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Effect of pH on direct electron transfer in the system gold electrode–recombinant horseradish peroxidase

Elena Ferapontova [a,*], Lo Gorton [b]

[a] *Department of Analytical Chemistry, Faculty of Pharmacy, University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain*
[b] *Department of Analytical Chemistry, University of Lund, P.O. Box 124, SE 221 00 Lund, Sweden*

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Abstract

The effect of pH on the kinetics of the bioelectrocatalytic reduction of H_2O_2 catalysed by horseradish peroxidase (HRP) has been studied at −50 mV vs. Ag|AgCl on HRP-modified Au electrodes placed in a wall-jet flow-through electrochemical cell. Native HRP (nHRP) and a nonglycosylated recombinant form containing a six-histidine tag at the C-terminus, C_{His}rHRP, produced by genetic engineering of nonglycosylated recombinant HRP using an *E. coli* expression system, have been used for adsorptive modification of Au electrodes. A favourable adsorption of C_{His}rHRP on preoxidised Au from a protein solution at pH 6.0 provided a high and stable current response to H_2O_2 due to its bioelectrocatalytic reduction based on direct (mediatorless) electron transfer (ET) between Au and the active site of HRP. The heterogeneous ET rate constant, k_s, calculated from experimental data on direct ET, on mediated ET in the presence of catechol as well as from microbalance data, increased more than 30 times when changing from nHRP to C_{His}rHRP. For both forms of HRP, the increasing efficiency of bioelectrocatalysis with increasing $[H_3O^+]$ was observed. The values of the apparent k_s between C_{His}rHRP and Au changed from a value of $12 \pm 2\ s^{-1}$ in PBS at pH 8.0 to a value of $434 \pm 62\ s^{-1}$ at pH 6.0; a similar k_s–pH dependence was also observed for nHRP, providing the possibility to consider the reaction mechanism involving the participation of a proton in the rate-determining step of the charge transfer.

Keywords: Bioelectrocatalysis; Recombinant horseradish peroxidase; Heterogeneous direct electron transfer; Proton transfer; Au electrode

1. Introduction

Reactions of coupled electron and proton transfer are of a great interest for investigation of the fundamentals of enzymatic redox catalysis and of the function of biological ET/respiratory chains implying proton transfer (PT) as well (Refs. [1,2] and references therein). Considering the kinetics of these redox-coupled reactions, the preceding chemical protonation, ET, PT itself, or coupled ET and PT, etc., may represent the rate-determining step of the overall reaction. Thus, the elucidation of a proper mechanism of the redox process enables to control and direct its efficiency in the most suitable manner.

Direct ET between the active site of horseradish peroxidase (HRP), a heme-containing redox enzyme (hydrogen peroxide oxidoreductase, EC 1.11.1.7), and the electrode surface has been extensively studied on a variety of electrode materials, e.g., carbon, Au, SnO_2, etc., and was shown to be kinetically slow (Refs. [3–6]. Kinetic models of the bioelectrocatalytical reduction of H_2O_2 catalysed by peroxidase, similar to the mechanism of enzymatic catalysis, consider the oxidation of the initial HRP with H_2O_2 to E1, representing oxidised HRP and consisting of oxyferryl iron ($Fe^{4+}=O$) and a porphyrin • cation radical ($P^{\bullet+}$) and further direct electroreduction of E1 at the electrode surface to the initial HRP state, ferriperoxidase, the electrode being considered as an electron donor [3]:

$$E(Fe^{3+}) + H_2O_2 \xrightarrow{k_1} E1(Fe^{4+}=O,\ P^{\bullet+})H_2O \quad (1)$$

$$E1(Fe^{4+}=O,\ P^{\bullet+}) + 2e^- + 2H^+ \xrightarrow{k_s} E(Fe^{3+}) + H_2O. \quad (2)$$

