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Identification of a Covalently Bound Flavoprotein in Rat Liver Mitochondria with Sarcosine Dehydrogenase

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SUMMARY

A covalently bound flavoprotein having highest molecular weight among four covalently bound flavoproteins found in rat liver mitochondria was partially purified and characterized. Its subunit molecular weight was estimated to be 94,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its absorption maxima were observed at 353 and 460 nm. Since this flavoprotein was reduced by either sarcosine or dimethylglycine and oxidized by phenazine methosulfate, it was identified with sarcosine dehydrogenase.

INTRODUCTION

In our previous study (1), we labelled flavin moiety of covalently bound flavoproteins in the liver by injecting ^{14}C-labelled riboflavin into a rat and demonstrated the occurrence of four covalently bound flavoproteins in rat liver mitochondria. The molecular weights of their subunits were estimated to be 91,000 (component I), 72,000 (component II), 60,000 (component III), and 44,000 (component IV). From the molecular weights reported (2 - 5), component II and III were assigned to succinate dehydrogenase and monoamine oxidase, respectively. The present paper deals with the characterization of component I.

MATERIALS AND METHODS

The materials were obtained from the following sources; Tween 20, SDS*, sarcosine, dimethylglycine, PMS, DCIP and all the reagents used for gel electrophoresis from Nakarai Chemicals Ltd., Kyoto; 2,3,4-triphenyltetrazolium chloride from Merck AG, Darmstadt; BSA from Sigma Chemicals Co., St. Louis; aldolase from

* Abbreviations : SDS, sodium dodecyl sulfate ; PMS, phenazine methosulfate ; DCIP, 2,6-dichlorophenolindophenol

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Boehringer, Mannheim. Myosin was kindly donated by Dr. T. Horigome, Juntendo University, Tokyo. The liver was excised from rat after decapitation and stored at -20°C after perfusing with ice-cold 0.9% NaCl.

Disc polyacrylamide gel electrophoresis with 7.5% of acrylamide was performed at pH 8.9 according to the method of Davis (6). The gel and buffer for electrophoresis contained 0.1% Tween 20 as described by Nishikimi et al. (7). Proteins were stained with Coomassie brilliant blue. Yellow fluorescence on the gel was detected under ultraviolet illumination (λ = 365 nm) as described previously (1). Staining of this protein in the gel for its enzymatic activity was performed according to Yagi and Ohishi (8). SDS-polyacrylamide gel electrophoresis was performed as described previously (1).

Sarcosine and dimethylglycine dehydrogenase activities were measured spectrophotometrically at 37°C with PMS as an immediate electron acceptor and DCIP as a terminal oxidant. The change in absorbance at 600 nm of DCIP upon reduction was used to follow the enzymatic activity using a molar extinction coefficient of 21 mM^{-1} cm^{-1}. The assay solution contained 1 M sarcosine or dimethylglycine as substrate, 50 mM potassium phosphate buffer (pH 7.2), 0.1% BSA, 1 mM potassium cyanide, 0.067% PMS and 0.01 mM DCIP. Absorption spectra were measured in a Union SM-401 spectrophotometer.

RESULTS AND DISCUSSION

In the previous study (1), two yellow fluorescent bands, corresponding to component I and II, were observed on the gel under ultraviolet illumination, after SDS-polyacrylamide gel electrophoresis of rat liver mitochondria. We utilized this yellow fluorescence to follow component I during its purification from the mitochondria, because its enzymatic activity had not been known.

Since component I was found in the solubilized fraction obtained by treating rat liver mitochondria by sonication, rat liver mitochondria were subjected to sonic oscillation, using Branson B-12 sonicator, in an ice-bath for 2 min. The sonicated solution was centrifuged at 100,000 × g for 60 min in a refrigerated preparative centrifuge. The supernatant solution was brought to 80% saturation of ammonium sulfate and the precipitates formed were dissolved in a small volume of 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% Tween 20, and dialyzed against the same buffer. The buffers used thereafter all contained 0.01% Tween 20. The dialyzed solution was chromatographed on a cellulose phosphate column (2.6 × 40 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2). Component I passed through the column. Then, the solution was applied onto a DEAE-Sephadex A-50 column (4.6 × 25 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.2). The column was washed with 70 mM potassium phosphate

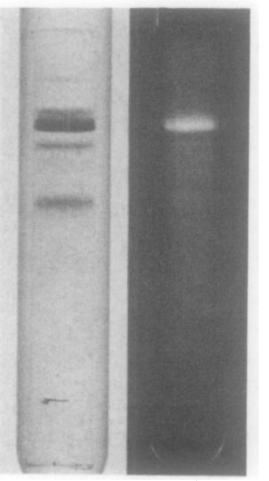

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified component I. The purified component I (46 μg protein) was heated in the mixture of 4 M urea, 1% SDS and 1% mercaptoethanol at pH 7.2 and subjected to electrophoresis. Gel a was stained with Coomassie brilliant blue. Gel b was photographed under ultraviolet illumination in 7% acetic acid.

buffer (pH 7.2), then eluted with 140 mM potassium phosphate buffer (pH 7.2). The yellow-colored fraction was chromatographed on a Sephacryl S-200 column (2.6 × 98 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.2), and eluted with the same buffer. The pooled protein fraction was placed on a hydroxylapatite column (1 × 5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.2). The column was washed with 50 mM potassium phosphate buffer (pH 7.2), and eluted with a linear gradient between 50 mM and 150 mM potassium phosphate buffer (pH 7.2). The yellow-colored fraction was obtained.

