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Synthesis and application of isotopically labeled flavin nucleotides

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Flavin nucleotides, i.e. flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are utilized as prosthetic groups and/or substrates by a myriad of proteins, ranging from metabolic enzymes to light receptors. Isotopically labeled flavins have served as invaluable tools in probing the structure and function of these flavoproteins. Here we present an enzymatic synthesis of several radio- and stable-isotope labeled flavin nucleotides from commercially available labeled riboflavin and ATP. The synthetic procedure employs a bifunctional enzyme, *Corynebacterium ammoniagenes* FAD synthetase, that sequentially converts riboflavin to FMN and then to FAD. The final flavin product (FMN or FAD) is controlled by the concentration of ATP in the reaction. Utility of the synthesized labeled FAD cofactors is demonstrated in flavin-dependent thymidylate synthase. The described synthetic approach can be easily applied to the production of flavin nucleotide analogues from riboflavin precursors.

Keywords: flavin adenine dinucleotide; FAD synthetase; isotopic labeling; thymidylate synthase

Introduction

Flavins are incredibly versatile compounds capable of carrying out one- and two-electron redox, nucleophilic and electrophilic chemistries.[1,2] This versatility makes flavins invaluable as electron-carriers, prosthetic groups of proteins and even precursors in the biosynthesis of other biologically important molecules.[3] The most widely utilized flavins in nature are the 'nucleotide' forms of the vitamin riboflavin: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), shown in Figure 1. Here we present the synthesis of several flavin nucleotides isotopically labeled at the isoalloxazine core or the adenyl tail, and their utility in analyzing an intermediate of an enzymatic reaction. Isotopically labeled flavins and flavin analogues have been used in the past to probe the structural dynamics of light receptors,[4,5] elucidate flavin transport and metabolism in healthy and diseased cells[6,7] and gain insight into the mechanisms of flavoproteins,[8–10] among other applications. In particular, reconstitution of cytochrome P450 reductase, the redox partner of P450 enzymes, with FMN analogues shed light on the mechanism of electron transfer in this protein.[11] FAD isotopologues labeled with ^{13}C in various positions of the isoalloxazine were employed in ultrafast time-resolved infrared spectroscopy studies of a Blue Light Using Flavin (BLUF)-domain protein AppA, a photoreceptor.[4,12] These studies demonstrated the direct response of AppA protein matrix to the photoexcitation of flavin chromophore, which affords a signaling state of the protein. Isotopic labeling of the adenyl tail of FAD, on the other hand, may prove useful in the flavoprotein systems where adenine is involved in protein function, e.g. DNA photolyase in which FAD assumes an unusual bent conformation and adenine mediates the electron transfer from isoalloxazine to the thymine dimer on DNA substrate.[13,14]

FMN is traditionally generated from riboflavin via chemical phosphorylation of 5′ hydroxyl.[15,16] However, this route invariably generates isomeric monophosphates and bisphosphates, which could be challenging to remove.[17] In fact, commercially available FMN synthesized this way contains ~30% of phosphorylated impurities. Enzymatic phosphorylation by riboflavin kinase (also known as flavokinase, FMN synthetase, or ATP:riboflavin 5′-phosphotransferase), on the other hand, yields pure FMN. A second enzyme, FAD synthetase (also known as FAD pyrophosphorylase, FMN adenylyl transferase or ATP:FMN adenylyltransferase), can subsequently be used to adenylylate FMN into FAD. This enzymatic FMN → FAD conversion (typical yields 85–93%,[18,19] based on the flavin) is much more efficient than the chemical routes to FAD (reported yields ranging from 6%[20,21] to 14%,[22] based on the flavin); however, it does require preparation of an additional protein. The advantage of the synthetic approach described here lies in using a single enzyme, *Corynebacterium ammoniagenes* FAD synthetase, to selectively convert riboflavin to either FMN or FAD, by varying the reagent concentrations.

