Enzymic formation of riboflavin 4',5'-cyclic phosphate from FAD: evidence for a specific low- K_m FMN cyclase in rat liver¹

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An enzyme activity splitting FAD to AMP and riboflavin 4',5'-cyclic phosphate (4',5'-cFMN), with a K_m of 6–8 μ M, was partially purified from the cytosolic fraction of rat liver homogenates. 4',5'-cFMN was characterized by enzyme, HPLC, UV-visible and NMR spectroscopic analyses. The data suggest that a novel enzyme, tentatively named FAD-AMP lyase

(cyclizing) or FMN cyclase, is involved. Also, 4',5'-cFMN was hydrolysed to 5'-FMN by a rat liver cyclic phosphodiesterase. The results indicate a novel enzymic pathway for flavins in mammals, and support the biological relevance of 4',5'-cFMN, perhaps as a flavocoenzyme or a regulatory signal.

INTRODUCTION

The common forms of riboflavin or vitamin B₂ in animals are FMN and FAD (prosthetic groups of many oxidoreductases [1,2]) formed from riboflavin in two steps catalysed by flavokinase [3–6] and FAD synthetase [6–8]). The intracellular fate of flavin nucleotides in mammals is the flavinylation of apoenzymes [9,10] and little intracellular FMN and FAD is found in the free state: less than 0.05 μ M and about 1 μ M respectively in mammalian liver [6]. The catabolism of flavocoenzymes is believed to depend on phosphohydrolases, FAD pyrophosphatase [11–15] and FMN phosphomonoesterase [14–17], which either act at extracellular surfaces as part of vitamin B₂-uptake pathways or are linked to (trapped in) intracellular membranes.

During a search for FAD-splitting enzymes in rat liver, we found a Mn²⁺-dependent activity which was partially purified from the cytosolic fraction of rat liver homogenates and was shown to produce AMP and riboflavin 4',5'-cyclic phosphate (4',5'-cFMN). Both the enzyme, tentatively named FMN cyclase, and its cyclic flavin product were previously unknown in biological materials.

MATERIALS AND METHODS

Materials

Female Wistar rats weighing around 200 g were used. FAD was from Sigma and MnCl₂ from Merck. Other (di)nucleotides and nucleosides were from standard commercial suppliers. Snake venom phosphodiesterase and alkaline phosphatase were from Boehringer (cat. no. 108260 and 108146 respectively). Rat liver nucleotide pyrophosphatase was purified by a shortened version of a published procedure [18]. In short, it was extracted from rat liver membranes with Triton X-100, solubilized by limited trypsin treatment, adsorbed on a DEAE-cellulose column in buffer G (20 mM Tris/HCl, pH 8.7, 5 mM MgCl₂), recovered with a 0–400 mM NaCl linear gradient in buffer G, concentrated by ultrafiltration through a PM30 membrane (Amicon), chromatographed on a Sephacryl S-200 column in buffer G, concentrated

again, and buffer-exchanged to 5 mM sodium phosphate, pH 8.25, on a Sephadex G-25 column (PD10; Pharmacia). The specific activity, with *p*-nitrophenyl-dTMP as substrate, was 13 units/mg. It was supplemented with 1 mg/ml BSA and kept at –20 °C.

Partial purification of FMN cyclase activity

The crude extract was the cytosolic fraction, i.e. the 100000 *g* supernatant of an isotonic rat liver homogenate in 3 vol. of 20 mM Tris/HCl, pH 7.5, containing 0.5 mM EDTA, 0.25 M sucrose and 1.3 mM PMSF. The 30–60 %-(NH₄)₂SO₄ fraction was filtered through a Sephadex G-100 column (119 cm × 2 cm) (Figure 1A), followed by negative adsorption chromatography through an in-series set of columns (Figure 1B) composed of DEAE-cellulose DE-52 (44 cm × 1.8 cm), Reactive Blue 2–Sephacrose CL6B (5.5 cm × 1.5 cm), Reactive Brown 10–agarose (3.2 cm × 1.7 cm) and Reactive Blue 4–agarose (3.3 cm × 1.7 cm), all equilibrated in and eluted with buffer T (20 mM Tris/HCl, pH 7.5, 0.5 mM EDTA) except for the DEAE-cellulose, which was equilibrated without EDTA, but eluted with buffer T as part of the in-series set. The active flow through fractions were adsorbed on a Reactive Green 19–agarose column (6.0 cm × 1.5 cm) in buffer T. The activity was eluted with a 250 ml KCl gradient (0–200 mM KCl) in buffer T (Figure 1C). This yielded partially purified enzyme (SDS/PAGE results not shown), enriched 40-fold with a yield of 16% (Table 1), and stable for at least 1 month at 4 °C. Protein was assayed by the method of Bradford [19].

