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Direct charge transfer to horseradish peroxidase revisited using a glassy carbon electrode

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ABSTRACT

This study reports the use of cyclic voltammetry (CV), hydrodynamic voltammetry, and electrochemical impedance spectroscopy (EIS) to investigate horseradish peroxidase (HRP) direct adsorption onto a glassy carbon (GC) surface and the adsorption of HRP in the presence of polymyxin (PM) forming Nafion®-covered HRP-PM films on a GC surface. The bioelectrocatalytic behavior of these electrodes toward H_2O_2 and O_2 reduction was also studied. The electrochemical reaction rate constant of HRP-PM/Nafion films was comparable to that of bare GC electrodes containing HRP and carbon nanotubes or HRP and graphene in their films. GC/HRP-PM/Nafion electrodes were sufficiently bioelectrocatalytic for H_2O_2 and O_2 reduction (even at pH 7 and 37 °C)—a feature that suggests their possible use as negative electrodes in biofuel cells. From EIS experiments, it was possible to estimate the resistance to electron hopping (R_{eh}) between heme Fe^{3+}/Fe^{2+} redox centers in HRP enzymes and between these and H_2O_2 or O_2 molecules.

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1. Introduction

A vast body of literature focuses on the horseradish peroxidase (HRP) enzyme, given its numerous applications [1] in diagnostic assays [2–5], nucleic acid analysis [6–8], biosensors for H_2O_2 [9–13], bioremediation [14,15], polymer synthesis [16], and other biotechnological processes [17]. One common method used for HRP immobilization at an electrode surface is electrostatic physical adsorption [14,18], which involves hydrophobic/hydrophilic effects [10,19,20]. Adsorption is often dependent on the inclusion of polyions. In particular, binding of proteins having negatively charged surfaces is greatly enhanced by the presence of polyamines, such as neomycin or polymyxin (PM), with very similar interactions between protein and electrode surface to those occurring at biological membranes [21]. In commercial preparations, HRP is usually available in a neutral form termed isoenzyme C (HRP-C) [18].

Pyrolytic graphite edge (PGE) electrodes are reported to have an appropriate surface—rich in acidic C—O functionalities—for adsorbing a variety of proteins. Also, maximum coverage achieved at PGE electrodes is one or two orders of magnitude greater than normally expected for typical electrode surfaces [21]. In terms of electrochemical phenomena, edge planes exhibits considerably faster electrode kinetics than basal planes [22]. Surfaces of polished glassy carbon (GC)—an electrochemically important variant of the graphite structure—typically have an 8–15% surface O/C ratio, and the GC structure is generally arranged as randomly intertwined ribbons of graphitic planes—a feature casting significant uncertainty over the detailed microstructure of this material [23].

In a review of the role played by electrode materials in the efficiency of direct (non-mediated) bioelectrocatalytic reduction of H_2O_2 catalyzed by HRP [18], the variability in direct peroxidase bioelectrocatalysis from carbon/graphite to metal electrodes and oxides, as well as at modified electrodes, was examined with regard to the variations in adsorption/orientation of peroxidase at the electrodes, interfacial electron transfer rates, and catalytic mechanism.

Few studies report results of HRP adsorption on GC electrodes. However, direct electrochemical changes of catalytically active HRP adsorbed on GC electrodes from HRP-DMSO and HRP-formamide solutions has been obtained in aqueous phosphate buffer (pH 7.0) at a surface formal potential ($E^{\circ\prime}$) of −0.365 V *vs.* Ag/AgCl and an apparent electron transfer rate constant of 0.655 s^{-1} [24].

