ENZYMIC CONVERSION OF RAT LIVER XANTHINE OXIDASE FROM DEHYDROGENASE (D FORM) TO OXIDASE (O FORM)

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1. Introduction

The xanthine oxidase (EC 1.2.3.2) of rat liver supernatant is actually an NAD^+-dependent dehydrogenase in its native form (form D) [1]. The enzyme can be converted into an oxidase (form O) by various treatments, which can be divided into two categories.

(i) Treatment with proteolytic enzymes, which causes an irreversible conversion of the dehydrogenase.

(ii) All other treatments, such as storage at $-25^\circ C$, treatment with sulphydryl reagents or preincubation of the whole homogenate the effect of which can be reversed by thiols [2].

The conversion following preincubation with subcellular fractions, seemed due to an enzyme [1], since it did not occur with boiled fractions. These experiments were performed to isolate and identify an enzyme capable of operating the conversion. It was observed that the conversion of the dehydrogenase into an oxidase is coupled to reduction of GSSG, and is catalysed by an enzyme of the thiol protein disulphide oxidoreductase type, which was partially purified from rat liver.

2. Experimental

2.1. *Purification of xanthine oxidase*

All buffers contained 0.1 mM EDTA and 0.15 mM 2-mercaptoethanol. Livers from Wistar rats were homogenised in a blender with 3 vol. 20 mM K-phosphate buffer (pH 6.1). The homogenate was centrifuged at 19 000 × *g* for 20 min, the supernatant was adjusted to pH 5.5 with 0.2 N acetic acid and centrifuged as above. The new supernatant was adjusted to 70% saturation with solid $(NH_4)_2SO_4$, and centrifuged as above. The precipitate was redissolved in the minimum volume of buffer, was desalted by passage through a column of Sephadex G-25 and then adsorbed on 100 g calcium phosphate gel treated as in [3]. After stirring for 20 min the gel was centrifuged and the supernatant was stored for the purification of the converting enzyme (see below). The gel was washed with 0.1 M K-phosphate buffer (pH 7.8) until no pink colour was present in the washing and then was eluted with 0.5 M K-phosphate buffer (pH 7.8) containing 15% $(NH_4)_2SO_4$. The eluate was dialysed against 10 mM K-phosphate buffer (pH 7.8) was incubated at 37°C for 20 min in the presence of 10 mM dithioerythritol and was stored at $-25^\circ C$. The enzyme prepared in this way was almost entirely in the D form. Immediately before use, the enzyme was equilibrated with 0.1 M Tris–HCl buffer (pH 8.1) containing 0.3 mM EDTA and 0.45 mM 2-mercaptoethanol, by passing through a column of Sephadex G-25.

2.2. *Determination of xanthine oxidase activity*

Xanthine oxidase and dehydrogenase activities were determined as in [1] in the presence of NAD^+. The % of enzyme in the D form was estimated from the ratio between the NAD^+ reduced in the presence of xanthine (which expressed the xanthine dehydrogenase activity) and the amount of uric acid formed aerobically in the presence of NAD^+ (which expressed the sum of dehydrogenase and of the oxidase activities).

2.3. *Purification and assay of other enzymes*

Sulphydryl oxidase was prepared from cow's milk and tested by DTNB reaction as in [4]. Protein disulphide isomerase was purified from rat liver and assayed with RNase as in [5]. Thiol transferase was prepared from rat liver and tested with cysteine

according to [6]. Glutathione reductase from yeast was purchased from Sigma and assayed with NADPH as in [9]. All the enzymes were active in their normal catalytic activities when tested for the converting activity on xanthine oxidase.

2.4. *Other methods*

SDS–polyacrylamide gel electrophoresis was performed as in [7]. Protein was determined by the method in [8].

3. Results

The experiments in which xanthine oxidase was converted from D into the O form by fresh, but not by boiled liver subcellular fractions led us to search for an enzyme capable of operating this conversion. No conversion was observed with purified sulphydryl oxidase [4] or with protein disulphide isomerase [5]. A supernatant enzyme catalysing the thiol–disulphide exchange is the thiol transferase, which was purified as in [6], except that liver supernatant was made free from xanthine oxidase by calcium phosphate gel. The preparation converted the xanthine oxidase from D into the O form, in the presence of GSSG up to the last step of purification. The peak of thiol transferase activity was retarded by Sephadex G-75 [6] and was unable to convert the xanthine oxidase. The converting fractions had some thiol transferase activity and were eluted in the void volume together with the glutathione reductase. This enzyme, however, was not responsible for the conversion of xanthine oxidase, since a Sigma preparation from yeast, tested as in [9], was inactive.