Enzyme assays

FMN cyclase activity was routinely assayed measuring colorimetrically the P_i liberated from the product AMP in 50 mM Tris/HCl, pH 7.5, containing 6 mM MnCl₂, 1.2 mM FAD, 1 mg/ml BSA and 7.5 unit/ml alkaline phosphatase. In the assays carried out to investigate the possible activity of FMN cyclase on NAD⁺, NADH or diadenosine 5',5''-diphosphate, the composition of the reaction mixtures was the same except for the substitution of one of these compounds for FAD. Samples were

Abbreviations used: cFMN, riboflavin cyclic phosphate; DEPT, distortionless enhancement by polarization transfer.

¹ R.M.P., M.J.C., J.C. and J.C.C. dedicate this paper to their teachers, Antonio Sillero and María Antonia Günther Sillero.

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Figure 1 Elution profiles of FAD-splitting activity

(A) Gel-filtration chromatography. (B) Multiple negative adsorption chromatography. Only the flow through fraction is shown. (C) Reactive Green 19–agarose affinity chromatography. ●, FMN cyclase activity; △, cFMN phosphodiesterase activity; ○, A_{280} ; —, KCl.

Table 1 Partial purification of the FMN cyclase activity from the cytosolic fraction of rat liver homogenates

The data correspond to a purification started from 32 g of fresh rat liver. Details of purification and activity assay (by the standard P_i -release assay) are given in the Materials and methods section.

Step	Protein (mg)	Activity (m-units)	Specific activity (m-units/mg)	Purification (fold)	Yield (%)
100 000 g supernatant (cytosolic fraction)	1152	13 464	11.7	1	100
30–60%-satd. $(NH_4)_2SO_4$ fractionation	750	9895	13.2	1.1	74
Gel-filtration chromatography	265	6972	26.3	2.3	52
Multiple negative adsorption chromatography	37.4	3438	92	7.9	26
Reactive Green 19–agarose affinity chromatography	4.4	2212	505	43.2	16

taken at 1–10 min intervals, quenched with 10 vol. of a P_i reagent (6 vol. of 3.4 mM ammonium molybdate in 0.5 M H_2SO_4 , 1 vol. of 570 mM ascorbic acid, 0.37 vol. of 350 mM SDS), and A_{280} was measured after 20 min at 45 °C. At FAD concentrations below 50 μ M (Figure 2C), the linear A_{265} increase due to the hyperchromicity of FAD splitting ($\epsilon = 7200 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was recorded in the absence of alkaline phosphatase. To validate the P_i -release and hyperchromicity assays, enzyme activity was also assayed by measuring the rate of 4',5'-cFMN accumulation by HPLC method 4 (see below), which afforded the rapid separation of FAD and cFMN (retention times 3.5 and 4.5 min respectively) and their quantification with detection at 430 nm (ϵ 9600 and 11300 $\text{M}^{-1} \cdot \text{cm}^{-1}$ respectively). In all cases, to correct for non-enzymic FAD splitting, every individual assay was run strictly in parallel with a control in which enzyme buffer was substituted for enzyme sample. Blanks without substrate were also run and subtracted as required. The rates measured in mixtures with enzyme less than that of no-enzyme controls were proportional to protein concentration (for example, see Figure 2A). Taking into account that at around pH 7.5 the enzymic and the non-enzymic reactions of FAD were very sensitive to pH (see Figure 2E), it must be emphasized that the buffers were adjusted to within ± 0.01 pH units of the desired value at 37 °C, and that all the controls and blanks were run strictly in parallel with enzyme-containing samples, using the same batches of buffers and other reagents. One unit of activity is defined as 1 μ mol of substrate split (P_i formed; see reaction 1 below)/min at 37 °C.

Phosphodiesterase activity on 4',5'-cFMN, 3',5'-cAMP or 2',3'-cAMP, and FMN phosphomonoesterase activity were assayed by a P_i -release method like that for FMN cyclase except that 0.5 mM substrate was used, 2.5 mM $MgCl_2$ was substituted for $MnCl_2$ and, when FMN was the substrate, alkaline phosphatase was omitted. The possible formation of 3',5'-cAMP or 3',5'-cGMP was assayed as for FMN cyclase activity except that 0.4 mM ATP or GTP was substituted for FAD, alkaline phosphatase was omitted and samples were analysed by HPLC method 3 (see below), which resolved 3',5'-cAMP (3',5'-cGMP) from adenosine (guanosine) phosphates. The possible formation of FAD from FMN and ATP was assayed as for both FMN cyclase (except that 0.5 mM FMN and 0.5 mM ATP were substituted for FAD and alkaline phosphatase was omitted) and rat liver FAD synthetase [7,8], measuring FAD formation by HPLC method 1 in both cases.

