Characterization of Alternative Molecular Forms of Xanthine Oxidase in the Mouse

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(Received 27 June 1972)

1. Two major forms of xanthine oxidase are demonstrated for the mouse. On polyacrylamide-gel electrophoresis the duodenal form migrates faster towards the anode than that of the liver. Both forms also differ in their $(\text{NH}_4)_2\text{SO}_4$ precipitation patterns and sucrose-density-gradient molecular-weight determinations. 2. The liver form is fully converted into the duodenal form by incubation at 37°C with 2.5 mg of crude trypsin/ml for 1½ h, without loss of activity. The trypsin-treated liver form behaves like the normal duodenal form as characterized by electrophoresis, $(\text{NH}_4)_2\text{SO}_4$ precipitation patterns, and sucrose-density-gradient molecular-weight determinations. 3. Partial conversion is also brought about by purified trypsin and chymotrypsin, but not with β -carboxypeptidase or lipase. The conversion is inhibited by soya-bean trypsin inhibitor. 4. In embryo mice the duodenal form is similar to the liver form on electrophoresis. 5. These studies indicate, as might be expected, that the duodenal form is a modified version of the liver enzyme, probably caused by proteolytic alteration.

Xanthine oxidase (xanthine–oxygen oxidoreductase, EC 1.2.3.2) catalyses both reactions in the sequence: hypoxanthine→xanthine→uric acid, and also oxidizes some other purines, pteridines and aldehydes (for references see Dixon & Webb, 1964). The xanthine-oxidizing system of micro-organisms and animal systems, in addition to having a wide range of specificity, is known to be influenced by both genetic and epigenetic factors. In *Drosophila melanogaster*, for example, there are at least three genes which regulate the activity of xanthine dehydrogenase (Forrest *et al.*, 1956; Glassman & Mitchell, 1959; Keller & Glassman, 1964). In addition, electrophoresis has shown that tissue-specific forms of the enzyme appear to exist in *Drosophila* and in the rat (Collins *et al.*, 1970; Sackler, 1965). Xanthine-oxidizing systems have therefore attracted much interest as models for investigating the various genetic and epigenetic factors which influence enzyme expression in animals. In the present paper experiments on the characterization of different forms of xanthine oxidase in the mouse are presented in an attempt to clarify their relationships and provide a basis for future analysis of factors involved in their control.

Materials and Methods

Animals

Female Q-strain mice (6 weeks old), weighing 25–30 g, were used in all experiments. This outbred strain was kindly supplied by Dr. J. C. McCarthy, University College, Dublin, who has also outlined their

history (McCarthy, 1967). The mice were fed *ad libitum* on a complete laboratory diet, and free access to water was always allowed.

Preparation of xanthine oxidase

Mice were killed by stunning and decapitation. The liver and duodenum were removed and washed in ice-cold homogenizing buffer (0.05 M-Tris–0.001 M-EDTA, adjusted to pH 7.0 with conc. HCl), with particular care being taken to evacuate the duodenal contents. The tissues were then blotted dry, weighed and homogenized in a Potter–Elvehjem hand homogenizer in 5 vol. (w/v) of buffer for liver and 10 vol. (w/v) for duodenum. The homogenates were spun at 30000g for 30 min at 4°C and the ‘crude’ supernatant fluids collected with a Pasteur pipette to avoid transfer of the overlying fatty layer. The enzyme was further purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$, in the range 0–55% satd. $(\text{NH}_4)_2\text{SO}_4$ for duodenum and 30–45% satd. $(\text{NH}_4)_2\text{SO}_4$ for liver. After 45 min the precipitates were separated by centrifugation at 30000g for 20 min and the pellets re-suspended in 2 ml of the homogenizing buffer and dialysed overnight against 2 litres of the same buffer at 4°C. These samples were then used for enzyme assays, electrophoresis and ultracentrifugational analysis.

