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PREPARATION AND PROPERTIES OF IMMOBILISED XANTHINE OXIDASE

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SUMMARY

The immobilization of milk xanthine oxidase (EC 1.2.3.2) has been investigated. The transition metals method of immobilization proved unsuccessful but the cyanogen-bromide method provided active preparations of xanthine oxidase bound to Sepharose and to cellulose. The immobilized enzyme oxidized xanthine, N^1-methylnicotinamide and NADH using O_2, dichlorophenolindophenol and ferricyanide as electron acceptors. The flavin prosthetic groups were readily removed from the immobilized enzyme by treatment with 3 M KI. Activity was restored to the KI-treated preparations by incubation with FAD. The results of KI treatment of xanthine- and NADH-pre-reduced enzyme indicate that the two flavin chromophores of xanthine oxidase are independent redox centres, *i.e.* are nonequivalent.

INTRODUCTION

The study of immobilized enzymes has grown because of their potential commercial and analytical uses[1,2] and because such studies may be of theoretical value. For example, information may be obtained about the microenvironment of membrane-bound enzymes[3] and immobilization has provided a novel method of investigating multi-subunit enzymes[4,5].

In the paper we describe the study of xanthine oxidase (EC 1.2.3.2) attachment to cellulose, Sepharose and glass, by the cyanogen bromide method of Porath *et al.*[6] and by the titanium method of Novais[7]. Studies on the removal of the flavin chromophores from the immobilized enzyme are also presented and the possible non-equivalence of these flavins is discussed.

MATERIALS AND METHODS

Xanthine oxidase was prepared by the method of Brady[8] from fresh un-

Abbreviation: DCIP, 2,4-dichlorophenolindophenol.

pasteurised cream kindly supplied by the Galway Milk Co. The purified enzyme had an activity: $A_{450\,nm}$ ratio of 140, indicating that approximately 70% of the active sites were functionally intact[9]. Samples of this enzyme were immobilized by the cyanogen bromide method[6] which was employed for the activation of Sepharose 4-B (Pharmacia) and cellulose (Sigmacell, Type 38). Typically, 1.8 g of activated cellulose or 5 ml of activated Sepharose 4-B were stirred with 20 I.U. of xanthine oxidase for 18 h at 4 °C.

Samples of cellulose were also activated with $TiCl_4$, both by the method of Novais[7] and by the following modified procedure which was also used to activate Sepharose 4-B and glass beads (Braun, 0.17–0.18 mM): glass beads (2.5 g), or cellulose (2.5 g) or Sepharose 4-B (5 ml) were shaken with a solution of $TiCl_4$ (15%; Hopkin and Williams) for 4 h, and then washed thoroughly with distilled water followed by phosphate buffer (0.05 M, pH 7.8). The activated materials were then stirred with enzyme (10 I.U.) in phosphate buffer (0.05 M, pH 7.8, containing 0.005% EDTA) for 18 h at 4 °C. Unbound enzyme was removed from the immobilized preparations by washing with 1 M NaCl followed by 0.05 M potassium phosphate buffer, pH 7.8.

Aliquots of immobilized enzyme were packed into columns (0.7 cm diameter) to a bed height of 1 cm or 2 cm. Enzyme activity was measured at room temperature by pumping buffered substrate through the column at a fixed rate and measuring the absorbance of the eluate (1-ml fractions were collected). In each case the buffered substrate served as the blank. The substrates were xanthine (0.15 mM), or *N'*-methylnicotinamide chloride (5.0 mM), or xanthine (0.15 mM) *plus* 2,4-dichlorophenolindophenol (DCIP) (50 μM), or NADH (0.5 mM) *plus* potassium ferricyanide (1 mM). Unless stated otherwise the buffer was 0.05 M phosphate, pH 7.8, containing 0.005% EDTA. The increased absorbance of the eluate at 295 nm or 300 nm or the decrease at 600 nm was a measure of xanthine: O_2 reductase, N'-methylnicotinamide: O_2 reductase and xanthine: DCIP reductase activity, respectively. The absorbances of the fractions were read immediately following their elution. NADH: ferricyanide reductase activity was measured as the decrease at 340 nm or at 420 nm using freshly prepared substrate each time and subtracting the decrease observed in the absence of enzyme.

