at least five equivalently prepared electrodes.

3. Results and Discussion

The activity of HRP for the bioelectrocatalytical reduction reaction of H_2O_2 was studied with two metal electrodes belonging to the Group IB elements, viz. silver and gold. These electrode materials display a broad double layer region, where the faradaic currents resulting solely from direct ET from the electrode to HRP can be detected [21]. Since electrostatic interactions between the electrode and the surface of the enzyme can significantly affect both the adsorption and the efficiency of ET [22, 23], optimal conditions for the bioelectrocatalysis of HRP were attained with polycrystalline gold bearing a slightly positive surface charge (potential of zero charge, pzc, close to −80 mV [24]) and with silver electrodes suggesting a strongly positive surface charge (pzc of −600 mV [24]). For this purpose, a chemical oxidative pretreatment of the electrodes was used providing surface charge and/or surface structure favorable for HRP adsorption and direct ET [16–19, 22].

Two forms of HRP, cationic at the pH used (isoelectric point pI = 8.8), were studied – glycosylated nHRP, the more hydrophilic of the two forms of the HRPs, due to the presence of its oligosaccharide overcoat, and recombinant wild type HRP containing an additional 6 histidine tag at the N-termius, N_{His}rHRP [15–17], which is nonglycosylated and thus more hydrophobic. It was therefore supposed that variations in the hydrophilicity/hydrophobicity of the electrode surface could, to some extent, be advantageous when changing from one HRP form to the other. The ability of engineered tags, such as the histidine tag, to chemisorb onto the surface of gold [25] or to produce covalent surface complexes with silver [26, 27] through the histidine imidazol ring was used to improve the adsorption/orientation of the recombinant form at the electrode surface as well. The

bioelectrocatalytical activity of the HRPs was analyzed at pH 6.0, providing an enhanced efficiency of the proton-coupled direct ET, compared to physiological pH 7.4 the more commonly pH used in previous investigations [12, 18, 19].

3.1. Bioelectrocatalytical Reduction of H_2O_2 at HRP-Modified Silver Electrodes

In phosphate buffer solutions containing 0.15 M NaCl, at pH 6.0, the HRP-modified gold electrodes exhibit well-defined polarization waves corresponding to the bioelectrocatalytical reduction of H_2O_2 starting from +700 mV [18]. For the case of silver electrodes the oxidation of a silver surface involving Cl^- anions should be taken into account when one works in the positive potential range. Figure 1 shows the current-voltage curves (CVs) of a bare Ag electrode in a phosphate buffer solution containing 0.15 M NaCl. Significant oxidation-reduction currents corresponding to the redox transformations of the electrode surface equal to:

$$Ag \rightleftarrows Ag^+ + Cl^- \rightleftarrows AgCl \quad (1)$$

are observed when scanning the potential from +80 mV and further in the positive direction up to +600 mV and then back to −500 mV (Fig. 1, curve a). The observed oxidation-reduction currents are so large that they mask the redox behavior of catechol, used as a mediator for some experiments reported on below, within the studied potential range (Fig. 1, curve b). When the potential range involved is restricted to −500 to +50 mV (Fig. 1, curve c), no redox transformations of the electrode surface can be observed, suggesting that within this potential range the bioelectrocatalysis of HRP for H_2O_2 can be studied. No currents corresponding to the direct electrochemical oxidation or reduction of H_2O_2 at bare Ag electrodes were observed within this potential range. Thus, further experiments on peroxidase bioelectrocatalysis were performed within the potential range from +50 to −300 mV, to avoid electrochemically- induced surface oxidation, which interferes with the studies.

