Substrate Specificity and Variables Affecting Efficiency of Mammalian Flavin Adenine Dinucleotide Synthetase†

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ABSTRACT: Substrate specificity and product inhibition have been evaluated by using purified rat liver FAD synthetase (ATP:FMN adenylyltransferase, EC 2.7.7.2), obtained by an improved purification protocol with optimized flavin affinity chromatography. FMN analogues studied fall into three general classifications: those with substitution on the pyrimidinoid ring and nitrogen replacement, those with substitution on the benzenoid ring, and those with N(10) side chain modifications. Substitutions on the pyrimidinoid ring and replacement of nitrogens have the greatest influence on binding to enzyme and FAD formation. When the hydrogen-bonding capacity of the NH group at position 3 is blocked or removed by substitution, such FMN analogues do not act as substrates or inhibitors of the enzyme. Substitutions on the benzenoid ring by small groups seem to be tolerated, while larger groups inhibit binding. Length of the N(10) side chain is optimal with five carbons and has greatest affinity for the natural ribityl side chain. Affinity matrices show similar binding characteristics in that the *N*(3)-(carboxymethyl)riboflavin–agarose does not bind enzyme, while agaroses linked to the flavin N(10) side chain provide varying degrees of purification. The C=O group at position 2, the NH group at position 3, and a five-carbon side chain at the N(10) position seem to be most crucial for flavin substrate binding to enzyme. Nucleoside triphosphates other than ATP do not act as substrates or inhibitors when sufficient Mg^{2+} is present. Products of the reaction, FAD and PP_i, act as inhibitors against both ATP and FMN. K_i values for FAD against ATP and FMN were found to be 1.3 and 0.75 μM, respectively. K_i values for PP_i against ATP and FMN were 200 and 66 μM, respectively.

Flavin adenine dinucleotide (FAD)[1] synthetase (ATP:FMN adenylyltransferase, FMN pyrophosphorylase; EC 2.7.7.2) has been purified from *Brevibacterium ammoniagenes* (Manstein & Pai, 1986) and from the cytosol of rat liver (Oka & McCormick, 1987). A comparison of the two isolated enzymes indicates that there are specific differences. The flavokinase/FAD synthetase from the bacterium catalyzes both the 5′-phosphorylation of riboflavin to FMN and the adenylylation of FMN to FAD. It has a reported molecular weight of 38 000 with an isoelectric point of 4.6. The FAD synthetase from rat liver catalyzes only the adenylylation of FMN to FAD, has a reported molecular weight of approximately 100 000 (composed of two 50 000 subunits), and an isoelectric point of 6.6. Both enzymes show similarity with regard to adenylyltransferase activity in that each requires ATP and Mg^{2+} for highest activity. The K_m values reported for ATP are 160 μM for the bacterial enzyme and 53 μM for the liver enzyme. The mammalian enzyme was also shown to exhibit no significant reversibility when FAD and PP_i were incubated with pure enzyme (Oka & McCormick, 1987).

Heretofore, only quite crude preparations of flavokinase/FAD synthetase from *Brevibacterium* had been used by different laboratories for formation of some flavocoenzymes from riboflavin analogues (Spencer et al., 1976; Walsh et al., 1978; Light et al., 1980; Hausinger et al., 1986). Thus, its two-step, viz., kinase and synthetase, flavin specificity has been indirectly and incompletely defined for some riboflavin analogues that undergo catalysis (no matter how slowly). Substrate-catalysis constants for the pure bacterial enzyme have only been quantitated for riboflavin and ATP (Manstein & Pai, 1986). Earlier work with crude mammalian synthetase had indicated a relative specificity for flavin 5′-phosphate and ATP (McCormick, 1964).

The present work examines the affinity of the liver synthetase for varying flavin affinity matrices with the achievement of an improved chromatographic purification, circumscribes the specificity of the purified liver FAD synthetase for both flavin monophosphates (FMN analogues) and nucleoside phosphates (ATP analogues), and quantitates product inhibition by FAD and PP_i.

Materials and Methods

Riboflavin, FMN, FAD, ATP, ADP, AMP, Ado, d-ATP, GTP, ITP, XTP, CTP, UTP, TTP, BSA, DAAO, DTT, and FMN–agarose (claimed to be attached through ribose hydroxyls) were all purchased from Sigma Chemical Co. D-Phenylglycine was obtained from Aldrich Chemical Co. Dye solution for protein assay and Affi-Gel 102 (with aminohexylagarose) were obtained from Bio-Rad. DEAE-Sepharose CL-6B, Sepharose CL-6B, and CH Sepharose 4B (with carboxyhexyl group) were purchased from Pharmacia. [U-^{14}C]-5′-ATP (500 mCi/mmol), [5-^{3}H]-5′-CTP (19.3 Ci/mmol), [8-^{3}H]-5′-GTP (11.5 Ci/mmol), [*methyl*-^{3}H]-5′-TTP (46 Ci/mmol), and aqueous counting scintillant were all

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; Ado, adenosine; dATP, 2′-deoxyadenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; XTP, xanthosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate; TTP, thymidine 5′-triphosphate; $NaPP_i$, sodium pyrophosphate; KP_i, potassium phosphate; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; DAAO, D-amino acid oxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS–Page, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; RP, reverse phase.

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Table I: Purification of FAD Synthetase from Rat Liver

step[a]	vol (mL)	act. (units)	protein (mg)	spec act. (units/mg)	purifn (*x*-fold)
(1) high-speed supernatant	154	14.9	2090	0.00698	1
(2) $(NH_4)_2SO_4$ fraction	33.5	10.4	663	0.0157	2.2
(3) DEAE-Sepharose 6B (pool)	8.0	20.9	43.4	0.483	69.2
(4) Sepharose CL-6B (pool)	7.2	19.2	6.80	2.83	405
(5) FMN–Sepharose 4B[b]	5.0	8.7	0.0233	374	53 600

[a] Unless noted otherwise in the text, details are as given in Oka and McCormick (1987). [b] Synthesized from FMN and CH Sepharose 4B.

purchased from Amersham Corp. All work reported here was completed under dim light conditions to minimize photodecomposition.

