Indian Journal of Chemistry
Vol. 36A, April 1997, pp. 335-338

Catalytic properties of xanthine oxidase immobilized on carbon materials

E Horozova*, N Dimcheva, Z Jordanova & L Kirova
Department of Physical and Theoretical Chemistry, University of Plovdiv, 24, Tsar Assen St, Plovdiv 4000, Bulgaria

Received 2 July 1996; revised 17 October 1996

The kinetics of catalysis by xanthine oxidase (XOD) immobilized on soot on the oxidation of xanthine obeys the laws of enzyme kinetics. The kinetics of catalysis with immobilized XOD has been determined by calculation of the rate constant (k), the constant of Michaelis (K_m), the maximum reaction rate (V), the energy of activation (E_a) and pre-exponential factor (Z_o). The values of the kinetic parameters depend on the nature of the adsorbent. The mechanism of action of the enzyme in the immobilized state has been explained on the basis of the calculated isobaric-isothermal potential of activation (ΔG^*), enthalpy of activation (ΔH^*) and entropy of activation (ΔS^*). The polarographic measurements on the electrooxidation of hydrogen peroxide formed in enzymatic oxidation of xanthine with immobilized XOD have provided the evidence that the rate limiting is due to electrochemical polarisation.

Xanthine oxidase (XOD) is a metal containing flavoproteide[1,2]. Two molecules of flavin-adenin-dinucleotide (FAD) and two atoms of molybdenum are bound to the molecule of the enzyme which form the prosthetic group of the enzyme. Furthermore, eight atoms of nonheme iron are bound to the molecule of xanthine oxidase.

The catalytic effect of xanthine oxidase is manifested on the oxidation of hypoxanthine to xanthine and of xanthine to uric acid with the participation of molecular oxygen, Eq. (1).

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{XOD}} \text{Urate} + H_2O_2 \quad \ldots (1)$$

The enzyme also catalyses the oxidation of other purines, pyridines and aldehydes. On the oxidation of these substrates xanthine oxidase can transfer electrons and hydrogen not only to O_2 but also to other acceptors.

In biocatalytic and electrochemical systems, the enzyme is usually used in immobilized state. Biocatalytic process of oxidation of the substrates with that enzyme was accomplished on glass-graphite electrode modified with adsorbed redox-polymers and TCNQ[3,4]. An enzyme-substrate system based on conducting organic salts was described in literature[5,6].

In electrochemical systems, xanthine oxidase is used in amperometric biosensors for determination of xanthine and hypoxanthine[7-11]. This way, it is possible to determinate the allopurinol-an inhibitor of the enzyme[12]. Herein we report the results of the study of catalytic activity of xanthine oxidase immobilized on carbon materials.

Experimental

The materials used were as follows:

—xanthine oxidase (EC 1.2.3.2), from milk of Fluka-Biochemika, with activity of 0.39 $U.mg^{-1}$ and $M_r = 275000$; xanthine ($C_5H_4N_4O_2$) of Fluka-Chemika, analytical grade (pa − 99%, UV), $M_r = 152.11$; disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4.12H_2O$) of Fluka-Chemika, analytical (pa − 99%, UV) $M_r = 358.14$.

All the solutions were prepared in doubly distilled water.

Two kinds of carbon materials were used − NORIT soot and PM-100 soot.

The immobilization of xanthine oxidase was carried out on both kinds of soot by an adsorption method in static conditions from 1 ml solution of xanthine oxidase per 10 mg of soot. The adsorption was performed in 24 h.

The quantity of the enzyme adsorbed was determined spectrophotometrically by the decrease of xanthine oxidase concentration in the solution after adsorption. The spectrophotometric measurements were performed on Specord UV VIS (Carl Zeiss Jena, GDR). The quantity of xanthine oxidase in the solution was determined by a calibration graph (for the maximum) at $\lambda_{max} = 278$ nm. The extinction coefficient value was $\varepsilon_{278} = 1.13 \times 10^5$ l. $mol^{-1}.cm^{-1}$. At 278 nm, the dependence of the adsorption on the concentration of xanthine oxdase keeps its linear character in the range of $0.5 \times 10^{-6} \div 6 \times 10^{-6}$ M and even further.