The peroxidase bioelectrocatalytic cycle implies the oxidation of the initial HRP (ferriperoxidase) with H_2O_2 to form compound I (E1), representing oxidized HRP and consisting of oxyferryl iron ($Fe^{4+}=O$) and a porphyrin π cation radical, followed by a $2e^-/2H^+$ direct electroreduction of E1 at the electrode surface to the initial HRP state, ferriperoxidase (direct ET), characterized by a heterogeneous ET rate constant, k_s. Therewith, the rate of the overall process is determined either by the ET step, when the ET rate is low compared to H_2O_2 reduction, or by the diffusion of H_2O_2 to the active site of HRP when the ET rate is high. Initially, amperometric detection of H_2O_2 was performed at −50 mV with nonoxidized silver electrodes modified with either nHRP or N_{His}rHRP. Calibration curves for the studied HRP-electrode systems, i.e., dependence of the current response on the concentration of H_2O_2, are presented in Figure 2 (curves 1, 2). As can be seen, the response of the nHRP-modified electrodes to H_2O_2 (residual current +0.15 pA, no reduction of H_2O_2 is registered with bare silver electrodes) through direct ET is extremely low. It is an expected result since the surface charges of the nHRP molecule and silver are the same and electrostatic interactions in this case hamper the adsorption of the enzyme at the electrode surface as was shown previously for gold [22]. The efficiency of direct ET is so low that when increasing the pH to 7.4, the concentration of protons at this pH provides additional kinetic restrictions for the proton-coupled ET reaction [12, 18, 19], with the result that no signal resulting from the bioelectrocatalytical reduction of H_2O_2 could be traced.

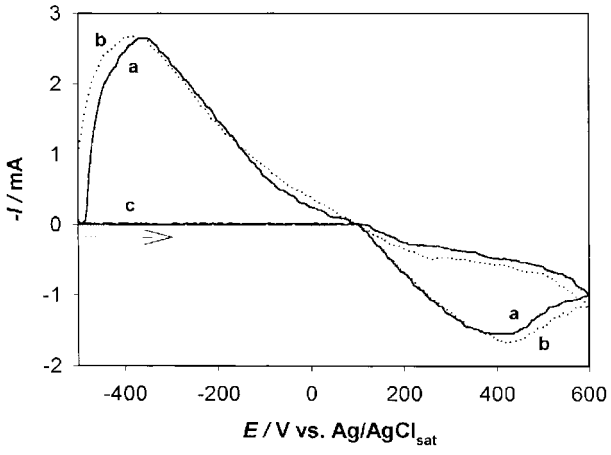

Fig. 1. Cyclic voltammograms of smooth silver electrodes in a, c) 0.01 M KH_2PO_4 containing 0.15 M NaCl, pH 6.0, and b) in the presence of 0.5 mM catechol. Scan rate 50 mV/s, scanning potential range a, b) from −500 to +600 mV and c) from −500 to +50 mV.

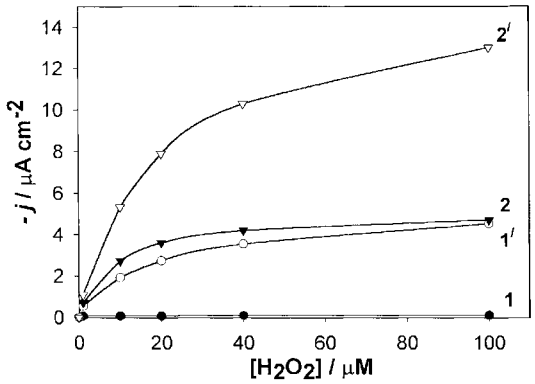

Fig. 2. Dependence of the steady-state current density on the H_2O_2 concentration determined with polished silver electrodes modified with 1,1′) nHRP and 2,2′) N_{His}rHRP; for the case of (1), (2) direct ET and (1′), (2′) mediated ET in the presence of 0.5 mM catechol. The electrodes were placed into a wall-jet cell. Flow rate of the carrier (PBS containing 0.15 M NaCl, pH 6.0) 0.9 mL min^{-1}. Applied potential was −50 mV (vs. Ag | AgCl in 0.1 M KCl).