Modification of Purification Steps. The original purification procedure (Oka & McCormick, 1987) required six steps. These included centrifugation for high-speed supernatant, $(NH_4)_2SO_4$ fractionation, and chromatographies on DEAE-Sephadex A-50, polybuffer exchanger 94, FMN–agarose, and Sephadex G-200. Enzyme with a specific activity[2] of 133 units/mg was usually obtained. We are now able routinely to prepare enzyme with a specific activity about 3-fold higher (Table I). The DEAE-Sephadex A-50 was replaced with DEAE-Sepharose CL-6B and the elution protocol modified. The column was equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 0.05 mM EDTA and 1 mM DTT. After the enzyme solution was applied, the column was washed with buffer containing 50 mM NaCl until absorbance at 280 nm decreased to near zero. The FAD synthetase fraction was eluted by buffer containing 100 mM NaCl. Deletion of the chromatofocusing step and substitution of Sepharose CL-6B for Sephadex G-200 gel filtration before affinity chromatography as well as changing the type of affinity gel (using the synthesized FMN–Sepharose 4B gel) have yielded an enzyme preparation with specific activity of 374 units/mg. To obtain this enzyme with higher specific activity, the affinity column was washed with equilibration buffer (10 mM potassium phosphate, pH 7.1, with 0.05 mM EDTA and 1 mM DTT) containing 200 mM NaCl. This removes the mixture of remaining nonspecifically bound proteins and possibly some partially denatured FAD synthetase. The enzyme is then released by competitive displacement by 400 μM FMN in the same buffer and assayed immediately to determine activity. It should be noted that the activity of the enzyme is highest immediately upon elution from the affinity gel and decreases to about 70% after 24 h. The enzyme obtained by using these modified purification procedures gave a single band with subunit molecular weight of 50 600 when evaluated by SDS–Page (6–12% gradient gels and Tris–glycine, pH 8.5, running buffer) and silver stain.

Standard Enzyme Incubation Method and Assay. The standard synthetase incubation mixture contained 50 mM potassium phosphate buffer (pH 7.1), 0.5 mM each of FMN and ATP, 1 mM $MgSO_4$, 1 mM DTT, 200 μg/mL BSA, and an aliquot of FAD synthetase in a total volume of 500 μL. The mixture was incubated for 1 h at 37 °C. The reaction was stopped by heating at 90 °C for 3 min. The samples were centrifuged, and the supernatants were frozen until analyzed for FAD. The FAD formed was then quantitated by using the indirect assay with apo-DAAO (Oka & McCormick, 1987), where apo-DAAO is converted to holoenzyme by the FAD produced in the synthetase incubation. Conversion of D-phenylglycine by the oxidase to benzoyl formate was monitored for 10 min at 252 nm. The constant velocity of the oxidase reaction was determined between 5 and 9 min and related to FAD content by using a calibration curve based on the reaction of apo-DAAO, standard FAD solutions, and D-phenylglycine.

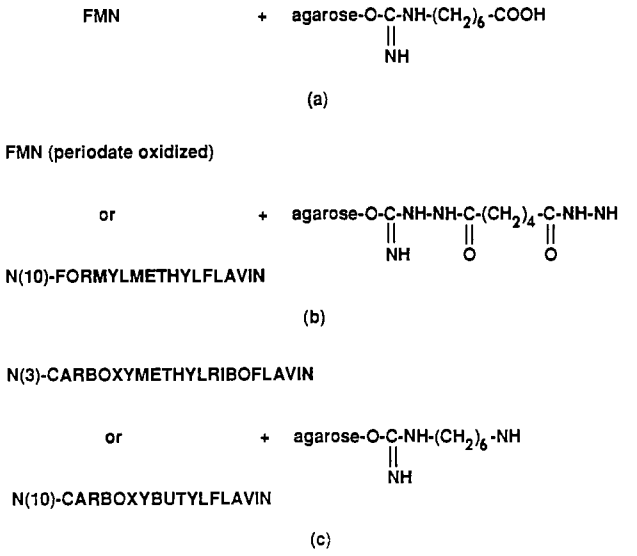

FIGURE 1: Syntheses of affinity matrices with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide linkage to agarose (a) through a carboxyhexyl group, (b) through adipic acid dihydrazide, and (c) through an aminohexyl group.

Affinity Matrices Syntheses and Specificity. Five affinity matrices were synthesized and compared to commercial FMN–agarose purchased from Sigma. The gels were synthesized by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide coupling of different flavins to activated agarose gels having various spacer arms as shown in Figure 1. Synthesis a was done according to the method of Kazarinoff et al. (1974) except that the reaction took place in water adjusted to pH 5.0 instead of in 20 mM KP_i at pH 7.0, synthesis b paralleled the procedure of Wilchek and Lamed (1974), and synthesis c followed that of Merrill and McCormick (1980). After the gels were thoroughly washed, the covalently bound flavin content was determined spectrophotometrically on a small aliquot of each matrix that had been acid-hydrolyzed for 1 h in 2 N HCl. The flavinylagaroses were then tested for their ability to bind FAD synthetase. One milliliter of each of the gels was first equilibrated with 10 mM potassium phosphate buffer (pH 7.1) containing 0.05 mM EDTA and 1 mM DTT. Then, 1.48 units of partially purified enzyme, specific activity of 0.676 unit/mg, was applied. After the gels were washed with equilibration buffer, the synthetase was released by competitive displacement by 400 μM FMN in this buffer and finally by buffer containing 400 μM FMN plus 1 M NaCl. All three fractions were saved and assayed for activity and protein content.

