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-adenyllyltransferase 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, non-denaturing gel electrophoresis, and isoelectric focusing. Edman degradation of the protein yielded a single N-terminal sequence.

**Experimental Procedures**

**Materials**—Adenosine 5'-triphosphate was obtained from Pharmacia (Piscataway, NJ). 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. Pharmalyte (4-6.5), DEAE-Sepharose CL-6B, and agarose-hexa-adenosine 5'-triphosphate AGATPTM Type 2 were obtained from Pharmacia, Freiburg. Blue Sepharose was prepared according to Böhnke et al. (5).

**Enzyme Assays**—FAD synthetase activity was assayed in a final volume of 50 µl of 50 mM Tris-HCl, pH 7.6, containing 50 µM 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, Abimed Analyseten-technik 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 260 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-adenyllyltransferase activity alone was assayed as above with 50 µM FMN as the flavin substrate. Under standard conditions and with homogenous enzyme, the synthetase and adenyllyltransferase reactions were linear for about 20 min and proportional to enzyme concentration through a range of 1.5-6 µg of protein/assay.

**Culture Conditions**—*B. ammoniagenes* (ATCC 6872) 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, 1 g of KH₂PO₄, 0.9 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 7.8 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 isopropl 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-isopropl alcohol cooling was applied to keep the temperature below 6 °C. After disrupting the cells, another aliquot of phenylmethylsulfonyl fluoride was added to intensify work on the enzyme.

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was added. All subsequent centrifugation steps were performed at 18,000 \( \times g \) and 4°C.

Centrifugation for 30 min removed cell debris and unbroken cells. The resulting reddish-brown supernatant was made 2 M in (NH\(_4\))\(_2\)SO\(_4\) and centrifuged for 30 min. The (NH\(_4\))\(_2\)SO\(_4\) concentration of the supernatant was brought to 3 M, and the solution was centrifuged for another 30 min. The precipitate was washed with 50 ml of buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol) and dialyzed for 1 h against four changes of 1 liter each of the same buffer. This buffer was used in all the following purification steps.

The retentate was diluted to a volume of 200 ml and loaded onto a column of DEAE-Sepharose CL-6B (2.5 × 25 cm) equilibrated with S buffer. The protein band was washed with 250 ml of buffer followed by 350 ml of buffer containing 175 mM NaCl. Elution was carried out by applying 500 ml of a linear gradient from 175 to 250 mM NaCl in buffer. Fractions containing FAD synthetase activity were combined and concentrated to about 20 ml in an Amicon ultrafiltration cell with a PM-10 membrane.

After dialysis against S buffer the concentrated solution was applied to a blue Sepharose column (2.5 × 25 cm). FAD synthetase activity was not absorbed by the column and was completely eluted with S buffer. Fractions containing FAD synthetase activity were pooled and concentrated to about 5 ml by ultrafiltration.

The affinity chromatography on N\(^6\)-coupled hexane-ATP agarose was the final purification step. After the material had been applied to the column (1.0 × 14 cm) it was washed with 25 ml of buffer. FAD synthetase activity was recovered by elution with 500 \( \mu \)M ATP dissolved in S buffer. The enzymatic activities eluted right after the breakthrough of ATP from the affinity column. 500-\( \mu \)l fractions were collected. The activities were combined and stored on ice.

**Protein Determination—** Protein was estimated by the method of Lowry et al. (6) and according to Bradford (7). Bovine serum albumin and lysozyme were used as standards.

**Determination of Molecular Weight—** Discontinuous SDS-polyacrylamide gel electrophoresis on 1.0-mm-thick and 15-cm-long slab gels was used. The protein was prepared according to Laemmli (8). The stacking gel contained 4% (w/v) acrylamide, and the separating gel contained 12.5% (w/v) acrylamide. As protein standards bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), carbonic anhydrase (bovine erythrocyte), soybean trypsin inhibitor, and lysozyme (hen egg white) were used.