A significantly higher efficiency of direct ET is achieved with N_{His}rHRP adsorbed on silver (Fig. 1, curve 2). The response of the N_{His}rHRP-modified electrode to H_2O_2 is more than 40 times higher than that obtained with nHRP. This is likely due to both a higher surface coverage with N_{His}rHRP as well as of a more favorable ET orientation of the enzyme molecules, both effects would be the result from the specific interaction of the His-tag with the silver electrode surface [26, 27]. However, it is difficult to elucidate whether the observed responses are determined by a variation of the surface coverage or by the orientation of the different forms of HRP at the electrode surface.

To estimate the fraction of the HRP molecules properly oriented for direct ET, bioelectrocatalytical reduction of H_2O_2 was studied in the presence of a second substrate-mediator (Fig. 2, curves 1′, 2′), catechol being chosen as mediator [17–19]. It had been shown that for glycosylated nHRP adsorbed at graphite/carbon surfaces mediated ET is, as a rule, more efficient than direct ET [9, 10, 12]. This fact was attributed to that less than 50% of all active HRP molecules adsorbed at the electrode surface were in direct ET contact with the electrode.

As can be seen from Figure 2, the efficiency of the biocatalytical reduction of H_2O_2 mediated by catechol increases significantly for the nHRP-modified electrodes (the response to H_2O_2 is 40–45 times higher) as well as for the N_{His}rHRP electrodes (3 times higher). Applying the Koutecky-Levich approach adapted for a flow through wall jet electrochemical cell and applied to the studied HRP-electrode systems [10, 18, 19], the part of the enzyme active in direct ET was estimated comparing the results from mediated versus direct ET. For these evaluations it was assumed that in the presence of a saturating concentration of the mediator (5×10^{-4} M catechol [9, 10, 12, 17–19]) all HRP molecules adsorbed at the electrode participate in mediated ET, whereas in the absence of the mediator only a fraction is available for direct ET [1, 9, 10]. For nHRP adsorbed at nonoxidized silver less than 4% ($3.2 \pm 0.4\%$) of the enzyme molecules were properly oriented to obtain direct ET, whereas close to 70% ($69 \pm 5\%$) for N_{His}rHRP. Even though the total amount of adsorbed peroxidase molecules was not taken into account as it remains unknown, it can be concluded that adsorption of N_{His}rHRP at polycrystalline silver evidently yields a large fraction of enzyme molecules that are correctly oriented through the His tag present at the N-terminus of the enzyme to obtain direct ET, in the same manner as was previously shown for His- and Cys-mutants of HRP adsorbed at gold surfaces [16, 17, 19, 28]. In contrast, direct ET between nHRP and silver is drastically hampered, likely due to surface interactions providing unfavorable adsorption of the enzyme at the electrode surface.

3.2. Bioelectrocatalytical Reduction of H_2O_2 with HRP-Modified Preoxidized Silver Electrodes

Preoxidation of gold was previously shown to crucially affect the adsorption/orientation of HRP molecules at gold electrodes, both for recombinant [16, 17, 19] and native forms of the enzyme [18, 22]. A silver surface bears sufficient positive charges compared with that of gold (due to the difference in the values of the pzc), which is electrostatically unfavorable for the adsorption of cationic forms of HRP. Thus, preoxidation of the surface of the silver electrode was expected to radically affect the adsorption and the efficiency of direct ET of the two forms of HRP at silver, both by inversion of the surface charge of the electrode and by variations of the intrinsic structure resulting from the formation of surface oxides. Preoxidation of the electrodes was performed with hot Piranha mixture, and the preoxidized electrodes were then in the next step modified with the enzyme.

The amperometric responses of the HRP-modified preoxidized silver electrodes to H_2O_2 (residual current from 0 to -1 pA, no electroreduction of H_2O_2 is registered) are presented in Figure 3. Compared with the nonoxidized electrode surfaces (Figure 2) a relative increase in the efficiency of both mediated and direct ET is observed for the nHRP-modified electrodes (curve 1, 1'). As was expected, the adsorption in this case is favored by electrostatic interactions between the positively charged enzyme surface and the preoxidized silver electrode. However, again a very low percentage of nHRP molecules active in direct ET was obtained, in the order of 2–4%. Thus it is evident, that preoxidation of silver provides an increase in the total amount of adsorbed nHRP, but not in a favorable orientation for direct ET, contrary to the results obtained with gold ($75 \pm 20\%$ of nHRP molecules active in direct ET [18]).