Examples involving other surfaces are reported much more frequently. In one study [25], films composed of dimyristoylphosphatidylcholine and *Mycobacterium tuberculosis* catalase-peroxidase (KatG), several peroxidases, myoglobin, or catalase showed reversible Fe^{3+}/Fe^{2+} conversion on pyrolytic graphite electrodes and exhibited catalytic current for H_2O_2 and O_2. The $E^{\circ\prime}$ value of heme Fe^{3+}/Fe^{2+} conversion for HRP-C was around −0.26 V

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vs. SCE at pH 5. Another investigation [26] reported the electrochemical behavior of Fe^{3+}/Fe^{2+} conversion in HRP entrapped within a solid matrix—*i.e.*, a tributylmethyl phosphonium chloride polymer (TBMPC)-bound anionic exchange resin (polystyrene crosslinked with 1% divinylbenzene)—using a PGE. This membrane-entrapped HRP takes part in a rapid one-electron transfer reaction at the PG electrode, even in the absence of mediators, at a calculated redox formal potential of around −0.27 V *vs*. SCE at pH 5 [26]. A further study [1] demonstrated deposition of a HRP monolayer onto an electrode surface using biotinylation of a carbon-based screen-printed electrode surface by means of a sacrificial biotinylated immunoglobulin, followed by anchoring of an avidin–enzyme conjugate. Cyclic voltammetry proved an efficient means of examining the catalytic reduction of H_2O_2 at such HRP monolayer electrodes in the presence of $[Os^{III}(bpy)_2pyCl]_2^+$ (where *bpy* stands for bipyridine and *py* for pyridine) as a one-electron reversible cosubstrate. The mediating redox couple $[Os^{III}(bpy)_2pyCl]_2^+$ in the solution was centered at around 0.21 V vs. SCE.

Peroxidases—a class of heme enzymes found in bacteria, fungi, plants, and animals—exploit the reduction of hydrogen peroxide to catalyze a number of oxidative reactions, involving a wide variety of organic and inorganic substrates [27]. The catalytic cycle of heme peroxidases is based on three consecutive redox steps, involving two high-valent intermediates (Compounds I and II) responsible for substrate oxidation. The thermodynamics and kinetics of the catalytic cycle are therefore influenced by the reduction potentials of three redox couples—namely, Compound I/Fe^{3+}, Compound I/Compound II, and Compound II/Fe^{3+}. In particular, the oxidative power of heme peroxidases is controlled by the (high) reduction potential of the latter two couples. Moreover, the rapid H_2O_2-mediated two-electron oxidation of peroxidases to Compound I requires a stable ferric state under physiological conditions, which depends on the reduction potential of the Fe^{3+}/Fe^{2+} couple [27]. Battistuzzi et al. [27] provide an overview of the data available on the redox properties of Fe^{3+}/Fe^{2+}, Compound I/Fe^{3+}, Compound I/Compound II, and Compound II/Fe^{3+} couples in native and mutated heme peroxidases.

Native ferric HRP (Fe^{3+}, ferriperoxidase) can be directly reduced at the electrode surface to a ferrous enzyme (Fe^{2+}, ferrous peroxidase) which involves $1e^-$ as well as protonation of the reduced Fe^{2+} form [18]. The ferrous form of HRP (but not ferriperoxidase itself) is capable of binding molecular oxygen, which results in formation of Compound III (a ferrous form of HRP containing an additional proton, relative to the native enzyme, plus dioxygen). Ferrous HRP and Compounds II and III have therefore an additional proton, relative to native ferriperoxidase and Compound I [18]. The bioelectrocatalytic reduction of molecular dioxygen and H_2O_2 taking place at the potentials at which Fe^{3+}/Fe^{2+} peroxidase transformation occurs may result either from the reaction between ferrous HRP and Compound III or from the reaction of ferrous enzyme with H_2O_2 [18]. However, the reaction between ferrous peroxidase and oxygen-bound Compound III yields the original ferriperoxidase, which can be successively re-reduced at the electrode surface back to its ferrous form [18]. Compound II can be reduced at the surface electrode to the initial ferriperoxidase state [18].

The main purpose of the present study was to use cyclic voltammetry (CV), hydrodynamic voltammetry, and electrochemical impedance spectroscopy (EIS) to investigate direct adsorption of HRP on a GC surface and the adsorption of HRP in the presence of polymyxin (PM) forming Nafion®-covered HRP-PM films on a GC surface. A further aim was to describe the bioelectrocatalytic behavior of modified GC electrodes toward H_2O_2 and O_2 electroreduction.

