ELECTRON EXCHANGE BETWEEN THE ENZYME ACTIVE CENTER AND ORGANIC METAL

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1. Introduction

The possibility of direct electron exchange between catalytically inactive proteins and electrodes has been demonstrated [1–5]. A reversible reduction-oxidation of FAD in the active center of glucose oxidase on a mercury electrode has been studied [6]. Berezin et al. [7] showed the possibility of electrocatalysis of oxygen reduction by laccase adsorbed on carbonous materials. The acceleration of electrochemical evolution of hydrogen from water by hydrogenase entrapped in a semiconductive matrix has been shown [8].

Available data indicate that the rate of electron exchange between the enzyme active center and the electrode is determined by the electrode material and the nature of the enzyme. Thus, the search for new electrode materials leading to effective electron transport is of theoretical and practical interest. Organic metals as such appeared to be the most promising ones for this purpose [9].

Organic metals are the organic complexes possessing metalic (not semiconductive) conductivity at room temperature [10,11]. Jaeger and Bard [12] studied the electrochemical behaviour of organic metal electrodes. The results of bioelectrocatalytic conversion of substrates by means of enzymes adsorbed on organic metals are presented in this work.

2. Experimental

2.1. *Enzymes used*

Cytochrome b_2 (L-lactate: ferricytochrome *c* oxidoreductase EC 1.1.2.3) was prepared from *Hansenula anomala* according to a modified method [13] (All-Union Research Institute of Applied Enzymology, USSR). Enzyme activity [13] varied from 10 to 473 U./mg in different batches. Crystalline glucose oxidase from *Penicillium vitale* (EC 1.1.3.4) with an activity of 180–250 U./mg was obtained according to [14]. Peroxidase (EC 1.11.1.7) was from horse radish (362 U./mg [15], Reanal, Hungary).

2.2. *Electrode preparation*

Organic metals – complexes of *N*-methylphenazinium (NMP^+) or *N*-methylacridinium (NMA^+) and the anion radical tetracyano-*p*-quinodimethane ($TCNQ^-$) composed of NMP^+TCNQ^-, $NMP^+(TCNQ^-)_2$ or NMA^+TCNQ^- – were synthesized according to [16]. Phenazinium methosulphate (Gee Lawson Chemicals Ltd, England) and TCNQ (Chemapol, Czechoslovakia) were used without additional purification. NMA^+Cl^- was synthesized as in [16].

To construct glucose oxidase and peroxidase electrodes organic metals were pressed (10^5 pounds force/in^2 $\approx$ 690 MPa) into discs (2.5 × 1 mm) which were glued to a glass tube. Electrical contact was obtained by means of a conductive compound. Enzyme solution (3 μl) containing 0.1 mg of protein was entrapped on the surface of an electrode using a dialysis membrane (35 μm thick). In kinetic measurements peroxidase was adsorbed directly from the protein solution (7.6 nM). Cytochrome b_2 (3 μl, 0.1 mg protein) was mixed with finely dispersed organic metal (1.0–1.2 mg) and the resulting suspension entrapped on the surface of a platinum electrode (4 mm in diameter).

2.3. *Electrochemical measurements*

Electrode current was recorded by an LP-7e polarograph (Czechoslovakia) in a three-electrode circuit, using an Ag/AgCl reference electrode (+205 mV vs. N.H.E) and a platinum electrode (56.2 mm^2, Radiometer; Denmark). The solutions were mixed using a disc magnetic stirrer (Radiometer; Denmark).

The fluorescence of NMP^+TCNQ^- organic metal was determined at a given potential in a thermostated cell of an MPF-4 spectrofluorimeter (Hitachi, Japan). The NMP^+TCNQ^- electrode (2 × 1 mm) was glued to a glass plate (1.3 × 2 cm) which was placed obliquely in a standard fluorescence cell. Electrical contact to the electrode was obtained using a platinum wire (20 μm diam). Electrochemical contact of the electrode with the reference and auxiliary electrodes was maintained by salt bridges (1.5% agar-agar in the buffer mentioned below).

Measurements were carried out in 0.1 M phosphate buffer, pH 7.0 or 7.2 in an anaerobic medium at 20 or 25°C. The enzyme electrode was kept in the buffer solution at a given potential until the establishment of a steady-state current (3–15 min) and then the substrate solution (10 μl) was introduced. The current of the bioelectrocatalytic process is equal to the difference between the basic current and that which is established after introduction of the substrate.