As can be seen from Figure 3 (curves 2, 2′), extremely interesting results are obtained with N_{His}rHRP immobilized on the preoxidized silver surface. The amperometric response to H_2O_2 is drastically increased compared with the nonoxidized surface, both in direct and mediated ET. The efficiency of direct ET is now so high, that the detected signal is determined solely by the diffusion of H_2O_2 to the

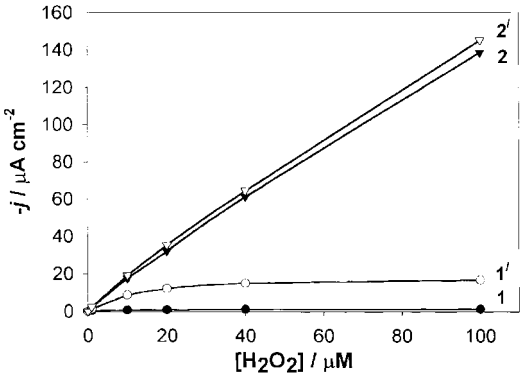

Fig. 3. Dependence of the steady-state current density on the H_2O_2 concentration determined with preoxidized silver electrodes modified with 1,1′) nHRP and 2,2′) N_{His}rHRP; for the case of (1), (2) direct ET and (1′), (2′) mediated ET in the presence of 0.5 mM catechol. The electrodes were placed into a wall-jet cell. Flow rate of the carrier (PBS containing 0.15 M NaCl, pH 6.0) 0.9 mL min^{-1}. Applied potential was -50 mV (vs. Ag | AgCl in 0.1 M KCl).

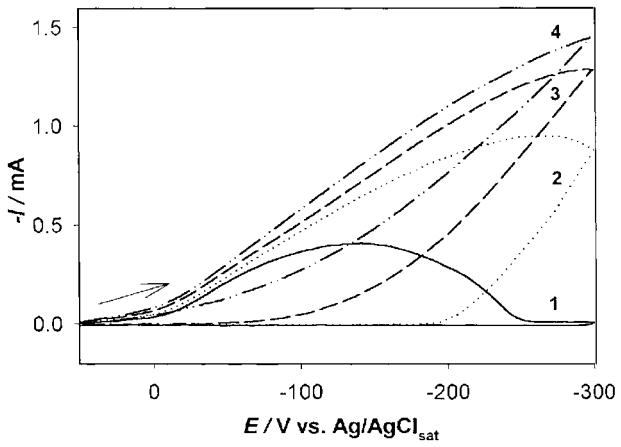

Fig. 4. Voltammograms of a N_{His}rHRP-modified preoxidized silver electrode in 1–4) 0.01 M KH_2PO_4 containing 0.15 M NaCl, pH 6.0, in the presence of 1) 10^{-5} M H_2O_2; 2) 5×10^{-5} M H_2O_2; 3) 10^{-4} M H_2O_2; and 4) 10^{-4} M $H_2O_2 + 5 \times 10^{-4}$ M catechol. Scan rate 500 mV/s.