**Nondenaturing Gel Electrophoresis—** was performed in 0.5-mm-thick and 8-cm-long slab gels following a modified procedure of Blackshear (9). Samples were developed on 2-10% gradient gels (pH 7.5) at 4°C. Bovine serum albumin (monomer, dimer, and trimer), ovalbumin (monomer), and a protein of molecular weight 30,000 purified from *B. ammonigenes* were used as molecular weight standards.

**Isoelectric Focusing—** Agarose isoelectric focusing was performed as described in Ref. 12 using two parts of Pharmalyte (4-6.5) and one part Servalyt (3-5) carrier ampholytes. The apparent PI of the sample was determined (pH 4.6) was found to be the same with both 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 both in agarose-isoelectric focusing and in the system described by O’Farrell (11) to form a single band, and the isoelectric point was reproducible within a narrow range.

**RESULTS**

**Enzyme Purification—** Table I shows the course of a typical preparation of *B. ammonigenes* 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 \( \text{min}^{-1} \) (400 \( \mu \)M \( \text{Zn}^{2+} \)) and 27 \( \text{min}^{-1} \) (10 mM 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\(^6\)-aminoethyl-ATP agarose column. This step led to a considerable increase in specific activity without substantial loss in total activity. No davalent 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 nondenaturing 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 both 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 1). These results altogether demonstrate that the *B. ammonigenes* 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, respectively. Normal activity of the ATP:riboflavin 5'-phosphotransferase, but no ATP:FMN adenylyltransferase activity was observed when 2'-deoxyadenosine 5'-triphosphate was used as substrate. Both \( R_F \) and \( S_p \) diastereomers of ATP\( _S \) 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\( _S \), \( S_p \)), 260 (Co-ATP\( _S \), \( S_p \)), 120 (Mg-ATP\( _S \), \( R_S \)), and 200 (Co-ATP\( _S \), \( R_S \)).

\(^1\) The abbreviations used are: SDS, sodium dodecyl sulfate; ATP\( _S \), adenosine 5'-O-(1-thiotriphosphate).
The 5'-phosphotransferase has a $K_a$ for Mg-ATP of approximately 5 $\mu$M whereas the $K_a$ (Mg-ATP) for the adenylyltransferase was found to be 160 $\mu$M. 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 MgCl$_2$ and of replacing MgCl$_2$ with ZnCl$_2$, Cd(CH$_3$COO)$_2$, Co(NO$_3$)$_2$, and MnCl$_2$ showed that the relative 5'-phosphotransferase activities with Zn$^{2+}$, Mg$^{2+}$, Cd$^{2+}$, Co$^{2+}$, and Mn$^{2+}$ 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, 406, and 400 $\mu$M, respectively. When divalent cations were omitted from the reaction mixture, 10% of the 5'-phosphotransferase activity observed in the presence of 200 $\mu$M MgCl$_2$ 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 I shows the effect of MgCl$_2$ and ZnCl$_2$ 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 adenylyltransferase activity was increased. At divalent ion concentrations optimal for the 5'-phosphotransferase reaction hardly any adenylyltransferase activity could be detected.

In the adenylyltransferase reaction highest activity was found in the order Mg$^{2+}$ (10 mM), Mn$^{2+}$ (20 mM), Co$^{2+}$ (2 mM), Zn$^{2+}$ (2 mM) (Table III); optimal concentrations for the respective cations are given in parentheses. Addition of more

**TABLE I**

**Purification of FAD synthetase from Brevibacterium ammoniagenes**

| Stage of purification | Volume $\mu l$ | Protein mg | Total activity $\mu$units | Specific activity units/mg | Recovery % | Purification-fold |
|-----------------------|---------------|------------|--------------------------|---------------------------|------------|------------------|
| Crude extract         | 750           | 10,500     | 200                      | 0.02                      | (100)*     | (1)              |
| 50-70% (NH$_4$)$_2$SO$_4$ fraction, di- | 235           | 2,490      | 210                      | 0.085                     | (105)      | (4.5)            |
| DEAE-Sepharose eluate, concentrated | 25            | 163        | 175                      | 1.07                      | 88         | 54               |
| Blue Sepharose eluate, concentrated | 5.5           | 21         | 134                      | 6.4                       | 67         | 320              |
| ATP-agarose eluate    | 2.4           | 0.7        | 96                       | 137                       | 48         | 6850             |

* 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 phosphoesterases.