Substrate Analogues and FAD Synthetase Efficiency. Riboflavin 5′-phosphothionate was a generous gift from Dr. Betty Sue Masters (Milwaukee) and synthesized according to published procedures (Calhoun et al., 1987). FMN analogues were prepared from riboflavin analogues by using the

[2] One unit of activity is defined as the amount of enzyme required to produce 1 nmol of FAD/min at 37 °C under standard assay conditions [see Materials and Methods and Oka and McCormick (1987)].

general monochlorophosphoric acid procedure (Flexser & Farkas, 1961). Since the phosphorylation reaction is only relatively specific for the terminal hydroxymethyl group of a flavin N(10) side chain, all FMN analogues were purified by RP chromatography using a preparative Dynamax C_{18} (8 μm, 60 Å, 21 × 250 mm) column and previously published elution protocol (Nielsen et al., 1986). The purified flavin 5′-phosphate was concentrated by using a speed vacuum and desalted by RP chromatography using a water–methanol linear gradient. Analogue concentrations were determined by UV and visible absorption and known molar extinction coefficients.

The activity of FMN analogues as substrates for FAD synthetase was evaluated in a two-step procedure. FAD synthetase (0.09 unit) was incubated with varying amounts (5 μM–1 mM) of FMN or one of its analogues (F′MN), 0.5 mM ATP, and 1 mM Mg^{2+} under standard assay conditions to form PP_i and FAD or F′AD. The reaction was initiated by the addition of enzyme, stopped after 1 h, and stored as described above. The FAD or F′AD was then quantitated by using direct assay with HPLC or by indirect assay with apo-DAAO.

The separation of FAD, FMN, and riboflavin, as well as their respective analogues, was achieved by the published RP-HPLC procedures (Light et al., 1980; Oka & McCormick, 1985) using a C_{18} μBondapak column (Waters, 3.9 × 250 nm) with appropriate guard (Brownlee, 4 × 30 mm) and various linear gradients between 5 mM aqueous ammonium acetate buffer, pH 6, and methanol as mobile phase. Elution of flavins was monitored by both fluorescence (Gilson 121 fluorometer; excitation filter, 305–395 nm; emission filter, 475–680 nm) and absorbance (Perkin-Elmer LC-85B variable-wavelength detector set at 260 nm or other appropriate wavelength). Quantitation of F′AD was achieved by using a calibration curve based on integrated peak areas corresponding to varying amounts of FAD run under similar chromatographic conditions.

To test substrate potential of various nucleoside triphosphates for FAD synthetase, the enzyme was incubated with each radiolabeled nucleoside triphosphate in a standard incubation mixture where the FMN, nucleoside triphosphate, and Mg^{2+} concentrations were 0.2, 0.2, and 0.4 mM, respectively. A typical 200-μL incubation contained 0.5 μCi of [^{14}C]ATP or 2 μCi of each tested analogue and 0.099 unit of enzyme. After incubation at 37 °C for 1 h, the reaction was terminated. A 20-μL aliquot of the supernatant was applied to HPLC for separation. The eluate was collected in 0.4-mL fractions and the radioactivity measured by using 4 mL of aqueous counting scintillant and a Beckman LS-3133 scintillation counter.

Inhibition of FMN to FAD Conversion by FMN Analogues, Nucleotides, and Products (FAD and PP_i). Various FMN analogues were tested as inhibitors of the FMN to FAD reaction by including constant amounts of each FMN analogue (20, 100, and 200 μM) in the standard reaction mixture containing FMN (5, 10, 20, 40, and 100 μM), 0.5 mM ATP, 1 mM Mg^{2+}, and 0.02 unit of FAD synthetase. The total volume of the reactions was 200 μL, and the FAD formed was quantitated by using the apo-DAAO assay.

For studies of inhibition by nucleotides and products, the enzyme was incubated in 50 mM Tris-HCl (pH 7.1), instead of KP_i, buffer since preliminary studies indicated that the concentration of Mg^{2+} and phosphate affects the FAD synthetase reaction. In addition, the ATP concentration was restricted to 0.1 mM to more clearly discern the influence of nucleotides and products on FAD production. To study the effects of selected adenine nucleotides and $NaPP_i$, enzyme (0.079 unit) was incubated at 37 °C for 1 h with 0.1 mM ATP, 0.1 mM Mg^{2+}, 0.2 mM FMN, and varying amounts of inhibitor (0.05, 0.1, 0.2, 0.3, 0.5, and 1.0 mM). The FAD formed was then assayed by using the apo-DAAO assay. These initial studies indicated that the Mg^{2+} concentration might be influencing FMN to FAD conversion; thus, extensive studies with a variety of effectors (0.5 mM) and three concentrations of Mg^{2+} (0.1, 0.2, and 1 mM) were initiated by using incubations containing 0.1 mM ATP, 0.2 mM FMN, and 0.079 unit of enzyme. The FAD formed was determined as stated previously.

To quantitate product inhibition, either FAD or PP_i was incubated against varying concentrations of each of the substrates (FMN and ATP) in 50 mM Tris-HCl, pH 7.1. For product inhibition against FMN, the standard incubation mixture contained 0.5 mM ATP, 1.0 mM Mg^{2+}, varying amounts of either FAD (0, 1.0, 1.5, 2.0, and 3.0 μM) or PP_i (0, 0.1, 0.2, and 0.3 mM), and 0.04 unit of enzyme. For product inhibition against ATP, the standard incubation mixture contained 0.2 mM FMN, a $[Mg^{2+}]/[ATP]$ of 2, varying amounts of either FAD (0, 1.0, 1.5, 2.0, 3.0, and 5.0 μM) or PP_i (0, 0.1, 0.2, and 0.3 mM), and 0.04 unit of enzyme. The FAD formed was determined as above.

Results

Initial attempts to purify FAD synthetase by affinity chromatography (Oka & McCormick, 1987) had revealed a certain selectivity on the part of FAD synthetase for the commercial (Sigma) so-called FMN–agarose. In an attempt to enhance the recovery of FAD synthetase from rat liver and to better understand affinity/specificity relationships, a variety of new affinity gels were synthesized as shown generally in Figure 1. After the gels were thoroughly washed and the flavin content was determined, they were tested for their ability to bind FAD synthetase. Each matrix was treated in the same manner, without optimization for efficiency, so that relative binding could be assessed. The enzyme was applied to the gels and then released by using three successive buffer changes (see Materials and Methods). The amounts of enzyme bound to each matrix and released were determined by assaying the activity in each of the three eluted fractions. The results are summarized in Table II.

