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Purification and General Characterization of FAD Synthetase from Rat Liver[1] (41554)

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Abstract. Flavin adenine dinucleotide synthetase (ATP:FMN adenylyltransferase, EC 2.7.7.2) has been enriched more extensively than previously from fresh rat liver. For this, 10% homogenates in sucrose-phosphate buffer were treated with 0.1% Tween-20 prior to high-speed centrifugation to obtain soluble proteins. Those precipitated by 40% saturation with ammonium sulfate were subjected to stepwise addition of calcium phosphate gel to remove pyrophosphatase, and the remaining synthetase was further enriched by passage through a tricalcium phosphate column. An apparent yield of greater than 70% and purification over 70-fold was achieved from the high-speed supernatant fraction. The synthetase activity in solution at 4° was largely lost within a week unless protected by thiols which could partly restore inactivated enzyme. The pH optimum for synthetase activity is near 7.7 when assayed with suitable concentrations of FMN, ATP, and Mg^{2+}. Purified enzyme could be separated into lower (140,000) and higher (325,000) molecular weight components when subjected to molecular sieving on a Sephadex G-200 column.

Earlier literature described the synthesis of flavin adenine dinucleotide from riboflavin *in vivo* in heart, liver, and blood in rats (1) and both *in vivo* and *in vitro* in human blood cells (2). The biosynthesis of the dinucleotide was further studied in rat liver and kidney by using ^{14}C-labeled riboflavin (3, 4). The enzyme responsible for the synthesis of the dinucleotide and pyrophosphate from flavin mononucleotide and ATP was partially purified for the first time from yeast (5). The occurrence of the synthetase (pyrophosphorylase) was also described in higher plants (6). Purification of the dinucleotide-forming enzyme from the rat liver soluble fraction was attempted but with limited success (7), and some of its properties were described (8). The substrate requirements, absolute for adenosine triphosphate and relative for riboflavin 5′-phosphate (FMN), were also reported for crude rat liver FAD synthetase (9). However, the difficulties in isolating the enzyme sufficiently free from FAD-hydrolyzing activity, principally pyrophosphatase, had greatly limited further study and characterization of FAD synthetase, especially from animal tissues.

In the present paper, we describe the means by which significant purification of pyrophosphatase-free FAD synthetase from rat liver can be achieved. Additionally some general characteristics of the enzyme are noted.

Materials and Methods. FMN, ATP, FAD, dithiothreitol, D-amino acid oxidase, cytochrome *c*, ferritin, bovine serum albumin, and polyoxyethylene sorbitan were purchased from Sigma Chemical Company, St. Louis, Missouri. D-Phenyl glycine was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Sucrose was the product of Mallincrodt, St. Louis, Missouri. Disodium-EDTA was purchased from Fisher Scientific Company, Pittsburgh, Pennsylvania. Sephadex G-200 and blue dextran were procured from Pharmacia Fine Chemicals, Piscataway, New Jersey. Other chemicals and reagents used were of analytical grade.

Calcium phosphate gel for batch treatment was prepared by the method of Keilin and Hartree (10) and for chromatography by Swingle and Tiselius (11). Protein was determined according to Lowry *et al.* (12) by using bovine serum albumin as the standard. D-Amino acid oxidase apoenzyme was prepared according to the method of Massey and Curti (13). Rat livers were obtained fresh from Sprague–Dawley adult males maintained on laboratory chow.

Assay of FAD synthetase activity. The assay was performed in two steps: (a) Incubation of

