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-adenylyltransferase 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 adenylylation 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.

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. Pharmalyte (4–6.5), DEAE-Sepharose CL-6B, and agarose-hexane-adenosine 5′-triphosphate AGATP™ 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 μl of 50 mM Tris·HCl, pH 7.6, containing 50 μM riboflavin, 3 mM ATP, and 15 mM $MgCl_2$. 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 × 250 mm, 5-μm particle size, Abimed Analysentechnik 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-adenylyltransferase activity alone was assayed as above with 50 μM FMN as the flavin substrate. Under standard conditions and with homogenous enzyme, the synthetase and adenylyltransferase 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, 3 g of KH_2PO_4, 3 g of K_2HPO_4, 2 g of $MgCl_2$, 0.1 g of $CaCl_2$, and 0.01 g of $FeCl_2$. 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 × *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 ¾-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

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 $(NH_4)_2SO_4$ and centrifuged for 30 min. The $(NH_4)_2SO_4$ concentration of the supernatant was brought to 3 M, and the solution was centrifuged for another 30 min. The precipitate was redissolved in 50 ml of S 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 bound protein 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.

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 μM ATP dissolved in S buffer. The enzymatic activities eluted right after the breakthrough of ATP from the affinity column. 500-μl fractions were collected. The active fractions 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[1]-polyacrylamide gel electrophoresis on 1.0-mm-thick and 15-cm-long slab gels was performed 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 erythrocytes), 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. ammoniagenes* were used as molecular weight standards.

Gels were stained for protein using Coomassie Brilliant Blue R-250 (0.2%)/G-250 (0.05%) in 10% acetic acid and 45% ethanol or the alkaline silver stain method (10).

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 using the protein test kit for pI determination from Serva (Heidelberg, Federal Republic of Germany).

Alternatively the isoelectric focusing procedure of O'Farrell (11) was used. The protein was prepared in sample buffer containing 8 M urea and 4 mM dithiothreitol and applied to 12-cm-long slab gels of 4% (w/v) polyacrylamide and 0.2% (w/v) bisacrylamide. After 5 h, electrofocusing gels were stained for protein with Coomassie Blue. For measurement of the pH gradient, part of the gel was removed before staining and cut into 5-mm sections which were triturated in distilled water for pH measurement.

Sequence Analysis—In order to determine the N-terminal amino acid sequence of FAD synthetase, 1 nmol of enzyme was subjected to automated Edman degradation in an Applied Biosystems 470A protein Sequencer. Analysis of the phenylthiohydantoins was performed on a Hewlett-Packard 1084B liquid chromatograph equipped with an automatic sampling system and a 254-nm fixed wavelength detector. The prepacked column (40 × 250 mm) Lichrospher 60 CH8/II was purchased from E. Merck, Darmstadt, Federal Republic of Germany. Elution was performed using the ternary isocratic solvent system described by Lottspeich (13).

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; ATPαS, adenosine 5′-O-(1-thiotriphosphate).

RESULTS

Enzyme Purification—Table I shows the course of a typical preparation of *B. 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 μM Zn^{2+}) and 27 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-aminohexyl-ATP agarose column. This step led to a considerable increase in specific activity without substantial loss in total activity. No divalent 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 I). These results altogether demonstrate that the *B. 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′-triphosphate was used as substrate. Both R_p and S_p diastereomers of ATPαS 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·ATPαS, S_p), 260 (Co·ATPαS, S_p), 120 (Mg·ATPαS, R_p), and 200 (Co·ATPαS, R_p).

TABLE I

Purification of FAD synthetase from Brevibacterium ammoniagenes

Experimental details are given under "Experimental Procedures."

Stage of purification	Volume	Protein	Total activity[a]	Specific activity	Recovery	Purification
	ml	*mg*	*units*	*units/mg*	*%*	*-fold*
Crude extract	750	10,500	200	0.02	(100)[b]	(1)
50–70% (NH$_4$)$_2$SO$_4$ fraction, dialyzed	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

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

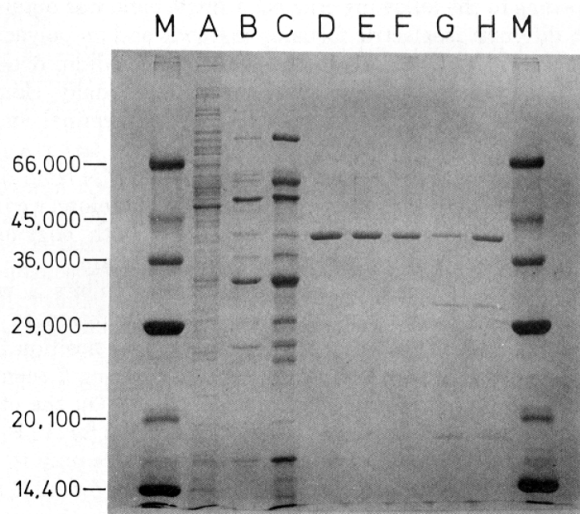[b] Numbers in parentheses could not be accurately determined due to the presence of phosphatases and phosphooliesterases.

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.