The enzymatic activity of the xanthine oxidase dissolved and immobilized on soot was evaluated by oxidation rate of its substrate, xanthine. The kinetics of the enzyme reaction was followed by the decrease in [substrate] with time at 275 nm,

or by the increase in the [product], uric acid, with time at 295 nm.

The electrochemical oxidation of hydrogen peroxide produced in enzymatic oxidation of xanthine with xanthine oxidase immobilized on the PM-100 soot was studied using the polarisation curves in potentiostate regime. The electrochemical measurements were conducted in three-electrode cell. Ag/AgCl reference electrode was used and platinum wire served as a counter electrode. The working electrode was in the form of a tablet of hydrophobized soot with a diameter of 1.44 cm and with an active layer of soot and immobilized xanthine oxidase on it. The tablet was placed into the cell as a floating electrode. The apparatus used for the electrochemical oxidation of H_2O_2 were: a potentiostat P-5848 (Zavod izmeritelnih priborov, Gomel, Russia), digital voltmeter IAB105 (instrument-making plant Pravets, Bulgaria), multimeter-1004.500 (RFT, veb mikroelektronic Karl Marx, Erfurt, GDR), *p*H-meter OP-208 (Radelkis, Budapest, Hungary) and, a recorder XY-Recorder (VEB, Messaparatewerk, Schlotheim, GDR).

All the measurements were carried out in phosphate buffer solution with *p*H = 8.4.

Results and discussion

Xanthine oxidase is adsorbed on PM-100 and NORIT soot. Maximum quantity of the adsorbed enzyme showed linear dependence on the concentration of the enzyme in the solution. At a concentration higher than 0.6 mg. ml^{-1} the quantity of the adsorbed enzyme comes up to 13 $mg.g^{-1}$. It shows that the adsorption takes place on a nonuniform energy surface, i.e. it is an adsorption of Tyomkin type.

In Fig. 1 is given dependence of the formation rate of the product of the enzyme oxidation of xanthine (curves 1 and 2) and the rate of [xanthine] decrease (curves 1′ and 2′) on time and temperature when xanthine oxidase is in immobilized state on NORIT soot. The hyperbolic character of the curve shows that the activity of the enzyme in immobilized state is retained.

The above data were analysed by Lineweaver and Burk method to obtain the kinetic parameters—Michaelis constant (K_m) and the maximum reaction rate (V) (Table 1). The catalytic rate constant was calculated from linear plot of ln A versus t.

From Table 1 it could be seen that the kinetic parameters of the enzymatic reaction depend on the state of the xanthine oxidase—whether it is dissolved or immobilized on soot. The adsorption of the enzyme on carbon carriers brings about certain decrease in its activity. The reaction rate V, of the molecules in enzyme-substrate complex (ES) is the same on both kinds of soot but considerably lower when xanthine oxidase is in solution. The values for K_m which characterise the enzyme affinity to substrate are quite different[3] but fall into the range of 10^{-2}-10^{-7} mol dm^{-3}. On NORIT, K_m is higher by an order than on PM-100 soot.

The dependence on carbon material used for immobilization indicates different reactivity of ES (Table 1).

On both kinds of soot the oxidation kinetics of xanthine obeys the kinetic equation of a first order reaction. The rate constant values calculated at various temperatures are given in Table 2. The data show that the temperature effect on the rate is greater when xanthine oxidase was immobilized on NORIT soot—a 20°C increase causes a 6 folds enhancement in the rate. With PM-100 soot, for the same temperature range the increase in rate is only 1.4 times.

The rate constant, as well as K_m depend also on the choice of a carbon carrier for xanthine oxidase immobilization. On the NORIT soot the rate constants are by an order higher than those on the PM-100 soot. Therefore both the kinetic ef-

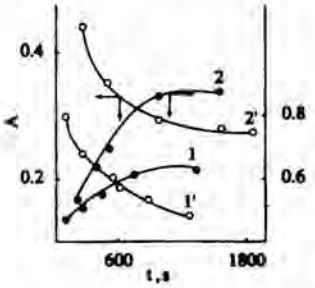

Fig. 1—Relationship between the formation rate of the product of xanthine enzymatic oxidation (1, 2), and the rate of decrease in xanthine quantity (1, 2) with time and temperature. Temperatures of (1, 1) 25°; (2, 2) 15°C; Quantity of XOD immobilized on soot = 0.064 mg