Preparation of enzymically formed cFMN

cFMN was purified from reaction mixtures with 1.2 mM FAD, 6 mM $MnCl_2$ and 25 m-units/ml FMN cyclase, in 80% (v/v) buffer T with 150 mM KCl. After 70 min at 37 °C, 0.5 ml samples were chromatographed on a Prep Nova-Pak HR C_{18} column (30 cm $\times$ 0.78 cm; Waters) with a Hypersil ODS precolumn (20 mm $\times$ 2.1 cm), at 1 ml/min, in 45 mM ammonium formate, pH 6.4, in 19% (v/v) methanol. cFMN was eluted (30 min) separated from FAD and AMP. Control mixtures like those used for synthesis, except that enzyme was omitted, were incubated and chromatographed under the same conditions, to check for non-enzymic FAD conversion into cFMN and AMP. These controls indicated that in fact less than 10% of the cFMN produced in the presence of enzyme was produced non-enzymically, i.e. more than 90% of total cFMN was synthesized by FMN cyclase. The pooled cFMN peaks from five to ten runs were repeatedly lyophilized to remove the eluent. The final molar recovery of cFMN was around 50% of FAD. Its purity was more than 99% by HPLC (method 1 with A_{250} detection as in Figures 3A–3F). 1H -NMR spectra showed, at δ 3.5 p.p.m. (Figure 5B), a minor contaminant, without any corresponding signal in the ^{13}C -NMR spectrum.

Analytical HPLC

A Hypersil ODS column (150 mm $\times$ 3.9 mm) and precolumn (20 mm $\times$ 2.1 mm) and a Hewlett–Packard HP-1090 chromatograph were used. Samples of 20–50 μ l, diluted as needed, were injected and eluted at 0.5 ml/min with a binary system dispensing

two buffers. For method 1 the conditions were as follows: buffer A, 10 mM sodium phosphate, pH 7; buffer B, 10 mM sodium phosphate, pH 7, 50 % (v/v) methanol; elution, 0–5 min, isocratic 100 % A, 5–5.5 min, linear gradient up to 70 % A and 30 % B, 5.5–16 min, isocratic 70 % A and 30 % B, 16–16.5 min, linear gradient up to 50 % A and 50 % B, after 16.5 min, isocratic 50 % A and 50 % B. For method 2 conditions were: buffer C, 5 mM sodium phosphate, pH 7, 20 % (v/v) methanol, 10 mM sodium tetrabutylammonium; buffer D, same as C with 100 mM phosphate; elution, linear gradient from 100 % C to 100 % D in 20 min. For method 3 conditions were: buffers C and D as in method 2; elution, linear gradient from 100 % C to 40 % C and 60 % D in 10 min, and then to 100 % D in another 10 min. For method 4 conditions were: isocratic elution with 30 % buffer A and 70 % buffer B.

RESULTS AND DISCUSSION

Non-enzymic splitting of FAD

It is known that Mn^{2+} catalyses the non-enzymic hydrolysis of FAD [20] and, in alkaline solutions, FAD decomposes giving

4',5'-cFMN [21,22]. Throughout this work, to ensure that the results represented true enzyme activity, the non-enzymic splitting of FAD was systematically controlled by running every individual enzyme assay mixture in parallel with a non-enzyme counterpart. The rate of non-enzymic FAD splitting increased linearly with FAD or Mn^{2+} concentration (at least up to 1 mM FAD and 10 mM MnCl_2 ; results not shown and Figure 2D respectively), and depended on pH as shown in Figure 2(E). Under the conditions of the standard FMN cyclase assay (1.2 mM FAD, 6 mM MnCl_2 and pH 7.5, at 37 °C), the non-enzymic rate was about 1.5 nmol/min per ml of reaction mixture, which allowed the reliable measurement of enzyme rates of similar magnitude after discounting the non-enzymic reaction. For instance, Figure 2(A) shows the time-linear increments of crude A_{820} values corresponding to FAD splitting in reaction mixtures that contained enzyme catalysing the reaction at 1-, 2- and 3-fold the non-enzymic rate. Furthermore, it should be emphasized that many experiments were performed at much higher ratios of enzyme/non-enzyme reaction, either by working at higher enzyme and/or at lower FAD and Mn^{2+} concentrations than in Figure 2(A). Particular examples are pointed out below.

