Enzymatic and Electrochemical Reactions of Catalase Immobilized on Carbon Materials

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It has been shown that catalase immobilized on graphite and soot undergoes an oxidationreduction transformation. Some results on the effect of the potential sweep rate, temperature and pH on this transformation are presented. On the basis of the relationship established, it has been proved that the redox transformation is related to the iron in the heme of the active center of the enzyme and takes place with a transfer of one electron.

Introduction

Catalase is regarded to as one of the most common enzymes in plant and animal tissues and has a protection function connected with the decomposition of hydrogen peroxide. The catalase macromolecule consists of four subunits – each of them involving ferriporphyrin as a prosthetic group (Metelitsa, 1984). The gross molecule mass of catalase is M_r=250000. Catalase is highly specific enzyme and its basic function is high performance catalysis of hydrogen peroxide decomposition with liberation of water and molecular oxygen. Besides, that catalase also shows a moderate peroxidase activity, i.e. it can speed up oxidation reactions with hydrogen peroxide (Artemchik *et al.*, 1985, 1986). It has been proved by spectroscopy that catalase, like peroxidase forms three compounds, when reacted with hydrogen peroxide. When catalase reacts with hydrogen peroxide compound I (an intermediate enzyme-substrate complex I) is formed. Further on it can oxidize the hydrogen peroxide. The loss of an oxidation equivalent in compound I leads to the formation of compound II. Compound III is formed on the oxidation of compound II with hydrogen peroxide and has three oxidation equivalents of Fe (III). (Dixon and Webb, 1966; Hughes, 1983). Compound I has high activity and takes part in the enzymatic process. Not only hydroperoxides but also other hydrogen donors can react with compound I – ethanol for example. The activity of compound II is by 10^4 lower than that of compound I, while compound III has no enzymatic activity at all.

In electrochemical systems catalase is used to produce enzyme membranes for the electrochemical determination of glucose (Liu *et al.*, 1979). Additions of catalase to glucose oxidase, in some cases, allow an expansion of the range of glucose concentrations that can be determined. This is due to the partial decomposition of the hydrogen peroxide being formed.

The objective of the present work was to study the adsorption of catalase on carbon materials and its electrochemical reactions in adsorbed state.

Materials and Methods

The catalase used in this work was EC 1.11.1.6 from *Penicillium chrysogenum* 245 ("Biovet"-Bulgaria). The specific activity of the enzyme is 1000 $U \times mg^{-1}$. The reagents for the solutions: $Na_2HPO_4 \times 12H_2O$, KOH, H_3PO_4, citric acid, $KMnO_4$ and H_2O_2, all with analytical grade qualification. The solutions were prepared with bidistilled water. The electrochemical measurements were carried out using cyclic voltammetry (Kulys and Razumas, 1986) in citrate buffer using a cell with separate of electrode compartments. The experimental system involved: Potentiostat PS-80 (Chemipan, Warszawa, Poland), generator, type EG-20 (Elpan, Lubawa, Poland), digital voltme-

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ter-type 1 AB 105 (Priborostroitelen zavod, Pravets, Bulgaria) and a recording device – XY-Recorder (VEB, Messapparatewerk, Schlotheim, GDR). A silver-silver chloride electrode was used as reference electrode, and a platinum wire as 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 of hydrophobized soot with deposited active layer (1–2 mg) of soot. During the voltammetric measurements the solution was purged with argon.

The adsorption of catalase was carried out under static conditions from 1 ml solution of catalase with concentration of enzyme $C=1\times10^{-4}$ M per 10 mg soot. The amount of enzyme adsorbed was determined spectrophotometrically by the decrease of the catalase concentration in the solution after adsorption. The spectrophotometer used was Specord UV VIS (Carl Zeiss, Jena, GDR). The amount of the catalase in the solution was determined on the basis of a calibration graph (Fig. 1 b) for the maximum at $\lambda_{max}=280$ nm. The value of the extinction coefficient is $\varepsilon_{280}=1.38\times10^5\ l\times mol^{-1}\times cm^{-1}$. The adsorption of the catalase on graphite was carried out as follows: the graphite electrode was pretreated electrochemically at a high anode potential $E=1.5$ V for $t=1$ min, cycled (cathode-anode) in the range of the potential change $E=-0.2-0.6$ V until a reproducible background curve was obtained. The adsorption was carried out by dipping the electrode into the solution of the enzyme. The catalase adsorption on soot and on graphite was conducted at room temperature.

The enzymatic activity of the catalase dissolved and immobilized on soot was determined by the decomposition reaction of hydrogen peroxide. The amount of H_2O_2 was determined by permanganometry.

