Purification, Characterization, and Substrate and Inhibitor Structure−Activity Studies of Rat Liver FAD-AMP Lyase (Cyclizing): Preference for FAD and Specificity for Splitting Ribonucleoside Diphosphate-X into Ribonucleotide and a Five-Atom Cyclic Phosphodiester of X, either a Monocyclic Compound or a *cis*-Bicyclic Phosphodiester−Pyranose Fusion†

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ABSTRACT: An enzyme with FAD-AMP lyase (cyclizing) activity, splitting FAD to AMP and riboflavin 4′,5′-phosphate (cFMN), was recently identified [Fraiz, F., et al. (1998) *Biochem. J. 330*, 881−888]. Now, it has been purified to apparent homogeneity from a rat liver supernatant, by a procedure that includes affinity for ADP-agarose (adsorption required the activating cation Mn^{2+} and desorption required its removal), to a final activity of 2.2 units/mg after a 240-fold purification with a 15% yield. By SDS−PAGE, only one protein band was observed (M_r = 59 000). The correspondence between protein and enzyme activity was demonstrated by renaturation after SDS−PAGE, by gradient ultracentrifugation followed by analytical SDS−PAGE, and by native PAGE with visualization of enzyme activity by fluorescence. A native M_r of 100 000 (ultracentrifugation) or 140 000 (gel filtration) indicated that FAD-AMP lyase could be a dimer. The enzyme required millimolar concentrations of Mn^{2+} or Co^{2+}, exhibited different optimum pH values with these cations (pH 8.5 or 7.3, respectively), and was strongly inhibited by ADP or ATP, but not by dADP, dATP, or the reaction products AMP and cFMN. A specificity study was conducted with 35 compounds related to FAD, mostly nucleoside diphosphate-X (NDP-X) derivatives. Besides FAD, the enzyme split 11 of these compounds with the pattern NDP-X → NMP + P═X. Structure−activity correlations of substrates, nonsubstrates, and inhibitors, and the comparison of the enzymic reactivities of NDP-X compounds with their susceptibilities to metal-dependent chemical degradation, pinpointed the following specificity pattern. FAD-AMP lyase splits ribonucleoside diphosphate-X compounds in which X is an acyclic or cyclic monosaccharide or derivative bearing an X-OH group that is able to attack internally the proximal phosphorus with the geometry necessary to form a P═X product, either a five-atom monocyclic phosphodiester or a *cis*-bicyclic phosphodiester−pyranose fusion. For instance, NDP-glucose and GDP-α-L-fucose were substrates, but dTDP-glucose, NDP-mannose, and GDP-β-L-fucose were not. Judging from k_{cat}/K_m ratios, we found the best substrate to be FAD, followed closely by ADP-glucose (k_{cat}/K_m only 2-fold lower, but not a physiological compound in mammals), whereas other substrates exhibited 50−500-fold lower k_{cat}/K_m values. However, there was no evidence for specific flavin recognition. Instead, what seems to be recognized is the NDP moiety of NDP-X, with a strong preference for ADP-X. Splitting would then depend on the presence of an adequate X-OH group. The possibility that, besides FAD, there could be in mammals other ADP-X substrates of FAD-AMP lyase is discussed, with emphasis placed on some ADP-ribose derivatives.

A Mn^{2+}-dependent enzyme that splits FAD to AMP and riboflavin 4′,5′-phosphate (cFMN)[1] was recently identified for the first time in rat liver supernatants (*1*). The flavin product, cFMN, was isolated, structurally characterized by chemical and enzymatic treatments, HPLC, ultraviolet, and NMR spectroscopies, and found to be a five-atom cyclic phosphodiester of riboflavin, with phosphate esterifying the 4′-OH and 5′-OH groups of the ribityl chain. This structure indicates that FAD is split by an internal attack of the 4′-OH on the proximal phosphorus, i.e., a cyclizing lyase (cyclase) type of reaction. A partially purified preparation of the cFMN-forming enzyme was devoid of detectable adenylate, guanylate, and cytidylate cyclase activities from the corresponding NTP, of FAD synthetase activity from

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FMN and ATP, and of FAD pyrophosphatase. In addition, the same preparation did not catalyze the hydrolysis of cFMN. This ruled out the possibility that the cFMN forming activity could represent an intermediate step or a side reaction of a hydrolytic reaction of FAD. On these grounds, and since the reaction specificity being studied was previously unknown, it was concluded that it could correspond to a novel cyclizing lyase which, by analogy to other nucleotidyl cyclases, could be tentatively named FAD-AMP lyase (cyclizing) or FMN cyclase (*1*).

Before the identification of this enzyme activity, cFMN was known only as a product of the chemical degradation of FAD (*2*, *3*) or as a minor contaminant of commercial samples of FAD (*4*). Very recently, its presence in extracts of food materials has been also described, although its possible formation by FAD degradation during the extraction procedure is not excluded (*5*, *6*). The occurrence of an enzyme with FAD-AMP lyase (cyclizing) activity in a mammalian organism speaks for a possible, if still unknown, biological role of cFMN.

As part of an effort started to investigate the identity and role of FAD-AMP lyase, here we describe its purification to apparent electrophoretic homogeneity and its enzymic characterization, including a detailed study of specificity using nucleoside diphosphate-X (NDP-X) compounds other than FAD as (potential) substrates. Several of the compounds that have been tested contained an X-OH group which could conceivably react with the proximal phosphorus and split the NDP-X, yielding a cyclic phosphodiester. In fact, some of these compounds were enzymically split, but not all. The structure−activity relationships inferred from the consideration of substrates, nonsubstrates, and inhibitors pinpointed a neat pattern of substrate and reaction specificity. In any case, kinetic data supported the proposal that FAD is the principal substrate of FAD-AMP lyase.

MATERIALS AND METHODS

Materials. Female Wistar rats weighing ~200 g were used. cFMN used as a standard was chemically prepared from FAD and purified as described previously (*7*). FAD used either for the nonenzymic preparation of cFMN or as the FAD-AMP lyase substrate was purchased from Sigma (catalog no. F 6625). Highly pure FMN (97%) was from Sigma (catalog no. F 8399). The rest of the compounds that were tested as substrates or inhibitors were also from Sigma, except for ATP (Roche). $MnCl_2$, $CoCl_2$, and other chemicals were highly pure preparations from Merck. BSA fraction V, alkaline phosphatase, and adenosine deaminase used in FAD-AMP lyase assays were from Roche (catalog nos. 735078, 108146, and 102113, respectively).

Purification of FAD-AMP Lyase. For each purification run, 38 ± 4 g of fresh liver (from four female Wistar rats) was cut with scissors, washed with cold saline, and homogenized in 3 volumes (3 mL/g) of TE-buffered (TE buffer, 20 mM Tris-HCl and 0.5 mM EDTA, adjusted to pH 8.2, at 4 °C) 0.25 M sucrose and 1.3 mM PMSF with a glass−glass homogenizer. The homogenate was filtered through several layers of gauze and successively centrifuged for 10 min at 1100*g*, for 20 min at 30000*g*, and for 60 min at 100000*g*. The final supernatant, filtered through glass wool, was the crude extract (cytosolic fraction) from which the purification was started. This extract was fractionated with ammonium sulfate; the fraction that precipitated between 30% and 60% saturation contained the FAD-AMP lyase activity, and it was resuspended in homogenization buffer devoid of sucrose, up to a final volume of ~10 mL. The resuspended precipitate was applied to a column {119 cm × 2 cm [inside diameter (i.d.)]} of Sephadex G-100 (Pharmacia Biotech) equilibrated in TE buffer. Chromatography was also carried out with TE buffer at 20 mL/h, and 3 mL fractions were collected. The FAD-AMP lyase activity was recovered as a single peak in the fractions with a V_e of ≈120−170 mL which were pooled. This preparation was applied to a column [44 cm × 1.8 cm (i.d.)] of DEAE-cellulose DE52 (Whatman) equilibrated in 20 mM Tris-HCl (pH 8.2, adjusted at 4 °C) followed by a wash with TE buffer at ~30 mL/h. Fractions of 3 mL were collected, and FAD-AMP lyase was recovered as a single peak (V_e ≈ 50−100 mL) with only a slight retardation with respect to the bulk of the unretained protein (V_e ≈ 35−90 mL). The protein bound to the exchanger [including two ADP-ribose pyrophosphatases with minor Mn^{2+}-dependent FAD hydrolase activity (*8*)] was eluted after FAD-AMP lyase with TE buffer supplemented with 1 M KCl. The preparation obtained from the anion-exchange chromatography step was then purified by dye−ligand affinity chromatography on a column [6 cm × 1.5 cm (i.d.)] of Reactive Green 19-agarose (Sigma, catalog no. R 4004) equilibrated in TE buffer. After application of the enzyme sample (~50 mL), chromatography was carried out at 35 mL/h with 65 mL of TE followed by a 250 mL linear gradient of KCl (from 0 to 0.2 M) in TE buffer. Fractions of 3 mL were collected. FAD-AMP lyase was retained by the dye−ligand matrix and eluted as a single peak centered in the fractions containing ~120 mM KCl. The active fractions were pooled, supplemented with $MnCl_2$ up to 5 mM, and applied to a column [3 cm × 1.5 cm (i.d.)] of ADP-agarose (Sigma, catalog no. A 4398) equilibrated in TE buffer also containing 5 mM $MnCl_2$. After the application of the enzyme sample, chromatography was carried out at 40 mL/h with successive washes of 22 mL of 1 M KCl and 5 mM $MnCl_2$ in TE buffer, 16 mL of 5 mM $MnCl_2$ in TE, and 56 mL of TE alone. Fractions of 3 mL were collected. The purified FAD-AMP lyase eluted with the last, Mn-free wash as a single peak (V_e ≈ 75−85 mL). The active fractions were pooled, aliquoted, and frozen at −80 °C until they were needed. Thawed aliquots were used the same day, and the leftover was discarded. The frozen enzyme was stable at least for 10 months. The summary of a typical purification run is shown in Table 1, and a purity analysis is shown in Figure 1A.