C. ammoniagenes FAD synthetase is a bifunctional enzyme that combines the activities of a riboflavin kinase and adenylyltransferase (Figure 2A). The recombinant enzyme has been overexpressed in *Escherichia coli* and purified in the past, and its steady-state kinetics have been characterized.[23] Here we use partially purified *C. ammoniagenes* FAD synthetase to produce FAD isotopically labeled at either the adenyl tail or the isoalloxazine core (Figure 2B) starting from commercially available isotopically

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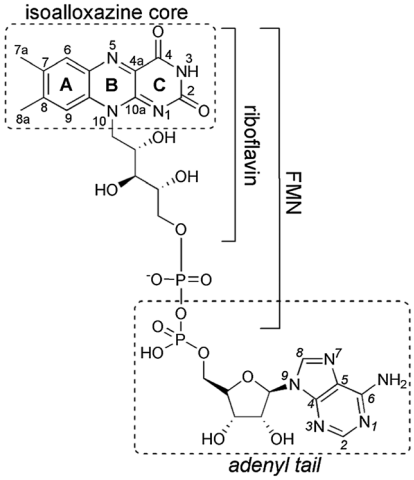

Figure 1. Structure and atomic numbering of flavin adenine dinucleotide (FAD) and shorter natural flavins.

labeled ATP or riboflavin, respectively. FMN labeled at isoalloxazine can also be obtained via this route, by using stoichiometric amounts of riboflavin and ATP in the synthetic mixture.

Following the description of the synthesis of FADs with four different labeling patterns (Figure 2B), one application is presented for the use of these labeled flavins in the mechanistic studies of an enzyme, flavin-dependent thymidylate synthase (FDTS). FDTS employs an FAD prosthetic group to reductively methylate 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP), a DNA precursor, in many human pathogens[24–27] (Figure 3). FDTS presents an exciting new target for antibiotics with low toxicity, considering that its mechanism of action differs drastically from 'classical' thymidylate synthase encoded by *thyA* gene in most organisms, including humans.[28,29] The details of FDTS chemical mechanism are still under investigation, and our recent acid-trapping of an intermediate in FDTS-catalyzed reaction has provided some insight into the timing of chemical events in this enzyme.[30] Surprisingly, a different derivative of an intermediate(s) is trapped under basic conditions, which contains the pyrimidine substrate plus an unknown adduct (Mishanina and Kohen, unpublished data). The currently reported isotopically labeled FAD molecules are employed in efforts to identify this mysterious adduct and add another piece to the mechanistic puzzle of FDTS.

Experimental

Materials

Chemicals were reagent grade and used as purchased unless specified otherwise. Unlabeled and [*dioxopyrimidine*-$^{13}C_4$,$^{15}N_2$]riboflavin, unlabeled and [$^{13}C_4$,$^{15}N_2$]ATP, phosphocreatine and creatine phosphokinase from rabbit muscle were obtained from Sigma-Aldrich. [2-^{14}C]dUMP (53.2 mCi/mmol), [2,8-^{3}H]ATP (32 Ci/mmol) and [7a,8a-^{3}H]riboflavin (6.2 Ci/mmol) were purchased from Moravek Biochemicals. The FDTS from *Thermatoga maritima* (TM0449, GenBank accession number NP228259) was expressed and purified as previously described.[31] The *E. coli* expression system for *C. ammoniagenes* FAD synthetase was a gracious gift from Prof. Dale E. Edmonson (Emory University). Magnesium chloride was purchased from BDH Chemicals, ammonium acetate, ammonium sulfate and sodium chloride from Fisher Scientific, and tris (hydroxymethyl)aminomethane [Tris] base from Research Products International Corp.

