Purification and Characterization of FAD Synthetase from Brevibacterium ammoniagenes*

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The bifunctional enzyme FAD synthetase from Brevibacterium ammoniagenes was purified by a method involving ATP-affinity chromatography. The final preparation was more than 95% pure. The apparent molecular weight of the enzyme was determined as 38,000 and the isoelectric point as 4.6.

Although previous attempts to separate the enzymatic activities had failed, ATP:riboflavin 5'-phosphotransferase and ATP:FMN-adenyllytransferase activities in B. ammoniagenes were believed to be located on two separate proteins with similar properties, possibly joined in a complex. The following evidence, however, suggests the presence of both activities on a single polypeptide chain. The two activities copurify in the same ratio through the purification scheme as presented. Only a single band could be detected when aliquots from the final purification step were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, nondenaturing gel electrophoresis, and isoelectric focusing. Edman degradation of the protein yielded a single N-terminal sequence.

FAD synthetase from the coryneform bacterium Brevibacterium ammoniagenes catalyzes the 5'-phosphorylation of riboflavin to FMN followed by the adenylation of FMN to FAD. Since the enzyme was first described by Spencer et al. (1) in the conversion of 5-deazariboflavin to 5-deaza-FAD, it became widely used in the preparation of the coenzyme forms of riboflavin analogues. This was due to its ability to catalyze both reactions with a broad variety of riboflavin isosteres (2) and its extraordinary stability which, in many cases, allows complete conversion of micromolar amounts of the analogues with a few milligrams of partially purified enzyme (1, 3, 4). It was also for this purpose that we started to work with the FAD synthetase. In trying to find a more effective procedure for separating the enzymatic activities from contaminating phosphatase and phosphodiesterase activities and in optimizing conditions for the conversion of 8-demethyl-8-OH-5-deazariboflavin to 8-demethyl-8-OH-5-deaza-FAD, evidence grew that both activities are catalyzed by a single polypeptide. The possibility of achieving the purification of a bifunctional enzyme of moderate molecular weight and our general interest in the structure and function of kinases and ATPases led us to intensify work on the enzyme.

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EXPERIMENTAL PROCEDURES

Materials—Adenosine 5'-triphosphate was obtained from Pharma Waldhof (Düsseldorf). Riboflavin, FMN, FAD, and lysozyme from chicken egg white were purchased from Sigma, Munich; phenylmethylsulfonyl fluoride, benzamidine, Servalyt (3–5), and dithiothreitol from Serva, Heidelberg. Phynylalane (4–6.8), DEAE-Sephrose CL-6B, and agarose-hexane-adenosine 5'-triphosphate AGATPM Type 2 were obtained from Pharmacia, Freiburg. Blue Sepharose was prepared according to Böhme et al. (5).

Enzyme Assays—FAD synthetase activity was assayed in a final volume of 50 ml of 50 mm Tris-HCl, pH 7.6, containing 50 mm riboflavin, 5 mm ATP, and 15 mm MgCl₂. The mixture was incubated at 37 °C, and the reaction was started by the addition of enzyme. After appropriate time intervals an aliquot was removed and applied directly to a high pressure liquid chromatography column (Shandon ODS Hypersil, 4.6 x 250 mm, 5-μm particle size, Ahimed Analysetechnik GmbH, Heidelberg, Federal Republic of Germany). The products of the reactions were analyzed at a flow rate of 2.5 ml/min applying a linear gradient from 5 to 22.5% acetonitrile in 50 mm potassium phosphate, pH 6.0. Absorbance at 280 nm was used for detection. Unless otherwise indicated 1 unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 nmol of FAD in 1 min at 37 °C. Under these conditions 5'-phosphotransferase was the rate-limiting step of the overall reaction and could, therefore, be measured by riboflavin conversion. ATP:FMN-adenyllytransferase activity alone was assayed as above with 50 μM FMN as the flavin substrate. Under standard conditions and with homogeneous enzyme, the synthetase and adenyllytransferase reactions were linear for about 20 min and proportional to enzyme concentration over a range of 1.5–6 μg of protein/assay.