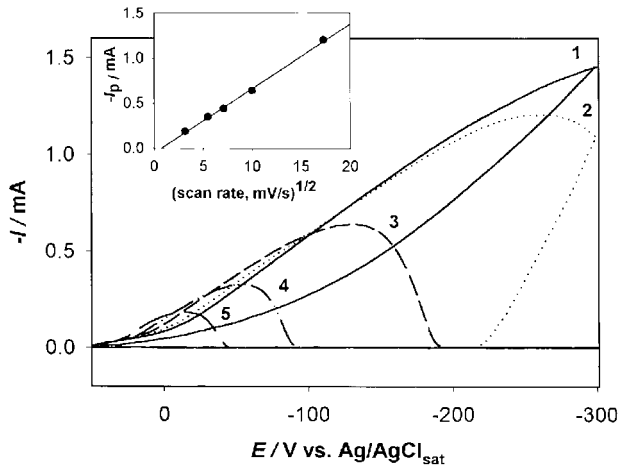

Fig. 5. Voltammograms of a N_{His}rHRP-modified preoxidized silver electrode in 1–5) 0.01 M KH_2PO_4 containing 0.15 M NaCl, pH 6.0, in the presence of 10^{-4} M H_2O_2. Potential scan rates 1) 500; 2) 300; 3) 100; 4) 30 and 5) 10 mV/s. Inset: the dependence of the peak current on the square root of scan rate.

active sites of adsorbed N_{His}rHRP molecules as practically no increase in the reduction current is observed upon the addition of the mediator to the studied system (Fig. 3, curve 2′). The efficiency of direct ET is now comparable with the best results obtained with recombinant forms of HRP adsorbed on gold electrodes [16, 17, 19, 28]. Cyclic voltammograms registered with an N_{His}rHRP-modified preoxidized Ag electrode in the presence of different concentrations of H_2O_2 (see Fig. 4) exhibit well-defined waves reflecting the efficiency of the bioelectrocatalysis of the H_2O_2 reduction reaction. As shown in Figure 4 (and similar to the data presented in Fig. 3), the introduction of the mediator to the studied system only slightly increases the catalytic current of H_2O_2 reduction (Fig. 4, curve 4) or does not increase it at all (data not shown) due to the efficient direct ET reaction. The high efficiency of peroxidase bioelectrocatalysis in the direct ET reaction causes the variation of the shape of the bioelectrocatalytic voltammograms. When increasing the concentration of H_2O_2 from 10^{-5} to 10^{-4} M, the peak-shaped wave obtained for low concentrations, for which strict diffusion limitations prevail, changes to a sigmoidal-shaped wave for high concentrations, where kinetic restrictions start to impair the shape of the voltammograms (compare curve 1 and 3 in Fig. 4). The diffusive nature of the observed currents is also supported by the variation of the shape of the voltammograms for a constant substrate concentration with scan rate shown in Figure 5. As seen a linear relationship between the cathodic peak current and the square root of the potential scan rate in the range 10–300 mV is obtained (see the inset of Fig. 5) giving evidence that the observed bioelectrocatalytical currents are determined by the slow mass transfer of H_2O_2 from the bulk solution to the active sites of HRP [29]. However, the complexity of the bioelectrocatalytical reduction reaction studied (chemical reaction – electron/proton transfer mechanisms) needs more profound quantitative analysis of the data obtained (in progress). Preliminary estimations suggest that the bioelectrocatalytic cycle in this case involves the oxidation of the electrode material in direct ET reaction between the silver surface and the porphyrine π cation radical formed in the reaction between heme and H_2O_2.

Several effects should be considered when discussing the observed phenomena. First, it is well known that the adsorption of His on the silver surface results in the formation of stable surface complexes through the nitrogen of the His imidazole ring [26, 27]. Second, it was previously shown that the binding of the HRP molecule onto the electrode surface through the N- or C-termini provides an extremely favorable orientation of the HRP molecule at the electrode surface suggesting high rates of proton-coupled ET [16, 17, 19, 28]. Thus, preoxidation of the electrode surface, providing surface charge/electrode structure variations, which are necessary for the optimal adsorption, in combination with the discussed binding-orientation factors result in the enhanced ET characteristics in the system N_{His}rHRP-preoxidized silver electrode.