3.1. *Purification of the converting enzyme*

All buffers contained 0.1 mM EDTA and 0.15 mM 2-mercaptoethanol. After calcium phosphate gel (see purification of xanthine oxidase) the unabsorbed material was passed on a CM-Sephadex C-50 column (3.4 × 25 cm) in 20 mM K-phosphate buffer (pH 6.1). After the washing the column was eluted with a linear NaCl gradient (0.1–0.3 M). Active fractions, eluted with ~0.175 M NaCl, were pooled, dialysed against 20 mM Tris–HCl buffer (pH 8.5) and applied to a DEAE–Sephadex A-50 column (2.6 × 26 cm). The activity was eluted with the washing and was concentrated by dialysis against solid polyvinylpyrrolidone to 1/50 of the original volume. A precipitate formed during this dialysis was discarded and the remaining solution was applied to a column (1.3 × 85 cm) of Sephacryl S-200 superfine in 20 mM Na-phosphate buffer (pH 7.2). The activity emerged with a peak at the 29th 1.5 ml fraction. A summary of the purification procedure is given in table 1.

Table 1
Purification of the converting enzyme

Step	Volume (ml)	Protein (mg/ml)	Specific activity (U[a]/μg protein)
Calcium phosphate washing	240	7.83	0.04
CM–Sephadex eluate	110	1.08	0.66
DEAE–Sephadex washing	120	0.3	1.50
DEAE–Sephadex concentrated	2.6	0.43	13.33
Sephacryl eluate	7.5	0.05	3.75

[a] Unit was arbitrarily defined as the amount of the enzyme producing a 1% reduction of the ratio NADH formed/uric acid formed, corrected for non-enzymic conversion.

Suitable amounts of each fraction were incubated at 37°C for 20 min, in a medium containing 35 mM Tris–HCl buffer (pH 8.1); 0.1 mM EDTA; 0.15 mM 2-mercaptoethanol; 0.5 mM GSSG and ~0.4 mg xanthine oxidase partially purified in 0.5 ml final vol. At each purification step the converting activity of the enzyme was proportional to the added amount of the enzymic preparation

The last passage brought about some loss of specific activity, presumably due to partial inactivation of the enzyme. However, with the passage through Sephacryl a considerable purification was obtained, as demonstrated by the disappearance of numerous electrophoretic bands, which were reduced to two (fig.1).

The activity of the enzyme in the presence of various concentrations of GSSG and the time courses of enzymic and non-enzymic conversion are shown in fig.2. Conversion of xanthine oxidase occurred to some extent in the presence of GSSG alone, but was accelerated by the converting enzyme, especially at low concentrations of GSSG. The optimum pH of the enzyme ranged from 7.0–8.5 (fig.3).

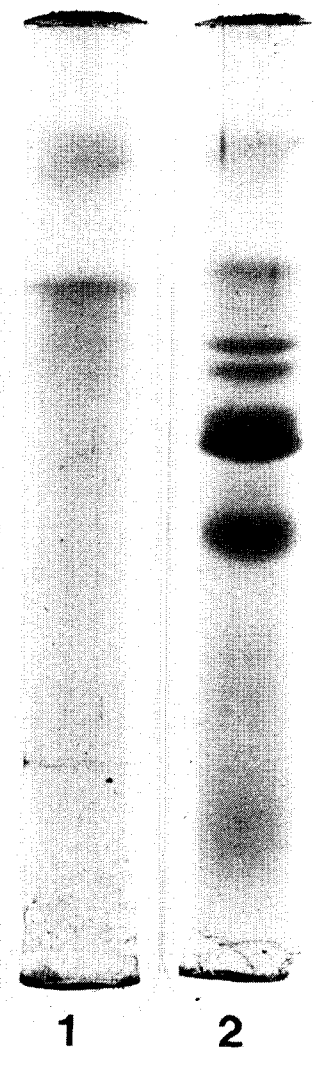

Fig.1. SDS–Polyacrylamide gel electrophoresis [7] of converting enzyme after (1) and before (2) Sephacryl chromatography, with 18 μg (1) and 43 μg (2) protein.

The converting enzyme was tested for other enzymic activity (see section 2): sulphydryl oxidase [4], protein disulphide isomerase [5] and glutathione reductase [9] were not detectable at a protein concentration 3-times that required for the conversion of xanthine oxidase. The converting enzyme showed thiol transferase activity with cysteine as substrate [6], but the purified thiol transferase at 10-times higher activity was unable to convert the xanthine oxidase.

We failed to obtain the reversibility of the enzymic reaction in the presence of GSH, glutathione reductase and NADPH, but the reverse conversion from O to D form, of the xanthine oxidase enzymatically transformed was always possible by dithioerythritol [2], thus excluding a proteolytic mechanism.

The activity of all preparations was destroyed by boiling.

4. Discussion

Conversion of rat liver xanthine oxidase from dehydrogenase (D form) into an oxidase (O form) occurs upon oxidation or binding of sulphydryl group(s) by several oxidizing agents or ligands [2,10]. These experiments demonstrate that the same conversion can be obtained by oxidation of the sulphydryl group(s) by GSSG in the presence of a rat liver enzyme of the type of those catalysing disulphide interchange (reviewed [11]), acting in the protein-oxidising direction [12]. Since the conversion of D into O form was obtained with ligands of sulphydryl groups such as *N*-ethylmaleimide [2], it is likely that formation of a mixed disulphide between xanthine oxidase and glutathione is sufficient to convert the enzyme.