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synthetase with FMN and ATP to form FAD and pyrophosphate and (b) quantitation of FAD formed by conversion of apo-D-amino acid oxidase to its active holoenzyme which was subsequently assayed by the spectrophotometric method of Fonda and Anderson (14). For the synthetase reaction, the mixture contained 1.67×10^{-4} *M* FMN, 4.17×10^{-4} *M* ATP, 6.94×10^{-4} *M* magnesium sulfate, and 0.033 *M* potassium phosphate, pH 7.5, to which enzyme was added for 3 ml final volume. The reaction mixture was incubated at 37° and aliquots of 0.3 ml were then withdrawn at 0, 30-, and 60-min intervals and added to 2.59 ml of 0.058 *M* sodium pyrophosphate buffer, pH 8.5. To this was added 10 μl (3–4 μg) of apo-D-amino acid oxidase. After 15 min 5 μmole of D-phenylglycine was added to make a final volume of 3 ml, and the activity of the reconstituted enzyme was monitored at 252 nm for 10–15 min at room temperature against a reagent blank. Under these conditions with excess apooxidase, rate of phenylglycine oxidation was directly proportional to synthetase-produced FAD, as compared to standard amounts of FAD added.

One unit of FAD synthetase is defined as the amount of enzyme that synthesized 1 nmole of FAD at pH 7.5 in one hour at 37°.

Assay of FAD pyrophosphatase activity. The FAD-destroying (pyrophosphatase) activity was determined by incubating at 37° a reaction mixture containing 0.6 μ*M* FAD and 0.5 ml of the enzyme preparation in a final volume of 3 ml of 0.05 *M* potassium phosphate buffer, pH 7.5. From the reaction mixture, 0.3-ml aliquots were withdrawn at the same time intervals as for the FAD synthetase assay, and the pyrophosphatase activity was calculated from the residual FAD as again determined by spectrophotometric assay of the reconstituted D-amino acid oxidase.

One unit of pyrophosphatase is that amount of enzyme responsible for destruction (hydrolysis) of 1 nmole of FAD at pH 7.5 in one hour at 37°.

Synthetase purification. The purification of FAD synthetase includes neutral detergent treatment of a suspension of rat liver homogenate in isotonic medium, centrifugation to obtain the soluble protein fraction, ammonium sulfate fractionation, removal of pyrophosphatase by stepwise addition of calcium phosphate gel, and tricalcium phosphate column chromatography. The protein content and the synthetase and pyrophosphatase activities were determined for every step as detailed in the following: 39 g of rat liver were sliced and homogenized in 0.05 *M* potassium phosphate buffer, pH 7.4, containing 0.25 *M* sucrose, by using the Potter–Elvehjem apparatus with a moderately tight Teflon pestle. The crude homogenate (10%, w/v) showed no FAD synthetase activity due to high masking pyrophosphatase.

The whole homogenate was stirred for 15 min after adding Tween-20 (0.1% final concentration), and was centrifuged at 100,000*g* for one hour in a Beckman Model L preparative ultracentrifuge.

To the clear supernatant, solid ammonium sulfate was stirred to 40% of saturation. The precipitate was recovered by centrifuging at 12,000*g* for 14 min. This was dissolved in 0.05 *M* phosphate buffer, pH 7.4, and dialyzed against three changes of 0.02 *M* buffer over a 15-hr period. The contents remaining in the dialysis bag were centrifuged to remove any precipitate which appeared during dialysis.

Calcium phosphate gel (13.2 mg/ml dry weight) was added to the dialyzed solution at an initial protein:gel ratio of 4. The mixture was slowly stirred for 10 min and then centrifuged (12,000*g*) for 10 min. The slightly yellow supernatant was recovered and the pellet (which contained mostly pyrophosphatase and little synthetase activity) was discarded. The procedure was repeated *four* more times. The clear and faintly yellow supernatant contained virtually all the synthetase and undetectable pyrophosphatase activity.

The supernatant from the preceeding step was poured on a 3.1 × 14-cm tricalcium phosphate gel column preequilibrated with 0.02 *M* phosphate, pH 7.4. The column was washed with 500 ml of the same buffer and then protein eluted with 0.05 *M* phosphate, pH 7.4, in 3.6-ml fractions.