Figure 2 Properties of FMN cyclase activity

Initial rates were assayed by measuring either the liberation of P_i (A_{820}) in 0.4 ml reaction mixtures (●) or the hyperchromicity (A_{265}) of FAD splitting in 1.2 ml reaction mixtures (○). All the enzymic initial rates are corrected for non-enzymic FAD splitting. Some non-enzymic reaction rates are also shown (▽) as well as some raw A_{820} values (■, ▨, □, ◇). (A) Time course of total FAD splitting measured by the increment of A_{820} with 1.40 (■), 0.70 (▨), 0.33 (□) µg of enzyme or no enzyme added (◇). The data are means $\pm$ S.D. ($n = 4$) for identical assay mixtures. The inset shows a plot of the enzyme-dependent slopes (i.e. the slopes measured in the presence of enzyme less the slope measured in its absence) versus enzyme amount. (B) Thermal inactivation of FMN cyclase. Enzyme samples were incubated for 2 min at the indicated temperatures, then cooled on ice and FMN cyclase was assayed as in (A). The A_{820} values measured after 30 min of incubation are shown. The arrows mark the A_{820} values reached by not-heated enzyme (nh) and a no-enzyme control (ne). (C) Saturation kinetics. The data are means $\pm$ S.D. ($n = 3$) for rates in independent mixtures with 1 m-unit (standard assay conditions) of FMN cyclase. The hyperbola drawn is the best computer fit [23] to rates up to 62 µM FAD. This gave a K_m of 6.0 ± 0.8 µM (S.E. for the adjustment [23]). The inset shows a linear transformation of the same data (△) and hyperbola. Another experiment gave similar results (K_m 7.2 ± 1.3 µM). For the sake of clarity, no-enzyme controls are not shown in this part; below 40 µM FAD they gave negligible rates ($< 5\%$ of enzyme activity). (D, E) Mn^{2+} requirement for activity and pH-activity profile (assayed with 5 and 2.5 m-units of FMN cyclase respectively). The rates are means $\pm$ range for two experiments. (F) Native M_r by gel filtration in a Sephadex G-150 column (87.5 cm $\times$ 1.2 cm) in buffer T and 100 mM KCl. The inset shows the calibration plot obtained with protein markers (◇): amylase (200 000), aldolase (158 000), BSA (66 000), egg albumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 400).

Enzymic characterization of the FAD-splitting activity

The FAD-splitting activity was studied with an alkaline phosphatase-coupled P_i -release assay or, when FAD concentrations lower than $50 \mu\text{M}$ were used (Figure 2C), with a spectrophotometric assay that measured the UV hyperchromicity due to FAD splitting. These two assays yielded similar initial rates (see Figure 2C at $40\text{--}50 \mu\text{M}$ FAD). In addition, the rate of P_i liberation was equal to the rate of conversion of FAD into reaction products, measured by HPLC. This was validated under initial rate conditions by sampling standard reaction mixtures simultaneously for colorimetric assay of P_i and for assay of cFMN formation by HPLC method 4. That the splitting of 1 mol of FAD gave equimolar cFMN and alkaline phosphatase-releasable P_i was also confirmed after exhaustion of FAD by incubation with FMN cyclase (see the next section).

The basic kinetic and molecular properties shown below demonstrated clearly the enzymic character of the FAD-splitting activity after discounting and in contrast with the non-enzymic process described in the preceding section. First of all, enzymic FAD splitting was inactivated by heating, whereas the rate of FAD splitting measured in the presence of heat-inactivated enzyme equalled that measured in no-enzyme controls (Figure 2B).

The enzyme-dependent activity displayed hyperbolic kinetics in the range $0\text{--}60 \mu\text{M}$ substrate, with a K_m of $6\text{--}8 \mu\text{M}$ for FAD, whereas FAD at concentrations higher than $100 \mu\text{M}$ was inhibitory (Figure 2C). In this experiment, below $40 \mu\text{M}$ FAD, non-enzymic splitting was undetectable ($< 5\%$ of enzymic reaction, i.e. the enzyme to non-enzyme rate ratio was > 20) whereas at higher concentration it increased linearly with FAD (not shown).

The enzymic activity required Mn^{2+} , not Mg^{2+} , and displayed saturation kinetics for Mn^{2+} in the range $0\text{--}10 \text{ mM}$ MnCl_2 , with an apparent K_m of 1.3 mM (Figure 2D). In this experiment, carried out at pH 7.5 and with 1.2 mM FAD, the enzyme/non-enzyme rate ratio was 7 at 6 mM MnCl_2 , i.e. considerably higher than in Figure 2(A), because of the higher enzyme concentration used. At less than 6 mM MnCl_2 , the ratio was even higher (e.g. 27 at 1 mM MnCl_2) because the enzyme and non-enzyme reactions responded differently to lowering MnCl_2 concentrations: they showed saturation and non-saturation kinetics respectively (Figure 2D).

The pH profile of the enzymic splitting of FAD resembled that of the non-enzymic reaction with a broad pH optimum around 9 (Figure 2E); this coincidence points to the two reactions sharing some mechanistic feature. However, the two profiles were not exactly equivalent: the ratio of enzyme to non-enzyme FAD splitting decreased with increasing pH, from 2.8 at pH 7.4–7.6 to 1.5 at pH 8.5–10.