[1] Abbreviations: A(2′)p(5′)A, adenosine 2′-phosphate 5′-adenosine; A(3′)p(5′)A, adenosine 3′-phosphate 5′-adenosine; A(5′)pp(5′)A, adenosine 5′-diphosphate 5′-adenosine; BSA, bovine serum albumin; CAPS, 3-cyclohexylamine 1-propanesulfonate; cFMN, riboflavin 4′,5′-phosphate or cyclic FMN; DEAE-cellulose, diethylaminoethylcellulose; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; IC_{50}, inhibitor concentration causing 50% inhibition; NDP, nucleoside 5′-diphosphate; NMP, nucleoside 5′-monophosphate; NMR, nuclear magnetic resonance; NTP, nucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; P_i, inorganic phosphate; PMSF, phenylmethanesulfonyl fluoride; P═X, cyclic phosphodiester of X; P-X, phosphomonoester of X; SDS, sodim dodecyl sufate; TBA, tetrabutylammonium bromide; TE, 20 mM Tris-HCl and 0.5 mM EDTA (adjusted to pH 8.2 at 4 °C); Tris, tris-(hydroxymethyl)aminomethane; X, any molecule that can form a phosphoric ester or anhydride, usually in this work a(n) (a)cyclic monosaccharide or derivative.

Table 1: Purification of FAD-AMP Lyase[a]

step	volume (mL)	activity[b] (units)	protein[c] (mg)	specific activity (units/mg)	purification (fold)	yield (%)
100000*g* supernatant	86	21.7	1740	0.013	1	100
30−60% ammonium sulfate precipitation	10.5	21.6	930	0.023	1.9	100
gel-filtration chromatography on Sephadex G-100	43	14.3	407	0.035	2.8	66
anion-exchange chromatography on DEAE-cellulose	52	5.4	76	0.071	5.7	25
affinity chromatography on Reactive Green 19-agarose	37.5	3.6	8	0.45	36	17
affinity chromatography on ADP-agarose	13.4	3.0	0.93	3.2	256	14

[a] The data correspond to a typical purification run from 36 g of fresh rat liver. [b] The activity was assayed by assessing the formation of cFMN by HPLC. [c] The protein was assayed according to the method of Bradford (*27*).

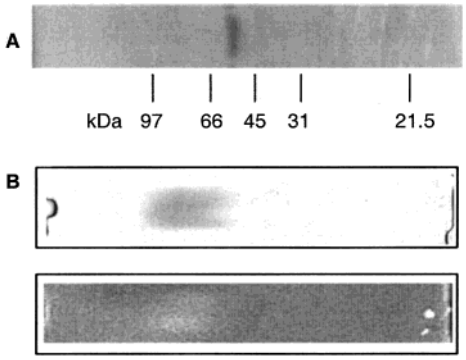

FIGURE 1: Electrophoretic analyses of purified FAD-AMP lyase. (A) SDS−PAGE analysis of purified FAD-AMP lyase under reducing conditions. The lane shown contained 0.56 μg of the purified enzyme (Table 1) loaded in a 9% acrylamide gel which was stained with silver (*28*). (B) Native gel electrophoresis of purified FAD-AMP lyase: correlation of protein content with enzyme activity. Two gel strips corresponding to two lanes of the same gel are shown with the electrophoretic origin on the left. Each lane was loaded with 1.2 μg of purified FAD-AMP lyase. The upper gel strip was stained with silver (*28*). The lower gel strip was incubated at 37 °C in a FAD-AMP lyase reaction mixture containing 20 mM Tris-HCl (pH 7.5), 6 mM $MnCl_2$, 1 mg/mL BSA, and 1.2 mM FAD. The incubation was carried out until the FAD-AMP lyase activity was manifest under a lamp emitting at ~360 nm due to the fluorescence of cFMN being more intense than that of FAD (see Figure 2).

Renaturation and Extraction of FAD-AMP Lyase after SDS−PAGE. For these experiments, SDS−PAGE was carried out in 9% acrylamide, 2.4% bisacrylamide, vertical slab gels that were 3 mm thick with 20 mm wide sample wells. After the electrophoretic run, to visualize the 59 kDa enzyme band without interferring with its renaturability, the gel slabs were immediately removed and incubated for 5 min in 0.25 M KCl, followed by a short wash with water and 45 min in 1 mM DTT (all solutions at 0 °C) (*9*). The band could then be observed for a while against a dark background as a whitish band which, depending on the amount of pure enzyme loaded onto the gel, allowed its photographic documentation and the cutting of the gel lane into pieces before the protein band faded away. SDS removal and enzyme renaturation were performed in parallel in all the cut gel pieces (each corresponding to 3−8 mm of gel lane length) with the following incubation steps (at room temperature except where indicated) (*10*): 30 min in 1 mL of 50 mM Tris-HCl (pH 8) and 200 g/L isopropyl alcohol (twice); 60 min in 2 mL of 50 mM Tris-HCl (pH 8) and 5 mM 2-mercaptoethanol; 30 min in 1 mL of 50 mM Tris-HCl (pH 8), 5 mM 2-mercaptoethanol, and 6 M guanidine HCl (twice); and 12−18 h at 4 °C in five changes of 2 mL of 50 mM Tris-HCl (pH 8), 5 mM 2-mercaptoethanol, and 0.4 g/L Tween 40. To extract the protein from the gel, each piece was washed with 1 mL of 20 mM Tris-HCl (pH 8) and 0.5 mM EDTA, and after this, it was homogenized with a glass pestle in the same buffer supplemented with 1.6 g/L BSA (0.17 mL of buffer per each millimeter of gel lane length, i.e., per ~60 mm^3 of gel). The homogenates were left at 4 °C for 72 h and were centrifuged off to recover the clear supernatants containing the extracted protein. The profile of FAD-AMP lyase along the gel lane was estimated by performing incubations of these supernatants with 10 μM FAD and 6 mM $MnCl_2$ at 37 °C, and measuring the extent of FAD conversion to cFMN by HPLC (HPLC method 1; see below).

Sucrose Gradient Ultracentrifugation. Gradients were prepared with a two-chamber mixer filled with 50 and 200 g/L sucrose solutions in 20 mM Tris-HCl and 0.5 mM EDTA, at either pH 6.8, 7.5, or 8.3, and with other additions as indicated. Samples of 0.22 mL were applied to the top of the gradients, and centrifugation was performed for 14−24 h at 38 000 rpm in a Beckman SW-41Ti rotor. The gradients were fractionated from the bottom, collecting fractions of ~0.4 mL.

Enzyme Assays. Except when otherwise indicated, reaction mixtures for the FAD-AMP lyase contained 50 mM Tris-HCl (pH 7.4, 37 °C), 6 mM $MnCl_2$, 1 mg/mL BSA, FAD, and an enzyme sample as required. The incubations were performed at 37 °C, and the mixtures were preincubated for 10 min at the same temperature before the reaction was started by the addition of FAD from a stock solution (also prewarmed at 37 °C). The extent of FAD conversion to cFMN was determined by one of three assay methods.

(i) Discontinuous colorimetric determination of phosphatase-sensitive P_i liberated from AMP (i.e., 1 mol of P_i/mol of FAD split). This required the inclusion of alkaline phosphatase (7 units/mL) in the assay mixtures. Reaction mixtures of up to 0.5 mL were sampled (aliquots of 50 or 100 μL) after different incubation times; the aliquots were immediately quenched with 1 mL of a P_i reagent (6 volumes of 3.4 mM ammonium molybdate in 0.5 M H_2SO_4, 1 volume of 570 mM ascorbic acid, and 1 volume of 130 mM SDS), and A_{820} was measured after 20 min at 45 °C.

(ii) Direct discontinuous measurement of cFMN by HPLC with A_{430} monitoring, which allowed detection and quantitation of substrate FAD and product cFMN. This was the standard method used unless otherwise indicated. Though not strictly necessary, alkaline phosphatase was also included in the reaction mixtures like in the P_i assay. Reaction mixtures of up to 0.5 mL were sampled at intervals, and either 20 μL aliquots were directly injected into the HPLC

column or 100 μL aliquots made up 33 mM in EDTA incubated for 2 min in boiling water, centrifuged for 2 min at 11 000 rpm, and frozen at −20 °C until they were ready for the HPLC assay (see below, method 1 or method 2). The following modifications were introduced as indicated. Alkaline phosphatase was omitted in all the reaction mixtures implemented for inhibition experiments (except when the product cFMN itself was tested as the inhibitor). For the analyses of reaction mixtures with added cFMN (tested as the inhibitor), A_{250} was monitored and the FAD splitting rate was quantitated from the alkaline phosphatase-mediated accumulation of adenosine. For the analyses of reaction mixtures with added FMN (tested as the inhibitor), HPLC method 2 (see below) was used as it improved the resolution of FAD, FMN, and cFMN.

(iii) Continuous recording at 265 nm of the hyperchromicity concomitant with FAD splitting ($\epsilon \approx 7200$ M^{-1} cm^{-1}) in reaction mixtures of 2.8 mL.

The activities of FAD-AMP lyase on non-FAD substrates were usually assayed by either the P_i assay or the HPLC method with modifications: omission of alkaline phosphatase when the substrate contained terminal phosphate groups and/or use of HPLC methods (see below) indicated to detect the (potential) reaction product(s) separated from the substrate. In addition, some of the assays of ADP-glucose splitting were performed by continuous recording of the A_{265} decrease ($\epsilon \approx 8500$ M^{-1} cm^{-1}) concomitant with adenosine deamination in incubations with alkaline phosphatase and adenosine deaminase as auxiliary enzymes.