The primary consideration used in designing the experiments to evaluate flavin substrate specificity was the sensitivity of the method for FAD quantitation and the intent to measure steady-state kinetic K_m and V_m values. The direct HPLC method (Light et al., 1980) used to separate flavins and flavocoenzyme analogues (synthesized by flavokinase/FAD synthetase from *B. ammoniagenes*) seemed appropriate. The separation of various flavin families was achieved by using a C_{18} RP column and applying 5 mM aqueous ammonium acetate buffer, pH 6, and methanol in a linear gradient from 5 to 70% methanol. For each analogue, the FAD and FMN coenzymes eluted before the free flavin at 4–6 and 2–3 min, respectively. Since the flavin 5′- or ω′-phosphates were synthesized from their respective riboflavin analogues, both free flavin and its monophosphate were available for estimating the expected retention time for the F′AD synthesized. In addition, an incubated control (40 μM F′MN) containg no FAD synthetase was compared to a similar incubation containing synthetase. This procedure served as a good indicator of any new flavin component formed during the initial synthetase incubation period.

One limitation anticipated was low detection sensitivity for FAD and F′AD by HPLC detection. Nielsen et al. (1987)

Table II: Comparison of Binding Efficiencies of Affinity Matrices for Liver FAD Synthetase

matrix	ligand (μmol/mL)	total act. bound[a] (units/μmol of ligand)	recovered act.[b] (%)	purifn (x-fold)
FMN–Sepharose 4B	1.11	0.72	51	15
FMN–agarose, commercial (Sigma)	0.710	1.44	48	6.0
oxidized FMN–dihydrazide agarose	0.509	0.93	29	12
(formylmethyl)flavinyl–dihydrazide agarose	0.568	0.76	25	9.2
N(3)-(carboxymethyl)riboflavinyl-Affi-Gel 102	5.80	0.093	34	2.0
N(10)-(carboxybutyl)flavinyl-Affi-Gel 102	7.09	0.21	0.0	0.0

[a] Activity calculated from difference in amount applied and that recovered with equilibration buffer. [b] Activity calculated from the competitive elution with 400 μM FMN in equilibration buffer.

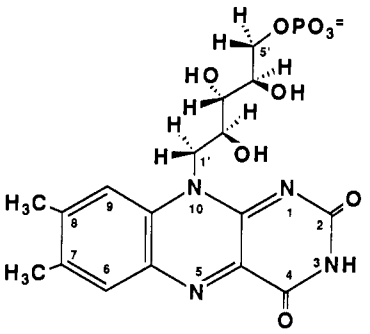

FIGURE 2: Structure of FMN with conventional numbering.

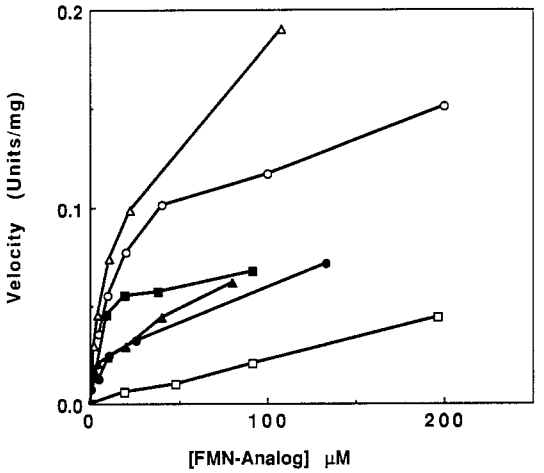

FIGURE 3: Substrate activity of representative FMN analogues with substitution on the benzenoid ring using 0.09 unit of FAD synthetase incubated for 1 h under standard conditions and assayed by HPLC. (○) FMN; (●) iso-FMN; (△) 7-chloro-FMN; (▲) 8-chloro-FMN; (□) 7,8-dichloro-FMN; (■) 7,8-dibromo-FMN.

reported a detection limit for FAD of about 100 and 30 pmol using photometric detection at 450 and 254 nm, respectively, and of less than 1 pmol using fluorometric detection (excitation, 470 nm; emission, 530 nm). On the basis of this knowledge, we concluded that our incubations would have to contain enough enzyme and proceed long enough to produce a 10 nM solution of FAD or F'AD (injection of 100 μL) to allow for detection and quantitation. Even then only those analogues that functioned as relatively efficient substrates could be detected.

Since it has been shown that some FAD analogues are much less efficient as coenzymes for apo-DAAO (Chassy & McCormick, 1965a), the indirect method using this enzyme for quantitating FAD or F'AD would not provide a true measure of FAD synthetase activity. In addition, the range of sensitivity for this assay is much less, namely, 0.25–5 μM (Oka & McCormick, 1987), than that anticipated for HPLC.

For flavin substrate specificity studies, the FMN analogues were divided into three major classes as can be related to the

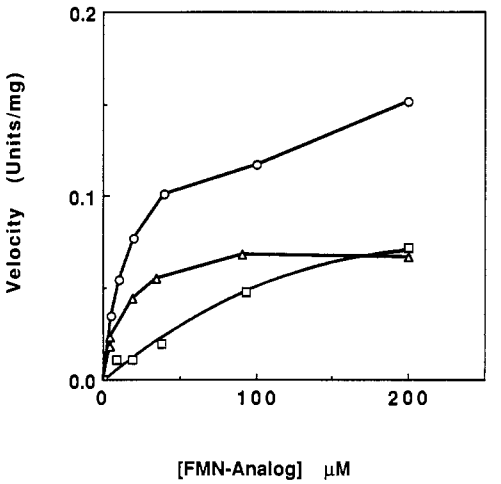

FIGURE 4: Substrate activity of representative FMN analogues with substitution on the N(10) side chain using 0.09 unit of FAD synthetase incubated for 1 h under standard conditions and assayed by HPLC. (○) FMN; (△) 5'-pentyl-FMN; (□) 4'-butyl-FMN.