The native M_r of the protein responsible for the enzymic splitting of FAD was estimated from the elution profile of the activity in a gel-filtration experiment. The results indicate an apparent M_r of approx. 140 000 (Figure 2F).

Products of the FAD-splitting activity

The reaction products of the enzymic splitting of FAD were detected by HPLC in standard reaction mixtures (see the Materials and methods section), except that FAD concentration was 0.4 mM and alkaline phosphatase was omitted. A high concentration of FMN cyclase was used to ensure a high yield of products and a high ratio of enzyme to non-enzyme reaction. The incubation was prolonged until the completion of FAD splitting. Under these conditions, FAD was completely converted into two UV-absorbing products. In the chromatographic system

Figure 3 HPLC analyses

Left panels: FMN cyclase products studied by HPLC method 1 (A–F) or 2 (G, H). (A) HPLC profile of a mixture of standard ADP, AMP, adenosine, FMN, FAD and riboflavin. (B–F) Samples with 50 mM Tris/HCl, pH 7.5, 6 mM MnCl_2 , 0.4 mM FAD and 30 m-units/ml FMN cyclase were analysed without incubation (B); or after 20 or 70 min of incubation at 37°C (C, D respectively); (E) same as (D) but treated with alkaline phosphatase; (F) same as (D) but with FMN cyclase replaced by enzyme buffer. (G) Rechromatography of the peak with a retention time of 3.8 min in (D); (H) same as (G), except that ADP was added to the FMN cyclase mixture at the end of the incubation period (the ADP peak represents a 100% recovery of the added compound). Right panels: conversion of cFMN in acid to 5'-FMN and 4'-FMN. Purified 3.5 mM cFMN was incubated in 1 M HCl and analysed by HPLC method 1. (I) Untreated cFMN. (J) cFMN immediately after dilution in acid. (K) cFMN after 45 min in acid at 45°C . (L) Control sample of high-purity 5'-FMN (97%; Sigma cat. no. F-8399) set up 3.5 mM in 1 M HCl and analysed immediately. (M) Same as (L), analysed after 8 h at 45°C . This sample shows the slow accumulation of 4'-FMN and 3'-FMN by phosphate migration in acid [24]. Shorter incubations (not shown) indicated that 3'-FMN started to form only after 4'-FMN had accumulated. (N) Impure 5'-FMN (76%; Sigma cat. no. F-2253) dissolved in water. The major contaminants of impure 5'-FMN preparations are reported to be 4'-FMN and, less abundantly, 3'-FMN [24–26]. The arrows mark retention times of standards, except cFMN, the FMN cyclase product. The identities of all the standards were confirmed by injecting them one at a time. The unlabelled peak in (A), with a retention time of 9 min , which actually appeared in all HPLC method-1 chromatograms (A–F, I–N), was an artifact related to the programmed entry of methanol in the column in advance. Its prominence in (A), relative to the other chromatograms, was due to the use of a larger-scale expansion.

of Figures 3A–3F (HPLC method 1), they eluted with retention times of about 3.8 min and 22 min (Figures 3C and 3D). Since the one eluted first was not detectable at 430 nm , whereas the second one clearly was (in fact the 22 min peak collected was deep yellow), it was concluded that they contained the adenine and isoalloxazine moieties of FAD respectively. Non-enzymic FAD splitting yielded the same product pattern but at much lower rate (compare Figures 3D and 3F), such that more than 90% of reaction products in Figure 3(D) were of enzymic origin. All the following evidence demonstrates that the products were AMP and cyclic FMN (cFMN).

Figure 4 4',5'-cFMN, the chemical structure of the cyclic nucleotide formed by rat liver FMN cyclase

By HPLC method 1 (Figures 3A–3F), adenosine was discounted as one of the products because of its retention time of about 12 min. However, in this system, AMP and ADP were barely retained (retention times 3–4 min), tended to overlap and it was difficult to discount either of them as being identical with the 3.8 min peak product of FMN cyclase. To distinguish unambiguously between AMP and ADP, the peak with a retention time of 3.8 min in Figure 3(D) was collected and rechromatographed in a different system (HPLC method 2) in which adenosine phosphates were retained and resolved, and which allowed the clear identification of the enzyme product as AMP (Figure 3G). This was confirmed by product co-elution with authentic AMP, and by its conversion by AMP deaminase into another compound which was co-eluted with IMP by HPLC method 2 (not shown). To discount the possibility that the 3.8 min peak of Figure 3(D) could have corresponded (in part) to ADP but was not seen in the chromatogram of Figure 3(G) because of degradation to AMP, a control experiment was carried out in which ADP was added to the FMN cyclase mixture at the end of the incubation period. After collection of the material eluted at 3–5 min by HPLC method 1, and rechromatographing it by method 2, 100% of the ADP added was recovered with the expected retention time, well separated from AMP (Figure 3H).