The procedures employed for removal of flavin from the immobilized enzyme were based on those of Kanda and Rajagopalan[10]. In order to maintain anaerobic conditions, all reactants were held at room temperature in reservoirs connected to a column of cellulose-bound enzyme, in a closed system. Reservoirs were flushed with O_2-free N_2 for 20 min before the experiment began and for the duration of the experiment. The column was irrigated thoroughly with 0.05 M potassium phosphate, pH 7.8, containing 0.005% EDTA from one reservoir then with 25 ml of 0.05 M potassium phosphate buffer, pH 7.8, containing 0.005% EDTA, 1 mM dithiothreitol and 1 mM xanthine (or 1 mM NADH) to effect reduction of the enzyme. Finally, 25 ml of 3 M KI were passed through the column. The column was disconnected from the anaerobic system and left at 4 °C overnight. It was then washed with 0.05 M potassium phosphate, pH 7.8, containing 0.005% EDTA, to remove KI.

RESULTS AND DISCUSSION

The transition metal methods described failed to provide an immobilized

enzyme preparation exhibiting xanthine oxidase activity, whether the assay was carried out by the column technique above or by shaking substrate with the preparation. Replacement of $TiCl_4$ in the activation process by $TiCl_3$, $SnCl_4$ or $FeCl_3$ also failed. Moreover, $TiCl_4$ treatment greatly impaired the flow properties of cellulose so that this method of attachment seems unsuited to the preparation of columns of cellulose-bound enzymes.

On the other hand, the CNBr method of attachment yielded active preparations of xanthine oxidase bound to Sepharose and to cellulose. Using these preparations apparent K_m values for xanthine ranging from $2.5 \cdot 10^{-5} \pm 0.9 \cdot 10^{-5}$ (S.E.) M to $4.5 \cdot 10^{-5} \pm 1.5 \cdot 10^{-5}$ M were obtained. These values should be compared with those obtained with the free enzyme, *e.g.* those of Massey *et al.*[11] of $1.18 \cdot 10^{-5}$ M at infinite O_2 concentration, pH 8.5 and 25 °C, of Fridovich[12] of $1.15 \cdot 10^{-6}$ M at $2.4 \cdot 10^{-4}$ M O_2, pH 7.0 and 25 °C, or of Greenlee and Handler[13] of $1.7 \cdot 10^{-6}$ M at pH 7.8, 25 °C and unstated O_2 concentration.

The flavin chromophores are readily removed from reduced forms of xanthine oxidase and related enzymes by treatment with high concentrations of salt[14]. Unlike native xanthine oxidase, the deflavo-enzyme can no longer utilize O_2 as an electron acceptor, but it continues to transfer electrons from xanthine to acceptors such as ferricyanide[15] or DCIP[14]. Treatment of immobilized xanthine oxidase with 3 M KI abolished the xanthine: O_2 reductase activity of xanthine- or NADH-prereduced enzymewhile the xanthine: DCIP reductase activity remained largely intact (Table I). Thus, it would seem that flavin may be removed from immobilized xanthine oxidase.

TABLE I

THE EFFECTS OF PROLONGED KI TREATMENT ON THE XANTHINE: O_2 REDUCTASE AND XANTHINE: DCIP REDUCTASE ACTIVITIES OF CELLULOSE-BOUND XANTHINE OXIDASE PREREDUCED WITH XANTHINE OR WITH NADH

The xanthine: O_2 reductase and xanthine: DCIP reductase activities were determined and compared with those obtained on the same column prior to KI treatment. Column height was 2 cm, flow rate 1 ml/min.

Enzyme-reducing agent	*Xanthine: O_2 reductase*			*Xanthine: DCIP reductase*		
	Control	*KI-treated*	*% control*	*Control*	*KI-treated*	*% of control*
Xanthine	0.54	0.00	0.00	0.62	0.49	79.0
NADH	0.68	0.08	12.00	0.70	0.73	104.0

Recently, it was postulated that the two flavin chromophores of chicken liver xanthine dehydrogenase are independent centres of transhydrogenase activity[10]. The evidence for this proposal included the fact that exposure of the avian enzyme, prereduced with NADH, to 3 M KI led to the dissociation of all of the FAD, whereas prereduction with xanthine prior to exposure led to the rapid dissociation of only 50% of the FAD.