According to the peroxidase catalytic cycle in homogeneous phase, reduction of E1 to $E(Fe^{3+})$ proceeds by two one-electron/proton transfer steps [7]:

$$E1(Fe^{4+}=O,\ P^{\bullet+}) + e^-(S) + H^+ \xrightarrow{k_2} + E2(Fe^{4+}=O) + S\bullet \quad (3)$$

$$E2(Fe^{4+}=O) + e^-(S) + H^+ \xrightarrow{k_3} E(Fe^{3+}) + S\bullet + H_2O \quad (4)$$

* Corresponding author. Fax: +34-91-885-4666.
E-mail address: elena.ferapontova@uah.es (E. Ferapontova).

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In the first step, the porphyrin π cation radical is reduced giving an intermediate compound E2 (E2 has an additional proton compared to E(Fe^{3+}) and E1), the latter is reduced in the step (4) to the initial peroxidase state, ferriperoxidase. The electron donor S may be also a proton donor (PD), as in the case of phenolics, or may display only electron donor properties, as in the case of iodide. From a great number of works on the kinetics of peroxidase oxidation of different substrates it follows that reaction (4) of the oxidation of S by E2 is the limiting step of the overall process ($10k_3 \cdot k_2$) [7]. As it is difficult currently to distinguish whether direct electroreduction of E1 to E proceeds stepwise through E2, the evaluation of the kinetics of E1–E(Fe^{3+}) reduction is considered in the general form (2). However, direct electroreduction of E2 to HRP may be regarded as a limiting step of reaction (2) similar to stage (4) of the enzymatic catalysis [3]. Taking into account that the electrode can display only electron donor properties, it could be supposed that the reduction of E1 to E(Fe^{3+}) may also proceed in a single two-electron transfer step, as in the case of iodide or hydrosulphite acting as two-electron donors [7], though data in Refs. [8,9] do not support the idea of a two-electron cooperative process. More data are required to judge on the kinetics of E1 electroreduction to the initial ferriperoxidase state, the study of the role of protons being of a particular interest.

As can be seen, the rate-determining direct electroreduction of E1 (direct ET) presented in general form (2) implies the participation of protons in the reaction cycle and may be referred to the type of ET reactions accompanied by PT. The necessary protons needed to complete the reaction must therefore be taken from the surrounding environment. Depending on the experimental conditions (pH, potential, solution composition, electrode material, etc.) this may cause kinetic restrictions, as the electrode is not prone to either accept or donate protons, and the rate-determining step may include the participation of a proton. Since the addition of protons in the reaction zone may provide a favoured proton-coupled ET pathway, the effect of the pH on the kinetic regularities of heterogeneous ET in the system HRP–Au electrode was studied.

2. Experimental

2.1. Instrumentation

Amperometric measurements with polycrystalline Au disk electrodes (CH-Instruments, Austin, TX, USA, 0.031 cm^2) were performed at room temperature (22 ± 1 °C) in a standard three-electrode wall-jet electrochemical flow through cell [4] connected to a potentiostat AUTOLAB (PGSTAT30, Eco Chemie, Netherlands) equipped with GPES 4.8 software. An Ag|AgCl|0.1 M KCl and a Pt wire were used as the reference and auxiliary electrodes, respectively. The distance between the nozzle and the working electrode was about 0.8 mm. The flow of the solutions was maintained by a peristaltic pump MINIPULS 2 (Gilson, Villiers-le-Bel, France).

2.2. Materials

Native HRP [nHRP, isoenzyme C, 1500 U mg^{-1} (ABTS)] was from Boehringer Mannheim (Mannheim, Germany), other reagents of analytical grade were provided by Sigma (St. Louis, USA). Recombinant wild type HRP containing a six-histidine tag at the C-terminus [C_{His}rHRP, 800 U mg^{-1} (ABTS)] was produced in *E. coli* strain BL21(DE3)pLysS transformed with the appropriate pET based expression vectors (Ref. [5] and references therein) and supplied by Professor Alexey Egorov of the M.V. Lomonosov Moscow State University. Deionised Milli-Q water (Millipore, Bedford, MA, USA) was used throughout this work.

