Enzymatic and Electrochemical Reactions of Xanthine Oxidase Immobilized on Carbon Materials

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An optimum way of immobilizing xanthine oxidase on graphite was found where a redox transformation of the enzyme was observed. The nature of the redox maxima was hypothesized on the basis of the dependence of the half-wave potential ($E_{1/2}$) on the pH of the solution. The enzymatic activity of xanthine oxidase adsorbed on two kinds of soot was studied by the oxidation of xanthine. The kinetic and activation parameters of the enzyme reaction were determined.

Introduction

Xanthine oxidase (XOD) is referred to the group of metal containing flavoproteids (Ganelin, 1994; Rubin and Ladigüena, 1974). Two molecules of flavin adenine dinucleotide (FAD) and two atoms of molybdenum are bound to the enzyme molecule which form its prosthetic group. Furthermore, eight atoms of nonheme iron are also bound to the molecule of xanthine oxidase. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid with the participation of molecular oxygen.

\[ \text{Xanthine oxidase} \ (XOD) \ \text{Xanthine} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{XOD} \text{Urate} + \text{H}_2\text{O}_2 \]

The enzyme also catalyses the oxidation of other purines, pteridines and aldehydes. By the oxidation of these substrates xanthine oxidase can transfer electrons and hydrogen not only to $\text{O}_2$ but also to other acceptors.

In biocatalytic and electrochemical systems the enzyme is generally used in the immobilized state. The biocatalytic process of oxidation of the substrates of this enzyme was carried out on a glass graphite electrode modified with redox-polymers and p-tetracyanoquinodimethane (TCNQ) (Kulys et al., 1983; Cenas et al., 1984). An enzyme-substrate system with xanthine oxidase based on conducting organic salts was described (Turner et al., 1992; Albery and Knowles, 1987). In this system the membrane electrode is sensitive to the increase of xanthine concentration.

In electrochemical systems xanthine oxidase is used in amperometric biosensors for determination of various substrates. A membraneless amperometric biosensor for hypoxanthine, based on immobilized xanthine oxidase, conducting organic salt and silicon oil was described (Korell and Spießbiger, 1994). A mediated amperometric biosensor for hypoxanthine, xanthine and phosphates based on deflavoxanthine oxidase was reported (Zhao and Luong, 1994). Xanthine oxidase and peroxidase, both immobilized on glass graphite electrodes (Kuly et al., 1983) were used to determine hypoxanthine and uric acid.

Both xanthine and hypoxanthine are important indicator compounds for determination of food freshness. They can be monitored through enzymatic oxidation which produces $\text{H}_2\text{O}_2$ and uric acid. For that purpose xanthine oxidase was immobilized on spectroscopically pure graphite (Lorenzo et al., 1991), on graphite soot (Martin and Rechnitz, 1990) and on carbon paste (Doblhoff-Dier and Rechnitz, 1989). The enzymatic reaction in these studies was followed by electrochemical oxidation of the uric acid formed. A sensor for determination of allopurinol, an inhibitor of xanthine oxidase, was described (Martin and Rechnitz, 1990).

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To study the enzymatic activity and electrochemical behaviour of xanthine oxidase in the immobilized state is the objective of the present study.

Materials and Methods

The materials used were as follows: xanthine oxidase EC 1.2.3.2 from milk (Fluka-Biochemica); activity — 0.39 U×mg⁻¹ (International Enzyme Unit — U = moles of substrate reacting or product produced per minute); \( M_r = 275,000 \); xanthine from Fluka-Chemika, with analytical grade qualification p.a. (99%-UV), \( M_r = 358.14 \). All solutions were prepared with bidistilled water.

Carbon materials: spectroscopically pure graphite and soot — “NORIT” and “PM -100”. The “NORIT” soot has fine-grained structure with an average size of particles of \( 5\times10^4 \) — \( 45\times10^4 \) \( \AA \) and the “PM-100” soot are built up of larger spherical particles with an average size of \( 21\times10^4 \) — \( 340\times10^4 \) \( \AA \). The two kinds of soot were kindly provided us from Institute of Electrochemistry in Moscow, Russia.

