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PURIFICATION OF HEPATIC XANTHINE DEHYDROGENASE FROM CHICKEN FED A HIGH-PROTEIN DIET

TAKESHI NISHINO

Department of Biochemistry, Yokohama City University School of Medicine, Yokohama (Japan)

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SUMMARY

Xanthine dehydrogenase (xanthine:NAD^+ oxidoreductase, EC 1.2.1.37) of high specific activity was obtained from chicken liver. As a starting material the livers of chicken fed a high-protein diet were adopted.

In the absorption spectrum of the purified enzyme the ratio $A_{280\ nm}/A_{450\ nm}$ was 5.54, which suggests a high purity. The specific activity of the purified enzyme was 1200 and 57 as expressed by the turnover number and the activity protein ratio, respectively.

INTRODUCTION

The purification of xanthine dehydrogenase from chicken liver has been reported in a number of laboratories, e.g., by Remy et al. [1], Rajagopalan and Handler [2], and Kanda and Rajagopalan [3]. Rajagopalan and Handler have purified this enzyme to near homogeneity, and the specific activity (as expressed by turnover number; moles of NADH/mole of FAD per min) of this enzyme preparation has been reported to be 506.

Kanda and Rajagopalan [3] have prepared the same enzyme preparation having a specific activity of 23 ± 2 from chicken liver according to the method of Rajagopalan and Handler [2] and presented the evidence leading to the conclusion that the two FAD moieties of this enzyme molecule may be catalytically nonequivalent, although the possibility of the presence of a nonfunctional enzyme molecule containing a full complement of prosthetic groups in their enzyme preparation has not been excluded.

In the purified xanthine oxidase preparation from bovine milk, the occurrence of inactive forms of enzyme has been suggested by Morell [4] to account for the biphasic course of the reduction of flavin moieties by xanthine as substrate. Hart et al. [5] also indicated the existence in the enzyme preparation of nonfunctional xanthine oxidase containing a full complement of the oxidation–reduction groups in addition to the inactive form lacking molybdenum. Recently Edmondson et al. [6] have resolved an inactive form of enzyme by affinity chromatography and suggested the possibility that the lability of a persulfide group required for catalysis might be responsible for the nonfunctional enzyme.

From the reports cited above, it might be probable that the purified xanthine

dehydrogenase preparations so far obtained by several workers contained inactive enzyme molecules to some extent.

For a proper understanding of the mechanism of action of xanthine dehydrogenase, it is important to isolate the enzyme having higher specific activity than that so far reported.

In this article, the purification of hepatic xanthine dehydrogenase having a high specific activity is described.

MATERIALS AND METHODS

Animals

1-day-old chickens were obtained from a local dealer and were fed a commercial chicken starter ration for 10 to 14 days before being fed a high-protein diet. The xanthine dehydrogenase activity was increased about 3- to 5-fold by treatment with a high-protein diet for 3 days. For enzyme purification, usually 50 to 70 chickens fed a high-protein diet were used. A high-protein diet was prepared by adding a small amount of water to soybean protein powder (about 63% protein content).

Chemicals

Xanthine was obtained from Nutritional Biochemical Co., NAD from Sigma, *o*-phenanthroline from Wako Junyaku. DEAE-cellulose was obtained from Brown, DEAE-Sephadex A-25 and Sephadex G-200 from Pharmacia. Calcium phosphate gel was prepared according to the method of Tsuboi and Hudson [7].

Enzyme assay

Xanthine dehydrogenase activity was measured routinely according to the method of Rajagopalan and Handler [2]. The value of the activity protein ratio and the activity flavin ratio defined by Avis et al. [8] were obtained by dividing the change in absorbance per min at 295 nm by the absorbance at 280 nm and 450 nm of xanthine dehydrogenase used in the assay, respectively. The change in absorbance per min at 295 nm was followed at 23.5 °C in the presence of 0.15 mM xanthine and 0.5 mM NAD.

Analytical methods

Molybdenum was determined colorimetrically by the method of Hart et al. [5], iron as the ferrous tris-*o*-phenanthroline after trichloroacetic acid denaturation of the enzyme by the method of Brumby and Massey [9], and flavin by the procedure described by Beinert and Page [10]. Protein was determined by the method of Lowry et al. [11] with crystalline bovine serum albumin as standard.

Analytical polyacrylamide gel (5.75% acrylamide in 0.039 M Tris–HCl buffer, pH 8.9) disc electrophoresis was performed according to the procedure of Davis [12] at 5 mA per tube for about 90 min.

RESULTS AND DISCUSSION

Purification of xanthine dehydrogenase

All preparative operations, unless otherwise stated, were carried out in a cold room at 4 °C. The enzyme solution in the course of purification was kept unfrozen.

Homogenization

310 g of freshly excised livers were homogenized in a Waring Blender for 2 min with 460 ml of 0.1 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 10 000 × *g* for 20 min, and the supernatant solution was centrifuged at 100 000 × *g* for 90 min.

First $(NH_4)_2SO_4$ fractionation

To the supernatant solution solid $(NH_4)_2SO_4$ (19 g/100 ml) was added with stirring. After gentle stirring for 30 min, the precipitate was removed by centrifugation and solid $(NH_4)_2SO_4$ (10.5 g/100 ml) was added to the supernatant solution. After stirring for 30 min, the precipitate was collected by centrifugation. It was dissolved in 0.05 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA in a final volume of 160 ml.

Acetone precipitation

The solution was placed in an ice bath and cold acetone (–20 °C) was added slowly to a concentration of 42% (v/v). The precipitate obtained by centrifugation at –10 °C, was dissolved in 140 ml of the same buffer as above, and insoluble material was removed by centrifugation.