2.3. Electrode modification and measurements

The surface of the Au 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, preoxidised in a hot Piranha solution for 2 min, quickly rinsed with water and immediately immersed in a 0.02-mg ml^{-1} HRP solution in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 6.0, for 2 h. After thorough washing with PBS, the modified electrodes were mounted in the wall-jet cell and steady state currents were measured at an applied potential of − 50 mV versus Ag|AgCl [3], PBS in the pH range from 6.0 to 8.0 being used as the electrolyte. The flow rates used were 1.1, 0.9, 0.7, 0.5 and 0.33 ml min^{-1}. The reproducibility of the data was verified by measurements with at least three equivalently prepared electrodes. An extensive 20 min sonication of the electrodes prior to the preoxidation and immobilisation procedure, performed to exclude the effect of alumina left after polishing, was shown not to affect the final results.

3. Results and discussion

The effect of pH on the kinetics of the bioelectrocatalytic reduction of H_2O_2 was studied with C_{His}rHRP and nHRP directly adsorbed on the bare Au surface. The favourable adsorption of HRPs on preoxidised Au from a protein solution in PBS, pH 6.0, provided a substantial current response to H_2O_2 due to its bioelectrocatalytic reduction based on direct ET between Au and the active site of HRP, deglycosylation and the histidine tags additionally increased the strength of binding of the enzyme with Au and therewith the stability of the signal, as was shown previously [5], making possible to perform kinetic studies at different pHs. Data on amperometric detection of H_2O_2 with HRP modi-

fied electrodes at different pHs are shown in Fig. 1. From these data, it is obvious that the efficiency of direct ET from Au to both C_{His}rHRP and nHRP increases essentially when increasing the H_3O^+ concentration. The enhancement of the direct ET rate in the system C_{His}rHRP–Au electrode in the pH range between 7.0 and 6.0 is so high that the introduction in the system of a mediator (5×10^{-4} M catechol) does not increase the current response, which is determined at this state solely by the diffusion of H_2O_2 at concentrations up to 40 μM (Fig. 2). To separate the kinetic and diffusion parts of the measured current, amperometric measurements were performed at different flow rates and data were plotted in Koutecky–Levich (KL) coordinates adapted for the flow-injection system according to Eq. (5) (the case of direct ET) and Eq. (6) (the case of mediated ET) [4]:

$$1/I = 1/I_k + 1/I_{lim} = 1/nFE_{DET}(1/(k_1 c_{H_2O_2}) + 1/k_s) + 1/0.898nFc_{H_2O_2}D^{2/3}A^{3/8}v^{-5/12}V^{3/4}a^{-1/2}, \quad (5)$$

$$1/I = 1/I_k + 1/I_{lim} = 1/2n_1FE_{MET}(1/k_1 c_{H_2O_2} + 1/k_3[S]) + 1/0.898nFc_{H_2O_2}D^{2/3}A^{3/8}v^{-5/12}V^{3/4}a^{-1/2}. \quad (6)$$

Here, I is the measured current of bioelectrocatalytic reduction of H_2O_2 on HRP-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_2O_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 $0.5n$ for catechol; F is the Faraday constant; $c_{H_2O_2}$ is the bulk concentration of H_2O_2; D is the H_2O_2 diffusion coefficient (1.6×10^{-5} cm^2 s^{-1}) [4]; 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 mol) participating in direct ET; E_{MET} is the total amount of active enzyme (in mol) on the electrode surface involved in the electrode reaction; k_s is the heterogeneous ET rate constant for direct ET; k_1 is the rate constant for H_2O_2 enzymatic reduction; [S] is the mediator concentration; k_3 is the rate constant of reaction (7) (mediated ET):

$$E2(Fe^{4+}=O) + S + H^+ \xrightarrow{k_3} E(Fe^{3+}) + S\bullet + H_2O, \quad (7)$$