The electrochemical measurements were carried out using cyclic voltammetry (Kulys and Razumas, 1986) in phosphate buffer solution, pH = 8.4. The measurements were carried out in a cell with separate electrode compartments. The experimental system involved: Bipotentiostate, type Bi-Pad (Tacussel, Villeurbanne, France), generator type EG-20 (Elpan, Lubawa, Poland) digital voltmeter — type 1AB105 (Priborostroителен завод, Pravets, Bulgaria) and a recording device — XY-Recorder (VEB, Messapparatwerk, Schlotheim, Germany). A silver-chloride electrode was used as a reference electrode, and a platinum wire as a counter electrode. The working electrode was prepared in the form of a disk with a diameter of 0.5—0.6 cm from spectroscopically pure graphite, pressed together with teflon and with a platinum current tap, or in the form of tablets hydrophobized soot with teflon and soot — “N ORIT” and “PM-100”. The amount of the enzyme adsorbed was determined on the basis of a calibration graph for the maximum at \( \lambda_{\text{max}} = 278 \) nm. The value of the extinction coefficient is \( \varepsilon_{278} = 1.13\times10^5\text{mol}^{-1}\text{cm}^{-1} \). The adsorption of xanthine oxidase on graphite was carried out as follows: the graphite electrode was pretreated electrochemically — an activation involving the polarization of the electrode at \( E = -0.8 \) V (1 min), at \( E = 2.0 \) V (1 min) and then again at \( E = -0.8 \) V (1 min); a cathode-anode cycling (3 min) in the range of the potential change \( E = 0.8 \) to -0.2 V until a reproducible background curve was obtained. Just before immobilization the graphite electrode was polarized at \( E = 1.5 \) V for \( t = 4 \) min and the reproducible background curve was recorded again. The adsorption of xanthine oxidase was carried out by dipping the graphite electrode into the enzyme solution. After the adsorption the electrode was kept in the air for 20 minutes. Both on soot and on graphite, the adsorption was carried out at room temperature.

The enzymatic activity of xanthine oxidase dissolved and immobilized on soot was determined by the oxidation rate of its substrate — xanthine. The enzyme reaction kinetic was monitored spectrophotometrically by the decrease of the substrate concentration with time at \( \lambda = 275 \) nm.

Results and Discussion

In Fig. 1 are shown typical voltammetric I.E — curves, recorded for graphite electrode with adsorbed xanthine oxidase. Reversible anode and cathode maxima (curves 2 and 3) are observed in the range from 0.55 to 0.35 V and from 0.25 to 0.15 V. Their reversibility was checked by using the following criteria (Kulys and Razumas, 1986):

\[
\frac{I^*}{I_c} = 1 \quad \text{and} \quad E^*_a - E^*_c = \Delta E^* = 2.3026 \frac{RT}{nF},
\]

where \( E^*_a \) and \( E^*_c \) are the potentials corresponding to \( I^*_a \) and \( I^*_c \) respectively — the peak current. For the maxima in Fig. 1 — curves 2 and 3, both criteria hold true. This fact is a proof for the complete reversibility of the electrochemical reaction which takes place.
on the graphite electrode with adsorbed xanthine oxidase. No such maxima are observed in the voltammetric curves for the pure graphite electrode (without adsorbed enzyme, curve 1). They are also absent from the background curves for the electrochemically treated, before the xanthine oxidase immobilization, graphite (curve 1'). It follows that their occurrence (curves 2 and 3) is caused by the xanthine oxidase adsorbed on the graphite. The finding that reproducible I, E – curves can be recorded for a long time without a change in the peak current values indicates that xanthine oxidase is firmly adsorbed.

The isoelectric point of xanthine oxidase, $I = 5.35$ (Jakubke, 1976), and the electrochemical behaviour of the enzyme was studied at $pH = 8.4$, i.e. the molecules of xanthine oxidase is negatively charged. By analogy (Bogdanolskaya et al., 1989; Bowden et al., 1982) we can suppose that the anode treatment of graphite before the adsorption at $E = 1.5$ V for $t = 4$ min allows an increase in the number of the surface oxygen-containing groups which take part in holding xanthine oxidase on the surface of the carbon material.