Table III: Substrate Efficiencies of FMN Analogues with Liver FAD Synthetase[a]

	direct assay[b]			indirect assay[c]	
substrate	K_m (μM)	V_m(meas) (units/mg)	V_m(corr)[d] (units/mg)	K_m (μM)	V_m (units/mg)
FMN	9.5	141		7.9	146
iso-FMN	15	56			
7-chloro-FMN	8.6	140		7.6	
8-chloro-FMN	19	68			
7,8-dichloro-FMN	~120	~200	~60	82	
7,8-dibromo-FMN	4.8	65		940	
4'-butyl-FMN	~250	~160	~600		
5'-pentyl-FMN	9.4	60	150		

[a] Number of data pairs used in Lineweaver–Burk regression analysis varied from four to six with the correlation coefficients reflecting 0.95 or greater for the degree of linear fit. [b] Assayed by using HPLC with fluorescence detection of flavins in pH 6 buffer. [c] Assayed by using apo-D-amino acid oxidase. [d] Velocity corrected for difference in fluorescence of FAD analogue from that of FAD at pH 6.

numbered structure given for FMN in Figure 2: those with substitutions on the pyrimidinoid ring (modifications at positions 1–4) and nitrogen replacement, those with substitutions on the benzenoid ring (modifications at positions 6–9), and those with modifications on the N(10) side chain both with regard to steric disposition of polyhydroxyl groups and length of the alkyl chain. The analogues that were found to form detectable quantities of F'AD by HPLC, using fluorescence and/or absorption detection, are presented in Figures 3 and 4. Whenever the analogue concentration exceeded 300 μM, the HPLC resolution of F'AD from the very large quantity

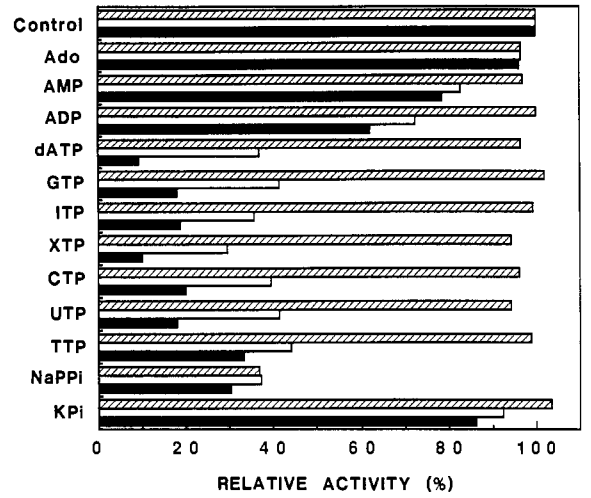

FIGURE 5: Effect of Mg^{2+} concentration on inhibition by nucleotides and product. Each reaction mixture contained 0.079 unit of FAD synthetase in Tris-HCl buffer. The concentrations of ATP, FMN, and various effectors were 0.1, 0.2, and 0.5 mM, respectively. The hashed bar shows the results with 1 mM Mg^{2+}, the open bar shows the results with 0.2 mM Mg^{2+}, and the solid bar shows the results with 0.1 mM Mg^{2+}. When ATP and Mg^{2+} were 0.5 and 1.0 mM, respectively, PP_i but not nucleotides shows inhibition.

of F'MN became the limiting factor in FAD quantitation. This was particularly true for 7,8-dichloro-FMN and 4'-butyl-FMN, which appear to be poor substrates. Thus, only concentrations in the 1–200 μM range are reported and used in a Lineweaver–Burk regression analysis to calculate K_m and V_m values. These values are summarized in Table III. The V_m values were then corrected for known fluorescence quenching due to pH (Chassy & McCormick, 1965b). It should be noted that the K_m values reported for 7,8-dichloro-FMN and 4'-butyl-FMN are relative indicators of enzyme efficiency and not based upon a substrate concentration range optimal for the enzyme. By use of the indirect assay with apo-DAAO, the K_m values calculated agree well except for that of 7,8-dibromo-FMN. As noted before, this indirect assay has some inherent difficulties. When alterations of the isoalloxazine system give rise to variations in the redox potential of the flavin moiety, the measured velocity of F'AD production by the synthetase is altered due to the decreased efficiency of apo-DAAO to bind flavin and convert its substrate to product. This may account for the large discrepency we observed for the 7,8-dibromo analogue.

When an excessive amount of FAD synthetase (0.09 unit as compared to typical 0.001 unit needed for sufficient apo-DAAO detection) was incubated with [^{14}C]ATP, 60% of the radiolabeled ATP was incorporated into the FAD formed. Yet, only trace amounts of radioactivity (<1%) were found at retention times for FAD when CTP, GTP, or TTP was used as the nucleotide substrate.

The ability of PP_i and selected adenine nucleotides to inhibit ATP binding and reduce FAD formation under a low Mg^{2+} concentration of 0.1 mM was tested. The extent of apparent inhibition was highest by dATP followed by PP_i, ADP, and AMP in descending order. However, this influence was superficial when sufficient Mg^{2+} was present as shown in Figure 5. When the Mg^{2+} concentration was 1 mM (exceeding the total combined concentrations of the effector and ATP, 0.5 and 0.1 mM, respectively) as indicated by hashed bar in Figure 5, only $NaPP_i$ inhibits the reaction. It appears that all the other tested compounds are depleting the essential Mg^{2+} cofactor and leading to reduced FAD formation as indicated by open and solid bars in Figure 5.

Table IV: Inhibitor Efficiencies of FMN Analogues with Liver FAD Synthetase

FMN analogue	$K_i \times 10^6$ (M)
Pyrimidinoid Ring Variations (Positions 1–4) and Nitrogen Replacement	
2-thio-	106
2-[(hydroxyethyl)amino]-	NDI[a]
2-morpholinyl-	NDI[a]
2-(phenylamino)-	NDI[a]
3-methyl-	NDI[a]
3-(carboxymethyl)-	NDI[a]
1-deaza-	283
1,5-dideaza-	29
Benzenoid Ring Variations (Position 8)	
8-hydroxy-7-methyl-	2.0
8α-imidazolyl-	NDI[a]
8α-(*N*-methylimidazolyl)-	NDI[a]
N(10) Variations (Positions 2'–5') side chains	
5'-sulfate	291
5'-phosphothionate	NDI[a]
polyhydroxy chains	
D-lyxose 5'-phosphate	184
D-glucose 6'-phosphate	19
D,L-glycerol 3'-phosphate	NDI[a]
ω'-hydroxyalkyl chains	
hexyl 6'-phosphate	266
propyl 3'-phosphate	NDI[a]

[a] NDI, no detectable inhibition.