In all enzymatic assays, for every individual incubation, controls without enzyme and/or substrate were processed in parallel to account for the metal-dependent, chemical degradation of FAD and other NDP-X compounds (*1*, *7*, *11*, *12*; see Table 6 in this work) such that the results correspond to initial reaction rates that are proportional to the amount of FAD-AMP lyase. One unit of activity equals 1 μmol of substrate split/min.

Determination of Reaction Product Patterns. All the FAD-AMP lyase substrates responded to the general description of a NDP-X compound, NDP being ADP, UDP, GDP, or CDP and X being an acyclic or cyclic monosaccharide or derivative. The product patterns were determined by performing parallel incubations of the enzyme with one of these substrates in the absence and presence of alkaline phosphatase and determining in every case the amount of P_i liberated (colorimetric assay) and the nature and amount of nucleotide or nucleoside formed (HPLC assay). In every case, blanks without FAD-AMP lyase were run and analyzed in parallel. The amounts of products detected in these controls were subtracted from the results obtained in the presence of enzyme. The HPLC methods that were used (see below) were as follows: method 2 (FAD), method 5 (ADP-glucose), method 7 (CDP-X), method 8 (GDP-X), and method 4 or 5 (UDP-X). All these methods were implemented with A_{250} monitoring (in the case of FAD, A_{430} and fluorescence tracings were also recorded). The HPLC methods were able to resolve and quantitate the substrate, and the potential nucleoside (phosphate) products which could result from the NDP moiety. In the case of FAD, method 2 did not fully resolve potential products of FAD splitting. AMP and ADP on one hand, or adenosine and FMN on the other, coeluted (see Figure 2). In this case, the product pattern was confirmed as explained in the Results and by reference to previous work (*1*).

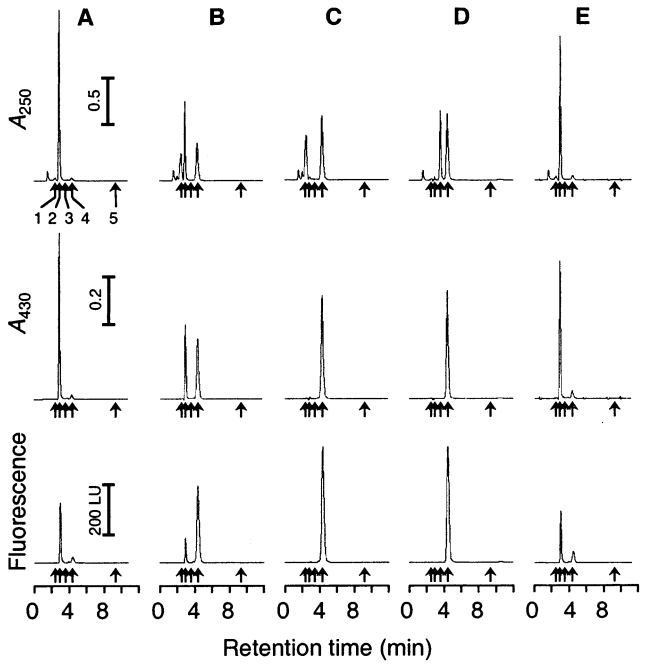

FIGURE 2: HPLC analyses of the products of FAD splitting by purified FAD-AMP lyase. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 6 mM $MnCl_2$, 1 mg/mL BSA, 0.1 mM FAD, and 7 milliunits/mL FAD-AMP lyase except as indicated below. Incubations were performed at 37 °C and stopped by injection in the HPLC column. Chromatographic conditions were as described in Materials and Methods (HPLC method 2): (A) full reaction mixture with no incubation (zero time), (B) 12 min incubation, (C) 45 min incubation, (D) 45 min incubation followed by addition of 7 units/mL alkaline phosphatase and a further 20 min incubation, and (E) 45 min incubation, reaction mixture without FAD-AMP lyase. The arrows denote the retention times of standards: (1) AMP and ADP, (2) FAD, (3) adenosine and FMN, (4) cFMN, and (5) riboflavin. The identification of AMP and cFMN as reaction products was confirmed as explained in the text and was in agreement with previous results (*1*). Under the conditions employed to record the chromatograms, the apparent molar extinction coefficients of FAD and cFMN at 430 nm were the same, which allowed us to use the relative area of the cFMN peak (versus FAD plus cFMN) to calculate the absolute reaction rate (standard FAD-AMP lyase assay). On the contrary, the intensity of the fluorescence of cFMN was ~5-fold larger than that of FAD, as indicated by the larger area of the product peak at the end of reaction than that of the substrate at the beginning (A−C).

HPLC Equipment and Methods. The equipment was from Hewlett-Packard and consisted of either a HP-1090 chromatograph with fixed-wavelength detection using a filter system or a HP-1100 chromatograph with fluorescence and diode array spectrophotometric detectors. Chromatogram tracings were obtained by monitoring A_{250}, A_{430}, and fluorescence (excitation at 450 nm, emission at 520 nm). Data were collected, elaborated, and stored with the aid of HP ChemStation software. Injection loops able to dose a 20 or 50 μL sample volumes were used. Three columns were used interchangeably: (a) Hypersil ODS 5 μm (150 mm × 3.9 mm) from Sugelabor, (b) Hypersil ODS 5 μm (150 mm × 4 mm) from Teknokroma, (c) Nova-Pak (150 mm × 3.9 mm) from Waters. In all cases, the column was protected with a guard Hypersil 5 μm precolumn (20 mm × 2.1 mm) from Hewlet-Packard. The chromatographies were carried out at a flow rate of 0.5 mL/min (except where otherwise indicated)

using combinations (see below) of the following solvent solutions: (A) 10 mM sodium phosphate (pH 7) and 35% (v/v) methanol, (B) 10 mM sodium phosphate (pH 7) and 30% (v/v) methanol, (C) 10 mM sodium phosphate (pH 7) and 22.5% (v/v) methanol, (D) 5 mM sodium phosphate (pH 7), 20% (v/v) methanol, and 20 mM TBA, (E) 100 mM sodium phosphate (pH 7), 20% (v/v) methanol, and 20 mM TBA, (F) 10 mM sodium phosphate (pH 7), 5% (v/v) methanol, and 10 mM TBA, (G) 50 mM sodium phosphate (pH 7), 5% (v/v) methanol, and 10 mM TBA, (H) 5 mM sodium phosphate (pH 7), 20% (v/v) methanol, and 10 mM TBA, (I) 100 mM sodium phosphate (pH 7), 20% (v/v) methanol, and 10 mM TBA, (J) 5 mM sodium phosphate (pH 7) and 10% (v/v) methanol, (K) 20 mM ammonium formiate and 5% (v/v) methanol; and (L) 20 mM ammonium formate and 50% (v/v) methanol. Depending on the composition of the enzyme incubation mixture to be analyzed and on the product(s) expected, we used different HPLC methods: method 1, 7 min isocratic wash with A; method 2, 7 min isocratic wash with B; method 3, 11 min isocratic wash with C at a rate of 1 mL/min; method 4, 15 min linear gradient from 100% D to 80% D and 20% E; method 5, 5 min linear gradient from 100% D to 60% D and 40% E; method 6, 5 min linear gradient from 100% D to 65% D and 35% E, followed by a 5 min isocratic wash under these conditions; method 7, 10 min linear gradient from 100% D to 95% D and 5% E; method 8, 10 min linear gradient from 100% D to 85% D and 15% E; method 9, 5 min linear gradient from 100% D to 100% E, followed by a 10 min isocratic wash with 100% E; method 10, 15 min linear gradient from 100% F to 100% G, followed by a 10 min isocratic wash with 100% G; method 11, 6 min isocratic wash with J; method 12, 5 min linear gradient from 100% K to 85% K and 15% L, then a 5 min linear gradient up to 80% K and 20% L, and then a 1 min linear gradient up to 50% K and 50% L, followed by a 5 min isocratic wash under these conditions; and method 13, 20 min linear gradient from 100% H to 50% H and 50% I.

RESULTS

Purification of FAD-AMP Lyase. The purification steps are described in detail in Materials and Methods, and the quantitative results are shown in Table 1. The starting material for purification was the 100000*g* supernatant of a rat liver homogenate prepared in an isotonic sucrose buffer ("cytosolic" fraction). From earlier work, we knew that this fraction, besides the cyclizing lyase, contains other Mn^{2+}-dependent, FAD-splitting activities which correspond to pyrophosphohydrolytic enzymes (*8*), and also a cyclic nucleotide phosphodiesterase of undefined specificity that hydrolyzes cFMN (*1*). Therefore, for the first part of the purification (up to the anion-exchange chromatography), we preferred to follow a series of steps in which the behavior of these enzymes was known. In the gel-filtration chromatography step, the cFMN phosphodiesterase was considerably retarded with respect to FAD-AMP lyase (*1*), and in the anion-exchange chromatography step, three ADP-ribose pyrophosphatases, two of them with minor FAD hydrolase activity in the presence of Mn^{2+} (*8*), became bound to the DEAE-cellulose exchanger, whereas FAD-AMP lyase was recovered in the flow-through fraction (results not shown). For the final part of the purification, two affinity chromatography steps were implemented using agarose matrixes containing immobilized Reactive Green-19 or ADP. FAD-AMP lyase was bound to the dye−ligand matrix at low ionic strength and was recovered with a KCl gradient wash. After this step, the pure enzyme was obtained by adsorption to ADP-agarose in the presence of Mn^{2+} (enzyme-activating cation) followed by specific desorption upon removal of Mn^{2+} from the mobile phase. By SDS−PAGE, this preparation exhibited a single protein band at 59 ± 2 kDa after Coomassie blue (not shown) or silver staining (Figure 1A).