which represents the rate-determining step of E1 reduction by some electron donor S (other than the electrode) to the initial peroxidase state through the formation of E2, similar to enzymatic catalysis (reaction (4)) [3,4]. The formed oxidised donor S• is then electrochemically reduced by the electrode. From the experiments on mediated ET the part of the enzyme active in direct ET can be calculated as it is assumed that in the presence of a saturating concentration of a mediator (5×10^{-4} M catechol; [3,4,6]) 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 [3,4]. The total amount of HRP adsorbed at the Au electrode, E_{MET}, was assumed to be 20 pmol cm^{-2} in the case of nHRP and 30 pmol cm^{-2} in the case of C_{His}rHRP (quartz crystal microbalance data) [5].

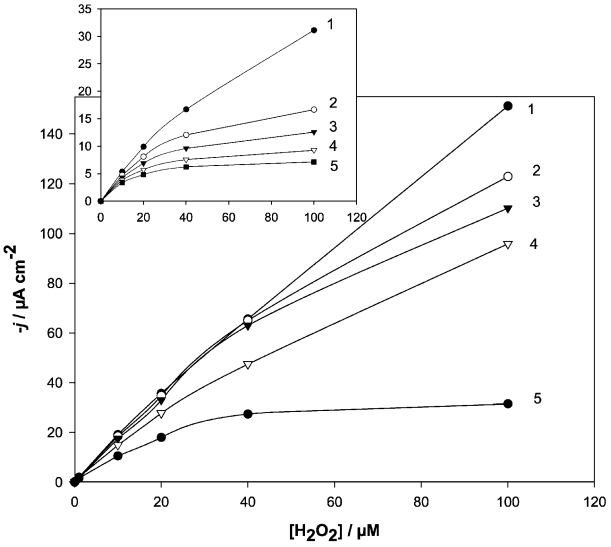

Fig. 1. Dependence of the steady-state current density on the H_2O_2 concentration determined with the gold disk electrodes modified with C_{His}rHRP at pH (1) 6.0, (2) 6.5, (3) 7.0, (4) 7.4, (5) 8.0. In the insert—data for native HRP. The electrodes were placed into a wall-jet cell: flow rate of the carrier (PBS containing 0.15 M NaCl) 0.9 ml min^{-1}. Applied potential was −50 mV vs. Ag|AgCl in 0.1 M KCl.

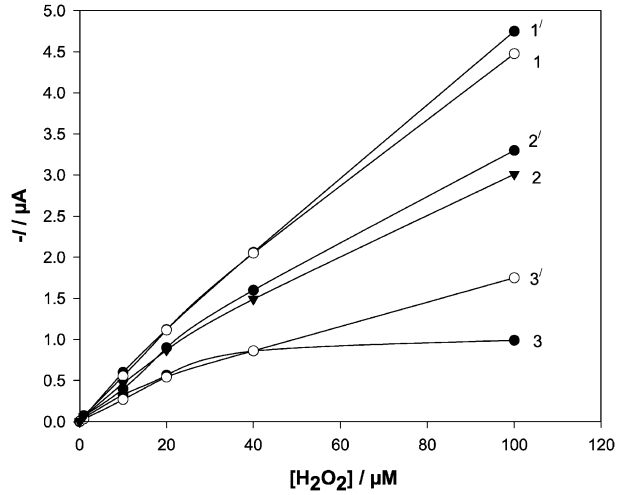

Fig. 2. Dependence of the steady-state currents on the H_2O_2 concentration determined with the gold disk electrodes modified with C_{His}rHRP at pH (1, 1′) 6.0, (2, 2′) 7.4, (3, 3′) 8.0. Curves (1′, 2′, 3′) correspond to mediated ET in the presence of 5×10^{-4} M catechol. All the rest conditions as in Fig. 1.