It was shown that the xanthine oxidase of rat organs exists mainly, if not entirely, in its D form [13], and so far no instances have been found of a prevalence of the O form. Consequently, it is difficult to assign a role to the converting enzyme in vivo, also because reversibility of the conversion, i.e., conversion, from the O into the D form, could not be obtained with our enzyme preparations, in the presence of GSH. It is possible that the converting enzyme is rather unspecific, and capable of modifying other enzymes which change their properties upon oxidation of their thiol groups [14–18].

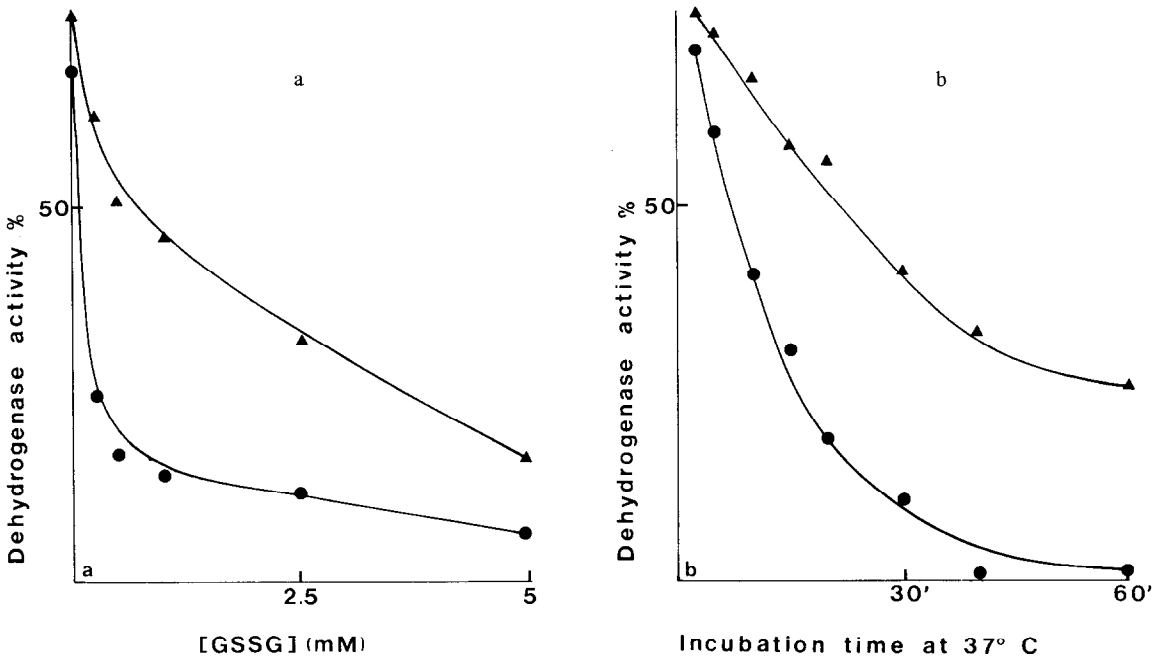

Fig.2. Enzymic (•) and non-enzymic (▲) conversion of xanthine oxidase as a function of [GSSG] (2a) and of incubation time (2b). Other incubation conditions are as in table 1, using a semi-purified preparation of the converting enzyme (after the DEAE–Sephadex chromatography) (360 μg protein in 1.2 ml final vol.). Xanthine oxidase and dehydrogenase activities were then determined as in the text. The dehydrogenase activity is given as % of the total activity (oxidase + dehydrogenase).

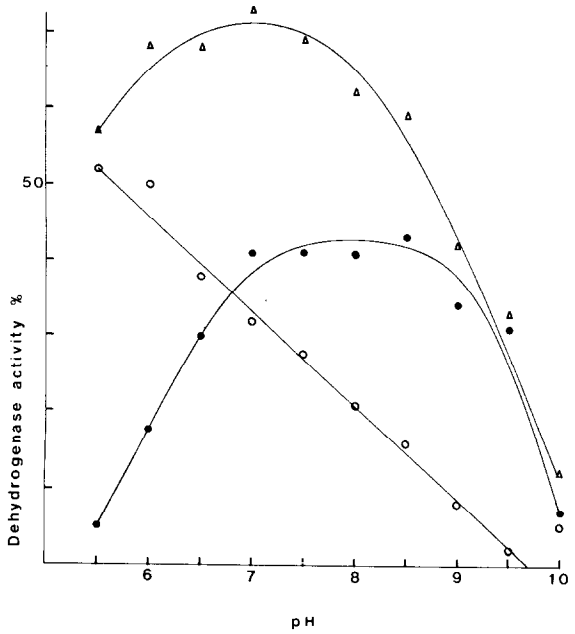

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Fig.3. Effect of pH on the enzymic and non-enzymic conversion of xanthine oxidase. Purified xanthine oxidase was preincubated with the converting enzyme (110 μg protein in 1.0 ml final vol.) and 0.5 mM GSSG at the pH values indicated in the abscissa. After preincubation the xanthine oxidase and dehydrogenase activities were assayed at pH 8.1. (○) Conversion in the absence of enzyme; (△) conversion in the presence of enzyme; (•) difference (enzymic conversion). Other details were as in fig.1.

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