By HPLC method 1, the yellow product of the FAD splitting, with a retention time of 22 min, was clearly different from FMN and riboflavin (Figures 3C and 3D).

When the enzymic splitting of FAD was carried out in the presence of alkaline phosphatase, as in the standard P_i -release assay, HPLC results showed the formation of adenosine (not AMP) and the same yellow product (Figure 3E), and 1 mol of P_i /mol of FAD split was detected colorimetrically (not shown). In contrast with the product pattern of FMN cyclase, FAD pyrophosphatases such as snake venom phosphodiesterase and rat liver nucleotide pyrophosphatase, after incubation in mixtures with the standard composition and conditions of FMN cyclase assays (see the Materials and methods section and Figure 3D), converted FAD into FMN and AMP in the absence of alkaline phosphatase or, after phosphatase treatment, into riboflavin and adenosine; in this case, 2 mol of P_i /mol of FAD split was measured.

An important conclusion of the above experiments was that FMN cyclase split FAD leaving one phosphatase-resistant phos-

phate bound to riboflavin, an indication that the unknown product was a cFMN. That it actually contained phosphate (1 mol/mol) was supported by comparisons of the ^1H - and ^{13}C -NMR spectra of cFMN and FMN, and it was directly demonstrated on use of cFMN as a substrate for a cyclic nucleotide phosphodiesterase activity, also identified in rat liver, which was assayed by coupling it to alkaline phosphatase and colorimetric determination of P_i (see below).

Structural characterization of the riboflavin-containing product as 4',5'-cFMN

The unknown product of FAD splitting that behaved as cFMN was purified by HPLC from reaction mixtures in which non-enzymic FAD splitting represented less than 10% of the enzymic reaction. The purified product was identified as 4',5'-cFMN (Figure 4) by UV-visible and NMR spectroscopy (Figure 5), and by HPLC analyses of the products of acid hydrolysis (Figures 3I–3N).

The UV-visible spectrum of cFMN at neutral pH was like that of FMN and riboflavin, demonstrating the presence of oxidized isoalloxazine (Figure 5A).

The ^1H - and ^{13}C -NMR spectra of cFMN (Figures 5B and 5C) were clearly related to FMN and riboflavin (Table 2, [27–29]); the resonances of their respective 7,8-dimethylisoalloxazine moieties showed no major differences. However, full assignment of the ribityl chain of cFMN required more informative, ^1H – ^1H (Figure 5E) and ^{13}C – ^1H (Figure 5F) correlated spectra. Selective irradiations of protons (not shown) were also carried out and, for proton couplings, led to the same conclusions as the ^1H – ^1H correlated spectrum. From these data, the assignment of the ribityl signals of cFMN can be explained as follows. (a) $\text{C}1'$ (δ 47.6 p.p.m.) and $\text{C}5'$ (δ 66.4 p.p.m.) showed chemical shifts similar to those of FMN (Table 2) and the inversion typical of $-\text{CH}_2$ -carbons in a DEPT experiment (Figure 5D). (b) The areas of the five proton signals at 3.9–5.0 p.p.m. indicate that they correspond to 1, 2, 1, 2 and 1 proton respectively (Figure 5B). (c) $\text{H}1'_a$ (δ 4.85 p.p.m.) was identified as the ribityl proton resonating at the lowest field under the influence of the 7,8-dimethylisoalloxazine ring, as in the case of FMN [27]. (d) The signal at 4.3 p.p.m. and part of the signal at 4.1 p.p.m. correspond to $\text{H}5'_a$ and $\text{H}5'_b$, as both are linked to $\text{C}5'$ (Figure 5F). (e) The other part of the signal at 4.1 p.p.m. corresponds to $\text{H}2'$, as it is coupled to $\text{H}1'_a$ (Figure 5E) but linked to one carbon different from $\text{C}1'$ (Figure 5F) which, at the same time, can be then identified as $\text{C}2'$ (δ 69.4 p.p.m.). (f) The signal at 4.5 p.p.m. corresponds to $\text{H}4'$ and $\text{H}1'_b$, as it is coupled to $\text{H}5'_a$, $\text{H}5'_b$ / $\text{H}2'$ and $\text{H}1'_a$ (Figure 5E). (g) The signal at 3.9 p.p.m. must then correspond to $\text{H}3'$, and it shows the expected couplings with the $\text{H}2'$ and $\text{H}4'$ signals (Figure 5E). (h) The signal at δ 72.6 p.p.m. can be assigned to $\text{C}3'$ by its linkage to $\text{H}3'$ (Figure 5F) and, finally, that at δ 75.3 p.p.m. to $\text{C}4'$.