Met Asp Ile — Tyr Gly Thr Ala Ala Val
Pro Lys Asp Leu — Asn — Ala — —

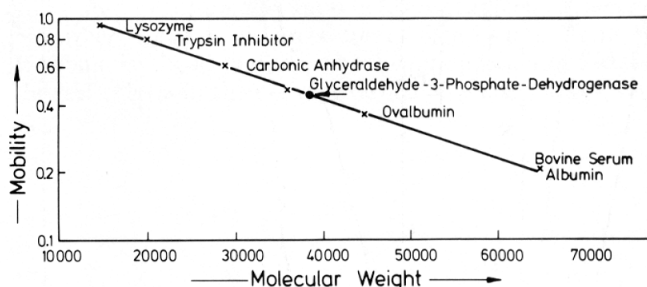SCHEME I. N-terminal amino acid sequence of FAD synthetase.

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.*

The 5′-phosphotransferase has a K_m for Mg·ATP of approximately 5 μM whereas the K_m (Mg·ATP) for the adenylyltransferase 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 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, 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 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 II 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 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	Activity[a]	Product	
			FMN	FAD
	mM	*units/mg*	*%*	
MgCl$_2$	0.25	341	97	3
	2.5	154	34	66
	20.0	145	3	97
ZnCl$_2$	0.1	673	100	0
	0.2	928	96	4
	2.0	446	60	40

[a] 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

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

Cation added	Concentration	Adenylyltransferase activity[a]	Relative rate
	mM	*units/mg*	*% of control*
$MgCl_2$	0.6	119	16.5
	2	648	90
	10	720	100
	20	670	93
$ZnCl_2$	2	475	66
$Co(NO_3)_2$	2	626	87
$MnCl_2$	20	677	94
$Cd(CH_3COO)_2$	0.5	26	3.6
$CaCl_2$	1	40	5.5
None		0	0

[a] 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 $ZnCl_2$, $Co(NO_3)_2$, $Cd(CH_3COO)_2$, or $CaCl_2$, respectively, led to the rapid and complete inactivation of both enzymatic activities. Of all the cations tested, Ca^{2+} 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 Zn^{2+} or Mg^{2+} is shown in Fig. 3. There were two major differences in the pH dependence according to whether either Mg^{2+} or Zn^{2+} was present. With Mg^{2+}, 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 Zn^{2+} 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 Zn^{2+} 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 Mg^{2+} or Zn^{2+}. We do, however, know from work with 8-OH-5-deaza-riboflavin, which only in its

neutral form ($pK_a = 6.0$) 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 $CaCl_2$ substantially counteracted both of these pH effects (16). When 1 mM $CaCl_2$ 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 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 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 Zn^{2+} 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 Mg^{2+}. The
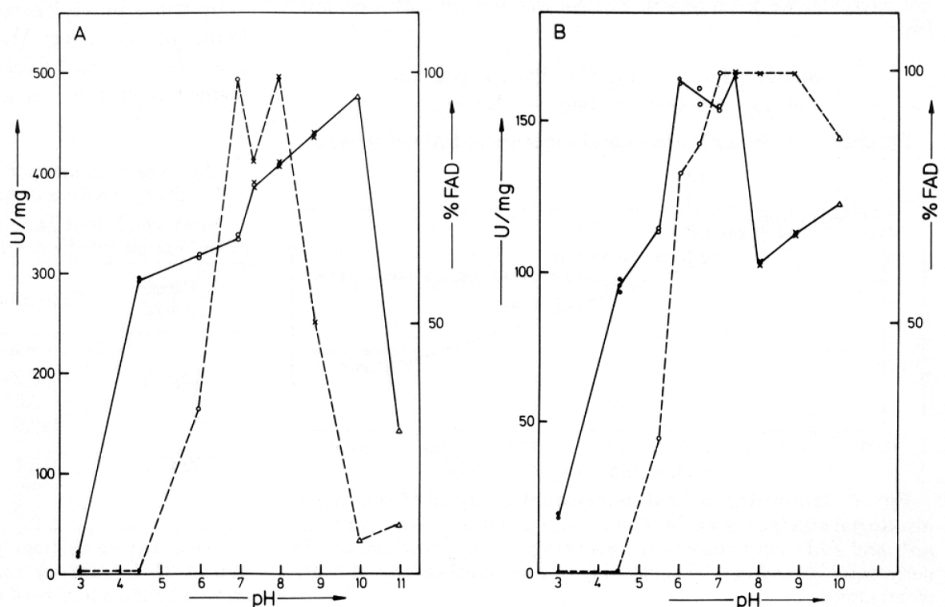

FIG. 3. **Effect of pH on the enzymatic activities of FAD synthetase.** The rate of riboflavin turnover (———) and the percentage of FAD formed (– – –) were measured in the presence of 3 mM ATP, 50 μM riboflavin, and 2 mM Zn^{2+} (*A*) or 20 mM Mg^{2+} (*B*). An aliquot of each reaction mixture was analyzed 20 min after the addition of enzyme. The following buffer solutions were used: 50 mM sodium acetate (●), 50 mM sodium phosphate (○), 50 mM Tris·HCl (×), and 50 mM sodium borate (△).

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 Ca^{2+} 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 Mg^{2+} similar but smaller effects could be obtained. Mg^{2+} 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 $MgCl_2$ was increased from 0.2 to 1 mM. The corresponding concentrations for $ZnCl_2$ 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 $MgCl_2$) 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 $MgCl_2$ 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 ATPαS is one of the substrates. However, no clear evidence is available on these points yet. At present, elucidation of further functional, *e.g.* thorough kinetic 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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