It was of a special interest to estimate quantitatively the efficiency of direct ET in the system N_{His}rHRP-preoxidized silver. This was done using the Koutecky-Levich approach as above and described previously [10, 18, 19]. It should be mentioned that for calculating the kinetic constants the surface concentration of active peroxidase molecules should be known. However, since it is unknown, as a first approximation, 30 pmol of peroxidase per cm^2 of the geometric electrode surface area was used, the same value as was experimentally obtained for the adsorption of N_{His}rHRP on gold using quartz crystal microbalance [16]. The calculations resulted in $95 \pm 5\%$ of the N_{His}rHRP molecules active in direct ET, and a heterogeneous ET constant, k_s, equal to 809 s^{-1}. This ET rate exceeds two-fold the values obtained with the recombinant His-form adsorbed on gold [19]. However, since we do not know exactly

the total amount of the enzyme molecules adsorbed at the preoxidized silver surface, the reported value for the direct ET must be taken with precaution.

3.3. Bioelectrocatalytical Detection of H_2O_2 with HRP-Modified Gold and Silver Electrodes

As presented above, preoxidized silver electrodes modified with N_{His}rHRP exhibit high sensitivity for H_2O_2, determined from the initial slopes of the calibration plots (Fig. 6). The sensitivity to H_2O_2 is close to 2.0 ± 0.2 A M^{-1} cm^{-2} at low H_2O_2 concentrations, and is in fact the same as the sensitivity of the corresponding gold electrodes modified with the same peroxidase form and using the same experimental conditions as for the silver electrodes (Fig. 7, Table 1). This sensitivity is determined solely by the diffusion of H_2O_2 to the active sites of HRP and is the best among the existing peroxidase-modified electrodes described in the literature and based on direct ET [30 and references therein, 31, 32]. Similar results (1.4 ± 0.1 A M^{-1} cm^{-2} at pH 7.4) were obtained earlier only with gold electrodes modified with various recombinant forms of HRP [16]. This sensitivity has already reached a maximal value, and does not change upon the addition of a mediator. The lower detection limit for H_2O_2 obtained with N_{His}rHRP-modified electrodes, determined as the value of that H_2O_2 concentration that gave a clear signal in the linear range of the calibration plots, was 10 nM H_2O_2 both for gold and silver (Table 1). In fact, the lowest H_2O_2 concentration that gave a significant current response corresponding to a signal to noise ratio of three, was even below 10 nM of H_2O_2, but not in the region of linearity.

Considering the sensitivity of the nHRP-modified electrodes, it reaches only 0.3 ± 0.1 A M^{-1} cm^{-2}, at pH 6.0, for gold, under optimal conditions for immobilization and detection, and is negligible for the preoxidized silver electrodes in the H_2O_2 concentration range studied. Additionally a sequential desorption of nHRP from the gold electrode surface was observed, which correlates to approximately a 10% loss of the response signal for 100 μM H_2O_2 per 1 hour, both in direct and mediated ET (Fig. 8, curve 3).

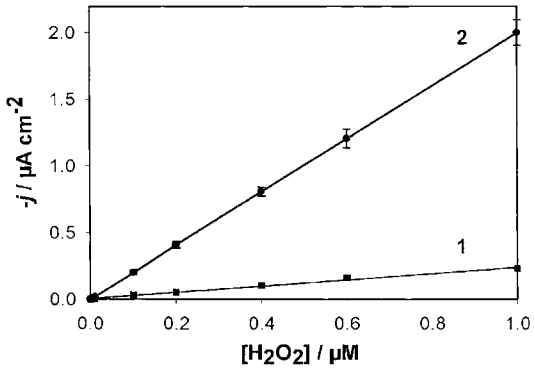

Fig. 7. Calibration plots for H_2O_2 (low concentration range) obtained with preoxidized gold electrodes modified with 1) nHRP and 2) N_{His}rHRP for the case of direct ET. All other conditions as in Figure 2.

