Intermediate Dehydrogenase–Oxidase Form of Xanthine Oxidoreductase in Rat Liver

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A spectrophotometric method for the determination of three forms of xanthine oxidoreductase, namely dehydrogenase (D), dehydrogenase–oxidase (D/O) and oxidase (O), is described. Enzymic fractions obtained from rat liver were found to contain either all three forms, or (under special conditions of preparation) only two forms, D and D/O. The conversion of form D→form D/O→form O in the presence of Cu^{2+} ions was shown. Form D/O acted with NAD^+ as well as with O_2 as electron acceptors, it exhibited greater affinity to NAD^+ than to O_2 , and NAD^+ abolished the oxidase activity of this form. Moreover, oxidase activity of form D/O was inhibited by NADH. These facts indicate that NAD^+ and O_2 compete for the same active site on the enzyme molecule.

The native form of xanthine oxidoreductase in rat liver has been found to act as a dehydrogenase (form D, EC 1.2.1.37) with NAD^+ as its natural electron acceptor, when xanthine or hypoxanthine were hydroxylated (Stirpe & Della Corte, 1969). Attempts to obtain a stable preparation of form D have not been successful; the dehydrogenase form was spontaneously transformed into the oxidase form (form O, EC 1.2.3.2) during purification, storage, heating and incubation (Stirpe & Della Corte, 1969; Waud & Rajagopalan, 1976*a,b*; Krenitsky & Tuttle, 1978). Moreover, a dehydrogenase–oxidase form (form D/O) of the enzyme, able to react with O_2 as well as with NAD^+ as electron acceptors, has been postulated (Della Corte & Stirpe, 1972; Krenitsky & Tuttle, 1978).

Owing to the unstability of form D it is difficult to study its properties and the transformation of form D into form O, which may possibly play some role in the regulation of the enzymic activity *in vivo*. The second difficulty is that for enzymic preparations from the rat liver the sum of the dehydrogenase+oxidase activities calculated from uric acid formation in the presence of $\text{NAD}^+ + \text{O}_2$ fails to equal the sum of the dehydrogenase activity, determined as NADH formation, and the oxidase activity, determined as uric acid formation in the absence of NAD^+ (Della Corte & Stirpe, 1972; Waud & Rajagopalan, 1976*a,b*).

In the present paper a spectrophotometric method for the determination of the activities of forms D, D/O and O of xanthine oxidoreductase is described. A method yielding the more stable form D of the enzyme from the rat liver is presented and some properties of the form D/O are reported.

Materials and Methods

Animals

Wistar male rats (weight about 300g) were given standard feed produced by Doświadczalny Zakład Produkcji Pasz, Borów, Poland. They were starved for 24 h (with water *ad libitum*), and then were killed.

Reagents

The sources of chemicals used were as follows: NAD^+ and dithiothreitol (both A grade), Calbiochem, Los Angeles, CA, U.S.A.; xanthine (p.A) and Tris, Serva, Heidelberg, Germany; sucrose (Ultrapure), Schwartz/Mann, Orangeburg, NY, U.S.A.; Chelex 100 (100–200 mesh), Bio-Rad Laboratories, Richmond, CA, U.S.A.; lactate dehydrogenase and pyruvate, Sigma, St. Louis, MO, U.S.A. $(\text{NH}_4)_2\text{SO}_4$ (p.A) from Polskie Odczynniki Chemiczne, Gliwice, Poland, was freed from heavy metals as follows: 5 litres of 3.8M- $(\text{NH}_4)_2\text{SO}_4$ solution were passed through a column (1.5cm×10cm) packed with Chelex 100 (NH_4^+ form). Redistilled methanol was added to the effluent (1:1, v/v), and the precipitate of $(\text{NH}_4)_2\text{SO}_4$ was separated on Whatman glass-fibre paper GF 83 and dried in a vacuum desiccator over P_2O_5 .

Redistilled water was used throughout.

The removal of heavy metals from $(\text{NH}_4)_2\text{SO}_4$ and water seems to be very important, because it is known that, for example, Cu^{2+} ions rapidly convert xanthine dehydrogenase into the oxidase form and later inactivate this enzyme almost completely (Della Corte & Stirpe, 1972).

Laboratory glassware used, including spectrophotometric cells, was soaked in 2% (w/v) EDTA

adjusted to pH 10 with Na_2CO_3 , and then rinsed with redistilled water.