Correlation between the Protein Content of the Purified Enzyme and Its FAD-Splitting Activity. Evidence that the unique 59 kDa band of the purified preparation corresponded to FAD-AMP lyase was obtained by several approaches.

First, the application of a protocol devised to extract and to renature the protein from nonstained SDS−PAGE gels (see Materials and Methods) indicated that only gel pieces coincident with the 59 kDa band contained FAD-AMP lyase activity (not shown). In these experiments, rather than by Coomassie blue or silver staining, the protein band was visualized by incubation of the gel in an ice-cold KCl solution (*9*), a low-sensitivity method which does not impede enzyme renaturation, and that allowed the gel to be cut into pieces of a few millimeters each, knowing exactly where the 59 kDa band was located. Two experiments of this kind with different amounts of protein were performed. The one with the smaller amount of FAD-AMP lyase (28 μg) exhibited significant FAD-splitting activity only in the single gel piece where the protein band was located. The other experiment, with a larger amount of protein (80 μg), exhibited significant FAD-splitting activity in three gel pieces: the one coinciding with the central part of the protein band, which showed the maximum of activity, and the two flanking ones. This activity was dependent on Mn^{2+} and was inhibited by ADP like that of the native enzyme. No FAD-AMP lyase activity was recovered from SDS−PAGE gels when the renaturation step was omitted, or when BSA or just buffer substituted for FAD-AMP lyase.

Second, native FAD-AMP lyase was submitted to sucrose gradient ultracentrifugation, and the fractions that were collected were analyzed by SDS−PAGE (not shown). The sedimentation profiles of the 59 kDa band and the FAD-AMP lyase activity coincided. The same results were obtained when (i) the enzyme sample and the gradient were supplemented with either 50 μM FAD or 50 μM ADP (FAD-AMP lyase inhibitor; see below) or (ii) the gradient was buffered at either pH 6.8, 7.5, or 8.3, in every case both in the absence and in the presence of 5 mM $MnCl_2$.

Third, the correlation between protein and activity was also tested by nondenaturing PAGE. Parallel gel strips containing the same amount of FAD-AMP lyase were stained for protein, with silver, or for activity, by incubation with FAD and illumination with an UV source, which revealed the position of FAD-AMP lyase by the intense fluorescence of cFMN as compared to FAD. The silver-stained and the activity spots were exactly coincident (Figure 1B).

Native Molecular Weight. The apparent molecular weight of native FAD-AMP lyase was determined both by ultracentrifugation in a sucrose gradient (*13*) and by gel-filtration chromatography in a Sephadex G-150 column, in both cases by comparison with protein standards with known molecular weights. The results obtained by both methods (not shown)

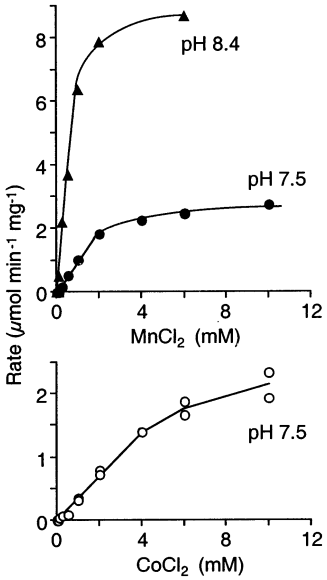

FIGURE 3: Response of FAD-AMP lyase activity to Mn^{2+} or Co^{2+} concentration. Initial reaction rates were assayed in mixtures with 50 mM Tris-HCl (pH 7.5 or 8.4), 100 μM FAD, 1 mg/mL BSA, alkaline phosphatase, FAD-AMP lyase, and the indicated concentrations of $MnCl_2$ or $CoCl_2$, by measuring the level of phosphatase-dependent accumulation of P_i. Similar results were obtained using 1.2 mM FAD as the substrate.

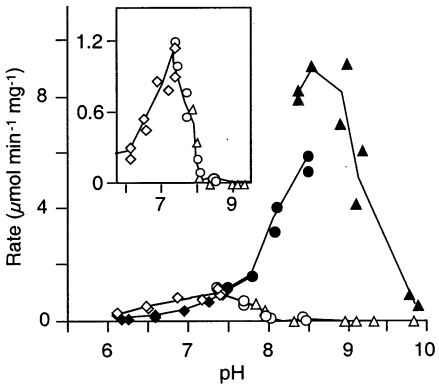

FIGURE 4: Response of FAD-AMP lyase activity to pH. Initial reaction rates were assayed by measuring the level of alkaline phosphatase-dependent accumulation of P_i in mixtures buffered at the indicated pH values with either (◆ and ◇) 100 mM Tris-acetate, (● and ○) 100 mM Tris-HCl, or (▲ and △) 100 mM CAPS-NaOH, also containing 500 μM FAD, 6 mM $MnCl_2$ (black symbols) or 6 mM $CoCl_2$ (white symbols), 1 mg/mL BSA, alkaline phosphatase, and FAD-AMP lyase. The amount of auxiliary alkaline phosphatase was enough to obtain rates proportional to that of FAD-AMP lyase at all the pH values that were studied. The inset is a vertical expansion of the Co^{2+}-dependent profile.

were somewhat different: 100 000 by ultracentrifugation and 140 000 by gel filtration.

Product Pattern of FAD Splitting. Earlier work performed with a less purified preparation of FAD-AMP lyase indicated that FAD was enzymically split to AMP and cFMN, the latter product being characterized in detail by ^1H and ^{13}C NMR studies (*1*). HPLC analyses of reaction mixtures of FAD with the purified FAD-AMP lyase showed the same reaction pattern. Figure 2 shows the results obtained by HPLC method 2, which allowed for a fast elution of substrate and products, and was the method of choice in this work as the standard for the assay of FAD-AMP lyase activity (A_{430} monitoring). Nonetheless, the identification of reaction products was confirmed by higher-resolution chromatographic methods like those used in (*1*). HPLC recordings of A_{250} showed the known conversion of FAD to AMP and cFMN, whereas recordings of A_{430} or fluorescence (excitation at 450 nm, emission at 520 nm), taken simultaneously in the same chromatographic run, showed only, as expected, the formation of cFMN, since AMP does not absorb visible radiation and it is not a fluorescent compound (Figure 2A−C). Enzyme reaction mixtures containing alkaline phosphatase showed the formation of adenosine rather than AMP, confirming that the adenosine-containing moiety of FAD left the active center of FAD-AMP lyase bearing a terminal phosphate group (only 1 mol of P_i was detected per mole of adenosine formed). On the contrary, the flavin-containing moiety was unaffected by the phosphatase (Figure 2D). Blank mixtures implemented without FAD-AMP lyase indicated that under the conditions of the experiment the chemical degradation of FAD, which also yields AMP and cFMN (*7*), occurred only to a small extent (Figure 2E).

Cation Requirements for FAD Splitting. FAD-AMP lyase showed a strict requirement for Mn^{2+} or Co^{2+} for activity (Figure 3). Millimolar concentrations of either of these cations were needed to obtain full activity. This requirement was not dependent on FAD concentration, since similar concentration−activity curves were obtained at 0.1 or 1.2 mM FAD, with Mn^{2+} and with Co^{2+}. Assays carried out at pH 7.5 and 8.4, with Mn^{2+} as the activating cation, also indicated similar concentration requirements for apparent saturation. High Mn^{2+} or Co^{2+} concentrations could not be replaced by concentrations of Mg^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{3+}, Li^+, Na^+, or K^+ (all as chloride salts) when tested under the following conditions: (i) each one of them individually, (ii) each one of them, including Mn^{2+} or Co^{2+}, at 100 μM, in combination with either 100 mM KCl, 1 mM $MgCl_2$, or both, (iii) all the possible binary combinations of those cations at 100 μM each, and (iv) the same binary combinations in the presence of 100 mM KCl and 1 mM $MgCl_2$.

pH−Activity Profile of FAD Splitting. The effect of varying the pH of assay mixtures on the FAD-AMP lyase activity was studied both with Mn^{2+} and with Co^{2+} as the activating cation. The results obtained with these cations were clearly different, as the Mn^{2+}-dependent profile displayed a maximum at pH 8.5 and the Co^{2+}-dependent one a maximum at pH 7.3. The highest activity reached with Mn^{2+} was considerably larger than that with Co^{2+}, although at pH ∼7.5 both cations were similarly effective as activators (Figure 4). The same difference between the Mn^{2+}- and Co^{2+}-dependent activity was observed using the partially purified preparation of FAD-AMP lyase previously described (*1*), in this case with 1.2 mM FAD as the substrate.