Fully swelled Sephadex G-200 gel capable of sieving proteins of higher molecular weight (above 200,000 daltons) (15) was used. A 2.5 × 50-cm gel column was equilibrated with 0.025 *M* phosphate, pH 7.4. Marker proteins used were cytochrome *c*, bovine serum albumin (dimeric), and ferritin. Blue dextran

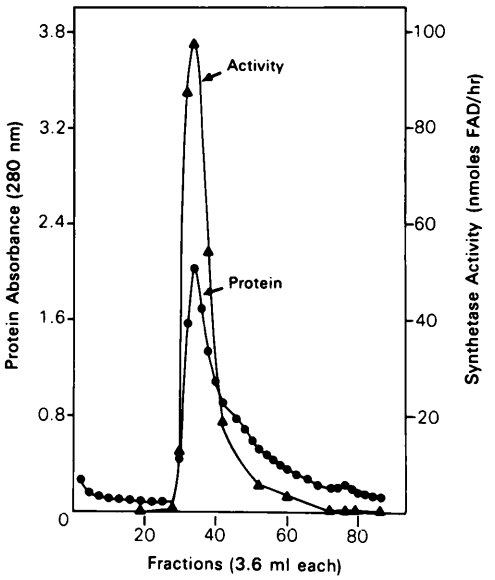

FIG. 1. Elution pattern of FAD synthetase on a tricalcium phosphate column. The fractions (31–36) that contained highest activity were pooled for further analysis. See text for details.

2000 was used to determine the void volume (V_0).

Results. As seen in Fig. 1, synthetase activity coeluted with the maximum of the principal protein fraction initially absorbed on the column. The eluted enzyme was found to be free from pyrophosphatase activity. The absorption spectrum with a principal maximum at 278 nm indicated absence of nucleic acids (A_{260}:A_{280} = 0.58), but a minor chromophoric component is reflected by a small maximum at 438 nm.

The activities and yields at each stage of purification are summarized in Table I. As seen from the data, much of the activity which was masked by interfering pyrophosphatase at earlier stages is recovered during an apparent enrichment of over 70-fold compared to the original supernatant.

Stability. To assure relative stability, FAD synthetase eluted from the tricalcium phosphate gel column was stored at 0–4° separately and after mixing with 0.2 μmole of FMN, 0.5 μmole of ATP, 1 μmole of magnesium sulfate, and 1 μmole of dithiothreitol in 1-ml portions. All samples unless frozen or treated with dithiothreitol lost most activity within 7 days. A frozen control retained 85%

TABLE I. PURIFICATION OF FAD SYNTHETASE FROM RAT LIVER

Purification stage	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity[a] (units/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (*n*-fold)
Tween-treated homogenate	390	21.5	8397	—	—	—	—	—
High-speed supernatant	350	10.8	3780	7.04	2464	0.65	100	1
Dialyzed solution following ammonium sulfate	115	10.1	1162	17.6	2024	1.74	82	2.7
Supernatant from calcium phosphate gel	170	2.5	425	22.9	3893	9.15	158	14.1
Effluent from tricalcium phosphate column	21.6	1.81	39.0	84.9	1835	47.0	74.4	72.4

[a] One unit equals 1 nmole of FAD synthesized per hour in 0.067 *M* potassium phosphate buffer, pH 7.5, at 37°.

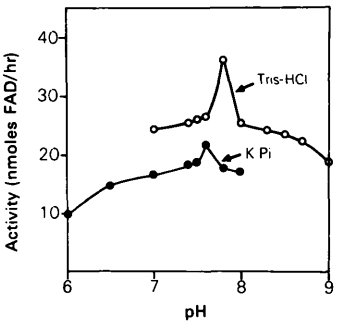

FIG. 2. pH dependence of FAD synthetase. Eighteen units of enzyme were used in 1.5 ml for the synthetase reaction at 37°, and 0.3-ml aliquots were taken for assay as usual (Methods). Buffers used were either 0.1 *M* potassium phosphate (●) or 0.1 *M* Tris-HCl (○).

of the original activity. Dithiothreitol (1 m*M*) not only protected but significantly enhanced (1.5 times) the synthetase activity examined on the second and fourth days. The thiol-treated enzyme even retained 90% of initial activity on the seventh day.

pH dependence. The effect of pH and buffer on activity of FAD synthetase was determined in 0.1 *M* phosphate (pH 6–8) and in 0.1 *M* Tris–HCl (pH 7–9) systems. The pH optima determined with these buffers were 7.6 and 7.8, respectively (Fig. 2).