Enzyme preparation

The homogenization solution contained 150 mM-sucrose and 100 mM-Tris/HCl buffer, pH 8.0; before use, dithiothreitol (final concn. 10 mM) was added to this solution. Freshly obtained rat livers were homogenized with 5 vol. of this solution in a Potter-Elvehjem homogenizer with a mechanically driven Teflon pestle, with cooling in an ice/water bath. The homogenate was centrifuged for 10 min at 25000g and 4°C. The supernatant was re-centrifuged for 60 min at 160000g and 4°C. The resulting supernatant, kept in an ice/water bath, was fractionated with a 3.8 M- $(\text{NH}_4)_2\text{SO}_4$ solution (Wood, 1976). To remove lipids adsorbed to protein, the fraction precipitated within the range of 1.6–2.4 M- $(\text{NH}_4)_2\text{SO}_4$ concentrations was suspended in 3.8 M- $(\text{NH}_4)_2\text{SO}_4$; after centrifugation of the suspension for 10 min at 10000g and 4°C, the opaque supernatant was discarded. The pellet was dissolved in 250 mM-sucrose (0.5 ml/g of liver) and kept at –25°C. This solution contained the enzyme purified about 5.5 times compared with the 25000g supernatant, and was used in experiments without further purification. If this solution became opaque during the storage (some material was precipitated), it was passed through a Sartorius cellulose nitrate membrane filter SM 11406 (pore size 0.45 μm).

The enzymic preparation obtained was free of the activities of uricase and aldehyde oxidase, the latter being tested with 6-hydroxy-3-methylpurine (1.5 mM) as substrate (Krenitsky *et al.*, 1972). No increase in A_{340} was observed when the enzyme preparation was incubated with NAD^+ alone.

Spectrophotometric measurements

The u.v. spectra and the increases in A_{302} and A_{340} were recorded on a Cary 118C spectrophotometer equipped with a Repetitive Scan.

Hydroxylation of xanthine was carried out in quartz cells, light-path 1 cm, under aerobic conditions. The standard incubation mixture contained 50 mM-Tris/HCl buffer, pH 8.0, 50 μM -xanthine, enzyme preparation [300 μg of protein (determined by the method of Stauffer, 1975)/ml] either without NAD^+ or with 150 μM - NAD^+ in a final volume of 3 ml. The blank contained no xanthine. In some experiments 40 nkat of lactate dehydrogenase and 0.5 mM-sodium pyruvate were added to oxidize NADH and thereby to prevent the inhibition of xanthine dehydrogenase by this compound (Della Corte & Stirpe, 1970).

The enzymic activity was measured as formation of uric acid and NADH (increases in A_{302} and A_{340} , respectively) and expressed in nmol of these sub-

stances/ml of the incubation mixture. The enzymic activity was calculated taking into account the initial rates of reactions. The molar absorption coefficient for NADH at 340 nm and pH 8.0 was $6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Uric acid formation was measured at 302 nm because its absorbance is still high there, whereas changes in NAD^+ concentration do not contribute. On the other hand, the molar absorption coefficient for NADH at 302 nm and pH 8.0 was $2.30 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Thus $\Delta A'_{302}$, the true A_{302} increase resulting from the formation of uric acid in the presence of simultaneously produced NADH, was calculated from the equation:

$$\Delta A'_{302} = \Delta A_{302} - \Delta A_{340} \cdot \frac{2.30}{6.22}$$

Moreover, a decrease in molar absorption coefficient at 302 nm for conversion of xanthine into uric acid was taken into account and $\epsilon_{302} = 7.12 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was used for calculation of uric acid amounts.

Determination of enzymic activities of forms D, D/O and O

Measurements of the increases in A_{302} and A_{340} permit determination of the activities of all three forms of the enzyme with the assumptions that: (a) the form D/O acts as an oxidase in the absence of NAD^+ and as a dehydrogenase at saturating concentrations of NAD^+ ; (b) both activities of the form D/O are equal at saturating concentrations of a suitable electron acceptor (see the Results and Discussion section). Then the enzymic activity (I) measured at 302 nm in the presence of O_2 , NAD^+ , lactate dehydrogenase and pyruvate consists of the uninhibited dehydrogenase activities of forms D and D/O and the oxidase activity of form O. The enzymic activity (II) measured at 302 nm in the presence of O_2 only comprises the oxidase activities of forms O and D/O. The enzymic activity (III) measured at 302 nm in the presence of O_2 and NAD^+ and in the absence of lactate dehydrogenase and pyruvate consists of dehydrogenase activities (inhibited partly by NADH) of forms D and D/O and the oxidase activity of form O. The enzymic activity (IV) measured at 340 nm in the presence of O_2 and NAD^+ and in the absence of lactate dehydrogenase and pyruvate consists of dehydrogenase activities (inhibited partly by NADH) of forms D and D/O. The enzymic activities (III) and (IV) were measured simultaneously in the same incubation mixture.