FAD Saturation Kinetics. Figure 5 shows the saturation kinetics of FAD-AMP lyase with increasing concentrations of FAD with Mn^{2+} or Co^{2+} as the activating cation. The initial rates obtained with Mn^{2+} at FAD concentrations higher than 0.3 mM pointed to the occurrence of inhibition by excess substrate, but data points obtained at <0.3 mM FAD were in good agreement with hyperbolic kinetics and, by nonlinear regression, gave a K_m of 9 μM and a k_{cat} of 289 min^{-1}. These values were not affected in any systematic manner when the concentration range being considered was progressively reduced starting from its upper limit (0.3 mM FAD). Similar inhibitory behavior of FAD at >0.3 mM was

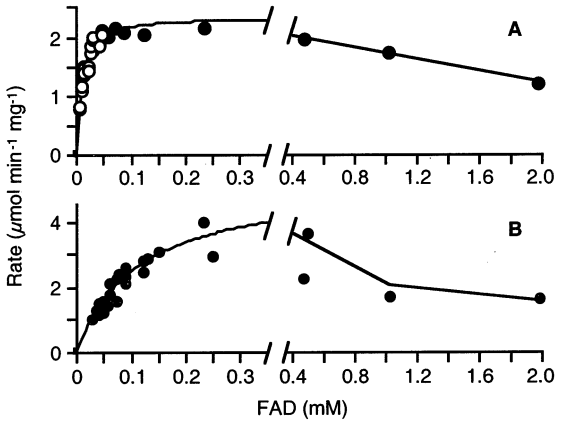

FIGURE 5: Saturation kinetics of FAD-AMP lyase with FAD as the substrate and Mn^{2+} or Co^{2+} as the activating cation. Initial rates were determined by the (●) alkaline phosphatase-sensitive P_i assay or the (○) hyperchromicity assay in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 1 mg/mL BSA, FAD-AMP lyase, the indicated concentration of FAD, and (A) 6 mM $MnCl_2$ or (B) 6 mM $CoCl_2$. Data of two independent experiments that yielded similar results are combined in each plot.

observed with Co^{2+} as the activating cation, although the K_m value obtained for FAD (considering only data up to 0.3 mM FAD) was ~10-fold higher than with Mn^{2+}.

Besides the experiments described above, carried out at pH 7.5, the saturation kinetics for FAD splitting were also studied at pH 8.3 in the presence of Mn^{2+}. Under these conditions, both k_{cat} and K_m were 4−5-fold higher than at pH 7.5; i.e., the catalytic efficiency was unaffected by the pH difference (not shown).

Inhibition of FAD Splitting by Nucleotidic Derivatives. Twelve nucleotidic compounds were tested as FAD-AMP lyase inhibitors, including (deoxy)nucleoside mono-, di-, and triphosphates. A comparison between all the compounds that have been tested is presented in Table 2. For some of the inhibitors, IC_{50} values were derived from dose−response curves at a fixed FAD concentration, and for the two most potent inhibitors that were found, ATP and ADP, the competitive character of the inhibition was demonstrated with K_i values of 50 and 25 nM, respectively (Figure 6). Both the 2′-OH group of the ribose ring and the presence of at least a diphosphate chain were required for strong inhibition, since AMP, dADP, and dATP were much weaker inhibitors than ADP and ATP. Remarkably, neither FMN nor cFMN, the product of FAD splitting, was a significant inhibitor of the reaction. In fact, there was no evidence of inhibition by these flavin nucleotides at concentrations 30-fold higher than that of the substrate FAD.

Activity of FAD-AMP Lyase toward Substrates Other Than FAD. An extensive study of in vitro substrate specificity was conducted. Thirty-five compounds structurally related to FAD, most of them nucleoside diphosphate-X (NDP-X) derivatives, were tested as potential substrates. Among them, there were other dinucleoside diphosphates, a large series of nucleoside diphospho sugars or diphospho alcohols, nucleoside mono-, di-, and triphosphates, and some phosphodiester derivatives (Table 3). Besides FAD, the enzyme split 11 of these compounds to different degrees (5−100% of the FAD-splitting reaction rate). All of them are NDP-X compounds in which the X group is an acyclic or cyclic monosaccharide or derivative. However, not all the compounds fitting this description were FAD-AMP lyase substrates. In fact, the results of the specificity study revealed a clear discrimination between NDP-X compounds of closely similar structures. Most remarkably, for instance, NDP-glucose and GDP-α-L-fucose were substrates, but NDP-mannose and GDP-β-L-fucose were not; i.e., FAD-AMP lyase could distinguish between substrates that differ in the configuration of a single asymmetric carbon. This and other aspects of the specificity of the enzyme are discussed below (see the Discussion).

Table 2: Inhibition of FAD-AMP Lyase by Nucleotidic Derivatives with Different Nitrogen Bases, Numbers of Phosphates, and the Presence or Absence of a 2′-OH Group

	experiment 1, with a fixed inhibitor concentration[a]			experiment 2, with a variable inhibitor concentration[b]	
	[inhibitor] (μM)	[FAD] (μM)	residual activity in the presence of inhibitor (%)	[FAD] (μM)	IC_{50} (μM)
ATP	20	50	<4	30	0.07
ADP	20	50	<4	30	0.10
AMP	20	50	103 ± 11	30	500
dATP	20	50	89 ± 2	−[c]	
dADP	20	50	74 ± 3	30	15
UDP	20	50	21 ± 1	30	3
UMP	20	50	94 ± 1	−[c]	
GDP	20	50	39 ± 1	−[c]	
CDP	20	50	26 ± 1	−[c]	
dTDP	20	50	110 ± 7	−[c]	
FMN	40	30	104 ± 2	30	>1000
cFMN	40	30	104 ± 2	30	>2000

[a] Initial rates were determined in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 6 mM $MnCl_2$, 1 mg/mL BSA, and the indicated concentrations of FAD and inhibitor. FAD splitting was assayed by assessing cFMN formation by HPLC method 1, except when testing the inhibition by FMN (HPLC method 2) or cFMN (HPLC method 3). Data are means ± the range of two independent assays. [b] Initial rates were determined in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 6 mM $MnCl_2$, 1 mg/mL BSA, FAD-AMP lyase, 30 μM FAD, and varying concentrations of the indicated inhibitor. FAD splitting was assayed by the hyperchromicity method, except when studying the inhibition by >40 μM AMP or FMN (assay of cFMN formation by HPLC methods 1 and 2, respectively), and when studying the inhibition by cFMN (assay of alkaline phosphatase-mediated adenosine formation by HPLC method 3). [c] Not studied.

The product patterns of these reactions were determined as explained in Materials and Methods (except for the minor activity on ADP-ribose, which was not investigated in this respect). In all cases, the results indicated that FAD-AMP lyase catalyzed the splitting of NDP-X to the cognate 5′-nucleotide and to a cyclic phosphodiester of X. This pattern was inferred from the fact that, in incubations of NDP-X with the purified enzyme, UV tracings of HPLC chromatograms indicated conversion to another compound with the same retention time of the relevant NMP, whereas the inclusion of alkaline phosphatase in the incubation mixture led to the formation of the corresponding nucleoside, accompanied by the accumulation of only 1 mol of P_i per mole of nucleoside (i.e., per mole of NDP-X split). This pattern corresponds to a cyclizing lyase that yields one alkaline phosphatase-sensitive and one phosphatase-resistant product, in the same way that the splitting of FAD to AMP and cFMN does.

In addition to the lyase-type reactions described above, a very minor (0.8% of the FAD splitting rate) ATPase activity [ADP was the product observed by UV-monitored HPLC

Table 3: Substrate Specificity of FAD-AMP Lyase

compound	activity (%) with 6 mM $MnCl_2$	activity (%) with 6 mM $CoCl_2$	compound	activity (%) with 6 mM $MnCl_2$	activity (%) with 6 mM $CoCl_2$
FAD[a]	100	100	CDP-glucose[a]	23	12
A(5′)pp(5′)A[b]	<1	<1	CDP-glycerol[a]	33	9
A(3′)p(5′)A[b]	<1	—[c]	CDP-choline[b]	<1	<1
A(2′)p(5′)A[b]	<1	—[c]	CDP-ethanolamine[b]	<2	<2
NADH[b]	<1	<1	CMP-*N*-acetylneuraminate[b]	<2	<2
NAD^+[b]	<4	<2	GDP-glucose[a]	20	4
dephosphocoenzyme A[b]	<1	<1	GDP-α-L-fucose[a]	12	2
ADP-ribose[b]	5	8	GDP-β-L-fucose[b]	<1	—[c]
ADP-glucose[a]	73	46	GDP-mannose[b]	<1	<1
ADP-mannose[b]	<2	<2	dTDP-glucose[b]	<1	<2
UDP-glucose[a]	87	36	ATP[b]	0.8	<0.1
UDP-galactose[a]	99	29	ADP[b]	<0.1	<1
UDP-xylose[a]	73	32	AMP[b]	<1	<1
UDP-glucuronate[a]	21	9	UDP[b]	<1	<1
UDP-galacturonate[a]	16	10	UMP[b]	<1	<1
UDP-mannose[b]	<1	<2	GTP[b]	<1	—[c]
UDP-*N*-acetylglucosamine[b]	<1	<1	CTP[b]	<1	—[c]
UDP-*N*-acetylgalactosamine[b]	<2	<2	cFMN[b]	<1	<1

[a] Limiting rates, *V*, expressed as percentages of those on FAD under similar conditions. For assay methods with these compounds as substrates, see Table 4. [b] Finite data given for ADP-ribose and ATP correspond to initial rates at a substrate concentration of 0.5 mM. All the other data (preceded by a < symbol) correspond to measurements which gave no detectable activity and represent the sensitivity limits of the assays. In these experiments, the substrate concentration was 0.5−1.2 mM, except for A(3′)p(5′)A and A(2′)p(5′)A which were tested at 80 μM. The activities were determined either by measuring the level of alkaline phosphatase-dependent accumulation of P_i (all cases except when indicated otherwise), by the same assay omitting the auxiliary phosphatase (AMP, UDP, or UMP), or by assessing the potential product formation by HPLC method 6 (GTP), method 9 (ATP and ADP), method 10 (CMP-*N*-acetylneuraminate), method 11 (GDP-β-L-fucose), method 12 [A(3′)p(5′)A and A(2′)p(5′)A], or method 13 (CTP). In every case, the number given is a percentage of the FAD-splitting activity determined under strictly equivalent conditions. [c] Not determined.