Results and Discussion

Catalase was adsorbed on soot and its maximum amount was 38 mg per gram of soot. The amount of the catalase adsorbed on spectroscopically pure graphite was evaluated by electrochemical measurements. Reversible anode and cathode maxima are observed in the current-potential curves recorded for the graphite electrode with adsorbed catalase (Fig. 2 a, curve 2) and for the electrode of soot with adsorbed catalase (Fig. 2 b) in the potential range of 0.5–0.1 V. The polarization capacity of the graphite electrode with adsorbed enzyme (Fig. 2 a), in the potential range of 0.6–0.3 V, decreases – another proof for the adsorption of catalase. The effect of the adsorption time and the enzyme concentration in the solution on the electric charge used for the redox transformation of the enzyme was studied on a spectroscopically

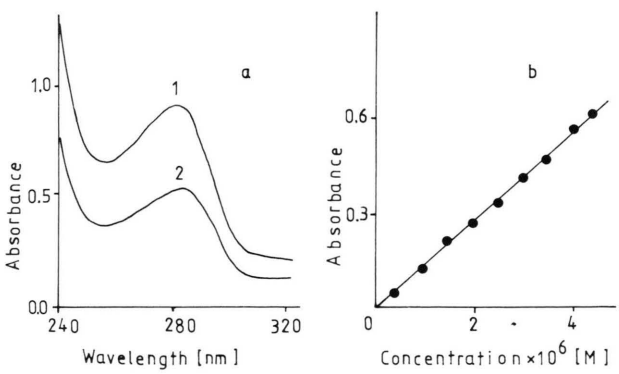

Fig. 1. Catalase absorption spectra (a) and a calibration curve for determination of the catalase concentration in the solution (b); a: catalase concentration in the citrate buffer – pH 7.0 , M:1–0.5×10^{-5}; 2–0.36×10^{-5}; 1cm cell.

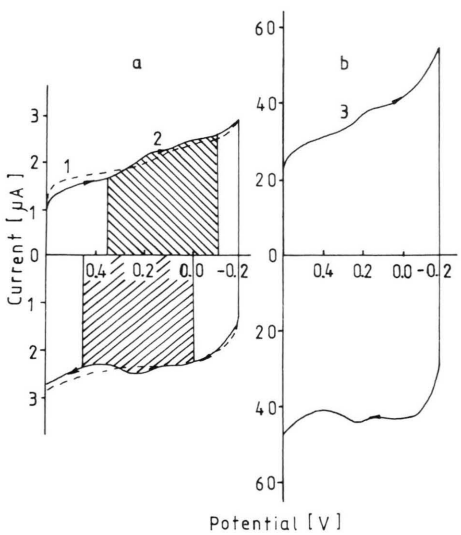

Fig. 2. Current-potential I,E – curves for pyrographite electrode (a-curve 1) for the same electrode with adsorbed catalase (a-curve 2) and on soot (b) with adsorbed catalase. The enzyme adsorption immobilization was carried out $t=5$ minutes using catalase solution with enzyme concentration $C=2.5\times10^{-4}$ M, $V=0.075\ V\times s^{-1}$.

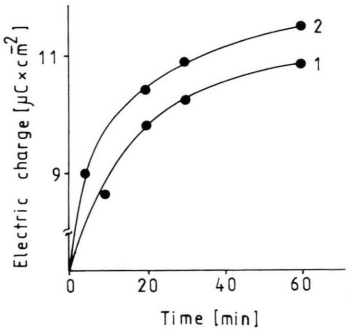

Fig. 3. Dependence of the electric charge needed for the redox transformation of the catalase on the adsorption time and the concentration of the enzyme,M:1 – 1×10^{-4}; 2 – 2.5×10^{-4}, V=0.075V×s^{-1}.

pure graphite electrode. The results are presented in Fig. 3. The maximum filling is reached in over 60 min and then it does not depend on the adsorption time. The electric charge is determined under the current-potential curve as it is shown in Fig. 2 without taking into consideration of the background curve. This assumption was made on the basis of previous experiments which had shown that the electric charge under the current-potential curve for graphite does not depend on the potential change rate. In this way, the contribution of electric charge from the background curve is constant. Moreover, the electric charge values were referred to the effective surface of the graphite electrode. The electrode surface was determined by the background current-potential curve in the potential range of 0.4–0.5 V, assuming that the carbon material capacity was 25 μF×cm^{-2} (Tarasevitch, 1984). Catalase is firmly adsorbed. This is indicated by the possibility of recording a number of current-potential curves, where the current values in the maxima remain constant.

The redox transformations observed in the current-potential curves are probably related to transformations of the iron in the active center of catalase. When potassium cyanide, a catalase inhibitor, is introduced into the solution, the current values in the maxima decrease and the electric charge used for the redox transformations of the enzyme drops from 12 μC, in the case without cyanide, to 10 μC at a concentration of 8×10^{-5} M KCN (The reduced values for the electric charge were calculated for a surface of 1 cm^2).