For flavin inhibition studies, FMN was incubated in the presence of FAD synthetase and varying concentrations of each of the FMN analogues. The FAD formed in the initial incubation was then quantitated by using the indirect apo-DAAO assay. The ability of the FMN analogues to inhibit the conversion of FMN to FAD is summarized in Table IV. The nature of this inhibition appears to be generally competitive.

It was of interest to see if either product of the synthetase reaction, FAD or PP_i, inhibited FMN to FAD conversion. The inhibitory effects of FAD and PP_i were studied under standard incubation conditions where both FMN and ATP concentrations were varied. In each case Lineweaver–Burk plots for the inhibition of FAD production indicated a family of intersecting lines from which inhibition constants were calculated. The inhibition of FAD against ATP was competitive in nature with a tight intersection on the *y* ordinate. The K_i was determined with an FMN concentration of 0.2 mM and $[Mg^{2+}]/[ATP]$ of 2 and calculated to be 1.3 μM. Similar studies of PP_i against ATP revealed a mixed-function inhibition with a calculated K_i of 200 μM. Mixed-function inhibition was also noted for both FAD and PP_i against FMN. The inhibition constants against FMN were determined at constant ATP and Mg^{2+} concentrations of 0.5 and 1.0 mM, respectively, and calculated to be 0.75 μM for FAD and 66 μM for PP_i. The strong inhibition by FAD was not prevented even when enzyme was preincubated with substrate.

Discussion

A comparison of commercial FMN–agarose (periodate-oxidized FMN coupled to agarose, Sigma) with the five matrices prepared in our laboratory (cf. Table II) revealed that there is a specificity for the flavin linkage. The FMN–Sepharose 4B [linked through the carboxyhexyl group presumably by ester linkage to the 5'-phosphate or one of the N(10) side chain hydroxyls], (Figure 1a) gave the highest recovered activity by competitive release of enzyme with FMN. Although the total activity bound to the commercial FMN–agarose gel is greater, the addition of 100 mM NaCl releases

over 50% of the active enzyme, whereas the FMN–Sepharose 4B gel retains the enzyme. This information enables one to improve purification efficiency (cf. Table I) since the FMN–Sepharose can be washed with buffer containing 200 mM NaCl without the release of active FAD synthetase. Both the FMN (periodate oxidized)–agarose and the *N*(10)-(formylmethyl)flavin–agarose have the adipic acid dihydrazide spacer (Figure 1b) and provide similar binding and recovery. Linkage through the N(3)-position (Figure 1c) of the isoalloxazine ring of 3-(carboxymethyl)riboflavin leads to lower specificity, especially when one considers the 15-fold reduction in recovered activity with a gel having 5-fold greater capacity. Changing the polyhydroxyl N(10) side chain to an ω'-alkyl side chain produces a gel that does not release active enzyme even under extreme conditions of buffer containing 400 μM FMN and 1 M NaCl.

FMN analogues substituted on the pyrimidinoid ring do not function as good substrates (substrate activity below our current detection limits), yet their ability to inhibit conversion of FMN to FAD implies critical spacial limitations. Replacing the nitrogen with methenic carbon at position 1 and at positions 1 and 5 (1-deaza- and 1,5-dideaza-FMN) allows the analogues to bind and inhibits the conversion of FMN to FAD. Since 1,5-dideaza-FMN (smaller K_i) appears to inhibit FMN conversion more strongly than 1-deaza-FMN, it may be that the *N*(5)-nitrogen is not crucial for substrate recognition and binding. However, the assay with apo-DAAO is dependent upon flavin binding and might also be influenced by these FMN analogues. Replacing the oxygen at position 2 with sulfur (2-thio-FMN) inhibits FAD formation, whereas replacing the oxygen with larger amine substituents [2-(hydroxyethyl)amino, 2-morpholinyl and 2-phenylamino] leads to inactive analogues. These findings may indicate a relatively tight fit along this edge of the flavin ring system and may reflect possible hydrogen-bond donation by the enzyme to the carbonyl oxygen at position 2. Substitution on the N(3)-position with a methyl or carboxymethyl group also produces a noninhibitory (and probably poorly bound) analogue. Thus, an apparent requirement for binding appears to be the ability of the 2-carbonyl and the 3-imino group to form hydrogen bonds with donor/acceptor groups on the enzyme. The role of the 3-imino group is additionally supported by the affinity matrices study, which indicated the inability of the *N*(3)-(carboxymethyl)riboflavin–agarose to affect purification of the enzyme. It is also compatible with the inability of 3-deaza-FMN to form the corresponding FAD analogue with the synthetase from *Brevibacterium* (Walsh et al., 1978).

Variations at positions 6–8 on the benzenoid ring do not markedly affect FAD production except when the group is bulky. Moving the methyl group from position 8 to position 6 (iso-FMN) or replacing the methyl with chloro or bromo (more electronegative groups) at positions 7 and/or 8 allow for generally similar substrate activity. The observation that 7,8-dichloro-FMN binds less tightly (higher K_m) than the 7,8-dibromo-FMN analogue may reflect some influence on the electron density at position N(5) imposed by the resonance-connected group attached at position 8. This same trend (higher K_m) is also observed when one compares the 8-chloro-FMN with the 7-chloro-FMN. Another possibility is that some of the synthetase is inactivated by covalent attachment of flavin via nucleophilic displacement of the halo substituent, which is expectedly greater with the bromo analogue that has a lower V_m. When a bulky and positively charged imidazole or the more neutral *N*-methylimidazole is attached at the 8α-position, the analogues do not appear to act as substrates nor do they inhibit FMN to FAD conversion.