The total number of electrons taking part in the redox transformations (Fig. 1) was determined by using the expression $E_p - E_{p_2} = 3.53 \frac{RT}{nF}$, where $E_p$ and $E_{p_2}$ are the potentials corresponding to $I_p$ and $I_{p_2}$; $I_p$ is the peak current. The expression given is valid to reversible redox systems undergoing redox transformations in adsorbed state (Kulys and Razumas, 1986). For the maximum at $E_p = 0.50$ V in the system we studied, $n = 1.5 - 2.06$, and for the one at $E_p = 0.16$ V, $n = 3.68 - 4.14$. For the first maximum ($E_p = 0.50$ V) the ratio is $\frac{\partial E_{p_2}}{\partial pH} = 0$, and for the second ($E_p = 0.16$ V) $\frac{\partial E_{p_2}}{\partial pH} = -0.029$. The data obtained for the number of the electrons $n$ and the ratio $\frac{\partial E_{p_2}}{\partial pH}$ brought about to some points concerning the nature of the redox maxima in Fig. 1. The $E_{p_2}$ independence of $pH$ for the first peak indicates that the electrochemical reaction takes place without a participation of any protons. The occurrence of this maximum is probably due to the reduction of some surface graphite groups which are activated by the adsorbed xanthine oxidase (Kuznetsov et al., 1977). For the reversible electrochemical reaction of the genus: $Ox + zH^+ + ne^- \leftrightarrow \text{Red}$, the dependence of $E_{p_2}$ on $pH$ is expressed by the equation (Kulys and Razumas, 1983): $\frac{\partial E_{p_2}}{\partial pH} = -0.059 \frac{z}{n}$, where $z$ is the number of protons and $n$ – the number of electrons. By using the value of $\frac{\partial E_{p_2}}{\partial pH} = -0.029$ in this expression we obtain that $z/n = 1/2$ i.e., one proton and two electrons take part in the redox process at $E_p = 0.16$ V. According to (Kulys and Razumas, 1983) at high values for $pH$ (in the present study $pH = 8.4$) a two-electron transfer takes place for each molecule of FAD from the active centre. Hence, the second redox maximum in Fig. 1, at $E_p = 0.16$ V, is probably related to the participation of FAD of the prosthetic group of xanthine oxidase.

The next stage in the investigation was to study the electrochemical oxidation of $H_2O_2$ obtained on the enzymatic oxidation of xanthine by xanthine oxidase adsorbed on “PM-100” soot. This reaction is an opportunity of principle for creating an amperometric biosensor in which the substrate (xanthine) can be detected by the value of the current on the oxidation of $H_2O_2$. In Fig. 2 are presented the polarization curves for the electrooxidation of $H_2O_2$ on soot electrode with adsorbed xanthine oxidase in a
solution of xanthine (curves 3–5). It could be seen that the electrooxidation rate of H$_2$O$_2$ is increased with concentration of the substrate (curves 4 and 5) and with temperature (curves 3 and 5). The considerably higher anode currents (curves 3 and 5) compared to those in the background polarization curve for soot in phosphate buffer (curve 1) and the curve for soot (without adsorbed enzyme) in a solution of xanthine (curve 2) are obviously due to the preceding oxidation reaction of xanthine with xanthine oxidase adsorbed on soot. The effective activation energy of the oxidation of H$_2$O$_2$ (Table I) was calculated by the basic equation in electrochemical kinetics

$$\ln I = \frac{E_{ef}}{RT} + B$$

where $E_{ef}$ is the effective activation energy and $I$ is the current. The activation parameters $\Delta H^*$ and $\Delta S^*$ of this process are also given in Table I.

Table I. Kinetic and activation parameters of the electrooxidation of H$_2$O$_2$, a product of the enzymatic oxidation of xanthine by xanthine oxidase adsorbed on soot.

<table>
<thead>
<tr>
<th>$E$ [V]</th>
<th>$I$ [µA]</th>
<th>$E_{ef}$ [kJ·mol$^{-1}$]</th>
<th>$-\Delta S^*$ [J·K$^{-1}$·mol$^{-1}$]</th>
<th>$\Delta H^*$ [kJ·mol$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>155</td>
<td>75.07</td>
<td>20.12</td>
<td>72.57</td>
</tr>
<tr>
<td>0.5</td>
<td>249</td>
<td>43.07</td>
<td>121.17</td>
<td>40.57</td>
</tr>
<tr>
<td>0.6</td>
<td>471</td>
<td>51.42</td>
<td>89.50</td>
<td>48.92</td>
</tr>
</tbody>
</table>

From the data in the Table is seen that the values for $E_{ef}$ are different for the various polarization potentials. The values for $E_{ef}$ and its dependence on the potential indicate that the rate of electrooxidation of H$_2$O$_2$ on soot electrode with immobilized xanthine oxidase is limited by the electrochemical polarization.