In Table II the effects of short exposure to 3 M KI on the activities of immobilized xanthine oxidase prereduced with xanthine or with NADH are shown. As before, the xanthine:O_2 reductase activity of both preparations has largely been

TABLE II

THE EFFECTS OF BRIEF KI TREATMENT ON THE XANTHINE:O_2 REDUCTASE, XANTHINE:DCIP REDUCTASE AND NADH:FERRICYANIDE REDUCTASE ACTIVITIES OF CELLULOSE-BOUND XANTHINE OXIDASE PREREDUCED WITH XANTHINE OR WITH NADH

The procedures employed in this study were as described earlier with the exception that incubation with 3 M KI was confined to 15 min. The various activities were determined and compared with those obtained on the same column before treatment. Column height was 1 cm, flow rate 2 ml/min.

Activity	*Assay wavelength (nm)*	*Activity left after KI treatment (%)*	
		Xanthine-prereduced	*NADH-prereduced*
Xanthine:O_2 reductase	295	13.3	14.3
Xanthine:DCIP reductase	600	79.0	98.5
NADH:ferricyanide reductase	340	58.3	17.6
NADH:ferricyanide reductase	420	57.1	14.3

abolished while the xanthine:DCIP reductase activity of both has been retained. On the other hand, short exposure to KI has affected the NADH:ferricyanide reductase activities of the two preparations differently. Thus, whereas almost 60% of the NADH:ferricyanide reductase activity of the xanthine-prereduced enzyme has been retained, less that 20% of that of the NADH-prereduced enzyme remains. Similar findings provided part of the evidence for the proposal that the flavins of the chicken liver enzyme were non-identical[10]. Thus, it would seem that the flavin moieties of xanthine oxidase also may be non-equivalent. It may be argued, that rather than being evidence in favour of non-identical flavin sites the above results merely reflect differential reduction of the immobilized enzyme (functional and non-functional) by xanthine and NADH. Xanthine can reduce only functional enzyme whereas NADH can reduce both functional and non-functional enzyme[9]. On this

TABLE III

RECONSTITUTION OF XANTHINE:O_2 REDUCTASE ACTIVITY BY INCUBATION OF PARTIALLY OR FULLY DEFLAVINATED ENZYME WITH FAD

The partially deflavinated (xanthine-prereduced) and fully deflavinated (NADH-prereduced) preparations from the previous study were incubated overnight at 4 °C with 1 mM FMN and 1 mM FAD, respectively. Unbound flavin was removed by exhaustive washing with 0.05 M potassium phosphate buffer, pH 7.8, containing 0.005% EDTA. The xanthine:O_2 reductase activity of each preparation was determined as before. Since incubation with FMN did not restore activity, the xanthine-prereduced preparation was subsequently incubated with 1 mM FAD for 2 h at room temperature. Unbound FAD was removed as before and again xanthine:O_2 reductase activity was determined. In all cases activities are expressed as percentages of that of the control.

Preparation	*Xanthine-prereduced*	*NADH-prereduced*
Control	100.0	100.0
KI treated	13.3	14.3
KI treated + FMN	14.6	—
KI treated + FAD	84.0	81.0

basis one might expect that ratio of loss of NADH:ferricyanide reductase activity (which is independent of the degree of functionality) from the xanthine- as opposed to the NADH-pretreated enzyme would be 7:10. Examination of the data in Table II show that such is not the case.

Incubation of soluble deflavo xanthine oxidase with FAD or with FMN restores xanthine:O_2 reductase activity[15]. Apparently, deflavoxanthine dehydrogenase from chicken liver can also be reactivated by incubation with FAD[14]. The possibility that the xanthine:O_2 reductase activities of the immobilized preparations above might be restored by incubation with flavin was investigated. It can be seen (Table III) that incubation of the NADH-prereduced (hence almost fully deflavinated) preparation with FAD restored its xanthine oxidase activity to about 80% of that of the control. Incubation of the half deflavinated (xanthine-prereduced) preparation with FMN gave no reactivation whereas subsequent incubation with FAD restored activity to more than 80% of that of the control. It may be concluded that FMN did not bind to the partially deflavinated enzyme. This and other possible interpretations of these results are currently being investigated.

The use of immobilized rather than free enzyme in studies such as flavin removal offers certain advantages. For example, reactants such as KI may easily be removed by irrigation with buffer. Similarly, the products of enzyme action are readily obtained free of enzyme. Such procedures, using soluble enzyme require extensive dialysis, gel filtration or ultrafiltration.

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