Variations in the N(10) side chain indicate some selectivity. As expected, riboflavin 5′-sulfate and riboflavin 5′-phosphothionate are not substrates for FAD synthetase, since they cannot form the pyrophosphate bond (Liang & Allen, 1987). However, riboflavin 5′-sulfate is a good inhibitor. Variations in the length of the phosphorylated N(10) side chain provide evidence for some selectivity. When the side chain is an alkyl ω'-phosphate, only the four- and five-carbon analogues act as substrates. The six-carbon alkyl analogue binds and inhibits the reaction, whereas the three-carbon analogue acts neither as substrate nor as inhibitor. Likewise, the short phosphorylated three-carbon polyhydroxyl analogue (D,L-glycerol 3′-phosphate) does not bind. Variations in the D configuration at position 3′ of the longer chained analogues (as in D-lyxose where 2′ and 3′ hydroxyls are L and the chain contains five carbons and in D-glucose where the 3′ hydroxyl is L and there are six carbons) lower substrate activity below detectable limits, while providing some moderate inhibition. In this connection, riboflavin analogues lacking the D configuration in the 3′- and 4′-positions of the pentyl side chain do not act as substrates or inhibitors for the rat liver flavokinase, yet the 5′-hydroxypentyl analogue binds and inhibits phosphorylation (McCormick & Butler, 1962; McCormick et al., 1963; Merrill & McCormick, 1980). The fact that the alkyl ω'-phosphate analogues with four and five carbons allow reactivity and that all five-carbon analogues more closely mimic FMN (lower K_m values) may indicate that chain length may be more significant than position of the hydroxyl groups. This specificity is supported by the affinity matrix binding. The *N*(10)-(carboxybutyl)flavin, linked through the aminohexyl group on the agarose, binds the enzyme most tightly, while the commercial FMN–agarose and the more directly synthesized *N*(10)-(formylmethyl)flavin–agarose, linked at the 2′-position, bind and release the enzyme with varying degrees of efficiency.

Of the numerous analogues of riboflavin where biological activities have been studied in the mammalian systems, only two analogues can moderately well replace the naturally occurring vitamin in at least most metabolic pathways (Lambooy, 1975). These two vitamin-like analogues are 7-ethyl-8-methylriboflavin and 7-methyl-8-ethylriboflavin. Such modest structural variations at positions 7 and 8 agree with our findings for FAD synthetase, viz., that analogues with small groups (chloro, bromo, hydroxyl) at these positions can bind and, in some cases act, as substrates. The reported ability of FAD synthetase from *B. ammoniagenes* to synthesize coenzymes (F′MN and F′AD) from various riboflavin analogues, cf. Table V, indicates that analogues with a large variation on the benzenoid ring can be accomodated by this enzyme as well. This may suggest that this portion of the flavin ring system is relatively less tightly circumscribed by the synthetase from either source. Likewise, the lack of activity of both bacterial and mammalian enzymes toward N(3)-substituted analogues indicates similar flavin binding sites as well as implies that a major recognition and perhaps anchor point for the isoalloxazine ring system is at the NH-3 locus.

The results obtained by using radiolabeled nucleotides as substrates, as well as the observation that none of the nucleotides inhibit FAD synthetase in the presence of 1 mM Mg^{2+}, imply that only ATP is able to support FMN to FAD conversion. This conclusion is compatible with results obtained by McCormick (1964) using crude enzyme. Similarly, Manstein and Pai (1986) found that FAD synthetase from *Brevibacterium* is specific for ATP since no measurable activity could be detected with dATP, ADP, GTP, CTP, ITP, or UTP.

Table V: Apparent Substrate Specificity of Flavokinase/FAD Synthetase from *B. ammoniagenes* for Riboflavin Analogues

riboflavin analogues	ref
riboflavin	Manstein and Pai (1986),[a] Walsh et al. (1978), Light et al. (1980)
Pyrimidine Ring Variations (Positions 1–4) and Nitrogen Replacement	
2-thio-	Light et al. (1980), Hausinger et al. (1986)
4-thio-	Hausinger et al. (1986)
1-deaza-	Walsh et al. (1978), Light et al. (1980), Hausinger et al. (1986)
3-deaza-	Walsh et al. (1978),[b] Hausinger et al. (1986)
5-deaza-	Walsh et al. (1978), Light et al. (1980), Hausinger et al. (1986), Spencer et al. (1976)
5-methyl-5-deaza-	Walsh et al. (1978)
7,8-didemethyl-8-hydroxy-5-deaza- (F_0)	Hausinger et al. (1986)[d]
5-methyl-7,8-didemethyl-8-hydroxy-5-deaza-(5-methyl-F_0)	Hausinger et al. (1986)[d]
5-oxide-	Walsh et al. (1978)
Benzenoid Ring Variations (Positions 6–9)	
6-amino-7,8-dimethyl-	Ghisla et al. (1986)
6-hydroxyl-7,8-dimethyl-	Hausinger et al. (1986)
6-methyl-7,8-dimethyl-	Walsh et al. (1978), Light et al. (1980), Hausinger et al. (1986)
6,7-dimethyl-8-demethyl- (isoriboflavin)	Walsh et al. (1978)
7-chloro-8-demethyl-	Hausinger et al. (1986)
7-bromo-8-methyl-	Hausinger et al. (1986)
7,8-dichloro-	Hausinger et al. (1986)
8-chloro-7-demethyl-	Light et al. (1980), Hausinger et al. (1986)
8-chloro-7-methyl-	Light et al. (1980), Hausinger et al. (1986)
8-fluoro-7-methyl-	Fitzpatrick et al. (1985)
8-hydroxy-7-methyl-	Walsh et al. (1978),[c] Light et al. (1980)[c]
8-(methylthio)-7-methyl-	Light et al. (1980), Hausinger et al. (1986)
8-(dimethylamino)-7-methyl- (roseoflavin)	Light et al. (1980)
9-aza-	Walsh et al. (1978), Hausinger et al. (1986)
N(10) Side Chain Variations (Positions 10 and 2′–5′)	
5′-sulfate	Walsh et al. (1978)[d]
5′-deoxy-	Walsh et al. (1978)[d]
10-(3-chlorobenzyl)-	Walsh et al. (1978)[d]
10-(hydroxyethyl)-5-deaza-	Walsh et al. (1978)[d]

[a] This is the only substrate for which kinetic parameters have been determined by using pure enzyme. [b] Rapidly converted to FMN but no FAD evident. [c] Reacts under modified reaction conditions. [d] Not a substrate.