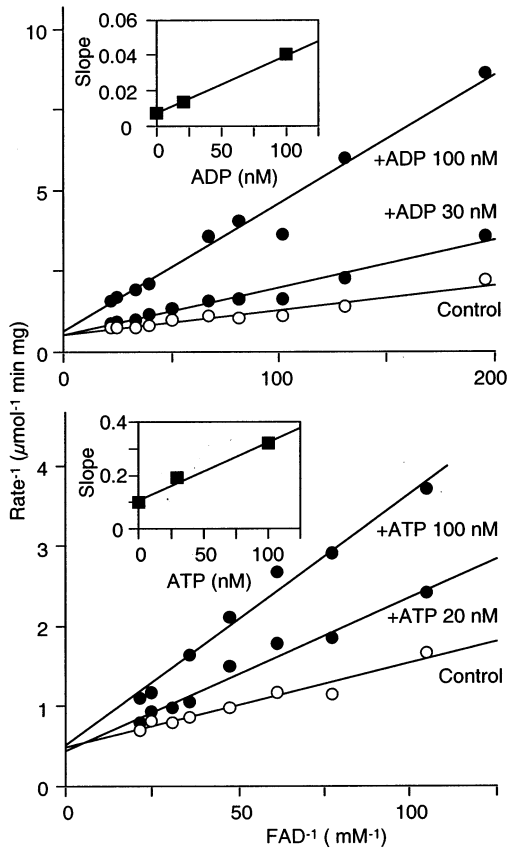

FIGURE 6: Competitive character of the inhibition of FAD-AMP lyase by ATP and ADP. Initial rates were determined by the hyperchromicity assay in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 6 mM $MnCl_2$, 1 mg/mL BSA, FAD-AMP lyase, and the indicated concentrations of FAD and inhibitors. The insets show replots of slopes vs inhibitor concentration.

(method 9); results not shown] was also detected in the purified preparation of FAD-AMP lyase. Whether this represents a minor hydrolytic activity of the same protein, or a contaminant activity, is not known. However, it must be stressed that the bivalent cation preferences of the ATPase activity (Mn^{2+} or Mg^{2+}) were different from those of FAD splitting (Mn^{2+} or Co^{2+}).

The kinetics of saturation of FAD-AMP lyase by 11 NDP-X substrates (including FAD) were studied both with Mn^{2+} and with Co^{2+} as the activating cation, at pH 7.5 (Table 4). In all non-FAD cases, and in contrast to the inhibition observed at FAD concentrations of >0.3 mM (Figure 5), the reactions displayed hyperbolic kinetics without signs of inhibition by excess substrate within the concentration ranges that were explored (Table 4). K_m and k_{cat} values were derived by nonlinear regression (*14*), considering an M_r of 120 000 for k_{cat} calculations. When the catalytic efficiencies or specificity constants (k_{cat}/K_m) were taken into account, the best FAD-AMP lyase substrate was FAD, followed closely by ADP-glucose (50% as efficient as FAD), whereas the rest of the substrates were much less efficiently split than FAD. In general, similar relative efficiencies were obtained with Mn^{2+} or Co^{2+}, although the absolute values were 4−10-fold higher in the first case (Table 4).

The effect of pH on the activities of FAD-AMP lyase on non-FAD substrates was studied with ADP-glucose, UDP-glucose, and UDP-glucuronate, both with Mn^{2+} and with Co^{2+} as the activating cation. The results (not shown) were similar to those obtained with FAD (Figure 4).

The requirement for high concentrations of Mn^{2+} for FAD-AMP lyase to be maximally active was studied with all the substrates of Table 4 except GDP-glucose and GDP-α-L-fucose. The results indicated that millimolar concentrations of this cation were required (results not shown; obtained with

Table 4: Kinetic Parameters of FAD-AMP Lyase with Different Substrates[a]

	Mn^{2+}-dependent activities				Co^{2+}-dependent activities			
substrate	concentration range (mM)	k_{cat}[b] (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})	concentration range (mM)	k_{cat}[b] (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
FAD[c]	0.005−2.0	289 ± 6	8.8 ± 1	32.9	0.03−2.0	643 ± 70	114 ± 24	5.6
ADP-glucose	0.007−0.07	211 ± 9	12.5 ± 2	16.9	0.04−1.0	293 ± 13	120 ± 14	2.4
UDP-glucose	0.19−4.7	251 ± 21	652 ± 130	0.38	0.19−4.7	234 ± 21	2550 ± 405	0.092
UDP-galactose	0.16−2.7	287 ± 24	606 ± 130	0.47	0.27−4.0	185 ± 18	3661 ± 589	0.050
UDP-xylose	0.25−5.8	210 ± 11	714 ± 111	0.29	0.25−5.8	209 ± 9	2354 ± 210	0.089
UDP-glucuronate	0.05−1.0	62 ± 3	107 ± 17	0.58	0.05−2.1	54 ± 4	539 ± 85	0.100
UDP-galacturonate	0.05−1.4	48 ± 1	108 ± 13	0.44	0.05−1.4	65 ± 6	759 ± 145	0.085
CDP-glucose	0.08−2.4	66 ± 4	416 ± 70	0.16	0.17−3.0	77 ± 17	3703 ± 1208	0.021
CDP-glycerol	0.06−1.9	95 ± 4	795 ± 74	0.12	0.09−1.9	55 ± 10	2031 ± 580	0.027
GDP-glucose	0.06−2.0	56 ± 3	784 ± 72	0.07	0.06−1.5	28 ± 5	2246 ± 536	0.012
GDP-α-L-fucose	0.08−1.0	34 ± 4	243 ± 68	0.14	0.1−1.0	11 ± 3	991 ± 408	0.011

[a] Initial rates were assayed at different substrate concentrations in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 6 mM $MnCl_2$ or 6 mM $CoCl_2$, and 1 mg/mL BSA. Substrate splitting was assessed by measuring the level of alkaline phosphatase-dependent accumulation of P_i, except for FAD at low concentrations (hyperchromicity assay; see Figure 9) and for ADP-glucose (in this case, the alkaline phosphatase-dependent formation of adenosine was monitored by including adenosine deaminase in the mixture and recording A_{265}). [b] For calculations of k_{cat} values, a molecular weight of 120 000 (the mean of the results obtained by gel filtration and ultracentrifugation) was assumed. [c] Kinetic parameters for FAD were derived from data obtained only at substrate concentrations of <0.3 mM, due to the inhibition observed at higher concentrations (see Figure 5 and the text).

the partially purified enzyme preparation described in ref *1*).

DISCUSSION

The enzyme purified in this work must be a relatively abundant protein in rat liver, as an apparently homogeneous preparation was obtained with an enrichment of only 250-fold with respect to the crude extract. At any rate, this low-level enrichment must be considered also in relation with the low 15% yield of the purification process (Table 1). The enzymatic protein may be a dimer composed of two subunits of similar size, as the M_r found by SDS−PAGE was 59 000, approximately half of the native M_r estimated by gel-filtration chromatography (140 000) or by sucrose gradient centrifugation (100 000). The different results obtained with these two techniques could be explained by the native dimer being of ellongated shape, such that it could deviate from the ideal behavior of spherical particles, with opposing biases upon molecular sieving and sedimentation.

Although several new substrates of the enzyme have been identified in this work, its definition as FAD-AMP lyase is now supported by the extensive specificity study that has been performed (Tables 3 and 4). On one hand, it has been previously reported that a partially purified preparation of the enzyme was devoid of adenylate cyclase, guanylate cyclase, cytidylate cyclase, FAD synthetase, FAD pyrophosphatase, and cFMN phosphodiesterase, and now it has been shown that the enzyme is also devoid of detectable 2′,3′-cyclic phosphodiester-forming activity typical of ribonucleases with A(3′)p(5′)A (Table 3) (*15*). On the other hand, the novel FAD-AMP lyase substrates identified in this work all have the NDP-X structure and are split following the pattern

$$\text{NDP-X} \rightarrow \text{NMP} + \text{P=X} \qquad (1)$$

where NMP stands for the 5′-nucleotide cognate of NDP-X and P=X for a cyclic phosphodiester of X. This reaction model also fits the earlier demonstrated splitting of FAD (*1*)

$$\text{FAD (“ADP-riboflavin”)} \rightarrow \text{AMP} + \text{cFMN} \qquad (2)$$

However, not every compound with the NDP-X structure is split by the enzyme, and those that are split exhibit a wide range of specificity constants. k_{cat}/K_m was 2-fold higher for FAD than for ADP-glucose and 50−500-fold higher than for the other substrates (Table 4). Since, in addition, ADP-glucose is not a physiological compound in mammals, FAD-AMP lyase (cyclizing) remains an appropriate denomination for the enzyme.

Structure−activity considerations on substrates or non-substrates (Table 5) and inhibitors (Table 2) pinpoint the following specificity pattern. *FAD-AMP lyase splits ribonucleoside diphosphate-X compounds in which X is an acyclic or cyclic monosaccharide or derivative bearing an X-OH group that is able to attack internally the proximal phosphorus with the geometry necessary to form a P=X product, either a five-atom monocyclic phosphodiester or a cis-bicyclic phosphodiester−pyranose fusion*. Figure 7 shows the two kinds of geometries that the reacting NDP-X substrates of FAD-AMP lyase and the P=X products may display: NDP-X substrates bearing an acyclic X monosaccharide and being split to a monocyclic P=X phosphodiester product (Figure 7B,D) and NDP-X substrates bearing a cyclic X monosaccharide and being split to a P=X *cis*-bicyclic phosphodiester−pyranose product (Figure 7C,E). All the reacting substrates of FAD-AMP lyase belong to one of these categories, as indicated by the answers to the three structural questions posed in the headings of the columns of Table 5 relative to the P−X moiety.