Culture Conditions—B. ammoniagenes (ATCC 8672) was grown on culture medium containing per liter 10 g of glucose, 10 g of glycerol, 3 g of yeast extract, 4 g of meat extract, 4 g of peptone from casein, 6 g of urea, 3 g of KH₂PO₄, 3 g of K₂HPO₄, 2 g of MgCl₂, 0.1 g of CaCl₂, and 0.01 g of FeCl₃. Large scale culture was performed in a vigorously aerated 150-liter fermentor at 32 °C, and the pH of the culture medium was kept constant at 7.6 by the addition of small aliquots of concentrated hydrochloric acid. Cells were harvested at the end of the exponential phase using a continuous-flow centrifuge cooled to 0 °C. They were frozen immediately and stored at ~80 °C. Approximately 10 g of cells (wet weight) was obtained per liter of culture medium.

Enzyme Purification—All manipulations were performed at 0–4 °C, except for the column chromatography steps which were performed at room temperature. All buffers and gels were degassed before use.

The enzymatic activities were typically purified starting with 400 g of frozen cell paste thawed in 2 liters of 1 mm EDTA, pH 8.0. After thawing was completed, 1.5 g of lysozyme was added, and the suspension was incubated at room temperature for 45 min with moderate stirring. After centrifugation (20 min, 5,000 x g, 4 °C) cells were resuspended in 500 ml of 100 mm Tris-HCl, pH 8.0, containing 10 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 0.1 mm benzamidine, and 12 mm 2-mercaptoethanol. The phenylmethylsulfonyl fluoride came from a 0.25 M stock solution in isopropyl alcohol that had been prepared immediately before use and was added under vigorous stirring. Cells were sonicated for 60 min in a Branson model 350 sonicator equipped with a 1/4-inch flat tip. Dry ice-isopropyl alcohol cooling was applied to keep the temperature below 6 °C. After disrupting the cells, another aliquot of phenylmethylsulfonyl fluoride
Enzyme Purification—Table I shows the course of a typical preparation of \( B. \textit{ammoniagenes} \) FAD synthetase. The enzyme was purified approximately 7000-fold from crude extract with a yield of 48% applying ammonium sulfate fractionation and column chromatography on DEAE-Sepharose, blue Sepharose, and ATP-agarose. Phosphotransferase and adenylyltransferase activity copurified together in a constant ratio through all steps of purification. Several preparations were performed, and the purification procedure was found to be reproducible within a narrow range.

Under standard assay conditions the 5'-phosphotransferase activity was about 6.7 times lower than the adenylyltransferase activity. When conditions for each reaction were optimized separately, the turnover numbers were 36 min\(^{-1}\) (400 \(\mu\text{M} \text{Zn}^{2+}\)) and 27 min\(^{-1}\) (10 \(\text{mM} \text{Mg}^{2+}\)) for the purified 5'-phosphotransferase and adenylyltransferase, respectively. Both activities could not be accurately determined in crude extract and ammonium sulfate fractions because of the presence of phosphatases and phosphodiesterases. However, after the DEAE-Sepharose step the bulk of these contaminating activities was removed. Partially purified protein from this stage of the purification was routinely used in the conversion of riboflavin analogues to the corresponding FAD derivatives. A further enrichment of FAD synthetase was achieved by the blue Sepharose column step. This step was of particular importance since it removed at least two proteins which showed binding to the ATP-affinity column similar to that of FAD synthetase (see Fig. 1). In the final purification step, the enzyme was bound to an \( N^6\)-aminohexyl-ATP agarose column. This step led to a considerable increase in specific activity without substantial loss in total activity. No divergent cations were required for binding to the affinity matrix.

Purity, Molecular Weight, and Subunit Structure—When the purified enzyme was submitted to electrophoresis on SDS-polyacrylamide gels, only one protein band was detectable. The observed band constituted more than 95% of the total stained protein, and the molecular weight was estimated as \( M_r = 38,000 \) (Fig. 2). FAD synthetase migrated in the non-denaturing gel system to a position corresponding to a \( R_f \) value of 0.54. For the protein markers the following \( R_f \) values were obtained: 0.29 (198 kDa), 0.34 (132 kDa), 0.435 (66 kDa), 0.49 (45 kDa), 0.57 (30 kDa). This indicated that the enzyme consists of a single polypeptide chain of approximate molecular weight 38,000. Spencer et al. (1) estimated the molecular weight of the enzyme from gel filtration of the partially purified enzyme as 40,000. The purified enzyme focused in agarose-isoelectric focusing and in the system described by O’Farrell (11) to form a single band, and the isoelectric point determined (pH 4.6) was found to be the same with both methods. Analysis of the N-terminal sequence by automated Edman degradation yielded a single sequence (Scheme I). These results altogether demonstrate that the \( B. \textit{ammoniagenes} \) FAD synthetase consists of a single polypeptide chain.