The above assignment of cFMN nuclei did not by itself allow us to ascertain which of the ribityl carbons were linked to phosphate. However, this question was answered by comparison of cFMN with FMN. The differences between the cFMN and FMN spectra (Table 2), for the ribityl chain, supported the notion that cFMN is 4',5'-cFMN. The chemical shifts of $\text{C}1'$, $\text{C}2'$, $\text{C}3'$, $\text{H}1'_a$, $\text{H}1'_b$, $\text{H}2'$ and $\text{H}3'$ atoms of cFMN were practically the same as in FMN: each carbon was only 0.3 p.p.m. downfield and each proton 0–0.1 p.p.m. up(down)field in cFMN with respect to FMN. This is concordant with cFMN positions $1'$, $2'$ and $3'$ being non-esterified, since if any of them were esterified, the corresponding nuclei should have shown strong downfield shifts relative to the same nuclei in FMN. Compared with the

Figure 5 Spectroscopic analyses and structure of enzymically formed cFMN

(A) UV-visible spectrum in 20 mM Tris/HCl, pH 7.5. (B) 400 MHz ^1H -NMR spectrum in $^2\text{H}_2\text{O}$ with water suppression. (C) 100 MHz, proton-decoupled ^{13}C -NMR spectrum. C3' was a broad singlet; the other signals resonated as singlets. (D) Distorsionless enhancement by polarization transfer (DEPT) spectrum. (E) ^1H - ^1H correlated spectrum in the region of the ribityl H1'-H5' protons. (F) ^1H - ^{13}C correlated spectrum. Only the region with the couplings of C2'-C5' is shown. Although the C1' signal (δ 47.6 p.p.m.) failed to show coupling with both H1' protons, all the cFMN resonances were assigned (see the text). The minor proton signal at 3.5 p.p.m. is an unknown contaminant.

other carbons, C5' showed a slight (0.8 p.p.m.) and C4' a strong (4.1 p.p.m.) downfield shift in cFMN relative to FMN. The same can be said of H5'_{ab} and H4' which were shifted downfield by 0.2–0.5 p.p.m. and 0.6–0.7 p.p.m. The stronger downfield shifts of C4' and H4' were in agreement with their expected unshielding on phosphate esterification of the 4'-OH group. That C3' resonated as a broad singlet in cFMN can then be explained by a small three-bond P-O-C4'-C3' coupling. That 5'-OH is also esterified by phosphate in cFMN, as it is in FMN, was indicated because otherwise one would expect stronger shielding, and therefore upfield shifts, of C5' and H5'_{ab} in cFMN relative to FMN. That the chemical shifts for C5' and H5'_{ab} were more affected than those for C3' and H3' by the esterification of the 4'-OH by phosphate may be because in 4',5'-cFMN the 5' position is part of the cycle whereas 3' is not. Finally, the absence or insignificance of phosphorus coupling to C4' and C5' of cFMN, which resonated as singlets in contrast with the doublets shown by these nuclei in FMN, is probably related to the conformation

of the phosphate-cyclized ribityl chain of cFMN compared with the open ribityl chain in FMN, since conformation is known to determine ^{13}C - ^{31}P coupling in other cyclic nucleotides [30,31].

The identification of cFMN as 4',5'-cFMN was confirmed by acid treatment and HPLC analyses (Figures 3I–3N). cFMN was easily and completely converted into two products (Figures 3H–3J), one at 18 min, coincident with 5'-FMN, and the other at 17 min, identified as 4'-FMN by its coincidence with (i) the first detectable product of the slow phosphate migration elicited by acid treatment of 5'-FMN (Figure 3L and shorter incubations not shown) [24,25] and (ii) the major contaminant of impure 5'-FMN preparations (Figure 3M) [24–26].

Specificity of the FAD-splitting activity

4',5'-cFMN was not a by-product or a stable catalytic intermediate of the hydrolysis of FAD by an FAD pyrophosphatase. FAD was converted stoichiometrically into 4',5'-cFMN

Table 2 Chemical shifts (p.p.m.) of carbon and proton nuclei of cFMN and FMN

cFMN data correspond to the spectra of Figures 5B and 5C. FMN data were collected in the same conditions, are in agreement with published ^1H -NMR [27,29] and ^{13}C -NMR spectra [28], and are shown with the only purpose of facilitating the interpretation of cFMN spectra. bs, broad singlet; d, doublet.