Table 1—Kinetic parameters for xanthine oxidation with XOD

Xanthine oxidase	K_m (mol dm^{-3})	V	k (s^{-1})
In the volume of the solution ($C = 1 \times 10^{-7}$ M)	1.4×10^{-2}	150	1.53×10^{-2}
Immobilized on NORIT (0.032 mg)	2.9×10^{-4}	40	2.40×10^{-4}
Immobilized on PM-100 (0.059 mg)	5.0×10^{-5}	40	7.10×10^{-5}

fects which are responsible for the substantial differences in the values for K_m and the rate constant can be associated with the effect of the immobilization on the state (conformation) of the enzyme.

For the complete description of the kinetic laws with immobilized xanthine oxidase the effects of reagent distribution in the system have to be clarified.

For that purpose, the activation energy (E_a) of the process (Table 2) was calculated from ln k versus $1/T$ plot. For both adsorbent the values for E_a fall into the range characteristic of enzymatic reactions[13]. For PM-100 soot E_a was 12 kJ.mol^{-1}. This value of E_a and the poor dependence of the reaction rate on temperature with PM-100 carrier confirm the assumption that on this adsorbent the process takes place in diffusion regime. The value of E_a = 68.7 kJ.mol^{-1} for the process with NORIT soot and the considerable effect of the temperature indicate that the total rate of the substrate transformation is determined by the very enzymatic reaction, i.e. the process is in the kinetic range. The difference in the rate setting stage of xanthine oxidation, depending on the adsorbent, can be explained with the two kinds of soot. The NORIT soot has a fine-grained structure with an average size of particles from 5×10^4 to 45×10^4 Å, and the PM-100 soot is built up of coarse globular particles with an average size of 21×10^4-340×10^4Å. The diffusion controllable reaction rate with immobilized enzyme decreases with the growth of the size of particles[14].

The analysis of the data in Table 2 shows a discrepancy between E_a and the rate constants. To explain it the entropy of activation ΔS^+ (Table 2) was calculated using the basic equation of the transition state theory. The data are indicative of its effect on the oxidation rate of xanthine with immobilized xanthine oxidase. The process on NORIT takes place with greater change of ΔS^+ than that on PM-100. On the oxidation of xanthine, the immobilized xanthine oxidases has no effect on E_a, but has definite influence on the pre-exponential factor (Z_0) and on ΔS^+, respectively. The data given in Table 2 also indicate the strength with which the xanthine oxidase is immobilized (adsorbed) on the soot.On PM-100 the enzyme is more firmly adsorbed because the process takes place with a relatively low entropy change $\Delta S^+ = -264$ J.K^{-1}.mol^{-1}. The identical values for ΔG^+ (Table 2) on both adsorbents are explained with the compensation relationship between ΔH^+ and $-T.\Delta S^+$.

The polarisation curves for electrochemical oxidation of hydrogen peroxide formed in enzyme oxidation of xanthine with immobilized XOD are given in Fig. 2 (curves 3-5). As it could be seen,

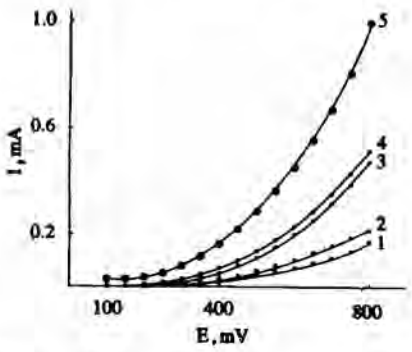

Fig. 2—Electrooxidation polarisation curves of H_2O_2 produced in enzyme oxidation of xanthine with immobilized XOD; (i) PM-100 soot in background electrolyte; (2) soot and xanthine; (3, 4, 5) soot with immobilsed XOD and xanthine; phosphate buffer $pH = 8.4$; [xanthine concentration] = (4) 1.75×10^{-5} mol dm^{-3}; (2, 3, 5) 4.76×10^{-5} mol dm^{-3}; temp. (3) 14°; (5) 28°C; quantity of the immobilized XOD = 12×10^5g

Table 2—Kinetic and activation parameters for xanthine oxidation with XOD immobilized on "NORIT" and "PM 100'