General Catalytic Properties of the Enzyme—FAD synthetase is specific for ATP, since there was no measurable activity observed when ATP was replaced by 3 mM ADP, GTP, CTP, ITP, or UTP, respectively. Normal activity of the ATP:riboflavin 5'-phosphotransferase, but no ATP:FMN adenylyltransferase activity was observed when 2'-deoxyadenosine 5'-triphasphate was used as substrate. Both \( R_8 \) and \( S_8 \) diastereomers of ATP\(\alpha\)S were good substrates for 5'-phosphotransferase, but no adenylyltransferase activity could be detected. The respective rates (units/mg) in the formation of FMN were determined as: 440 (Mg-ATP\(\alpha\)S, \(S_8\)), 280 (Co-ATP\(\alpha\)S, \(S_8\)), 120 (Mg-ATP\(\alpha\)S, \(R_8\)), and 200 (Co-ATP\(\alpha\)S, \(R_8\)).
TABLE I

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>Crude extract</td>
<td>750</td>
<td>10,500</td>
<td>200</td>
<td>0.02</td>
<td>(100)</td>
<td>(1)</td>
</tr>
<tr>
<td>50-70% (NH₄)₂SO₄ fraction, dialyzed</td>
<td>235</td>
<td>2,490</td>
<td>210</td>
<td>0.085</td>
<td>(105)</td>
<td>(4.5)</td>
</tr>
<tr>
<td>DEAE-Sepharose eluate, concentrated</td>
<td>25</td>
<td>163</td>
<td>175</td>
<td>1.07</td>
<td>88</td>
<td>54</td>
</tr>
<tr>
<td>Blue Sepharose eluate, concentrated</td>
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<td>21</td>
<td>134</td>
<td>6.4</td>
<td>67</td>
<td>320</td>
</tr>
<tr>
<td>ATP-agarose eluate</td>
<td>2.4</td>
<td>0.7</td>
<td>96</td>
<td>137</td>
<td>48</td>
<td>6850</td>
</tr>
</tbody>
</table>

*One enzyme unit catalyzes the formation of 1 nmol of FAD/min at 37 °C.

Numbers in parentheses could not be accurately determined due to the presence of phosphatases and phosphohistases.

The 5'-phosphotransferase has a Kₘ for Mg·ATP of approximately 5 μM whereas the Kₘ (Mg·ATP) for the adenyllyltransferase was found to be 160 μM. In general, both enzymatic activities showed considerable differences in their substrate requirements.

Not surprisingly, the concentration dependence and specificity for divalent cations differed, too. Studies on the effect of varying the concentration of MgCl₂ and of replacing MgCl₂ with ZnCl₂, Cd(CH₃COO)₂, Co(NO₃)₂, and MnCl₂ showed that the relative 5'-phosphotransferase activities with Zn²⁺, Mg²⁺, Cd²⁺, Co²⁺, and Mn²⁺ were 1, 0.38, 0.38, 0.34, and 0.31; these values were obtained at the optimal divalent cation concentration for this reaction, which were 300, 200, 400, 400, and 400 μM, respectively. When divalent cations were omitted from the reaction mixture, 10% of the 5'-phosphotransferase activity observed in the presence of 200 μM MgCl₂ was still measurable, and no effect was seen upon addition of 5 mM EDTA. Similar findings have been described for rat liver flavokinase (14) and reduced-riboflavin kinase from Bacillus subtilis (15).

Table II shows the effect of MgCl₂ and ZnCl₂ concentration on the initial rate of product formation in the reactions catalyzed by FAD synthetase. In general, higher cation concentrations led to a decrease in the turnover of riboflavin and the 5'-phosphotransferase activity, while the adenyllyltransferase activity increased. At divalent ion concentrations optimal for the 5'-phosphotransferase reaction hardly any adenyllyltransferase activity could be detected.

