Proteins with Covalently-Bound Flavin in Rat Liver Mitochondria

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SUMMARY

By SDS-polyacrylamide gel electrophoresis, mitochondrial proteins having covalently-bound flavin were analyzed. Mitochondria were prepared from the liver of rat injected with radioactive riboflavin. Radioactivity was found to be associated with four protein components. Their subunit molecular weights were 91,000, 72,000, 60,000 and 44,000. The first two components exhibited yellowish fluorescence on a gel under ultraviolet illumination. The component of the highest molecular weight seems to be a new protein containing covalently-bound flavin.

INTRODUCTION

Recently, much knowledge has been accumulated on the enzymes having covalently-bound flavin as prothetic group. In rat liver, four such enzymes, which catalyze biologically important reactions are known, viz., succinate dehydrogenase [EC 1.3.99.1] (1), monoamine oxidase [EC 1.4.3.4] (2) and sarcosine dehydrogenase [EC 1.5.99.1] (3) localized in mitochondria, and L-gulono-γ-lactone oxidase [EC 1.1.3.8] (4) localized in microsomes.

The analysis of covalently-bound flavin has usually been carried out with extensively purified enzyme as material. In order to detect the proteins having covalently-bound flavin and to clarify their physiological significance, we tried to analyze them with crude materials such as cell organelles. For this purpose, the flavin covalently-bound to apoproteins in rat liver mitochondria was labeled with ^{14}C-riboflavin (5) and was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. In addition, we attempted to detect the flavin covalently-bound to apoprotein on a gel by their intrinsic fluorescence.

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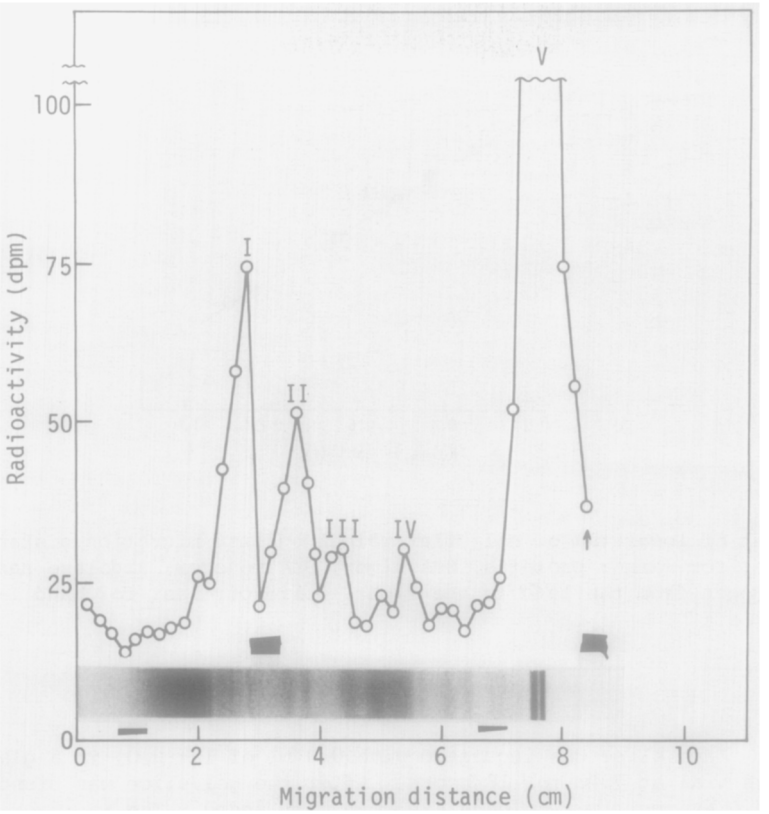

Fig. 1. Pattern of radioactivity and Coomassie blue staining profile after slab gel electrophoresis of rat liver mitochondria. Mitochondrial suspension (8.8 mg protein) was heated in the mixture of 8 M urea and 2% SDS at pH 7.2 and subjected to electrophoresis.

