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ALIPHATIC L-α-HYDROXYACID OXIDASE FROM RAT LIVER

II. A FLAVOPROTEIN

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SUMMARY

An aliphatic L-α-hydroxyacid oxidase present in a light mitochondrial fraction of rat liver cells was purified some 600-fold. At this stage the enzyme had a purity of 67%. The enzyme was still contaminated with catalase, and its absorption spectrum did not show a typical flavoprotein curve. Absorbance at 460 mμ was rapidly reduced by the addition of dithionite in air or of substrate during anaerobiosis. The difference spectrum of the reduced enzyme suggested that the enzyme is a flavoprotein. Spectrophotometry and paper chromatography of the extract of the enzyme after heat treatment indicated that the prosthetic group is FMN.

INTRODUCTION

We have shown that a partially purified L-α-hydroxyacid oxidase obtained from rat liver catalyzes the dehydrogenation of glycolate and of many aliphatic L-α-hydroxy acids by molecular oxygen[1]. The production of hydrogen peroxide suggests that the enzyme is not a heme protein. There was no spectrophotometric evidence of flavoprotein after 300-fold purification of the enzyme.

We now report further purification of the enzyme and its identification as a FMN-containing protein.

EXPERIMENTAL

Materials

FMN and FAD were purchased from the Sigma Chemical Co. Rat liver catalase, purified according to HIGASHI AND PETERS[2], was kindly provided by Dr. M. Saga in our laboratory. The absorbance curve for the purified catalase showed peaks at 276, 407, 540 and 625 mμ. $A_{407\ m\mu}/A_{276\ m\mu}$ was 1.04; $A_{312\ m\mu}/A_{407\ m\mu}$, 0.2; $A_{252\ m\mu}/A_{276\ m\mu}$, 0.60 and $A_{407\ m\mu}$ per mg of protein per ml, 1.6. These data agree with those reported by other authors[2]. Anticatalase serum was obtained from a rabbit immunized with rat liver catalase[2].

Methods

The activity of the enzyme was measured by the 3-hydrazinoquinoline method using L-α-hydroxyisocaproate as the substrate[1]. One unit of activity is defined as the amount of enzyme that produces 1 mμmole of α-ketoisocaproate per 20 min under the assay condition. The specific activity is stated in units per mg of protein. The concentration of protein was measured by the phenol method[3]. Absorbance spectra were obtained with a Hitachi model 139 spectrophotometer and a Hitachi model EPS-2U spectrophotometer with automatic recorder. Fluorimetric determination was carried out in a Hitachi model G-2 spectrofluorimeter with automatic recorder. Antibody titration with purified catalase was carried out by the incubation of rat liver catalase (5 to 30 μg) with a known amount of anticatalase serum (0.2 ml), and the estimation of the protein concentration of precipitable catalase–antibody complex according to a modification of the procedure of HIGASHI AND PETERS[2]. The standard precipitin curve was then obtained by plotting the amount of the precipitate against catalase added. Catalase present in the enzyme preparation was precipitated with the anticatalase serum by the antibody titration method and calculated from the amount of precipitate using the standard precipitin curve. The purity of the enzyme preparation was examined by acrylamide-disc electrophoresis[4]. Protein components in the acrylamide gel were stained with Amido Black, and relative protein concentrations were determined by densitometry of the photograph of stained protein pattern (indirect densitometry). The enzyme was extracted from the gel, by repeated freezing and thawing, into a small amount of 0.005 M sodium phosphate buffer which contained 1 mg of human serum albumin per ml at pH 7.9. The enzyme activity of the extract was measured as described above.