In the adenyllyltransferase reaction highest activity was found in the order Mg²⁺ (10 mM) > Mn²⁺ (20 mM) > Co²⁺ (2 mM) > Zn²⁺ (2 mM) (Table III); optimal concentrations for the respective cations are given in parentheses. Addition of more

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**Scheme I.** N-terminal amino acid sequence of FAD synthetase.

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of FAD synthetase at various stages of purification. Lane A, 50-75% ammonium sulfate fraction, 50 μg of protein; lane B, DEAE-Sepharose fraction, 40 μg of protein; lane C, blue Sepharose fraction, 70 μg of protein; lanes D-F, peak fractions from the ATP-agarose column containing 7, 5, and 3 μg of purified FAD synthetase, respectively; lanes G and H, ATP-agarose peak fractions from a preparation where a blue Sepharose column of lower capacity had been used, showing two additional bands originating from protein with a similar affinity for the ATP-affinity column as FAD synthetase. Lanes marked M are molecular weight markers. The gel was stained with Coomassie Blue.

**Fig. 2.** Molecular weight determination by SDS-polyacrylamide gel electrophoresis. The relative mobilities of protein standards and FAD synthetase were determined as described under "Experimental Procedures." The position of FAD synthetase is indicated by an arrow.
**TABLE III**

Effect of divalent cations on adenylyltransferase activity

Homogeneous FAD synthetase was added to the reaction mixture containing 50 μM FMN (for details see "Experimental Procedures"). Traces of cations were removed by passing all the solutions used through a Cellex column. Assay conditions were as described under "Experimental Procedures" except for the divalent cations used.

<table>
<thead>
<tr>
<th>Cation added</th>
<th>Concentration (mM)</th>
<th>Activity (units/mg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>0.6</td>
<td>119</td>
<td>16.5</td>
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<tr>
<td></td>
<td>2</td>
<td>648</td>
<td>90</td>
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<tr>
<td>ZnCl₂</td>
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<td>475</td>
<td>66</td>
</tr>
<tr>
<td>Co(NO₃)₂</td>
<td>2</td>
<td>626</td>
<td>87</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>20</td>
<td>677</td>
<td>94</td>
</tr>
<tr>
<td>Cd(CH₃COO)₂</td>
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<tr>
<td>CaCl₂</td>
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<tr>
<td>None</td>
<td></td>
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</tbody>
</table>

*One enzyme unit catalyzes the formation of 1 nmol of FAD/min at 37 °C. Activity was measured 3 min after the reaction was started by the addition of enzyme.

Discussion

Although FAD is a ubiquitous coenzyme, attempts to isolate the enzyme that catalyzes the last step in its biosynthesis, the ATP-dependent adenylylation of FMN, have failed. Only partial purification of the enzyme from bacteria (1, 15), yeast (17), higher plants (18), and rat liver (19) has been achieved. We report a purification of the enzymatic activity from *B. ammoniagenes* leading to an enzyme which is homogeneous according to the following criteria: a single band was obtained with different isoelectric focusing methods and on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and under non-denaturing conditions. Finally, Edman degradation of the protein gave a single N-terminal amino acid sequence. The protein obtained from the last stage of purification catalyzed both the formation of FMN from riboflavin and the conversion of FMN to FAD. Therefore, we take the above findings also as evidence that both enzymatic activities are located on a single polypeptide chain.

It is well known that FAD synthetase exhibits a wide specificity for flavin substrates (3, 20). In addition to the absolute requirement for the 5'-hydroxyl, only position 3 of the isoalloxazine ring and substitution at position 7 seem to be important for substrate recognition (2, 21). On the other hand, the enzyme seems to be absolutely specific for ATP. 2'-Deoxyadenosine 5'-triphosphate was a substrate only in the 5'-phosphotransferase reaction. The specificity for ATP and a number of other properties were also observed with pure rat liver flavokinase (19) and the partially purified enzymatic activities from *B. subtilis* (15). As for the rat liver enzyme, maximum activation of 5'-phosphotransferase activity was observed when Zn²⁺ was added. Analogous to the *B. subtilis* enzymes, the substrate requirements are generally more stringent for the adenylyltransferase reaction than for the phosphotransferase reaction. Again, the highest activity in the formation of FAD was observed in the presence of Mg²⁺. The