**TABLE II**

**Effect of concentration of divalent cations on the initial rate of product formation in the reaction catalyzed by FAD synthetase**

Assay conditions were as described under "Experimental Procedures" except for the divalent cations used.

| Cation Added | Concentration $mM$ | Activity $\mu$units/mg | Product $\%$ |
|--------------|---------------------|------------------------|--------------|
| MgCl$_2$    | 0.25                | 341                    | 97           |
|             | 2.5                 | 154                    | 34           |
|             | 20.0                | 145                    | 3            |
| ZnCl$_2$    | 0.1                 | 673                    | 100          |
|             | 0.2                 | 928                    | 96           |
|             | 2.0                 | 446                    | 60           |

* One enzyme unit catalyzes the conversion of 1 nmol of riboflavin/min at 37 °C. Activity was measured 3 min after the reaction was started by the addition of enzyme.
TABLE III
Effect of divalent cations on adenylyltransferase activity

| Cation added | Concentration (mM) | Adenylyltransferase activity* | Relative rate |
|--------------|--------------------|-------------------------------|--------------|
| MgCl₂        | 0.6                | 119                           | 16.5         |
|              | 2                  | 648                           | 90           |
|              | 10                 | 670                           | 100          |
|              | 20                 | 670                           | 93           |
| ZnCl₂        | 2                  | 475                           | 66           |
| Co(NO₃)₂     | 2                  | 626                           | 87           |
| MnCl₂        | 20                 | 677                           | 94           |
| Cd(CH₃COO)₂  | 0.5                | 26                            | 3.6          |
| CaCl₂        | 1                  | 40                            | 5.5          |
| None         |                    | 0                             | 0            |

*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.

than equimolar amounts, relative to ATP, of ZnCl₂, Co(NO₃)₂, Cd(CH₃COO)₂, or CaCl₂, respectively, led to the rapid and complete inactivation of both enzymatic activities. Of all the cations tested, Ca²⁺ gave the lowest rates and a 5'-phosphotransferase activity even smaller than had been found in the absence of divalent cations.

The effect of pH on the enzymatic activities in the presence of Zn²⁺ or Mg²⁺ is shown in Fig. 3. There were two major differences in the pH dependence according to whether either Mg²⁺ or Zn²⁺ was present. With Mg²⁺, the highest turnover of riboflavin was observed in the range between pH 6.0 and 7.5, and FAD was the only product which could be detected in the test solution between pH 7.0 and 9.0. When Zn²⁺ was used for activation we observed a steady increase in the initial rate of riboflavin turnover between pH 4.5 and 10. Again FAD was the major product at pH 7.0, but here the percentage of FAD formed decreased rapidly above pH 8.0. While this finding might simply reflect the reduced effective concentration of Zn²⁺ due to the formation of Zn(OH)₂, we cannot easily explain why at pH values below 5.0 only the 5'-phosphotransferase was activated by Mg²⁺ or Zn²⁺. We do, however, know from work with 8-OH-5-deaza-riboflavin, which only in its neutral form (pKₑ = 6.0) was accepted as a substrate by FAD synthetase, that the stability of the enzyme rapidly decreased at pH values below 6, with a parallel decrease in the ratio of FAD to FMN formation. Curiously, addition of 1 mM CaCl₂ substantially counteracted both of these pH effects (16). When 1 mM CaCl₂ was added to a standard assay no change in the initial rate of product formation was detected.

**DISCUSSION**

Although FAD is a ubiquitous coenzyme, attempts to isolate the enzyme that catalyzes the last step in its biosynthesis, the ATP-dependent adenylation 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 nondenaturing 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
**B. ammoniagenes FAD Synthetase**

*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-5-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 nM 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 ATPaS is one of the substrates. However, no clear evidence is available on these points yet. At present, elucidation of further functional, e.g. thorough kinektical 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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