RESULTS AND DISCUSSION

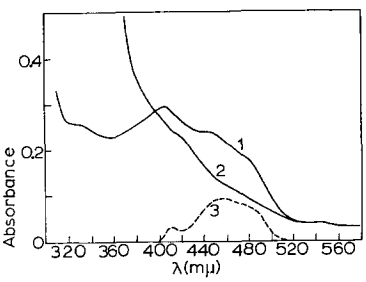
The initial stages of purification (extraction of the enzyme from the light mitochondrial fractions, the first ammonium sulfate treatment, the first and second DEAE-cellulose column chromatography, and gel filtration on Sephadex G-200) were performed as described in the previous report[1]. In the experiments described herein the increased amounts of the enzyme preparation obtained after the second DEAE-cellulose column chromatography, 25–100 mg as protein, were subjected to gel filtration on a Sephadex G-200 column (2.5 cm × 53 cm). The column was equilibrated and eluted with 0.005 M sodium phosphate buffer containing 10^{-3} M EDTA at pH 7.9. Effluent fractions containing approx. 3.5 ml were collected. The active fractions (more than 30 000 units per $A_{280\ m\mu}$) were combined, and the protein was precipitated by the addition of solid ammonium sulfate to 0.8 saturation. The yellowish precipitate obtained was dissolved in a small amount of 0.05 M sodium phosphate buffer containing 10^{-3} M EDTA at pH 7.9 and again passed through a Sephadex G-200 column. Active fractions (more than 50 000 units per $A_{280\ m\mu}$) were combined, and the enzyme was then precipitated with ammonium sulfate as described above. The precipitate was dissolved in a small amount of 0.05 M sodium phosphate buffer containing 10^{-3} M EDTA (pH 7.9) and dialyzed against 3 l of 0.005 M sodium phosphate buffer containing 10^{-3} M EDTA at pH 7.9. The specific activity of the highly purified enzyme was 55 000–61 000 units per mg protein.

Purity of the L-α-*hydroxyacid oxidase*

Examination of the highly purified enzyme with a specific activity of 61 000 units per mg protein by acrylamide-disc electrophoresis with Amido Black stain revealed one main component and at least three additional components. Estimates based on color densities suggested that the main band comprised approx. 67% of the protein total. Only the main component was enzymically active.

Study of the prosthetic Group

The absorbance spectra of the highly purified oxidase with a specific activity of 61 000 units per mg protein before and after reduction with dithionite are presented in Fig. 1. The difference spectrum of the reduced enzyme showed maxima at 407 and



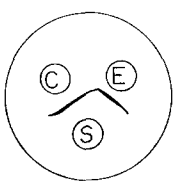


Fig. 1. Absorbance spectra of the enzyme (specific activity, 61 000; protein, 1.96 mg/ml) before and after the addition of dithionite at pH 7.9. Curve 1, oxidized form of the enzyme; Curve 2, same solution as that used to establish Curve 1, 10 min after the addition of dithionite in air; Curve 3, difference spectrum of Curve 2 (Curve 1 — Curve 2).

Fig. 2. Agar diffusion analysis of anticatalase serum interaction. The well marked E contained 2.5 mg/ml of L-α-hydroxyacid oxidase (specific activity, 35 000) which may be contaminated with 178 μg of catalase per ml; C contained 1000 μg of pure catalase per ml; S contained antiserum. Both bands in the plate gave a catalytic reaction when hydrogen peroxide was applied.

450 mμ, suggesting that the enzyme is a flavoprotein contaminated by iron protein.

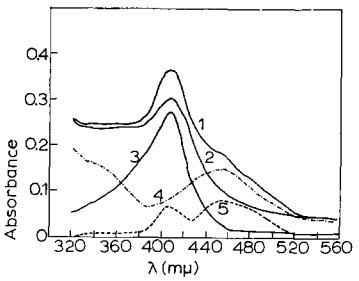
The presence of catalase in the enzyme preparation was demonstrated by agar diffusion tests[2] using anticatalase serum as antibody (Fig. 2). Since a protein other than catalase could not be detected as precipitation upon agar diffusion of the purified catalase or the enzyme preparations against the anticatalase serum, the concentration of catalase in the enzyme preparation was easily determined by precipitin reaction between the enzyme preparation and anticatalase followed by calculation from the standard precipitin curve for the purified catalase–anticatalase interaction.

The absorbance spectrum of catalase present in the enzyme preparation, calculated from the concentration of catalase in this preparation (0.178 mg/ml) and the absorption spectrum of purified catalase from rat liver ($A_{407\ m\mu}$, 1.6 per mg protein per ml), and that after incubation of the enzyme with substrate under anaerobic conditions, are shown in Fig. 3. An increased absorption ratio, $A_{407\ m\mu}/A_{450\ m\mu}$, was observed in this preparation together with a lower specific activity. The plots of the difference between the curves for catalase in this preparation and the oxidized preparation (Curve 4 in Fig. 3) represent the absence of a peak absorbance at 407 mμ and a low $A_{410\ m\mu}/A_{450\ m\mu}$ ratio, approx. 0.6.