From the intercepts of the KL plots (Eqs. (5) and (6), Fig. 3) with the y-axis the $1/I_k$ values at different pHs were obtained and plotted versus the reciprocal H_2O_2 concentrations (Fig. 3). The data obtained show a linear dependence, from the slope of these plots k_1 can be determined; from the intercepts with the y-axis the value of k_s was evaluated in the case of direct ET and k_3 in the case of mediated ET (Eqs. (5) and (6), Fig. 3). The ratio of the slopes in the presence and in the absence of a mediator gave the ratio between E_{DET} and E_{MET} ($90 \pm 10\%$ for C_{His}rHRP and $75 \pm 20\%$ for nHRP, in good agreement with previous findings [6]), making possible to calculate the values of k_s and giving evidence of a tight and intimate contact of the HRP molecules with the preoxidised Au surface under the conditions of immobilisation from solutions at pH 6.0 where favoured electrostatic and hydrophobic interactions between the enzyme molecule and the electrode surface are achieved.

Equal slopes (within experimental error) of curves 1–5 presented in Fig. 3 corresponds to the same value of k_1 ($0.53 \pm 0.02 \times 10^6$ $M^{-1}s^{-1}$ for C_{His}rHRP and $0.13 \pm 0.02 \times 10^6$ $M^{-1}s^{-1}$ for nHRP, data not shown) at different pHs and implies that H_3O^+ does not affect the rate of reaction (1). In contrast, the apparent heterogeneous rate constant of direct ET to C_{His}rHRP increases more than 40 times when changing the pH from 8.0 (12 ± 2 s^{-1}) to 6.0 (434 ± 62 s^{-1}) and 10 times—in the case of nHRP (from 1.2 ± 0.2 to 11.6 ± 3.8 s^{-1}) (Fig. 4). It can be followed from Fig. 4 that k_s–$[H_3O^+]$ dependence approaches a linear relationship at pHs lower than 7.4, when H_3O^+ can be considered as a PD, with slopes of $11.5 \pm 0.4 \times 10^6$ for nHRP and $396.7 \pm 13.9 \times 10^6$ M^{-1} s^{-1}—for C_{His} rHRP and with the values of k_s extrapolated to $[H_3O^+]=0$ (when solvent (water)

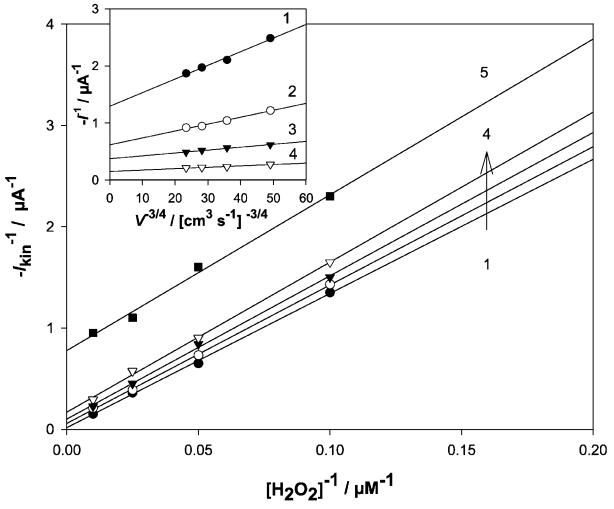

Fig. 3. Dependence of the kinetically limited currents on the H_2O_2 concentration in inverted coordinates for the gold disk electrodes modified with C_{His}rHRP at pH (1) 6.0; (2) 6.5; (3) 7.0; (4) 7.4; (5) 8.0, direct ET. $92 \pm 5\%$ of HRP molecules active in direct ET; k_1 equals (0.53 ± 0.03) 10^6 M^{-1} s^{-1}. In the insert–KL plots at pH 6.0, direct ET, for (1) 10; (2) 20; (3) 40; (4) 100 μM H_2O_2.