Analysis of the kinetic parameters of the redox-transformations of catalase immobilized on graphite and concepts of the mechanism of this process could be obtained on the basis of the dependence of the anode and cathode potentials on pH and on the potential change rate. When pH is changed from 3.5 to 8, where the enzyme retains its properties, the potential values of the anode and cathode maxima E^* are shifted (Fig. 4). The ratio $\frac{\partial E^*}{\partial pH} = -0.06$ V for both anode and cathode maxima is an indication of a proton participation in the reaction.

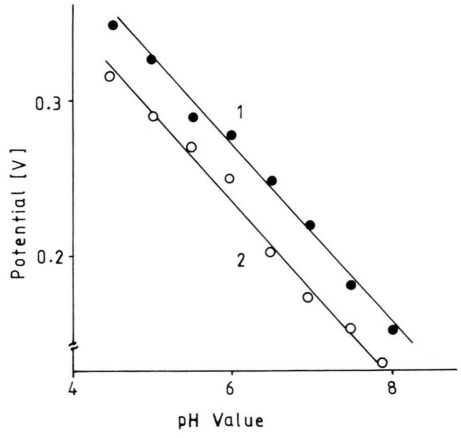

Fig. 4. Dependence of the potentials of the anode (1) and cathode (2) maxima in the redox transformation of catalase on pH of the solution.

The dependence of the anode E_a^* and cathode E_c^* potential maxima in the redox transformation of catalase on the logarithm of potential change rate lg$V$ is given in Fig. 5. The ratio $\frac{\partial E_a^*}{\partial \lg V} = \frac{\partial E_c^*}{\partial \lg V} = 0.030$ V. From the figure presented, it is seen that in the range of the rate change from 0.01 to 0.10 V×s^{-1} the potential shift in the anode (of the anode maximum) and in the cathode (of the cathode maximum) side is of 0.04 V, i.e. up to a rate of 0.10 V×s^{-1} the potential value at the maxima is insignificantly shifted with is rate. This fact and the independence of E_a^* and E_c^* on V up to 0.01 V×s^{-1} testify that the reaction rate was not very high. The extrapolation of the cathode and anode areas of the relationship E*-lgV up to the intersection point allowed the determination of the equilibrium potential 0.19 V. The voltammetric measurements of the graphite electrode with im-

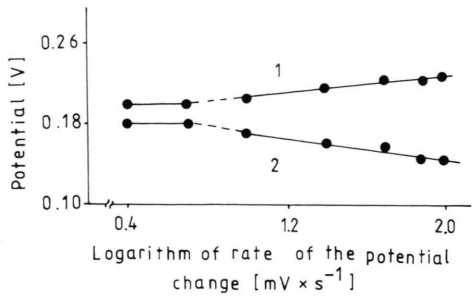

Fig. 5. Dependence of the potentials of the anode (1) and cathode (2) maxima in the redox transformation of catalase on the rate of the potential change.

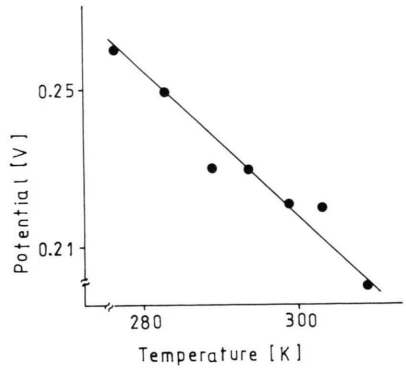

Fig. 6. Dependence of the potentials of the anode maximum in the redox transformation of catalase on temperature.

mobilized catalase (Fig. 2) show that the oxidation and reduction of the enzyme takes place at a potential of 0.20 V. The potential value of 0.19 V coincides with the redox potential which is another proof that the redox reaction takes place in the active center of the protein which, after the adsorption, retained the properties of the native molecule. The number of electrons taking part in the redox transformation of the adsorbed catalase was determined using the following expression $E_p - E_{p/2} = 3.53\ RT/nF$, where E_p and $E_{p/2}$ are the potentials corresponding to the current peak I_p and $I_{p/2}$. This expression is valid for reversible redox systems in adsorbed state (Kulys and Razumas, 1986). For the system we studied $n=1$. In that case $E^* = E_p$. From the values obtained for $n=1$ and for the kinetic parameter (0.03 V) with in the boundaries of the formal electrochemical kinetics (Damaskin and Petrii, 1983) it can be concluded that the transfer of electrons does not appear as a slow stage. It is most likely that the process rate is limited by the conformation changes in the protein globule in the course of its transformation. It can be concluded on the basis of the data found in literature, that they could be changes in the volume of the macromolecule in the transition from oxidatized to reduced state and vice versa (Taniguchi *et al.*, 1984).