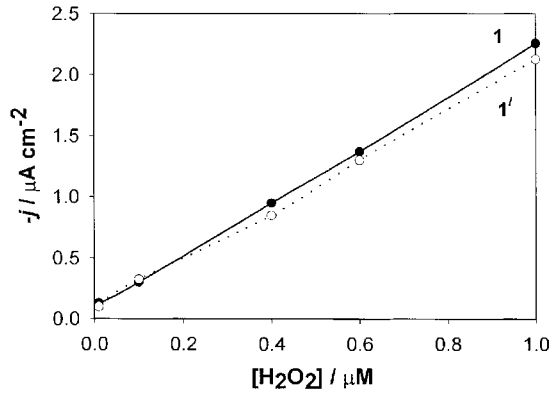

Fig. 6. Calibration plots for H_2O_2 (low concentration range) obtained with preoxidized silver electrodes modified with N_{His} rHRP for the case of 1) direct ET and 1′) mediated ET in the presence of 0.5 mM catechol. All other conditions as in Figure 2.

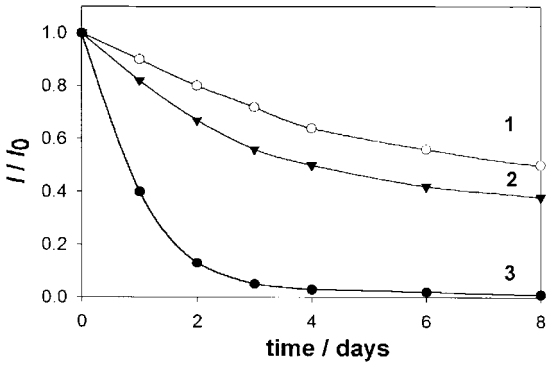

Fig. 8. Stability of the amperometric response to 100 μM H_2O_2 for preoxidized gold electrodes modified with 1,2) N_{His}rHRP and 3) nHRP in 1,3) direct and 2) mediated ET in the presence of 0.5 mM catechol. Data are normalized to the initial current response. Other conditions as in Figure 2.

Table 1. Bioelectrocatalytical detection of H_2O_2 based on direct ET between gold/silver electrodes and HRPs.

Electrode modification	*Conditions[a]*	*Sensitivity (A M^{-1} cm^{-2})*	*Detection limit (M)*
Au_{ox}/nHRP (1300 U/mg)	pH 6.0, −50 mV	0.3 ± 0.1	10^{-7}
Au_{ox}/N_{His}HRP (820 U/mg)	pH 6.0, −50 mV	2.0 ± 0.1	10^{-8}
Ag_{ox}/N_{His}HRP (820 U/mg)	pH 6.0, −50 mV	2.0 ± 0.2	10^{-8}

[a] Potentials are cited with respect to Ag | AgCl reference electrode.

In contrast, the stability of the signal achieved with N_{His}rHRP adsorbed on gold is significantly higher, though a sequential, relatively slow desorption of the enzyme from the electrode surface should be considered as well (Fig. 8, curves 1, 2). The stability of the response of the modified electrodes was tested with the same electrode operated daily for 2 h and stored in buffer at 4 °C between days. Thus, despite some similarities in the adsorption and bioelectrocatalytical activity characteristics of N_{His}rHRP on preoxidized gold and silver electrodes, evidently resulting from the oriented adsorption through the His tag, the adsorption and direct ET characteristics for nHRP at these two surfaces are principally different. This conclusion is most pronounced when one compares the percentage of nHRP molecules active in direct ET when changing from gold ($75 \pm 20\%$) to silver (less than 4%).