MATERIALS AND METHODS

[2-^{14}C]Riboflavin (specific radioactivity, 32 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. Omnifluor and protosol were the products of New England Nuclear, Boston. Chymotrypsinogen and aldolase were obtained from Boehringer, Mannheim, and bovine serum albumin (BSA) from Sigma Chemicals Co., St. Louis. Dansyl (DNS-) labeled proteins were prepared according to the method of Talbot and Yphantis (6). All other chemicals were of analytical grade.

For slab gel electrophoresis, the discontinuous buffer system described by Laemmli (7) was used with a slight modification. The separation gel was prepared with 1% SDS and 9% acrylamide in 0.375 M Tris-HCl (pH 8.8), and the stacking gel with 1% SDS and 3% acrylamide in 0.125 M Tris-HCl (pH 6.8). The sample for electrophoresis was prepared as follows: 0.4 ml (8.8 mg protein) of mitochondrial suspension was mixed with 1.0 ml of 0.02 M sodium phosphate buffer (pH 7.2) containing 8 M urea and 2% SDS, and the mixture was heated in boiling water for 3 minutes. After gel electrophoresis at 50 V for 18 hours, 5 cm portion of the gel (corresponding to 3.3 mg protein) was cut into approximately

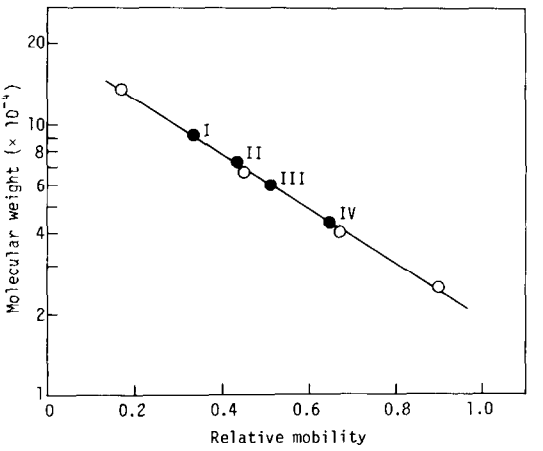

Fig. 2. Plot of logarithm of molecular weight against migration distance. Open circles: for marker proteins, BSA dimer, BSA monomer, aldolase and chymotrypsinogen from the left to the right; solid circles: for band I-IV of Fig. 1.

2 mm slices. Each slice was incubated with 0.4 ml of 15% H_2O_2 in a glass scintillation vial at 70°C for 16 hours. After the gel slice was dissolved, protosol and 0.4% omnifluor-toluene solution were added. The radioactivity was estimated in a Beckman LSC-355 scintillation counter.

Disc gel electrophoresis in the presence of SDS was performed by a slightly modified method (8) of Dunker and Rueckert (9). The continuous gel system was used: the gel consisted of 1% SDS and 5% acrylamide in 0.1 M sodium phosphate buffer (pH 7.2). The preparation of the sample protein was the same with that for the slab gel system. The protein (166 µg) of SDS-treated mitochondria was applied to each disc gel. After electrophoresis, the gel was soaked in 7% acetic acid for 1 hour or overnight. Yellowish fluorescence was observed on a gel under ultraviolet illumination (λ = 365 nm), which was photographed with highly sensitive film (Tri-X pan, ASA 400, Eastman Kodak Co., Rochester) and a green filter (Kenko PO1) (f = 8; exposure, 0.5-10 minutes).

The protein band on a gel was stained with 0.01% Coomassie brilliant blue in 7% acetic acid and 50% methanol solution for overnight, and then destained with 7% acetic acid.

RESULTS AND DISCUSSION

After fed on the flavin-deficient diet (10) for 2 weeks, a rat was injected daily with 0.2 ml of radioactive riboflavin solution (0.2 mg of [2-^{14}C] riboflavin dissolved in 1.0 ml of 0.9% NaCl) through tail vein for 6 days. During this period, the rat was still fed on the flavin-deficient diet. At 24 hours after the last injection, the rat was sacrificed and the liver was excised