In order to obtain some information about the structural transformations taking place in the enzyme molecule the temperature dependence of the enzyme redox potential was studied (Fig. 6). The ratio $\frac{\partial E_a^*}{\partial T} = -18.7\times10^{-4}\,V\times K^{-1}$. The entropy of the redox transformation of the catalase immobilized on graphite was calculated by the formula (Kulys and Razumas, 1986): $\Delta S = F\left(\frac{\partial E_a^*}{\partial T}\right) - 15.6$.

Using the data for ΔS and $E^*(E_p)$ at $t=25$ °C, we calculated ΔG and ΔH from the following equations: $\Delta G = -nFE^*$; $\Delta H = \Delta G + T\Delta S$.

The results obtained are given in Table I.

Table I. Kinetic and thermodynamic parameters of the redox transformation of catalase immobilized on graphite.

Kinetic parameters		Thermodynamic parameters	
$\partial E_a^*/\partial T$	-18.7×10^{-4} V×K^{-1}	ΔG	−21.14 kJ
$\partial E^*/\partial$pH	−0.06 V	ΔH	−92.66 kJ
$\partial E^*/\partial \lg V$	0.03 V	ΔS	−0.24 kJ×K^{-1}×mol^{-1}
E_a	24.45 kJ×mol^{-1}		

The dependence of the current in the maximum (the pure graphite current was eliminated) on the temperature is given in Fig. 7. In the range of 5–35 °C, where the enzyme retains its activity, the Arrhenius plot is linear. The activation energy value determined in that range was 24.45 kJ×mol^{-1}, considerably higher than the value of the activation energy in the redox transformation of cytochrome *c* adsorbed on carbon material (Bogdanovskaja *et al.*, 1989), but close to that of cytochrome *c* on modified electrodes (Koller and Hawkridge, 1985). The values of the kinetic parameters – E_a and $\partial E_a^*/\partial T$ in

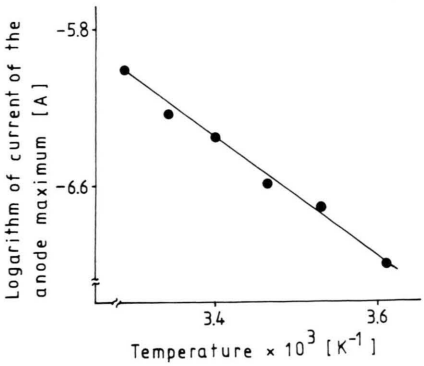

Fig. 7. Dependence of the current of the anode maximum in the redox transformation of catalase on temperature.

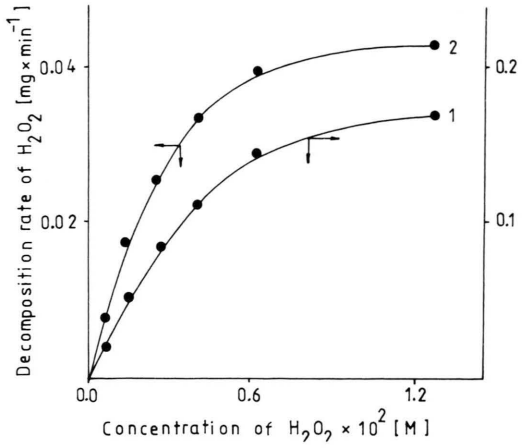

Fig. 8. Dependence of the decomposition rate of H_2O_2 on the concentration of the substrate:
1. catalase in solution, enzyme concentration $C=0.22\times10^{-6}$ M;
2. catalase immobilized on soot, enzyme amount $g=0.54$ mg.

Table I, compared to the ones for cytochrome *c* (Taniguchi *et al.*, 1984; Bowden *et al.*, 1982) express the conformation changes taking place in the protein macromolecule. At the same time, however, the data for ΔS in Table I give evidence for a firmly immobilized catalase because the enzyme redox transformation takes place with a relatively low change of the entropy.

Any increase in temperature over 30 °C causes partial desorption of the catalase immobilized on graphite. This is also confirmed by the data obtained with the enzyme immobilized on soot. When the solution containing soot with adsorbed catalase is heated up to 40 °C, 65–70% of the compound adsorbed passes into the volume of the solution (the amount was determined by spectrophotometry).

The next step in the study was to compare the catalase enzymatic activity in solution to that in adsorbed 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 reaction rate of hydrogen peroxide decomposition and the concentration of the substrate (Fig. 8). The kinetic parameters are as follows: for catalase in solution – $K_m=2.0\times10^{-2}$ M and $V=7.14$, and for catalase immobilized on soot – $K_m=2.5\times10^{-2}$ M and $V=2.5$. From these data it follows that the enzyme activity decreases after immobilization.

Acknowledgement

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