This difference spectrum resembles that of the short-chain L-α-hydroxyacid oxidase from pig renal cortex, a flavoprotein with FMN as prosthetic group[5]. The difference spectrum of the enzyme treated with substrate was similar to that of the enzyme reduced by dithionite. A maximum at 375 mμ, one of the peak absorbances for flavin analogs, was not present in this difference spectrum.

An aqueous extract of a heat-treated preparation of the enzyme (pH 7.5), prepared by the method of BLANCHARD *et al.*[6], exhibited three maxima at 265, 375 and 450 mμ, and gave a maximal response of fluorescence energy at 530 mμ when excited at 462 mμ (excitation maximum).

Paper chromatography of this extract in *n*-butanol–acetic acid–water (4:1:5, by vol.) (ref. 7) revealed only one fluorescent band under ultraviolet irradiation. This



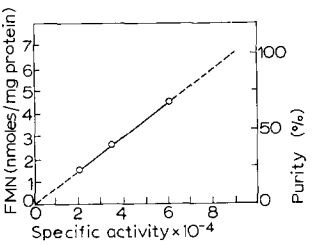


Fig. 3. Absorbance spectra of the enzyme (specific activity, 35 000; protein 2.5 mg/ml) before and after the addition of L-α-hydroxyisocaproate (0.2 M) during anaerobiosis, and absorbance spectrum of catalase in the enzyme preparation (plots of the calculated values). When anaerobiosis was required, the enzyme was placed in a spectrophotometer cuvette fitted with a Thunberg tube side arm in which the substrate was placed. The tube was evacuated and flushed several times with oxygen-free nitrogen. The reaction was initiated by mixing the enzyme with substrate. Curve 1, oxidized form of the enzyme; Curve 2, same solution as used for Curve 1, 10 min after the addition of L-α-hydroxyisocaproate; Curve 2, rat liver catalase (0.178 mg/ml as pure catalase); Curve 4, difference spectrum of the catalase (Curve 1 — Curve 3); Curve 5, difference spectrum of the enzyme treated with L-α-hydroxyisocaproate (Curve 1 — Curve 2).

Fig. 4. FMN content and specific activity of the enzyme preparation. FMN was measured by spectrophotometric assay of extracts of the heat-treated enzyme as described in the text.

band had the same migration rate as that of an authentic sample of FMN. A control experiment was carried out to check the hydrolysis of FAD to FMN with an authentic sample of FAD under the condition used to split its prosthetic group from the enzyme preparation. Judging by the results of paper chromatography, breakdown of FAD to FMN did not occur under these conditions. When the absorbance of the extract of the heat-treated enzyme was calculated as FMN with a millimolar extinction coefficient at 450 mμ of 12.2 (ref. 8), the plot of the data between 370 and 520 mμ virtually coincided with that from an authentic sample of FMN. The much higher absorbance of this extract in the region of shorter wavelengths may indicate the presence of some impurity. We have reported that, compared with the trichloroacetic acid procedure, the heat extraction method gives a 14% lower value in the determination of FMN of crystalline L-aminoacid oxidase from rat kidney[9]. On the other hand, little difference between the heat extraction and the trichloroacetic acid extraction, if any, has been demonstrated in the determination of FAD in xanthine oxidase or aldehyde oxidase[10]. In the present experiment, a millimolar extinction

coefficient at 450 mμ of 11.0 (rather than 12.2) was tentatively used for the colorimetric determination of FMN in L-α-hydroxyacid oxidase by the heat extraction method, assuming that nearly 90% of the total FMN in this enzyme would be extractable under this condition.

If FMN content and protein concentration of the enzyme preparations, measured by the methods applied here, are correct, the data in Fig. 4 may offer presumptive evidence that one mole of enzyme with specific activity of 90 000 has approx. 2 moles of FMN, assuming a mol. wt. of 300 000 (ref. 1).

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