T(K)	Kinetic parameters				Activation parameters		
	k(s^{-1})	k(s^{-1}, mg^{-1})	E_a (kJ.mol^{-1})	Z_0 (s^{-1})	ΔS^+ (J.K^{-1}.mol^{-1})	ΔH^+ (kJ^{-1}.mol^{-1})	ΔG^+ (kJ^{-1}.mol^{-1})
				NORIT			
283	1.65×10^{-4}	3.18×10^{-2}	68.77	8.15×10^8	−38.3	66.415	77.269
288	2.37×10^{-4}	4.60×10^{-2}	68.77	7.05×10^8	−42.3	65.658	77.853
298	5.50×10^{-4}	10.60×10^{-2}	68.77	6.24×10^8	−41.1	66.292	78.529
303	11.30×10^{-4}	21.83×10^{-2}	68.77	8.11×10^8	−39.0	66.228	78.068
				PM-100			
278	5.72×10^{-3}	1.35×10^{-3}	12.04	1.05×10^{-2}	−264.2	9.743	83.209
288	7.10×10^{-5}	1.68×10^{-3}	12.04	1.08×10^{-2}	−264.1	9.544	85.737
308	9.63×10^{-5}	2.28×10^{-3}	12.04	1.06×10^{-2}	−264.0	9.482	91.097

Table 3—The rate of H_2O_2 electrooxidation (I, μA) formed in enzymatic oxidation of xanthine with XOD immobilized on PM-100 depending on temperature and substrate concentration

$C_{xan}(M)$	T(K)	I, μA					
		Polarization potential, V					
		0.15	0.2	0.4	0.5	0.6	0.7
2×10^{-5}	298	19	15	63	132	225	341
5×10^{-5}	288	11	11	40	135	186	292
5×10^{-5}	301	22	29	155	294	471	675

the electrooxidation rate increases with the increase in both the concentration of the substrate (curves 4 and 5) and the temperature (curves 3 and 5). The curve for soot in xanthine solution (curve 2) is a little higher than the background polarisation curve on PM-100 (curve 1). However, because of formation of hydrogen peroxide, a considerable acceleration of the process could be reached when xanthine oxidase is adsorbed on PM-100 (curves 3-5) in the presence of xanthine.

The effective activation energy (E_{ef}) was calculated by the basic equation of the electrochemical kinetics, $\ln I = -(E_{ef}/RT) + B$ (Table 3). From the data in Table 3 it is seen that the E_{ef} values are different for the different polarisation potentials. The values of E_{ef} and their dependence on the potential show that H_2O_2 electrooxidation rate on soot with immobilized xanthine oxidase is limited by the electrochemical polarisation.

References

1 Ganelin V L & Lvov I I, *Uspechi biol chim*, 16 (1994) 68.
2 Rubin B A & Ladiguina M E, *Physiol biochim dihanya rasten, Moskva, Moskovskogo Universiteta* (1974) 512.
3 Kulys J J & Razumas V J, *Biocatal electrochim organiches soed, Vilnjus, Mosklas*, (1983) 86.
4 Cenas N K & Kulys J J, *Bioelectrochem Bioenerg*, 12 (1984) 583.
5 Turner E, Karube I & Wilson J, *Biosensory-osnovy i prilozhenya*, trnaslated by G Abidora, Moskva, Mir, (1992).
6 Albery W J & Knowles J R, *J Theoret Biol*, 124 (1987) 137.
7 Korell U & Spicbiger U E, *Electroanalysis*, 6 (1994) 305.
8 Doblhoff-Diel O & Rechnitz G A, *Anal Let*, 22 (1989) 1047.
9 Zhao S & Luong J H T, *Electroanalysis*, 6 (1994) 830.
10 Kulys J J, Laurinavicius V S A, Pesliakiene M V & Gureviciene V V, *Anal Chim Acta*, 148, (1983) 13.
11 Lorenzo E, Gonzalez E, Pariente F & Hernandez L, *Electroanalysis*, 3 (1991) 319.
12 Martin G B & Rechnitz G A, *Anal Chim Acta*, 237 (1990) 91.
13 Klove D, *Enzymologya, Sofia, Nauka i Izkustwo*, (1988) 158.
14 Berezin I V & Klyachko N L, *Immobilizovannye fermenty, Moskva, Visshaya Shkola*, (1987).