One of the compounds not split by FAD-AMP lyase, dTDP-glucose, fulfils exactly the three structural conditions of Table 5, and its expected P=X product of splitting would contain a phosphodiester−pyranose fusion. Like the reacting NDP-glucose substrates, dTDP-glucose can in theory adopt the geometry shown in Figure 7C and form the kind of P=X product shown in Figure 7E. However, it does not react in the FAD-AMP lyase active site. Since ADP-glucose, GDP-glucose, CDP-glucose, and UDP-glucose were all substrates, the lack of reactivity of dTDP-glucose must be attributed to either the extra methyl group of thymine versus uracil or to the absence of the 2′-OH group in the deoxyribofuranose

Table 5: Structure−Reactivity Relationship of NDP-X or NMP-X Compounds Tested as Substrates of FAD-AMP Lyase[a]

	reactivity	structural features of the P-X moiety		
compound	Is the compound a FAD-AMP lyase substrate?	Is there in X an OH group that can be oriented to attack the proximal phosphorus?	Would this attack lead to a P═X product containing a five-atom cyclic phosphodiester?	If this five-atom cycle forms a bicyclic fusion, would it adopt the *cis* configuration?
FAD	yes	yes	yes	−
ADP-ribose (open chain)	yes[b]	yes	yes	−
CDP-glycerol	yes	yes	yes	−
ADP-glucose	yes	yes	yes	yes[f]
UDP-glucose	yes	yes	yes	yes[f]
UDP-galactose	yes	yes	yes	yes[f]
UDP-xylose	yes	yes	yes	yes[f]
UDP-glucuronate	yes	yes	yes	yes[f]
UDP-galacturonate	yes	yes	yes	yes[f]
CDP-glucose	yes	yes	yes	yes[f]
GDP-glucose	yes	yes	yes	yes[f]
GDP-α-L-fucose	yes	yes	yes	yes[f]
dTDP-glucose	no	yes	yes	yes[f]
A(3′)p(5′)A	no	yes	yes	yes[g]
A(2′)p(5′)A	no	yes	yes	yes[g]
ADP-mannose	no	yes	yes	no[h]
UDP-mannose	no	yes	yes	no[h]
GDP-mannose	no	yes	yes	no[h]
GDP-β-L-fucose	no	yes	yes	no[h]
CMP-*N*-acetylneuraminate	no	yes	no[d]	−
dephosphocoenzyme A	no	yes	no[e]	−
ADP-ribose (furanose)	no[b]	yes	no[e]	−
A(5′)pp(5′)A	no	yes	no[e]	−
NADH	no	yes	no[e]	−
NAD^+	no	yes	no[e]	−
UDP-*N*-acetylglucosamine	no	no	−	−
UDP-*N*-acetylgalactosamine	no	no	−	−
CDP-choline	no	no	−	−
CDP-ethanolamine	no	no	−	−
ATP	no[c]	no	−	−
ADP	no	no	−	−
GTP	no	no	−	−
CTP	no	no	−	−
UDP	no	no	−	−
AMP	no	−	−	−
UMP	no	−	−	−
cFMN	no	−	−	−

[a] The answers to the "reactivity" question are experimental (Table 3), and those to the "structural" questions were obtained by molecular model building. [b] ADP-ribose was a weak substrate of FAD-AMP lyase (Table 3). The X moiety (ribose) of this compound may exist in solution in open chain or furanose forms. In fact, it is not known which one of these forms of ADP-ribose is the actual substrate of FAD-AMP lyase; however, we assume that the ADP-ribose open chain form is likely to be the substrate, because its structural features fit with those of other substrates (Figure 12) better than those of ADP-ribofuranose form. [c] The FAD-AMP lyase preparation exhibits a very minor ATPase activity (Table 3) which by its hydrolytic character and by its metal preferences is different from the cyclizing lyase activities (see the text). [d] In this case, the potential P═X product would contain a six-atom cyclic phosphodiester. [e] In these cases, the potential P═X product would contain a seven-atom cyclic phosphodiester. [f] In these cases, the P═X product would contain a *cis* phosphodiester−pyranose bicyclic fusion. [g] In these cases, the P═X product would contain a *cis* phosphodiester−furanose bicyclic fusion. [h] In these cases, the P═X product would contain a *trans* phosphodiester−pyranose bicyclic fusion.

versus the ribofuranose ring. It seems that the second possibility is more realistic, because it appears unlikely that thymine is excluded from the active site while adenine or guanine is accepted. In addition, although neither dADP-glucose, dGDP-glucose, dUDP-glucose, dCDP-glucose, nor ribo-TDP-glucose was available for confirmatory testing, it could be clearly demonstrated that dADP and dATP were much weaker inhibitors of FAD-AMP lyase than ADP and ATP (Table 2). Therefore, it can be inferred that the 2′-OH group of the NMP moiety plays an important role in the interaction of NDP-X substrates or nucleotidic inhibitors with the active site.

The behavior of A(3′)p(5′)A and A(2′)p(5′)A is also worthy of comment. On one hand, they are not NDP-X but NMP-X derivatives. On the other, in their 3′-AMP or 2′-AMP moieties, they bear ribofuranose OH groups (the 2′-OH or 3′-OH ones, respectively) that can attack the unique phosphorus of these compounds, forming a five-atom P═X phosphodiester which would constitute a *cis*-bicyclic fusion with the furanose ring of either the 3′-AMP or 2′-AMP moiety, respectively. The attribution of a particular rational basis to the lack of reactivity of A(3′)p(5′)A and A(2′)p(5′)A is less straightforward than in the case of dTDP-glucose, as it can be due to the absence of the diphosphate, to the presence of an adenosine residue in the X moiety, or to the phosphodiester−furanose (rather than −pyranose) bicyclic fusion that would result from the reaction. In any case, the results of inhibition experiments indicate that, like the 2′-OH group of NMP, the diphosphate group is essential for the interaction of nucleotidic compounds with the active site. This can be inferred from the inhibitory potency of the following series: ATP ≈ ADP ≫ AMP (Table 2).

The rest of the compounds not split by FAD-AMP lyase (Table 5) fit well in the picture, either because they lack the

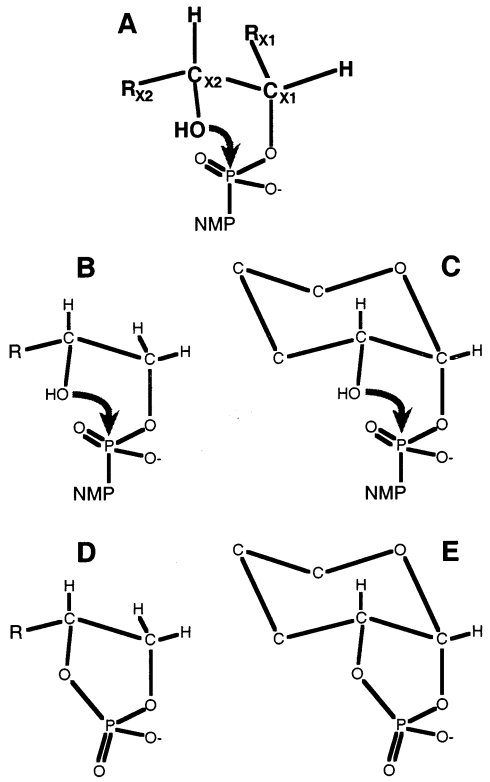

FIGURE 7: Geometries of the reacting NDP-X substrates of FAD-AMP lyase and their corresponding P═X products. (A) General structure of a NDP-X substrate in which there is an X-OH group (the one bound to C_{X2}) that is able to perform a nucleophilic attack on the proximal phosphorus. It can be either an open chain or a cyclic monosaccharide or derivative. (B) Structure of NDP-X with X being an acyclic monosaccharide or derivative, like in the substrates FAD (P-X is riboflavin 5′-monophosphate), ADP-ribose (P-X is D-ribose 5-phosphate, open chain form of the sugar) and CDP-glycerol (P-X is *sn*-glycerol 3-phosphate). (C) Structure of NDP-X with X being a cyclic monosaccharide or derivative, like in the other substrates, NDP-glucose, -galactose, -xylose, -glucuronate, -galacturonate, or -α-L-fucose (P-X is α-pyranose 1-phosphate in all cases). (D) Five-atom monocyclic P═X phosphodiester formed from substrates such as in panel B. (E) *cis*-Phosphodiester−pyranose bicyclic P═X product formed from substrates such as those in panel C.

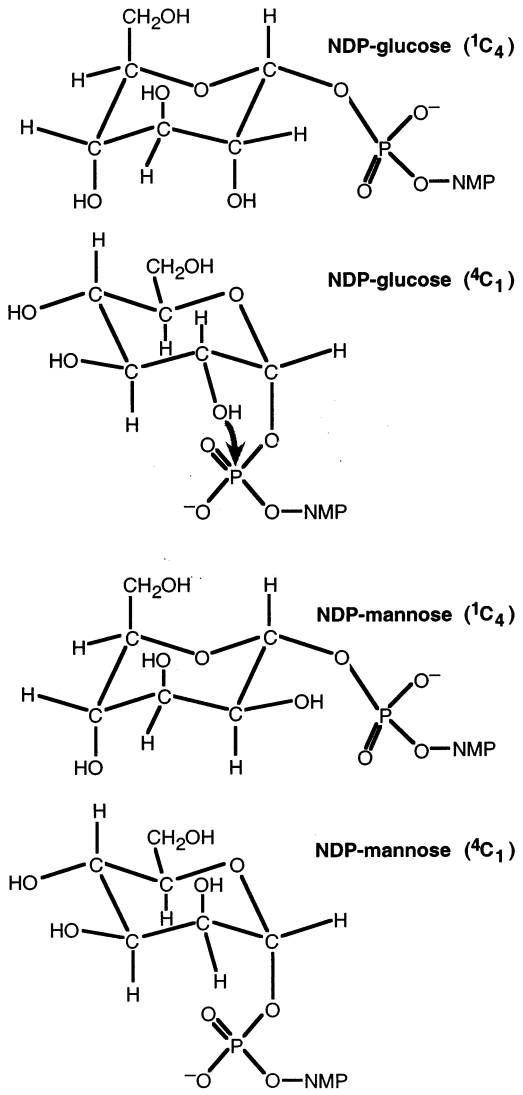

FIGURE 8: Comparison of the 2-OH positions of the pyranose rings of NDP-glucose and NDP-mannose with regard to their possible participation in a nucleophilic attack on the proximal phosphorus. The arrow indicates the attack effectively taking place in the case of NDP-glucose, which was not observed with NDP-mannose.