Carbons			Protons		
Nucleus	cFMN	FMN	Nucleus	cFMN	FMN
7,8-Dimethylisalloxazine moiety					
C2	157.9	157.5	H6	7.42	7.40
C4	161.1	160.8	H9	7.54	7.58
C6	130.3	130.1	CH ₃ (C7)	2.20	2.19
C7	133.9	133.7	CH ₃ (C8)	2.34	2.32
C8	134.3	134.1			
C9	116.8	116.7			
C11	131.5	131.4			
C12	139.3	139.2			
C13	150.7	150.5			
C14	149.9	149.6			
CH ₃ (C7)	18.6	18.4			
CH ₃ (C8)	20.7	20.6			
Ribityl chain					
C1'	47.6	47.3	H1' _a	4.85	4.86
C2'	69.4	69.1	H1' _b	4.52–4.55	4.50
C3'	72.6 bs	72.3	H2'	4.09–4.14	4.19
C4'	75.3	71.2 d	H3'	3.94	3.83–3.92
C5'	66.4	65.6 d	H4'	4.52–4.55	3.83–3.92
			H5' _a	4.26	3.83–3.92
			H5' _b	4.09–4.14	3.83–3.92

without riboflavin phosphomonoester formation, and 4',5'-cFMN itself, tested as a substrate instead of FAD under the standard assay conditions for FMN cyclase (with 6 mM MnCl_2 ; see the Materials and methods section), was not attacked by the enzyme preparation that formed it. Thus the enzyme reaction is

$$\text{FAD} \rightarrow \text{AMP} + 4',5'\text{-cFMN} \quad (1)$$

This specificity is not recorded by the Enzyme Commission nor has it been reported previously. The partially purified enzyme did not split NAD^+ , NADH or diadenosine 5',5'''-diphosphate, nor did it show any adenylate cyclase, guanylate cyclase or FAD synthetase activity (each < 1%, except NAD^+ splitting, which was < 3% of FAD-splitting activity). Therefore a novel enzyme which should be named FAD-AMP lyase (cyclizing) or FMN cyclase may exist in rat liver.

This enzyme was inactive in the absence of added bivalent cations, although it should be stressed that the extraction and purification were carried out in 0.5 mM EDTA. The EDTA-containing cyclase, assayed with 1.2 mM FAD, was activated by millimolar concentrations MnCl_2 but not MgCl_2 . Since the total and free Mn^{2+} content of rat liver are estimated to be lower than 50 μM and 1 μM respectively [32,33], it seems that either Mn^{2+} is not the physiological activator of the enzyme or optimal conditions for (re)activation have not yet been found. Further explorations of the bivalent cation specificity or attempts to purify the FMN cyclase activity from EDTA-free extracts have not been performed.

Hydrolysis of 4',5'-cFMN by a cyclic phosphodiesterase of rat liver

4',5'-cFMN was resistant to rat liver nucleotide pyrophosphatase and snake venom phosphodiesterase, but it was hydrolysed by a

cyclic nucleotide phosphodiesterase separated from FMN cyclase in the gel-filtration step of Figure 1(A). The phosphodiesterase was adsorbed on a DEAE-cellulose column in 20 mM Tris/HCl, pH 7.5, and eluted with a 200 ml KCl gradient (0–600 mM) in buffer T, as a peak centred around 300 mM KCl (not shown). It was probably active with other cyclic nucleotides, as in the DEAE-cellulose column it was co-eluted with phosphodiesterase activity on 3',5'-cAMP and 2',3'-cAMP (about 25 and 50% of the activity with 4',5'-cFMN). The products of 4',5'-cFMN hydrolysis were 5'-FMN and riboflavin (HPLC results not shown), indicating hydrolysis to 5'-FMN and the presence of a contaminating FMN phosphomonoesterase, the activity of which was also detected using FMN as the substrate.

A novel enzymic pathway for flavins and potential roles for 4',5'-cFMN

So far, 4',5'-cFMN has been known only as a non-enzymic product of FAD in alkaline solutions [21,22] and a contaminant in commercial FAD [25]. In biological systems, there is no antecedent for 4',5'-cFMN other than 2',5'-cFMN, which occurs in *Rhizopus* molds and displays some cytotoxic activity on tumour cells [34,35]. 2',5'-cFMN is formed from riboflavin by a GTP-dependent synthetase of *Rhizopus* [36,37], a specificity different from reaction (1). Thus this work suggests a novel enzymic pathway for flavins in mammals:

The two enzyme activities involved, cyclase and phosphodiesterase, have been identified in the cytosolic fraction of rat liver homogenates, but other cellular locations cannot be ruled out, as the activities were not assayed in the particulate fraction.

The very existence of FMN cyclase, its specificity and low K_m near the intracellular free concentration of the substrate [6] demonstrate the biological relevance of 4',5'-cFMN, which is perhaps formed *in vivo* at low concentrations that so far have escaped detection.

Elucidation of the biological role(s) of 4',5'-cFMN will require further investigation, but two possibilities should be mentioned here. One is that it may be a redox flavocoenzyme. The second, revealed by comparison with other well-known cyclic nucleotides (cAMP, GMP, cADP-ribose [38,39]) also produced from co-enzymes (ATP, GTP and NAD^+) [40–42], is that 4',5'-cFMN may have a role in cell regulation as a signal molecule.

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