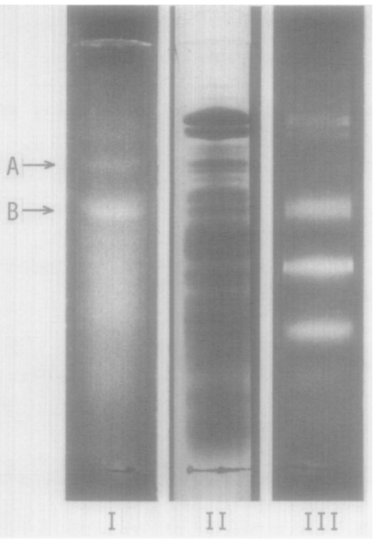

Fig. 3. SDS-polyacrylamide gel electrophoresis of rat liver mitochondria. Mitochondrial suspension (166 μg protein) treated as described in Fig. 1 and DNS-labeled marker proteins (5 μg of each protein) were subjected to electrophoresis. I: fluorescent bands of mitochondrial proteins; II: Coomassie blue-staining bands of mitochondrial proteins; III: fluorescent bands of marker proteins, BSA dimer, BSA monomer, aldolase and chymotrypsinogen from the top to the bottom.

Mitochondrial fraction was obtained by the ordinary method (11), and subjected to the analysis by slab gel electrophoresis.

Figure 1 shows the pattern of the radioactivity and that of the protein distribution. Five peaks are observed in the radioactivity pattern and numbered I to V, from the top to the bottom. The highest peak, V, observed near the tracking dye, is due to free flavin. The molecular weights of the protein subunits corresponding to peaks I to IV were calculated to be 91,000, 72,000, 60,000 and 44,000, respectively, from the calibration curve obtained with the marker proteins (Fig. 2).

Since flavinyl peptides such as 8α-histidyl-FMN were reported to be fluorescent at an acidic pH, SDS-treated protein band containing covalently-bound flavin is considered to be fluorescent in 7% acetic acid. As shown in Fig. 3-I, two yellow fluorescent bands A and B were observed, when the disc gel after SDS-electrophoresis was illuminated with an ultraviolet lamp. The fluorescence

intensity of band B was higher than that of band A. The broad fluorescent band in the lower part of the gel disappeared by soaking in 7% acetic acid overnight. This indicates that the band is of free flavin. The molecular weights of band A and B were estimated to be 90,000 and 68,000, respectively, by using DNS-labeled marker proteins (Fig. 3-III). On the basis of the molecular weights obtained, band A and B seem to correspond to peak I and II observed by slab gel electrophoresis.

It is known that in mitochondrial flavoproteins, succinate dehydrogenase, monoamine oxidase and sarcosine dehydrogenase contain covalently-bound flavin as coenzyme. The subunit molecular weight of succinate dehydrogenase was estimated to be 70,000 (12,13). This value is in good accord with that of peak II in our system. As to the subunit of monoamine oxidase, the experimental data using radioactive inhibitors were reported. Collins and Youdim (14) reported that ^{14}C-phenethylhydrazine-binding monoamine oxidase has a molecular weight of 60,500. More recently, it was also shown by using a specific inhibitor, ^{14}C-pargylin, that the radioactivity was associated with the protein of 60,000 in outer membranes of rat liver mitochondria (15). Taking these results into consideration, the protein of peak III is assigned to monoamine oxidase.

The subunit molecular weight of sarcosine dehydrogenase is not yet available in the literature. However, Hoskins and Bjur (16) reported that the preparation solubilized from monkey liver revealed a single major boundary with a sedimentation coefficient of 4.3 S in the sedimentation pattern. The peak IV seems to be a plausible candidate for sarcosine dehydrogenase, though further investigation is necessary.

In the present study, it is noticed that rat liver mitochondria contain a protein component having covalently-bound flavin, whose molecular weight is of the order of 90,000. Since the strongly dissociating conditions were used for the preparation of the sample of mitochondrial proteins, the possibility that peak I protein is an aggregate of known covalently-bound flavin-containing enzyme seems to be excluded. Thus the protein is probably a new protein with covalently

bound flavin. Since this fluorescent band was also detected in rabbit and human liver, its distribution in mammals seems to be wide. It is of great interest whether it is a enzyme or a precursor of some known enzyme possessing covalently bound flavin.

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