As a consequence:

the dehydrogenase activity of form D = (I) – (II)

the oxidase activity of form O = (III) – (IV)

the oxidase activity of form D/O

= (II) – [(III) – (IV)]

the total dehydrogenase activity (the sum of that of forms D+D/O) = (I) - [(III) - (IV)].

Results and Discussion

Freshly obtained enzyme preparations showed a relative dehydrogenase content, defined as 'D/O' ratio (Waud & Rajagopalan, 1976a), of 7.5–8.8, comparable with the values for the best preparations obtained as antibody complexes by Waud & Rajagopalan (1976b). However, our measurements were carried out at 302nm in the presence of NAD⁺, lactate dehydrogenase and pyruvate, whereas Waud & Rajagopalan (1976a,b) measured the activity at 295nm and did not specify whether the inhibitory effect of NADH was overcome. In contrast with their enzyme preparations, the 'D/O' ratio of our enzyme preparations did not change during storage at -25°C for at least 6 days, and it fell to 4.9 only after 8 weeks.

Presence of form D/O of the enzyme

Typical progress curves of the reactions catalysed by xanthine oxidoreductase in freshly obtained enzyme preparations are presented in Fig. 1. At the concentrations of xanthine and NAD⁺ used, the hydroxylation of xanthine and reduction of NAD⁺

Fig. 1. Xanthine hydroxylation by freshly obtained enzymic preparation from rat liver

Experimental conditions were as described in the Materials and Methods section. Curves represent xanthine oxidoreductase activities measured as: (a) uric acid at 302nm (O₂ + NAD⁺ + lactate dehydrogenase + pyruvate); (b) uric acid at 302nm (O₂ + NAD⁺); (c) NADH at 340nm (O₂ + NAD⁺); (d) uric acid at 302nm (O₂). Enzyme forms were as follows: D, difference between curves (a) and (d); D/O, curve (d); O, absent (lack of difference between curves b and c).

were linear functions of time, irrespective of the kind of the spectrophotometric measurements performed (see the Materials and Methods section). When O₂ and NAD⁺ were present and lactate dehydrogenase + pyruvate were omitted from the incubation mixture, the activity measured at 302nm (curve b) appeared to equal the activity measured at 340nm (curve c). This showed that the enzyme preparation did not contain the form O. The low activity in the absence of NAD⁺ (curve d) must correspond to the oxidase activity of form D/O, which in the presence of NAD⁺ acted as dehydrogenase. Thus curve (a) obtained in the presence of O₂, NAD⁺, lactate dehydrogenase and pyruvate represents the sum of uninhibited dehydrogenase activities of forms D and D/O. Comparison of curves (a) and (b) shows that the inhibition of the dehydrogenase activity of the enzyme preparation by NADH accounted for 48% and appeared immediately when NADH began to accumulate. The fresh enzyme preparations contained 87% of form D and 13% of form D/O, and form O was absent. Such distribution of the total xanthine oxidoreductase activity persisted for at least 6 days of storage at -25°C.

In enzyme preparations stored for several weeks at -25°C or in fresh enzyme preparations obtained without dithiothreitol in the homogenization solution the activity measured at 302nm in the presence of NAD⁺ and the absence of lactate dehydrogenase and pyruvate (Fig. 2, curve b) was higher than the

Fig. 2. Xanthine hydroxylation by enzymic preparation from rat liver after storage for several weeks at -25°C. Experimental conditions were as described in the Materials and Methods section. Curves (a), (b), (c) and (d) are as in Fig. 1; curve (e) is the difference between curves (b) and (c). Enzyme forms were as follows: D, difference between curves (a) and (d); D/O, difference between curves (d) and (e); O, curve (e).

activity measured at 340 nm (curve *c*); this difference (curve *e*) shows that form O of the enzyme was present. This activity of form O was lower than the oxidase activity measured at 302 nm in the absence of NAD^+ (curve *d*), which showed that the form D/O was also present. It may be noted that form O is not inhibited by NAD^+ (Waud & Rajagopalan, 1976a) or by NADH (Della Corte & Stirpe, 1970), and in fact the activity of form O in their presence is linear with time (curve *e*).