At low Mg^{2+} concentration, nucleotides tend to inhibit the mammalian enzyme. The extent of inhibition is triphosphate > diphosphate > monophosphate, indicating that nucleotides deprive the enzyme of essential Mg^{2+}. This cation may not only bind as a nucleotide chelate to the catalytic site but may also affect activity by altering protein structure.

Conversion of riboflavin to coenzymes occurs within the cytosol of most mammalian tissues, e.g., liver, kidney, heart, brain, spleen, and intestine. Quantitation of the salt-dissociable flavin content of liver indicates that FAD predominates at 20–60 μg/g wet weight (McCormick, 1975; Yagi, 1971). In the cytosol, this would account for a total FAD concentration of approximately 50–150 μM.[3] In vivo, this FAD can associate to form numerous catalytically active flavoproteins (K_d values range from 10^{-6} to 10^{-10} M) (Dixon, 1971), or it can remain in equilibrium with FAD synthetase. Thus, it is not surprising that under normal physiological conditions FAD synthetase is regulated primarily by its flavocoenzyme product. The FAD inhibition constants of 0.75 μM against FMN and 1.3 μM against ATP are about $^1/_{10}$ the K_m values of substrates, which are 9.5 μM for FMN and 53 μM for ATP. This regulation parallels that found for the pyridoxal 5′-phosphate forming system. Pyridoxamine and pyridoxine are first phosphorylated by pyridoxal kinase (EC 2.7.1.35) and then converted into pyridoxal 5′-phosphate by pyridoxamine (pyridoxine) 5′-phosphate oxidase (EC 1.4.3.5). The latter oxidase is strongly inhibited by product formed not only as the pure enzyme but also in vivo (Merrill et al., 1978). The K_i of pyridoxal 5′-phosphate, 3 μM, is considerably lower than the K_m for either naturally occurring substrate, 10 μM for pyridoxamine 5′-phosphate and 30 μM for pyridoxine 5′-phosphate. Hence, it appears that the final step in the synthetic process of these most crucial coenzymes is regulated by end-product formation.

[3] Conversion of wet weight of liver to equivalent volume of cytosol is based on isolated hepatocyte studies in this laboratory, where 10 mg wet weight is equivalent to 10^6 cells or 5–6 μL of cytosol.

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Effect of *S*-Adenosyl-1,12-diamino-3-thio-9-azadodecane, a Multisubstrate Adduct Inhibitor of Spermine Synthase, on Polyamine Metabolism in Mammalian Cells†

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ABSTRACT: The effects of the potent spermine synthase inhibitor *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDatad) on polyamine biosynthesis have been studied in transformed mouse fibroblasts (SV 3T3 cells) and in mouse leukemia cells (L1210). A dose-dependent decrease in intracellular spermine concentration was observed in both cell lines when grown in the presence of the inhibitor. A major difference in the effects seen in these two cell lines was the cytotoxicity observed in L1210 cells exposed to the inhibitor, which contrasted with little or no effects on growth of SV 3T3 cells treated similarly. Oxidative metabolism of the drug in L1210 cells was suggested by the fact that addition of aminoguanidine, an amine oxidase inhibitor, to the cell cultures ablated the cytotoxic effects of the inhibitor. Complete analysis of intracellular polyamines was carried out, together with analysis of *S*-adenosylmethionine, decarboxylated *S*-adenosylmethionine, and the inhibitor. These analyses revealed that, although the inhibitor had a dramatic effect on spermine biosynthesis in the cells studied, a compensatory increase in spermidine biosynthesis was observed. This resulted in no change in total polyamine concentrations in cells treated with inhibitors of either spermine synthase or spermidine synthase (Pegg et al., 1982) alone or in combination. In all cases, the concentration of the aminopropyl donor decarboxylated *S*-adenosylmethionine increased dramatically, thus allowing for the observed maintenance of total polyamine levels even in the presence of either one or both potent inhibitors of the aminopropyltransferases. Oxidative metabolism of the inhibitor complicates the interpretation of experiments carried out in the absence of amine oxidase inhibitors such as aminoguanidine. In addition, the results suggest that the extent of polyamine depletion achieved with AdoDatad and other aminopropyltransferase inhibitors could be significantly improved by combination with specific inhibitors of *S*-adenosylmethionine decarboxylase, which would prevent the compensatory increase in the decarboxylated *S*-adenosylmethionine pool size.

Inhibitors of polyamine biosynthesis have potential as chemotherapeutic agents and are also of considerable value as research tools to investigate the physiological function of polyamines. Potent and specific inhibitors have now been described for most of these enzymes, and the effects of exposure to cells to inhibitors of ornithine decarboxylase, *S*-adenosylmethionine (AdoMet)[1] decarboxylase, and spermidine synthase are well documented (Pegg & McCann, 1982, 1988; Tabor & Tabor, 1984; Porter & Sufrin, 1986; Pegg, 1986,

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; AdoS$^+$(CH$_3$)$_2$, *S*-methyl-5′-deoxy-5′-(methylthio)adenosine [dimethyl(5′-adenosyl)-sulfonium perchlorate]; AdoDatad, *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane; DFMO, α-(difluoromethyl)ornithine; AdoDato, *S*-adenosyl-1,8-diamino-3-thiooctane; MTA, 5′-(methylthio)adenosine; MAOEA, 5′-deoxy-5′-[*N*-methyl-*N*-[2-(aminooxy)ethyl]amino]-adenosine; MHZPA, 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)-amino]adenosine.

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