Thus, both silver and gold surfaces virtually reveal a strong effect of the oriented immobilization of N_{His}rHRP on the rates of the direct heterogeneous ET. However, the enhanced electronic communication between the heme containing active site of N_{His}rHRP and a silver surface has a dramatic impact on the bioelectrocatalytic activity of HRP in direct ET. While the N_{His}rHRP-modified gold electrodes exhibit a satisfactory stability both in direct and mediated ET (Fig. 8), a pronounced decrease in the response of the N_{His}rHRP-modified silver electrodes to H_2O_2 in direct ET is observed (Fig. 9). Contrary to the simultaneously decreasing activity of the N_{His}rHRP-modified gold electrodes both in direct and mediated ET, connected with a possible gradual removal of the enzyme from the electrode surface, a catastrophic 90% inhibition of the activity of the N_{His}rHRP-modified silver electrodes in direct ET (but not mediated one!) is obtained within the first 2 days of electrode operation. Since the stability of the N_{His}rHRP-silver electrodes in mediated ET, after the initial 10 – 40% fall (dependent on the H_2O_2 concentration), stayed the same during the whole test period (up to three months), then the rapid loss of the electrode activity in direct ET is likely to be connected with enzyme inactivation in the course of the direct ET reaction. Thus, it could be supposed that enhanced interactions between the silver surface and N_{His}rHRP, resulting initially in a highly efficient ET from the electrode to the active site of HRP would with time result in rapid inactivation of the enzyme. However, a relatively high stability and sensitivity of the amperometric response of the N_{His}rHRP-modified preoxidized silver electrodes to H_2O_2 in mediated ET may be considered extremely promising for the development of HRP-based biosensors for the detection of different types of phenolics, as well as of some other classes of environmental pollutants included in priority pollutant list of the EEC and US-Environmental Protection Agency and representing potential substrates for peroxidases [33, 34].

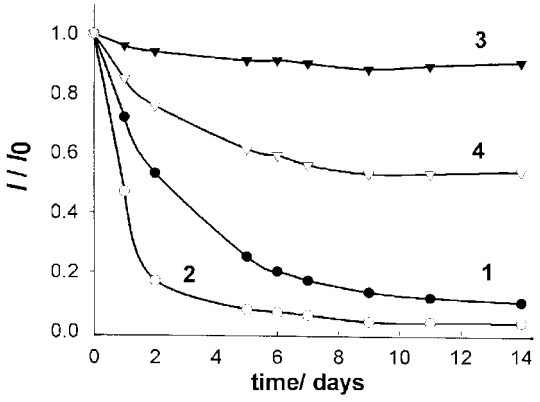

Fig. 9. Stability of the current signal in time for preoxidized silver electrodes modified with N_{His}rHRP in 1, 2) direct ET and 3, 4) mediated ET. Data are normalized to the initial current response The concentration of H_2O_2: 1, 3) 1 μM, 2, 4) 100 μM. Other conditions as in Figure 2.

4. Conclusions

Two metal electrode materials, specifically, polycrystalline silver and gold electrodes were studied and compared with respect to the efficiency of bioelectrocatalytical reduction of H_2O_2 based on direct ET between the electrode surface and the heme containing active site of HRP. From the results shown above it can be concluded that:

Gold electrodes are characterized by 1). A high efficiency of direct ET for recombinant nonglycosylated N_{His}rHRP; 2). A high percentage of both native and recombinant forms of HRP properly oriented for direct ET; 3). A high sensitivity for H_2O_2 attained with N_{His}rHRP resulting from strong effects of deglycosylation/surface mutations on the efficiency of direct ET. However, a gold surface needs obligatory preoxidation for better adsorption of HRPs, as well as for efficient direct ET.

Silver electrodes are characterized by 1). A high efficiency of direct ET for N_{His}rHRP, evidently connected with the effect of deglycosylation and the presence of the His tag, providing an improved adsorption of this form of HRP on silver, 2). A high percentage of properly oriented molecules of the recombinant form of HRP for direct ET, 3). A low efficiency of direct ET for the glycosylated native form, as a result from an extremely low percentage of enzyme molecules properly oriented for direct ET; 4). A high sensitivity for H_2O_2 with N_{His}rHRP, as a result of the strong effects of deglycosylation/surface mutations on the efficiency of direct ET, as well as on the amount of molecules of HRP properly oriented for direct ET. A silver surface needs obligatory as well preoxidation to obtain efficient direct ET between the electrode and adsorbed N_{His}rHRP. However, a rapid loss of the bioelectrocatalytic activity of N_{His}rHRP in direct ET is observed (not in mediated one), likely to be connected with the direct ET inactivation of the enzyme molecules involved in direct electronic communication with silver.

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6. References

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