3. Results

3.1. *Electrocatalytic oxidation of L-lactate by cytochrome* b_2 *adsorbed on* NMP^+TCNQ^- *and* $NMP^+(TCNQ^-)_2$ *electrodes*

Lactate oxidation in the presence of cytochrome b_2 occurs in the interval from –0.2 to 0.5 V (fig.1). The dependence of the stationary anodic current which is established in the course of 1.5–2 min has a hyperbolic character. $K_{M\ (app)}$ values are almost the same for both complexes. In the interval from 0.4 to –0.03 V $K_{M\ (app)}$ changes from 2.1 to 1.9 mM (enzyme activity 91 U./mg; D,L-lactate). The dependence of the lactate oxidation current on pH (0.1 M KCl) shows a bell-shaped form, the maximum of which is shifted towards the acid region by 0.6 units as compared with the pH-optimum of native enzyme. Raising the solution temperature from 14 to 32°C leads to an increase in the lactate oxidation current. Further temperature rise inactivates the electrode irreversibly.

Other metabolites: glucose (2.0 mM) or sucrose (2.0 mM) are not oxidized on this electrode.

$K_{M\ (app)}$ of the electrode does not change in the aerobic medium. The maximal current of the electrode is reduced by 53% (0.08 V) or by 44% (0.3 V).

The electrode retains its activity for 2–6 days depending on the enzyme batch.

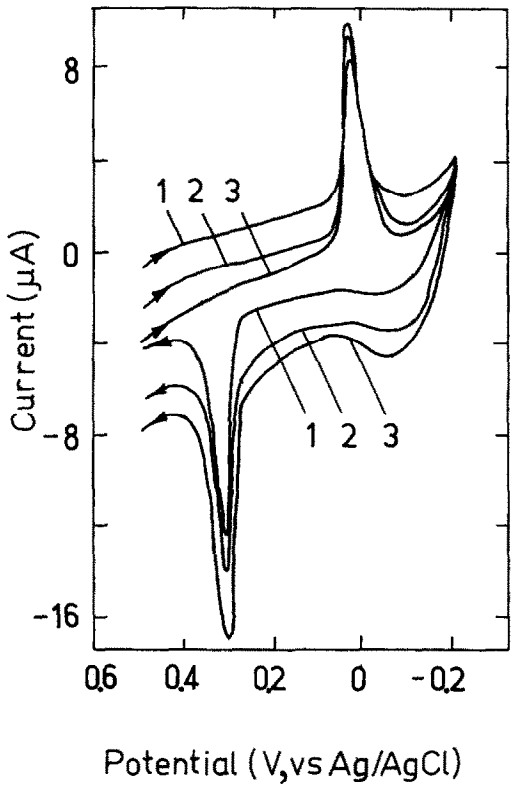

Fig.1. Cyclic voltammogram of cytochrome b_2 adsorbed on an NMP^+TCNQ^- electrode. D,L-lactate concentration 0 (1), 1.77 (2) and 5.90 mM (3). Scanning rate 200 mV/min, 0.1 M phosphate buffer, pH 7.2, anaerobic medium, enzyme activity 72 U./mg, 20°C.

3.2. *Electrocatalytic oxidation of glucose by glucose oxidase adsorbed on an* NMP^+TCNQ^- *electrode*

Biocatalytic oxidation of glucose is observed at the potentials higher than –0.08 V. The anodic current limits itself at high substrate concentrations (fig.2). Electrode response shows little dependence upon the electrode potential. However, at low potentials the limitation of the calibration graph is observed at lower glucose concentrations.

In aerobic medium at glucose concentrations lower than 3 mM and 0.078 V electrocatalytic oxidation of substrate does not take place (fig.2). At 0.6 V the effectivity of glucose electrocatalytic oxidation is also reduced at low concentrations.

The electrode retains its efficiency for more than 100 days. Replacing the enzyme reduces the electrode sensitivity.

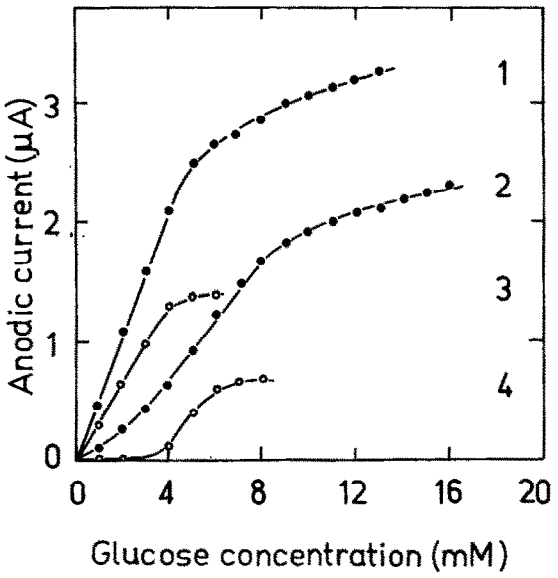

Fig.2. D-Glucose oxidation by glucose oxidase adsorbed on an NMP^+TCNQ^- electrode in anaerobic (1,3) and aerobic (2,4) medium. Anode potential 0.6 V (1,2) or 0.078 V (3,4) vs Ag/AgCl electrode, 0.1 M phosphate buffer, pH 7.2, 20°C.