**Fig. 3. Effect of pH on the enzymatic activities of FAD synthetase.**

The rate of flavin turnover (---) and the percentage of FAD formed (---) were measured in the presence of 3 mM ATP, 50 μM riboflavin, and 2 mM Zn²⁺ (A) or 20 mM Mg²⁺ (B). An aliquot of each reaction mixture was analyzed 20 min after the addition of enzyme. The following buffer solutions were used: 50 mM sodium acetate (Ο), 50 mM sodium phosphate (Ο), 50 mM Tris-HCl (Ο), and 50 mM sodium borate (Ο).
B. ammoniagenes enzyme clearly uses oxidized flavins as substrates. In addition we and others (1) have obtained preliminary evidence that reduced flavins are also accepted. The B. subtilis enzymes exclusively work on reduced flavins (15). This seems to be the major difference between the enzymes from the two species giving the impression that they could be closely related. It might be worthwhile to reconsider whether the enzymatic activities in B. subtilis are also located on a single protein chain.

As noted above, FAD synthetase is very useful in the preparation of the coenzyme forms of riboflavin analogues. Partial purification by ammonium sulfate fractionation and DEAE-Sepharose column chromatography was found to be a fast and efficient method of separating the enzyme from contaminating phosphatase and phosphodiesterase activities. The pool fractions from the DEAE-Sepharose step could be stored at −20 °C without apparent loss of activity for several months.

It is important to point out that the values for FAD synthetase activity given in Table II or Fig. 3 shed little light on the optimal conditions of FAD formation when the enzyme is used in a coenzyme preparation. This is because in many preparative experiments the limiting factor in product formation is the stability of the enzyme rather than the activity measured during the first minutes of incubation. We found that the best conditions for the conversion of uncharged flavin analogues were achieved at neutral pH, using substrate concentrations similar to or lower than in the standard assay described above. With flavin analogues possessing a negative charge like 8-OH-riboflavin or 8-OH-6-deaza-riboflavin, optimal turnover was obtained in the range between pH 6.0 and 5.5.

The metal dependence of FAD synthetase showed some interesting features. 1 mM Ca2+ had a pronounced stabilizing effect on the enzyme at pH values below 6.0 and shifted the product ratio in favor of FAD. By addition of 30 mM Mg2+ similar but smaller effects could be obtained. Mg2+ concentrations, optimal for 5'-phosphotransferase activity (0.4 mM), were appreciably lower when compared with those optimal for adenylyltransferase activity (10 mM). A decrease in 5'-phosphotransferase activity was observed when MgCl2 was increased from 0.2 to 1 mM. The corresponding concentrations for ZnCl2 were 0.4 and 0.8 mM, respectively. In contrast, the adenylyltransferase is activated when the cation concentration is increased in the same range. At higher concentrations of cations (2–20 mM MgCl2) 5'-phosphotransferase activity which under these conditions is the rate-limiting step and can, therefore, be measured as overall turnover of riboflavin (Table II) as well as adenylyltransferase (Table III) do not show distinct dependence on cation concentration, when determined separately. However, in the overall reaction there is still a shift in the product ratio in favor of FAD by a factor of ∼15 when MgCl2 is increased from 2.5 to 20 mM (Table II).

It should be noted that the actual concentration of FMN in the test tube is in the mM range when 5'-phosphotransferase or overall reaction rates are determined, whereas in the case of adenylyltransferase assays 50 μM FMN is applied. As the cation dependence of the adenylyltransferase reaction might well change when the FMN concentration varies by 3 orders of magnitude, a definite explanation of the shift in product ratio shown in Table III cannot be given at the moment.

From the results presented here one might speculate about the presence of two active sites on the protein, or two ATP binding pockets, and a possible two-step mechanism in the adenylylation of FMN, since the reaction is much slower when ATPo5S is one of the substrates. However, no clear evidence is available on these points yet. At present, elucidation of further functional, e.g. thorough kinetical studies, and structural details is mainly hindered by the fact that the protein is expressed only in small amounts in the bacterial cell. To overcome this problem, we have recently started cloning of the FAD synthetase gene in order to get overproduction from the cloned gene.

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REFERENCES