Second $(NH_4)_2SO_4$ precipitation

Solid $(NH_4)_2SO_4$ (34.2 g/100 ml) was added to the solution and after stirring for 30 min, the precipitate was collected by centrifugation. It was dissolved in a minimum amount of 0.01 M potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA, and dialysed against 5 l of the same buffer overnight.

Calcium phosphate gel absorption

To the dialysed solution calcium phosphate gel (2 mg dry weight/mg protein) was added and the mixture was gently stirred for 30 min. The gel was collected by centrifugation and was washed twice with 500 ml of 0.05 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The enzyme was eluted by successive treatment with 300 ml and 200 ml of 0.2 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The enzyme solution was concentrated by $(NH_4)_2SO_4$ precipitation (38 g/100 ml) and was dialysed against 5 l of 0.05 M Tris–HCl buffer (pH 8.5) containing 0.1 mM EDTA.

DEAE-cellulose column chromatography

The dialysed solution was absorbed on a DEAE-cellulose column of 3 cm × 35 cm which had been equilibrated with 0.05 M Tris–HCl buffer (pH 8.5) containing 0.1 mM EDTA. The column was washed with 1 l of the same buffer, and was developed by a linear gradient of 0 to 0.2 M KCl. The enzyme was eluted as a single peak at about 0.1 M KCl.

Sephadex G-200 column chromatography

The enzyme fractions from the previous step were pooled and concentrated with an Amicon ultrafiltration cell to 6 ml and this was applied to a Sephadex G-200 column of 5 cm × 90 cm which had been equilibrated with 0.02 M Tris–HCl buffer

(pH 8.2) containing 0.1 mM EDTA. The column was developed upward with the same buffer. In this column chromatography, the enzyme activity was eluted as a major peak at the fraction corresponding to the molecular weight of about 300 000 with a minor peak having a somewhat lower specific activity at the void volume.

The presumed polymeric forms of the enzyme in the fraction of the minor peak could not be depolymerized by the treatment of 6 M urea or 0.1 % sodium dodecylsulphate, but depolymerization occurred with 10 mM mercaptoethanol or 1 mM dithiothreitol using polyacrylamide gel electrophoresis and Sephadex G-200 gel filtration, which suggests that a disulfide bond is formed in polymerization.

The content of the polymeric forms was found to vary with different batches of the preparation.

DEAE-Sephadex column chromatography

The main fraction from the previous step was applied to a DEAE–Sephadex column of 2.5 cm × 40 cm equilibrated with 0.02 M Tris–HCl buffer (pH 8.2) containing 0.1 mM EDTA. The column was developed using a linear gradient of 0 to 0.4 M KCl. The enzyme activity was eluted as a single peak coincident with the protein peak. The fractions containing the enzyme activity were pooled.

In the polyacrylamide gel electrophoresis of the enzyme purified in this step, slowly moving contaminants having enzyme activity, which are supposed to be polymeric forms were still detectable, but the $A_{280\ nm}/A_{450\ nm}$ ratio was lowered by this column chromatography.

A representative purification is summarized in Table I.

TABLE I

PURIFICATION OF HEPATIC XANTHINE DEHYDROGENASE FROM CHICKEN FED A HIGH-PROTEIN DIET

Fraction	Total activity (μmoles/min)	Total protein (mg)	Specific activity (μmoles/min per mg)	Yield (%)
100 000 × *g* supernatant	887	27 500	0.032	100
1st $(NH_4)_2SO_4$-fraction	829	11 700	0.071	93
Acetone ppt.	680	6 420	0.106	77
2nd $(NH_4)_2SO_4$-fraction	675	5 170	0.130	76
Calcium phosphate gel	439	1 350	0.325	49
DEAE-cellulose	352	281	1.25	40
Sephadex G-200	318	62.4	5.09	36
DEAE-Sephadex	250	49.0	5.10	28

Enzyme analyses

The specific activity and other analytical data for the purified enzyme are summarized in Table II.

The absorption spectrum of the purified enzyme was similar to that reported by Rajagopalan and Handler [2], except that the ratio of the absorbances, $A_{280\ nm}/A_{450\ nm}$, was rather lower than the value reported by them. The contents of molyb-

TABLE II

ANALYTICAL DATA OF XANTHINE DEHYDROGENASE

Specific activity (μmoles/min per mg of protein)*	5.10
Turnover number (moles/mole of FAD per min)**	1 200
Activity protein ratio (23.5 °C)	57
Activity flavin ratio (23.5 °C)	313
$A_{280\ nm}/A_{450\ nm}$	5.54
$A_{450\ nm}/A_{550\ nm}$	2.8
Molybdenum content (gatoms/mole of FAD)	0.86
Iron content (gatoms/mole of FAD)	3.99

* Protein determination by the method of Lowry et al. [11].
** Apparent V with 0.15 mM xanthine as substrate and 0.5 mM NAD as electron acceptor.

denum and iron per mole of FAD were consistent with those reported by the same authors.

The apparent turn-over number (1200 moles of xanthine oxidized per mole of FAD) is considerably higher than that obtained by Rajagopalan and Handler [2]. Although the reason for the difference in the specific activity between these two enzyme preparations of chicken liver is uncertain, one reason is supposed to be that liver of high enzyme concentration was used as a starting material in this experiment, as Remy et al. [1] described that the specific activity of the purified enzyme depended on the activity of the original liver.

Recently Kanda and Rajagopalan [3] have reported that their xanthine dehydrogenase preparation of chicken liver containing a full complement of prosthetic groups, exhibits properties which indicate a nonequivalence of the two FAD moieties of this enzyme molecule. However, as they have described, the presence of a non-functional enzyme molecule containing a full complement of prosthetic groups in their enzyme preparation is still possible, since the specific activity of the enzyme preparation obtained in the present experiment is about twice as high as that obtained by them.

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