SDS-polyacrylamide gel electrophoresis of the purified sample of component I thus obtained is shown in Fig. 1. Fig. 1-a shows the gel stained with Coomassie brilliant blue, and Fig. 1-b the gel photographed under ultraviolet illumination in 7% acetic acid. The relative mobility of the yellow fluorescent band of Fig. 1-b corresponds to that of the major protein band of the gel of Fig. 1-a. Besides the major band, four minor bands were observed in Fig. 1-a. They have no fluorescence. The molecular weight of the major protein with yellow fluorescence was estimated

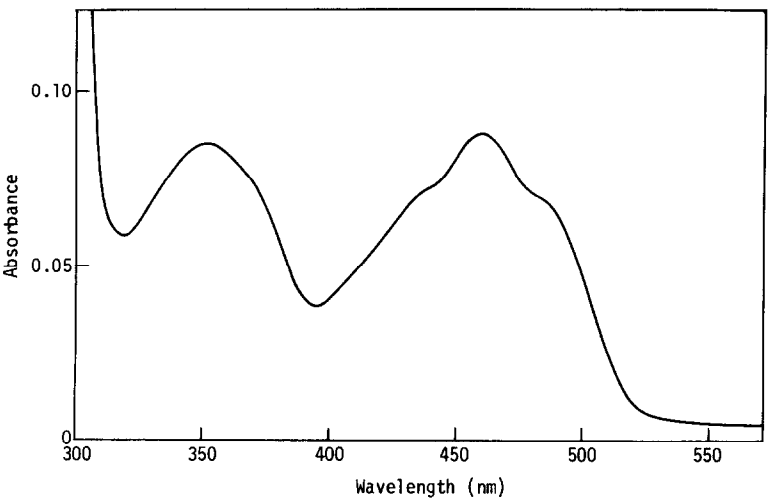

Fig. 2. Absorption spectrum of purified component I. The concentration of the protein in 20 mM potassium phosphate buffer (pH 7.2) was 1.84 mg/ml.

to be 94,000 on the basis of the marker proteins, myosin (200,000), BSA dimer (136,000), BSA monomer (68,000) and aldolase (40,000).

The visible absorption spectrum of the solution of the purified component I had two peaks at 353 and 460 nm and shoulders at 436 and 492 nm (Fig. 2). The second absorption maximum showed a typical hypsochromic shift. This behavior proved that the flavin is attached to the peptide through the 8α-position of the isoalloxazine ring. It was found that the absorption spectrum was changed to its reduced form upon addition of either sarcosine or dimethylglycine to the solution. The enzymatic activity was further confirmed by staining the gel after disc polyacrylamide gel electrophoresis. The gels were incubated in a solution containing a substrate, PMS and 2,3,4-triphenyltetrazolium chloride in 50 mM potassium phosphate buffer (pH 7.2). Fig. 3-a shows the staining of the enzymatic activity for sarcosine, and Fig. 3-b that for dimethylglycine. Fig. 3-c shows the yellow fluorescent band, which corresponds to each band of Fig. 3-a and 3-b having enzymatic activity. Betain, succinate and tyramine could not serve as substrate of this enzyme. From these results, component I is identified with sarcosine dehydrogenase.

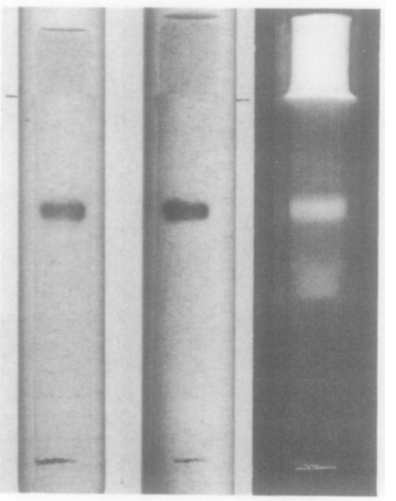

Fig. 3. Disc polyacrylamide gel electrophoresis of purified component I. The purified component I (60 μg protein) was applied onto each gel. After electrophoresis, gel a and b were incubated in a solution containing a substrate (1 M), PMS (0.33 mM), 2,3,4-triphenyltetrazolium chloride (0.12 mM) and potassium phosphate buffer (50 mM, pH 7.2) at 37°C for 30 min. a: sarcosine; b: dimethylglycine. Gel c was photographed under ultraviolet illumination in 7% acetic acid.

The values of K_m and V_{max} were measured kinetically. From the Lineweaver-Burk plots, the K_m values for the two substrates were found to have equal values of 0.38 M. The values of V_{max} for sarcosine and dimethylglycine were estimated to be 16 mmol/hr/mg protein and 50 mmol/hr/mg protein, respectively.

Since this enzyme appears to catalyze the dehydrogenation of both dimethylglycine and sarcosine, it may be concluded that the reactions of two steps, dimethylglycine → sarcosine → glycine, are catalyzed by a single protein.

Although sarcosine and dimethylglycine dehydrogenases of rat liver mitochondria have been thought to contain covalently bound flavin since 1962 (9), they have been considered to be different enzymes. Our present results clearly demonstrated that these reactions are catalyzed by the same enzyme. In the previous paper (1), we suspected from the reported sedimentation coefficient of sarcosine dehydrogenase (10) that component IV might be sarcosine dehydrogenase, which was supported by Addison and McCormick (11). However, the present results clearly

indicate that component I is sarcosine dehydrogenase. Component IV remains to be identified.

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