3.3. *Electrocatalytic reduction of H_2O_2 by peroxidase adsorbed on NMP^+TCNQ^- or NMA^+TCNQ^- electrodes*

Electroreduction of H_2O_2 on organic metals in the absence of peroxidase proceeds at more negative potentials than –0.2 V. On adsorbing the enzyme on an NMP^+TCNQ^- electrode H_2O_2 reduction begins at 0.25 V (fig.3). The kinetics of the development of bioelectrocatalytic current (0.1 V) during enzyme (7.6 nM) adsorption is close to first order with a rate constant of 0.13/min (25°C). The dependence of the H_2O_2 reduction current (0.15 V) on the protein concentration is described by the Langmuir adsorption isotherm for which Θ = 15.4 nM. $K_{M\,(app)}$ in the case of peroxidase entrapped in the layer near the electrode surface changes from 0.33 to 3.3 mM H_2O_2 in the range 0.25–0.05 V.

Electrocatalytic reduction of H_2O_2 on an NMA^+TCNQ^- electrode proceeds in the same way except for a constant $K_{M\,(app)}$ = 2.5 mM in the interval from –0.15 to 0.2 V.

3.4. *Fluorimetric investigations*

Equilibrium concentrations of NMP^+ and NMA^+ determined fluorimetrically and established by dissolving the organic metals NMP^+TCNQ^- or NMA^+TCNQ^- (in the buffer solution) are equal to 13–18 μM or 9 μM, respectively.

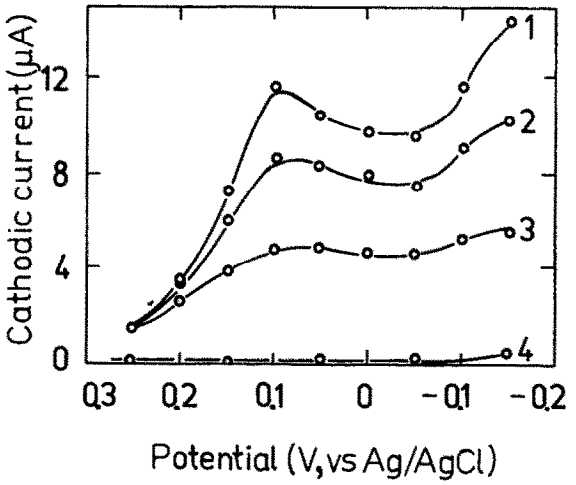

Fig.3. The dependence of the current of H_2O_2 reduction by peroxidase adsorbed on an NMP^+TCNQ^- electrode upon the electrode potential. H_2O_2 concentration 3 (1,4), 2 (2) and 1 mM (3). In (4) peroxidase is absent. 0.1 M phosphate buffer, pH 7.0, 20°C.

A very rapid increase in *N*-methylphenazinium concentration (at potentials higher than 0.49 V) or decrease (at potentials lower than –0.21 V) is observed on determining the intensity of NMP^+ fluorescence in the layer near the NMP^+TCNQ^- electrode surface. These potential values lie on the electrode electrochemical dissolution boundaries. The stationary concentration of NMP^+ in the layer near the surface of the electrode does not change much at intermediate values of the electrode potential, increasing at electrode potentials higher than 0.19 V or diminishing at lower potentials.

4. Discussion

Oxidoreductases containing FMN and heme (cytochrome b_2), FAD (glucose oxidase) or heme (peroxidase) when adsorbed on organic metals accelerate the electrochemical change of substrates. Electrocatalysis proceeds in the absence of external mediators at potentials higher than –0.2 or –0.08 V in the case of L-lactate or glucose oxidation. Bioelectrocatalytic reduction of H_2O_2 under the action of peroxidase occurs at a potential lower than 0.25 V. In

the range of these potentials specific electrochemical changes take place on the surface of the electrodes: (i) at potentials higher than 0.19 V NMP^+ frees itself from the NMP^+TCNQ^- electrode, whereas at negative potentials the stationary concentration of NMP^+ in the layer near the electrode surface is decreased; (ii) In the region 0.07 to 0.33 V the reduction of TCNQ or the oxidation of $TCNQ^-$ takes place [12], forming peaks on the voltammograms (fig.1). As a result of these changes the surface concentrations of NMP^+ and $TCNQ^-$ which serve as the mediators of electron transport [9] vary. However, the bioelectrocatalytic current does not depend on the varying concentration of these substances. So it follows, that the electron exchange between the cytochrome b_2 or the peroxidase active center and the organic metal electrode proceeds directly. The influence of oxygen on the glucose oxidase electrode current indicates that the reduced form of the enzyme is formed, in the oxidation of which NMP^+ and TCNQ may take part.

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