The fact that the enzyme preparations initially contained only forms D and D/O, whereas form O appeared later, supports the hypothesis assuming that form D/O is an intermediate stage in the transformation of form D into form O, as Della Corte & Stirpe (1972) have postulated. In the hypothetical scheme presented by those authors, form D is transformed into intermediate form D/O, and further into two forms O (reversible and irreversible); this transformation occurred when Cu^{2+} or thiol reagents were added to the enzymic preparation. In subsequent experiments the effect of Cu^{2+} ions on the content of forms D, D/O and O in the enzyme preparation was investigated.

Conversion of the three forms of the enzyme induced by Cu^{2+} ions

The enzyme preparation was preincubated at various Cu^{2+} concentrations for 5 min, whereupon the dehydrogenase and oxidase activities were measured as usual. Contents of the individual forms of the enzyme as a function of CuSO_4 concentration are presented in Fig. 3. The preparation used did not initially contain form O (curve *c*), and form D/O (curve *b*) accounted for 12.5% of the total activity. With an increase in the Cu^{2+} concentration, the content of form D (curve *a*) decreased and at $7 \mu\text{M}$ - CuSO_4 disappeared completely. However, the preparation continued to exhibit the dehydrogenase activity caused by the presence of form D/O (curve *d*, sum of forms D+D/O). Initially the content of this form increased, reaching a maximum at $3 \mu\text{M}$ - CuSO_4 concentration, and then decreased. The content of form D/O predominated over that of form D at CuSO_4 concentrations higher than $3 \mu\text{M}$; thus form D/O must arise from form D. On the other hand, the content of form O (curve *c*) steadily increased with CuSO_4 concentration, and above $7 \mu\text{M}$ - CuSO_4 form O arose exclusively from form D/O. The shapes of the curves therefore showed that the sequence of the transformation process is: form D $\rightarrow$ form D/O $\rightarrow$ form O. The final oxidase activity of the enzymic preparation was equal to its initial dehydrogenase activity; this fact may support the arbitrary assumption that the dehydrogenase activity of form D/O is equal to its oxidase activity (see the Materials and Methods section).

Fig. 3. Conversion of forms D, D/O and O, induced by Cu^{2+} ions

The enzymic preparation was incubated with various concentrations of CuSO_4 for 5 min, and then added to standard incubation mixtures. Data calculated from absorbance increases after 2.5 min of xanthine hydroxylation are presented. Curves represent activities of: (a) form D, (b) form D/O, (c) form O, and (d) forms D+D/O (total dehydrogenase activity). It is evident that the initial dehydrogenase activity was equal to the final oxidase activity.

At higher Cu^{2+} concentrations the oxidase form was transformed into the inactive form, as reported previously (Della Corte & Stirpe, 1972).

A similar but less complete transformation of form D to forms D/O and O was obtained with Pb^{2+} ions within the concentration range 20–100 μM .

The ratio 'D/O', defined as stated above and by Waud & Rajagopalan (1976a), seems to be an inadequate measure of the efficiency of NAD^+ as electron acceptor. Preparations with 'D/O' ratio = 1 still exhibited the dehydrogenase activity owing to the presence of the intermediate form D/O, if NADH formed was determined and taken into account.

Competition between NAD^+ and O_2 for form D/O

The dependence of the oxidase activity of form D/O on the NAD^+ concentration in the incubation mixture is presented in Fig. 4. Since the preparation used failed to exhibit oxidase activity at saturating concentration of NAD^+ (curve *a*), it did not contain form O. Oxidase activity found in the absence of NAD^+ (curve *a*, first point) corresponded then to the activity of form D/O, and it decreased with an increase in the concentration of NAD^+ . The dehydrogenase activity (measured as NADH formation) rose much more quickly (curve *b*) than the oxidase activity decreased, because form D/O represented

only 10% of the total xanthine oxidoreductase in the enzymic preparation, the remaining part consisting of form D. The oxidase activity of form D/O disappeared at about $40\mu\text{M-NAD}^+$. Since the concentration of O_2 in the incubation mixture was about 6 times as high (approx. $240\mu\text{M}$), it can be assumed that the affinity of form D/O is greater for NAD^+ than for O_2 . This difference would be in agreement with the difference between the Michaelis constants for appropriate electron acceptors of forms D and O of the milk xanthine oxidoreductase: K_m for $\text{NAD}^+ = 15\mu\text{M}$ (Battelli *et al.*, 1973) and K_m for $\text{O}_2 = 50\mu\text{M}$ (Massey *et al.*, 1969), respectively.