Assay of xanthine oxidase activity

The fluorimetric method described by Haining & Legan (1967) was followed. In the procedure used

here, the reaction mixture contained $5\text{ }\mu\text{M}$ -2-amino-4-hydroxypteridine in 0.05 M -Tris- 0.001 M -EDTA buffer (adjusted with conc. HCl to pH 6.75 at 37°C , which is the pH optimum of all the enzyme forms studied). An appropriate amount of the enzyme (usually 0.05 ml) was added at zero time to make a final volume of 1 ml . Readings were taken at 1 min intervals for up to 10 min in a Photovolt fluorimeter no. 540 (Photovolt Inc., New York, N.Y., U.S.A.), equipped with a 347 nm primary filter and a 405 nm secondary filter (Balzers, Liechtenstein). The sensitivity of the instrument was adjusted so that a solution of $1.6\text{ }\mu\text{M}$ -quinine in 0.1 M - H_2SO_4 gave a reading of 100 on the scale. The initial reading of the reaction mixtures were then decreased to about 10–15 by zero suppression. Cuvettes were returned to a dry bath (Thermolyne Corp., Dubuque, Iowa, U.S.A.) kept at 37°C between readings. Under these conditions a change of one division on the fluorimeter corresponds to the oxidation of 2.2 pM -2-amino-4-hydroxypteridine to give isoxanthopterin, and one unit of xanthine oxidase activity corresponds to the oxidation of 1 pM -2-amino-4-hydroxypteridine.

Electrophoresis

Polyacrylamide-gel electrophoresis at pH 9.0 was carried out by the method of Yen & Glassman (1965), by using the EC-470 apparatus of Raymond (1962). Gels were prepared by using 7.5 g of Cyanogum-41 (BDH Chemicals Ltd., Poole, Dorset, U.K.), 0.1 g of ammonium persulphate and 0.1 ml of Temed (*NNN'*-tetramethylethylenediamine) per 150 ml of 0.1 M -Tris buffer, adjusted to pH 9.0 with conc. HCl. Electrophoresis of samples containing 1 M -sucrose and a trace of Bromophenol Blue at a constant voltage of 300 V was carried out for $3\frac{1}{2}\text{ h}$ at 4°C . The gel was then removed, trimmed and stained for xanthine oxidase with 100 ml of 0.2 M - Na_2HPO_4 buffer (adjusted to pH 7.4 with 0.2 M - NaH_2PO_4) containing 10 mg of hypoxanthine and 40 mg of Iodonitrotetrazolium Violet for 2 h at 37°C .

Sephadex G-200 column chromatography

Samples (2 ml) of purified xanthine oxidase extracts, containing 2000 units of activity, were applied to a column ($2.5\text{ cm} \times 50\text{ cm}$) of Sephadex G-200 and eluted with 0.05 M -Tris- 0.001 M -EDTA buffer (adjusted to pH 7.5 with conc. HCl). Fractions (2 ml) were collected and assayed for activity.

Sucrose-density-gradient centrifugation

Samples (1 ml), purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, were layered on to 22 ml of a linear 5–20% (w/v) sucrose density gradient and centrifuged in a $3 \times 23\text{ ml}$ aluminium swing-out rotor (model 59590) of

the MSE Superspeed 65 ultracentrifuge for 20 h at $100000g$ and 4°C . Fractions (1 ml ; 12 drops) were then collected from the bottom of the tubes and assayed for xanthine oxidase activity.

Analysis of dissociation and reassociation of subunits by various treatments

1. *Sodium dodecyl sulphate*. The sodium dodecyl sulphate was dissolved in 0.05 M -Tris- 0.001 M -EDTA buffer (adjusted to pH 7.0 with conc. HCl) and added to liver and duodenal extracts to give final concns. of 0.05%, 0.1%, and 0.2% (w/v) of detergent, respectively. The solutions were kept on ice for 1 h and then subjected to electrophoresis.

2. *Mercaptoethanol*. 2-Mercaptoethanol was added to the extracts to give a final concn. of 0.014 M (Kirkman & Hanna, 1968). The solutions were kept on ice for 25 min and then subjected to electrophoresis.

3. *Freezing and thawing with NaCl*. Liver and duodenal extracts of xanthine oxidase were prepared in 0.2 M - Na_2HPO_4 buffer (adjusted to pH 7.4 with 0.2 M - NaH_2PO_4). Then 0.5 M -NaCl was added and the solution frozen at -30°C for 18 h , allowed to thaw spontaneously at room temperature and subjected to electrophoresis (Goldberg, 1966).