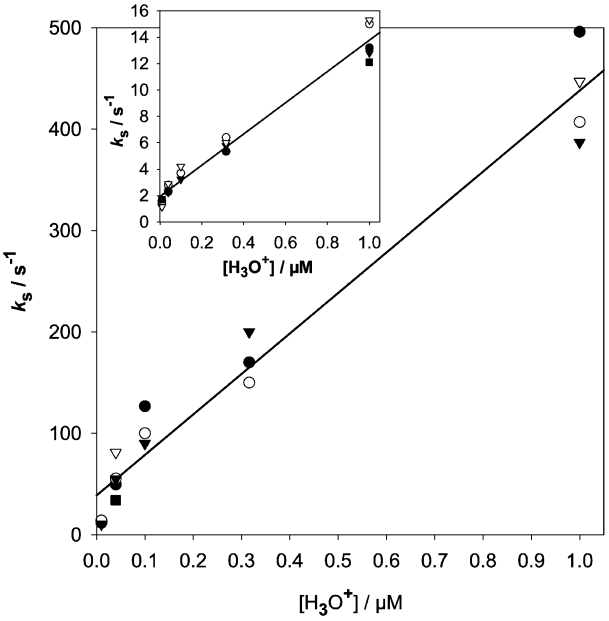

Fig. 4. Dependence of the apparent heterogeneous rate constant of direct ET between gold and C_{His}rHRP on the concentration of hydroxonium ions. In the insert—data for nHRP.

molecules should display PD properties) corresponding to 1.9 and 40.5 s^{-1}, respectively. The data obtained indicates the following.

(1) Deglycosylation of HRP enhances the efficiency of direct ET. This fact is likely to be connected with a decreased ET distance between the electrode and the active site of the enzyme (as the structure of the active site of HRPs under study is virtually the same).

(2) $[H_3O^+]$ strongly affects the rate of reaction (2), thus, controlling I_k, the k_s–$[H_3O^+]$ dependence being close to linear under the conditions when H_3O^+ can be regarded as a PD. Thus, it can be assumed that at a considered constant potential the kinetic regularities of direct ET in the system HRP–Au electrode can be presented in the following general form (when H_3O^+ is a PD):

$$I_k = nFk_s^{\bullet}[H_3O^+]E_{DET}, \quad (8)$$

where $k_s^{\bullet}$ corresponds to the slope of the k_s–$[H_3O^+]$ dependence, the obtained values approaching the values of k_3 in the most efficient reactions of peroxidase oxidation (reaction (4)) of p-cresol and o-phenylendiamine (5×10^7 $M^{-1}s^{-1}$) obtained in stop-flow mode [7].

(3) The oligosaccharide overcoat present in nHRP not only makes direct ET less efficient, but also hinders PT to the active site of the enzyme (obvious when comparing the slopes of k_s–$[H_3O^+]$ dependencies).

Thus, it can be assumed that at a considered overvoltage the rate of electroreduction of the oxidised form of HRP is likely to be controlled by a coupled electron and proton transfer from some PD (in the considered case—H_3O^+)

localised in the reaction zone. As a result, the increased efficiency of direct ET with increasing $[H_3O^+]$ is achieved.

4. Conclusions

Studies of the effect of pH on the direct ET between Au electrodes and adsorbed HRP demonstrated that changing $[H_3O^+]$ from pH 8.0 to 6.0 does not affect the rate of the bioelectrocatalytic reduction of H_2O_2 (k_1), but drastically increases the rate of direct ET between the electrode and HRP (k_s). The dependence of k_s on $[H_3O^+]$ gave evidence that the rate-determining step of ET is likely to occur through a coupled proton and ET. Deglycosylation of nHRP enhances direct ET and, in parallel, facilitates PT to the active site of the enzyme. Further studies of the effect of PDs of different nature on the kinetics of the electroreduction of HRP at different overvoltages is necessary to reveal a detailed mechanism of ET between the active site of HRP and the electrode surface [10].

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