Waud & Rajagopalan (1976a) have found that the activity of form O is not affected by NAD^+ . On the other hand, Krenitsky & Tuttle (1978) have found that O_2 did not interfere with electron acceptance by NAD^+ during xanthine-dependent NAD^+ reduction. These latter authors supposed that separate sites for O_2 and NAD^+ exists, or two FAD sites are non-equivalent. The abolition of the oxidase activity of form D/O by NAD^+ found in our experiments seems to indicate that NAD^+ and O_2 compete for the same site on the molecule of form D/O. This is in agreement with the results obtained with the deflavo forms of bovine xanthine oxidase (Komai *et al.*, 1969) and avian xanthine dehydrogenase (Brady *et al.*, 1971); these investigations have shown that electron

transfer to O_2 and NAD^+ occurs almost exclusively from FAD. Probably the flavin site changes during the transformation of form D into form O in such a manner that in form D it is able to react only with NAD^+ , in form D/O with both electron acceptors, and in form O with O_2 only.

Inhibition of the form-D/O activity by NADH

A dual effect of NADH on form D/O could be expected: NADH may inhibit either both dehydrogenase and oxidase activities or only the dehydrogenase activity, perhaps promoting the oxidase activity, despite the high concentration of NAD^+ .

The enzyme preparation used contained all three forms of xanthine oxidoreductase. Enzyme activities were plotted against the increasing concentrations of NADH added to the incubation mixtures (Fig. 5). Because the activity of form O is not inhibited by NADH (Della Corte & Stirpe, 1972), its constancy (curve *a*) showed that no oxidase activity of form D/O was promoted by NADH. However, at the highest possible concentration ($93\mu\text{M}$) of NADH that could be used under the present spectrophotometric conditions, the dehydrogenase activity of forms D+D/O (curve *b*) was inhibited by only 80%; thus the dehydrogenase activity of form D/O, representing only 14% of total enzyme activity, could still be non-inhibited. On the other hand, in the absence of NAD^+ the oxidase activity of forms

Fig. 4. Dependence of the oxidase activity of form D/O on the NAD^+ concentration

Experimental conditions were as described in the Materials and Methods section, except that various concentrations of NAD^+ were added to the incubation mixtures. Reaction time was 5 min. Form O was absent (as shown by the lack of difference between uric acid and NADH formation at saturating NAD^+ concentrations). Curve (a), oxidase activity of form D/O; curve (b), dehydrogenase activity of forms D+D/O.

Fig. 5. Effect of NADH on the activity of form D/O. Experimental conditions were as described in the Materials and Methods section, except that various concentrations of NADH were added to the incubation mixtures. Data calculated from absorbance increases obtained after 2 min of xanthine hydroxylation are presented. Curves represent activities of: (a), form O; (b), forms D+D/O; (c), forms D/O+O; (d), form D/O.

D/O+O (curve *c*) decreased with increasing concentrations of NADH; this decrease must be due to the inhibition of form D/O by NADH (curve *d*) and not to the diaphorase activity of form O, because O₂ does not act as electron acceptor for NADH oxidation by the enzyme (Waud & Rajagopalan, 1976b). In comparison with the inhibition of the dehydrogenase activity of forms D+D/O (curve *b*), the inhibition of oxidase activity of form D/O was pronouncedly slower at low concentrations of NADH, but attained 87% at 93 μ M-NADH.

The inhibition of the oxidase activity of form D/O by NADH supports the conclusion in the previous paragraph that NAD⁺ and O₂ react with the same site of the enzyme molecule.

Although we were not able to demonstrate the inhibition of the dehydrogenase activity of form D/O by NADH, it seems reasonable to assume that NADH inhibits both dehydrogenase and oxidase activities of this form.

Della Corte & Stirpe (1972) have postulated that xanthine oxidoreductase from rat liver may occur in four active forms: native dehydrogenase, intermediate dehydrogenase-oxidase, and two oxidases, reversible and irreversible. In the present report the existence of the intermediate form D/O was demonstrated for the first time by using the spectrophotometric method; moreover changes in its activity under various conditions were determined. The intermediate form D/O appeared to be a transient form in the transformation of form D to the reversible form O, as postulated by Della Corte & Stirpe (1972). Form D/O may represent the first step of the degradation

of native form D in the living cell, the reversible form O being the next step. The form D/O is inhibited by NADH similarly to the native form D; therefore its activity may still be regulated by the [NAD⁺]/[NADH] ratio, this regulation seeming to be important *in vivo*.

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