Treatment with urea

Various amounts of urea were added to liver and duodenal xanthine oxidase extracts to give final concns. of 2, 4 and 6 M . The solutions were then dialysed overnight against 2 litres of 0.05 M -Tris- 0.001 M -EDTA buffer (adjusted to pH 7.0 with conc. HCl) containing 0.001 M -2-mercaptoethanol, and subsequently subjected to electrophoresis (Epstein & Schnechter, 1968).

Results

Electrophoresis

Polyacrylamide- and starch-gel electrophoresis reveal the presence of two major forms of mouse xanthine oxidase, both of which migrate towards the anode (Plate 1a). The liver enzyme migrates more slowly than that of the duodenum, which has an additional minor isoenzyme. An analysis, however, of the two tissue forms in 16-day-old embryo mice yielded similar 'liver-type' patterns for both tissues.

Precipitation with $(\text{NH}_4)_2\text{SO}_4$

The crude supernatant extracts were fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, by using salt saturations of 0–30, 30–40, 40–50, 50–60 and 60–70%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitates in each case were

EXPLANATION OF PLATE I

Electrophoresis of xanthine oxidase extracts

The purified enzyme preparations were subjected to electrophoresis on 4% polyacrylamide gel and stained as described in the Materials and Methods section. The trypsin-treated extracts were treated with 2.5mg of crude trypsin/ml and incubated at 37°C for 1½h. The arrow indicates the direction of electrophoretic mobility. (a) The samples were extracts from (i) liver and (ii) duodenum. (b) The samples were extracts from (i) trypsin-treated liver, (ii) trypsin-treated duodenum, (iii) and (iv) untreated liver and (v) untreated duodenum. O marks the position of the origin.

collected and resuspended as described in the Materials and Methods section. Fig. 1 shows the percentage of total activity for each 'cut' for the normal liver, the normal duodenum and the trypsin-treated liver respectively. All of the normal liver xanthine oxidase activity is precipitated in the range 30–50% satd. $(\text{NH}_4)_2\text{SO}_4$, whereas the normal duodenal xanthine oxidase activity is precipitated mainly in the range 40–60% satd. $(\text{NH}_4)_2\text{SO}_4$. The effect of trypsin treatment on the liver enzyme is noteworthy, in that the trypsin-treated form is precipitated mainly in the range 40–60% satd. $(\text{NH}_4)_2\text{SO}_4$, which is the range in which the duodenal form is precipitated.

Structural relationship between the liver and duodenal forms of xanthine oxidase

It is conceivable that the observed electrophoretic differences between the two forms could be due to proteolytic degradation of one or other form, either within the cell, or during the extraction procedure. To test if this was the case both enzymes, purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, were treated with 2.5 mg of a crude trypsin preparation/ml (from ox pancreas; BDH Chemicals Ltd., Poole, Dorset, U.K.), incubated at 37°C for 1½ h and subsequently subjected to electrophoresis. This trypsin treatment converted the electrophoretic mobility of the liver form into that of the control duodenal form, whereas the treated duodenal form was only slightly changed, showing a small increase in electrophoretic mobility (Plate 1b). When the liver form was treated with 0.625 mg and 1.25 mg of the crude trypsin/ml respectively, the former concentration failed to bring about conversion, whereas the latter increased the electrophoretic mobility to approximately one-half that obtained with the 2.5 mg of crude trypsin/ml. On the other hand, concentrations of up to 5 mg/ml of a pure crystalline trypsin preparation [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] caused only a partial conversion, even though this preparation was ten times more active than the crude preparation. However, the fact that enzyme activity (rather than some impurity) is essential seems clear from the observation that boiled preparations, or the addition of soya-bean trypsin inhibitor [Sigma (London) Chemical Co. Ltd.] completely inhibited the conversion. Again, when the duodenal enzyme was extracted in buffer containing 8 mg of the inhibitor/ml, on subsequent electrophoresis the mobility was considerably slower than that of the control and closely resembled the liver form. Incubation of the liver form with 2.5 mg of chymotrypsin/ml [Sigma (London) Chemical Co. Ltd.] also altered the electrophoretic mobility but, like pure trypsin, no complete conversion into a duodenal-like form was obtained. Treatment of liver enzyme with concentrations from 0.1–5.0 mg/ml, of a toluene suspension of