Molecular weight. Two active components of FAD synthetase were eluted from each of two separate preparations, and their approximate molecular weights were estimated from the line obtained by plotting the ratio of elution to void volumes (V_e/V_0) against log molecular weights (log MW) of the markers (Fig. 3) (16). The molecular weight of the lighter component 1 and the heavier component 2 were in the ranges of 130,000–150,000, and 316,000–339,000, respectively.

Discussion. As noted previously (5, 7, 8), FAD synthetase activity could not be readily detected in whole homogenates from rat liver (and other animal tissues) due to the presence of high amounts of pyrophosphatase. Treatment of the homogenate with Tween-20 seemed to liberate more synthetase and partially retard pyrophosphatase. Greater quantities of this FAD-destroying enzyme were eliminated in the insoluble cell debris and particulates by high-speed centrifugation. However, pyrophosphatase activity returned when Tween-20 was removed during ammonium sulfate fractionation. The most effective step for removing almost all the pyrophosphatase and much other protein impurities was the calcium phosphate batch treatment which raised the apparent synthetase activity in the supernatant to about 14-fold over that obtained in the soluble fraction (cf. Table I). Tricalcium phosphate chromatography removed more impurities and yielded about 75% of the synthetase with 72-fold purification compared to that measured in the initial soluble protein fraction. Actually, this amount represented about half of the total units of synthetase present in the supernatant following the calcium phosphate batch treatment.

The purified enzyme was faintly yellow in color. When stored at 0–4° it progressively lost most activity within a week. Although freezing offered reasonable protection, dramatic effects were achieved by adding dithiothreitol which not only protected the enzyme from inactivation but also caused reactivation. Preparations that had lost up to half the activity upon storage regained this when treated with millimolar dithiothreitol. This suggests that the thiol can protect sulfhydryl groups of the enzyme that may be essential

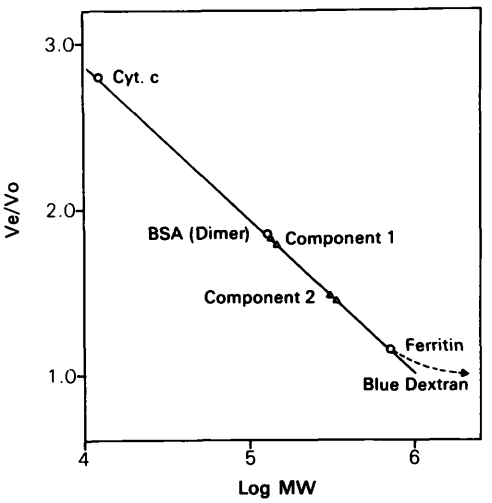

FIG. 3. Molecular weight estimation of FAD synthetase by Sephadex G-200 gel filtration. Two milliliters (1.81 mg/ml for one preparation and 1.73 mg/ml for another) were used for each run. Markers proteins (○) and synthetase preparations (△) are indicated.

for synthetase activity or conformational requirement. Substrates namely FMN, ATP, and/or Mg^{2+}, did not protect the enzyme from inactivation.

The pH dependence curves showed optima at pH 7.6 with phosphate and pH 7.8 with Tris–HCl buffers. Relatively greater activity was observed with Tris–HCl as compared with phosphate which might have complexed some Mg ions required for synthetase activity.

The spectrum of the purified enzyme is typical for a tryptophanyl-containing protein with an absorption maximum at 278 nm. The faintly yellow color attributed to a chromophore with absorption maximum at 438 nm may be due to impurity or possibly a prosthetic group which might be essential for activity. The A_{260}:A_{280} ratio of 0.583 indicated the absence of nucleic acids/nucleotides in the preparation.

Sephadex G-200 gel chromatography allowed separation of two active components of FAD synthetase in roughly equal amounts at the present stage of purification. As the smaller component has an estimated molecular weight of 130,000–150,000 and the heavier component one of 316,00–340,000, it is possible that the synthetase exists in polymeric forms.

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