2. Experimental

CV, hydrodynamic voltammetry, and EIS measurements were made using a three-electrode glass cell with a working electrode consisting of a GC disk (0.25 cm^2 geometric area) embedded in Teflon (Pine Research Instrumentation). A Pt plate (Degussa) was employed as the counter-electrode and a saturated calomel electrode (SCE) served as the reference electrode. All experiments were carried out at controlled temperature. The GC electrode surface was polished before use to a mirror finish by abrasion with emery paper, sequential polishing in 1.0 and 0.05 μm alumina slurries, and a final cleaning step by sonicating twice in Milli-Q water (Milli-pore) (15 min each).

The electrode named GC/HRP was prepared from a freshly polished GC surface to which 100 μL of HRP solution (2–3.5 mg mL^{-1} of HRP in 0.1 M solution) was applied at selected pH values. Subsequently, the electrode surface was covered with laboratory film (ensuring their surfaces did not touch) and refrigerated for 3 h. Before the electrochemical experiments were begun, the electrode was washed with 0.1 M solution at the desired pH [28].

The electrode named GC/PM-HRP/Nafion was prepared using a freshly polished GC surface to which 22 μL of HRP solution in water (11 μL each of HRP solution at 10 mg mL^{-1} and PM solution at 1 mg mL^{-1}) was applied and dried at room temperature. The electrode was then covered with 11 μL of Nafion solution at 5 wt.% in lower aliphatic alcohols and water, and again dried at room temperature [29].

Additional electrode variations were prepared according to the same principle—*e.g.*, a GC/HRP/Nafion electrode required 22 μL of HRP solution containing 11 μL each of HRP solution at 10 mg mL^{-1} and water, followed by the steps described above.

An Autolab potentiostat/galvanostat (model PGSTAT128N) equipped with a FRA2.X module was used in the EIS and other electrochemical experiments. For hydrodynamic voltammetry, the potentiostat/galvanostat was employed in conjunction with a Pine Instrument rotation rate module (model AFMSRX). The EIS experiments were conducted at fixed potentials—namely, an open-circuit potential (OCP) of around 0.21 V (on average) in the presence of $Fe(CN)_6^{3-}$ and −0.35 V in the presence of H_2O_2 or O_2, with potential perturbation of 25 mV (rms) within a frequency range of 10 mHz to 100 kHz. Care was taken to ensure that AC impedance data corresponded to the interfaces being investigated at high frequencies—namely, the potentiostat employed had faster performance; the highest cutoff frequency was limited to 160 kHz for the FRA2 module, which used a fixed filter when the applied frequency exceeded 19 kHz; cables were short and, as with the connections, were shielded; the electrochemical cell was placed inside a Faraday cage; the working electrode was positioned in front of and close to a larger Pt plate; and the SCE was placed near the working electrode. Nova 1.5 Autolab (2009) software was used to simulate the behavior of equivalent circuits of the interface in the presence of different redox probes, and the parameters of these circuits were fitted to the measured spectra using a non-linear least-squares program. A Fisher Scientific thermostatic flow bath (model 8001) was used for measurements under controlled temperature.

Horseradish peroxidase (EC 232-668-6) (Sigma), polymyxin B sulfate salt (Fluka BioChemika), 5 wt.% Nafion perfluorinated resin solution in lower aliphatic alcohols and water (Aldrich), 30% hydrogen peroxide solution (Merck)—concentration confirmed by permanganometry (titration against $KMnO_4$ [30])—and $K_3Fe(CN)_6$ (Dinâmica) were used as received. A 0.1 M KH_2PO_4 (Synth) solution was adjusted to different pH values with 1 M KOH (Vetec). A pH 4 solution was obtained by mixing 0.1 M acetic acid (Vetec) with 0.1 M sodium acetate (Merck).

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