X-OH group at the correct distance to react with the proximal phosphorus or because, having one, it cannot give rise to a five-atom cyclic phosphodiester or it can do it but only with the geometry that leads to a *trans*-phosphodiester−pyranose P═X product. In this regard, it is most remarkable that, whereas NDP-glucose and GDP-α-L-fucose were substrates of FAD-AMP lyase, NDP-mannose and GDP-*β*-L-fucose were not. Figure 8 shows, for example, the structures of the NDP-glucose and NDP-mannose derivatives, emphasizing the two possible conformations, $^1C_4$ and $^4C_1$, of their pyranose rings. Observe as, for the 2-OH group of glucopyranose to be able to reach the proximal phosphorus, the pyranose ring has to adopt the $^4C_1$ conformation, and if reaction takes place it will give rise to the phosphodiester−pyranose fusion in the *cis* configuration (like in Figure 7C,E). On the contrary, for the 2-OH group of mannopyranose to complete the same kind of reaction, the pyranose ring has to adopt the $^1C_4$ conformation which, upon reaction, would produce a phosphodiester−pyranose fusion with the *trans* configuration. Similar circumstances are observed when the structures of GDP-α-L-fucose (FAD-AMP lyase substrate) and GDP-*β*-L-fucose (not substrate) are considered (not shown).

Table 6 shows that there is a clear parallelism, with a few deviations, between the specificity of FAD-AMP lyase toward NDP-X substrates and the susceptibilities of the same compounds to metal-dependent chemical degradation. The parallelism must reflect mechanistic features common to both processes, like the nucleophilic attack depicted in Figure 7 and possibly the formation of metal−NDP-X complexes, whereas the deviations point to aspects of the binding of NDP-X to the enzyme active site. The major deviations between the enzymic and chemical splitting are (i) the different behavior of dTDP-glucose, not an enzyme substrate but susceptible to chemical degradation, which argues again for the proposed role of the 2′-OH group of NDP-X in the interaction with the FAD-AMP lyase active site, and (ii) the relative discrimination against UDP-hexuronic, GDP-X, and, to some extent, CDP-X compounds in the enzymic versus the chemical process, which suggests that the presence of a carboxylic group in X versus its absence, and the arrangement of the substituents of the purine and pyrimidine rings

Table 6: Metal-Dependent Chemical Decomposition of NDP-X Compounds: Comparison to Enzymic Reactivities as FAD-AMP Lyase Substrates

	with 6 mM $MnCl_2$			with 6 mM $CoCl_2$		
NDP-X	k_{cat}[a] (min^{-1})	k[b] ($\times 10^3$ min^{-1})	$10^{-5} \times k_{cat}/k$	k_{cat}[a] (min^{-1})	k[b] ($\times 10^3$ min^{-1})	$10^{-5} \times k_{cat}/k$
FAD	289	1.25	2.31	643	0.75	8.57
ADP-glucose	211	1.21	1.74	293	0.59	4.97
UDP-glucose	251	1.16	2.16	234	0.25	9.36
UDP-galactose	287	2.18	1.32	185	0.25	7.40
UDP-xylose	210	1.34	1.57	209	0.22	9.50
UDP-glucuronate	62	2.10	0.30	54	0.59	0.92
UDP-galacturonate	48	2.43	0.20	65	0.77	0.84
CDP-glucose	66	0.93	0.71	77	0.23	3.35
CDP-glycerol	95	0.65	1.46	55	0.32	1.72
GDP-glucose	56	1.66	0.34	28	1.52	0.18
GDP-α-L-fucose	34	6.67	0.05	11	10.42	0.11
GDP-β-L-fucose	nd[c]	0.28	nd[c]	—[d]	—[d]	—[d]
ADP-ribose	—[d]	0.15	—[d]	—[d]	0.26	—[d]
ADP-mannose	nd[c]	nd[c]	—[d]	nd[c]	nd[c]	—[d]
UDP-mannose	nd[c]	nd[c]	—[d]	nd[c]	nd[c]	—[d]
UDP-*N*-acetylglucosamine	nd[c]	nd[c]	—[d]	nd[c]	nd[c]	—[d]
UDP-*N*-acetylgalactosamine	nd[c]	nd[c]	—[d]	nd[c]	nd[c]	—[d]
CDP-choline	nd[c]	nd[c]	—[d]	nd[c]	nd[c]	—[d]
CDP-ethanolamine	nd[c]	0.15	nd[c]	nd[c]	0.11	nd[c]
GDP-mannose	nd[c]	nd[c]	—[d]	nd[c]	nd[c]	—[d]
dTDP-glucose	nd[c]	1.00	nd[c]	nd[c]	0.35	nd[c]

[a] FAD-AMP lyase catalytic constants (k_{cat}) taken from Table 4. [b] First-order rate constants (k) for the metal-dependent decomposition of NDP-X compounds were determined by measuring the rate of alkaline phosphatase-dependent liberation of P_i in reaction mixtures without FAD-AMP lyase, at pH 7.5, with 1.2 mM NDP-X compound and either 6 mM $MnCl_2$ or 6 mM $CoCl_2$. [c] Not detectable. [d] Not determined.

of guanine and cytosine versus adenine and uracil, hamper binding to the enzyme active site or catalysis.

Altogether, the evidence indicates that what FAD-AMP lyase recognizes is the NDP moiety of NDP-X compounds with a marked preference for the ADP moiety of ADP-X. This is supported by the substrate structure−reactivity relationships discussed above (Figure 7 and Tables 5 and 6), by the relative potencies of the compounds tested as inhibitors (Table 2), and by the Mn^{2+}-dependent adsorption of the enzyme to ADP-agarose (the same chromatographic behavior was observed with Co^{2+}, the other enzyme-activating cation; results not shown). Once the NDP-X ligand is bound, the manifestation of a splitting activity would depend on whether there is an X-OH group in the ligand which can be properly oriented. With regard to FAD, the best substrate of FAD-AMP lyase, it is important to state that there is little or no evidence for specific recognition of the flavin moiety of this substrate by the enzyme. Under conditions in which ADP inhibited strongly, there was no evidence of inhibition by the reaction product cFMN or its noncyclic analogue FMN. In contrast, AMP inhibition was detectable at high concentrations (Table 2).

Finally, one wonders whether, besides FAD, there is any other ADP-X compound which could be considered as a possible in vivo substrate of FAD-AMP lyase in mammals (ADP-glucose is not physiological in these organisms). One answer of uncertain significance, but one that should be discussed, is the open chain form of ADP-ribose. ADP-ribose is the only compound among those tested as substrates (Tables 3 and 5) which contains a monosaccharide X moiety that is able to undergo anomeric conversion. Whereas in all other NDP-sugars the linkage between NDP and X involves the anomeric OH group, in ADP-ribose the group esterified by NDP is the 5-OH group. This means that ADP-ribose must exist in solution as a mixture of two ADP-ribofuranose forms and one ADP-ribose open chain form. In fact, free ADP-ribose, due to its reducing character, has a considerable potential for nonenzymatic protein glycation (*16*−*23*), and it is considered a harmful metabolite whose cellular accumulation should be avoided (*22*−*25*). When the structure of the furanose and the open chain forms is considered, only the latter fits the structural conditions summarized in Figure 7 and Table 5, which made us believe that this is the form split by FAD-AMP lyase (Table 5). FAD-AMP lyase could bind open chain ADP-ribose or, perhaps more likely, ADP-ribofuranose, which could undergo the transition to the open chain form while bound to the enzyme. At any rate, we do not intend to suggest that free ADP-ribose splitting is a major role of FAD-AMP lyase, since this seems to be a rather inefficient way to eliminate this dangerous metabolite. The FAD-AMP lyase activity toward free ADP-ribose is very low, and the same liver extract from which the enzyme was purified contains several hydrolytic ADP-ribose pyrophosphatases, one of them a Mg^{2+}-dependent enzyme with a high specificity and a low micromolar K_m for ADP-ribose, which probably can do the job more efficiently (*8*). Alternatively, one can think of free ADP-ribose as the source of possible substrates for FAD-AMP lyase. In fact, ADP-ribose conjugates formed by nonenzymatic reactions which include the opening of the ribofuranose ring (*19*, *21*−*23*) would fulfill the structural requirements shown in Figure 7A, including the presence of the critical C_{X2}-OH group, which is absent in ADP-ribofuranose due to its participation in furanose cyclization. Also, it is worth mentioning the reported occurrence of ADP-ribulose in human erythrocytes, possibly formed by isomerization of ADP-ribose (*20*, *26*). ADP-ribulose cannot cyclize as furanose and must occur predominantly in the ribulose open chain form that also fits the model of Figure 7A. The hypothesis that FAD-AMP lyase could split these ADP-ribose derivatives was out of the scope of this work, since they are not immediately available. This question remains to be studied in future developments, which

should also contemplate how the enzyme could override in vivo the strong ADP and ATP inhibition and the requirement for high Mn^{2+} or Co^{2+} concentrations, along with the cloning and expression studies needed to achieve the molecular identification of FAD-AMP lyase.

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