The enzymatic activity of xanthine oxidase on the oxidation of xanthine was studied with the enzyme adsorbed on both kinds of soot – “NORIT” and “PM-100”. The activity of the enzyme in solution was compared to that in immobilized state. It has been shown that the enzyme in immobilized state retains its activity and the dependence of the reaction rate on the concentration of the substrate has a hyperbolic character in both cases. The kinetic parameters of the enzyme reaction were calculated by the relationship between the oxidation rate of xanthine and the concentration of the substrate (Fig. 3). The kinetic parameters are as follows: for xanthine oxidase in solution - $K_m = 1.4\times10^{-2}$ m and $V = 150$; for xanthine oxidase immobilized on the “NORIT” soot $K_m = 2.9\times10^{-4}$ m and $V = 40$, and on the “PM-100” soot $K_m = 5\times10^{-5}$ m and $V = 40$. From these data follows that the enzyme activity decreases after immobilization.
In order to clarify the kinetic laws of xanthine oxidation with xanthine oxidase immobilized on both kinds of soot, the effect of the temperature on the rate of the process was studied. By the kinetic equation for a reaction of first order, \( k = \frac{I}{t} \ln \frac{[A_0]}{[A]} \), where \([A_0]\) is absorbance corresponding to the start concentration of the substrate; \([A]\) corresponds to current concentration, given in the coordinates \( \ln A - t \) (Fig. 4), the rate constants of the catalytic process at various temperatures were calculated. The effect of the temperature on the oxidation rate of xanthine was found stronger when the enzyme was adsorbed on “NORIT” (for \( T = 283 \) K, \( k = 3.18 \times 10^{-2} \text{s}^{-1} \text{mg}^{-1} \) and for \( T = 303 \) K, \( k = 21.83 \times 10^{-2} \text{s}^{-1} \text{mg}^{-1} \)), i.e., with an increase of 20 °C the rate is increased 6 times). For “PM-100”, at the same temperature range, the rate increased only 1.4 times (for \( T = 278 \) K, \( k = 1.35 \times 10^{-3} \text{s}^{-1} \text{mg}^{-1} \), and for \( T = 308 \) K, \( k = 2.28 \times 10^{-3} \text{s}^{-1} \text{mg}^{-1} \)). From these data for the specific rate constants it follows that the process rate is higher when xanthine oxidase is immobilized on “NORIT” soot. The activation energy of the enzymatic oxidation of xanthine with xanthine oxidase in immobilized state was calculated by the relationship \( k = \frac{k_B T}{h} e^{\frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT}} \), where \( k_B \) is the Boltzmann constant; \( h \) is the Planck constant; \( \Delta S^* \) is the activation entropy change; and taking into consideration that \( E_a = \Delta H^* + RT \), we calculated the activation parameters of xanthine oxidation with xanthine oxidase adsorbed on soot (Table II).

The data for \( \Delta S^* \) in Table II indicate that the oxidation of xanthine with xanthine oxidase immobilized on “PM-100” takes place with a smaller

![Fig. 5. Relationship \( \log k - 1/T \) for oxidation of xanthine on soot “NORIT” (1) and on soot “PM-100” (2) with adsorbed xanthine oxidase.](image)

<table>
<thead>
<tr>
<th>Kind of soot</th>
<th>Kinetic parameters</th>
<th>Activation parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>( k = 4.60 \times 10^{-2} \text{s}^{-1} \text{mg}^{-1} )</td>
<td>( \Delta S^* = -42.37 \text{J}\text{K}^{-1}\text{mol}^{-1} )</td>
</tr>
<tr>
<td>O</td>
<td>( E_a = 68.77 \text{kJ}\text{mol}^{-1} )</td>
<td>( \Delta H^* = 65.66 \text{kJ}\text{mol}^{-1} )</td>
</tr>
<tr>
<td>R</td>
<td>( Z_0 = 1.37 \times 10^{11} \text{s}^{-1} )</td>
<td>( \Delta G^* = 77.85 \text{kJ}\text{mol}^{-1} )</td>
</tr>
<tr>
<td>I</td>
<td>( k = 1.68 \times 10^{-3} \text{s}^{-1} \text{mg}^{-1} )</td>
<td>( \Delta S^* = -264.1 \text{J}\text{K}^{-1}\text{mol}^{-1} )</td>
</tr>
<tr>
<td>P</td>
<td>( E_a = 12.04 \text{kJ}\text{mol}^{-1} )</td>
<td>( \Delta H^* = 9.54 \text{kJ}\text{mol}^{-1} )</td>
</tr>
<tr>
<td>M</td>
<td>( Z_0 = 0.25 \text{s}^{-1} )</td>
<td>( \Delta G^* = 85.73 \text{kJ}\text{mol}^{-1} )</td>
</tr>
</tbody>
</table>
change in the entropy of activation \( \Delta S^* = -264.1 \text{ J} \times \text{K}^{-1} \times \text{mol}^{-1} \) than on "NORIT". This finding explains the lower rate of the process on these soot and the discrepancy between the values for the activation energy and the rate constants for the two kinds of soot.

The data for \( \Delta G^* \) in Table II are very close for both adsorbent. This fact can be explained with the compensatory relationship between \( \Delta H^* \) and \( -T \Delta S^* \) (Fig. 6). From the graph it could be seen that the two activation parameters \( \Delta H^* \) and \( -T \Delta S^* \) are mutually compensated and the values of \( \Delta G^* \) for both adsorbent are approximately the same.

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