Fig. 1. *Precipitation of xanthine oxidase with $(\text{NH}_4)_2\text{SO}_4$*

The crude supernatant solutions were treated with $(\text{NH}_4)_2\text{SO}_4$ as described in the text. Each fraction was assayed for xanthine oxidase activity, and this is expressed as the percentage of total activity. □, Untreated liver; ■, untreated duodenum; ▨, trypsin-treated liver.

β -carboxypeptidase [Sigma (London) Chemical Co. Ltd.], failed to bring about any conversion. Also, incubation for up to 2 h at 37°C with concentrations of lipase [wheat-germ, type I; Sigma (London) Chemical Co. Ltd.] up to 10 mg/ml failed to bring about conversion of the liver enzyme.

The existence of other mechanisms, apart from proteolytic degradation, which could produce the two molecular forms of xanthine oxidase are very unlikely. Experiments were carried out, however, to test whether possible dissociation of subunits with subsequent reassociation, or different aggregations of subunits (as tested by Sephadex G-200 chromatography), or conformational alterations by treatment with urea, had any effects. In no case was any inter-conversion of isoenzymes or formation of new forms observed.

A comparison of the molecular weights of the various forms was carried out by using sucrose-density-gradient analysis. This, surprisingly, indicated that the trypsin-treated liver enzyme, although similar to the duodenal form, had an apparently higher molecular weight than that of the untreated molecule.

Discussion

Sackler (1965) has shown that at least two forms of xanthine oxidase exist in the rat. On starch-gel electrophoresis the liver enzyme had a slower mobility than that of the duodenum. Similar results, with polyacrylamide-gel electrophoresis have been reported for the mouse (Joyce & Duke, 1971). In recent years it has become apparent that enzymes can exist in a variety of forms as a consequence of proteolytic degradation. Hexokinase of yeast cells affords an

excellent example of this phenomenon (Gazith *et al.*, 1968). Conversion of isoenzymes by proteinases into active forms of different electrophoretic mobility is well known. Several enzyme systems have been studied including glucose 6-phosphate dehydrogenase (Hori & Noda, 1971), cholinesterase (Saeed *et al.*, 1971) and aldolase (Masters, 1967). In the case of aldolase, at least, the modified forms seem to be of physiological significance. Results from the present study show clearly that mouse liver xanthine oxidase can be fully converted into a duodenal-like form, as determined by electrophoresis, $(\text{NH}_4)_2\text{SO}_4$ precipitation, and sucrose-density-gradient molecular-weight determination, when incubated with crude trypsin. On the other hand, pure trypsin is only partially effective in converting the liver form into the duodenal form. Also the fact that chymotrypsin, but not β -carboxypeptidase, partially promotes this conversion could indicate that some specific peptide or peptides are removed. Kaplan (1968) suggests that modification of isoenzymes by proteases probably involves only the breakage of a few peptide linkages. The fact that tryptic action does not affect the activity of xanthine oxidase would seem to confirm this. The finding that trypsin treatment converts the liver enzyme into a molecule of apparently higher molecular weight as determined by sucrose-density-gradient centrifugation could be due to the removal of a peptide group to which a lipid moiety is attached. The fact that lipase has no overt effect on the enzyme would not invalidate this suggestion.

It can be concluded from this study that the duodenal form of xanthine oxidase is most likely an altered form of the liver enzyme. The evidence suggests, as expected, that the differences observed are probably due to proteolytic degradation during the extraction procedure, rather than to effects which occur within the intact cell. This is strongly supported by the fact that both tissues in embryo mice have

similar electrophoretic properties, presumably caused by the inadequate development of intestinal proteolytic enzymes in embryo tissue.

This work was in part supported by National Science Council of Ireland grant no. 48/71, which also supported J. P. R. as a post-doctoral research fellow. The technical assistance of Kay McKenna is acknowledged.

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