Expression and partial purification of FAD synthetase

C. ammoniagenes FAD synthetase was produced by *E. coli* following a modified procedure of ref. 23. Bacteria were grown overnight at 30 °C in 6-L Luria Broth medium containing 200 mg/L ampicillin. The cells (34 g paste) were harvested and lysed by passing the cell suspension through French press at 4 °C in Lysis Buffer [100 mL of 100 mM Tris, pH 7.45, 10 mM EDTA, 1 mM DTT, 400 mM NaCl, 20 mM $MgCl_2$, 3 mg/mL lysozyme, 0.1 mg/mL DNAase I, EDTA-free protease inhibitor pellets (Roche)]. The cell debris was removed by centrifugation at 40 000 ×*g*, and the soluble fraction was treated with solid ammonium sulfate to 50% saturation, i.e. 30.11 g solid ammonium sulfate added per 100 mL solution at 4 °C. The precipitated proteins were pelleted by centrifugation, and ammonium sulfate was added to the supernatant to 80% saturation, i.e. an additional 19.98 g solid ammonium sulfate per 100 mL solution at 4 °C. After centrifugation, the pellet containing partially purified FAD synthetase was dissolved in 36 mL of 100 mM Tris, pH 7.45, 10 mM EDTA and 1 mM DTT, and then dialyzed against 1 L of water for 1 h and finally against 1 L of 50 mM Tris, pH 7.45 overnight at 4 °C. This crude preparation (total protein concentration of ca. 2.7 mg/mL as determined by Bradford assay)[32] was used in the synthesis of flavins.

Synthesis of labeled flavins

Reactions for the synthesis of labeled FAD contained the following: 50 μM riboflavin, 8 mM $MgCl_2$, 1 mM ATP, 196 mM phosphocreatine, 800 units/mL of creatine phosphokinase in 50 mM Tris buffer, pH 7.6. The reactions were initiated by addition of 100 μL partially purified FAD synthetase (total protein concentration of ca. 2.7 mg/mL) per mL of synthetic mixture and incubated at 25 °C until completion (typically 24 h), as determined by analytical HPLC. Radiolabeled FAD synthesis was carried out in a final volume of 500 μL. For the synthesis of compound **1**, 50 μCi of tritiated ATP was used along with non-radioactive ATP (final ATP concentration 1 mM, i.e. 20-fold excess over riboflavin). Addition of unlabeled ATP was necessary to push the reaction toward FAD production. As a result, the radioactive FAD yield was quite low (~3%). However, unreacted ^{3}H-ATP could be recovered in the HPLC purification, lyophilized and re-used in additional cycles of synthesis. In the synthesis of compound **3**, 30 μCi of tritiated riboflavin was employed in addition to non-radiolabeled riboflavin (final total concentration 50 μM riboflavin). ^{13}C,^{15}N-labeled FAD was synthesized in a final reaction volume of 10 mL with ^{13}C,^{15}N-labeled ATP (compound **2**; no unlabeled ATP added to the synthesis mixture), or ^{13}C,^{15}N-labeled riboflavin (compound **4**; no unlabeled riboflavin added to the synthesis mixture).

Analytical methods

Separations were carried out on an Agilent 1100 series HPLC, with UV/vis diode array detector, flow-scintillation analyzer (FSA, model RT505 from Packard, now PerkinElmer) for radioactive synthetic mixtures, or liquid scintillation counting (LSC, Tri-Carb model 2900 TR from PerkinElmer) for base-quenched FDTS reactions containing ^{3}H-labeled FAD. An Ultima Flo™ AP scintillation cocktail (PerkinElmer) was employed in flow-scintillation analyses and Ultima Gold™ in LSC. An analytical reverse phase Supelco column (Discovery series 250 mm × 4.6 mm, 5 μm) was used at 0.8 mL/min for monitoring the progress of FAD synthetase reactions. The column was pre-equilibrated in 85:15 buffer:methanol mixture, and the following method was employed for separation of flavins (5 mM ammonium acetate buffer, pH 6.5 as solvent A; methanol as solvent B): 0–5 min 15% B; 5–25 min 15–75% B; 25–26 min 100% B. Elution of the flavins was followed by

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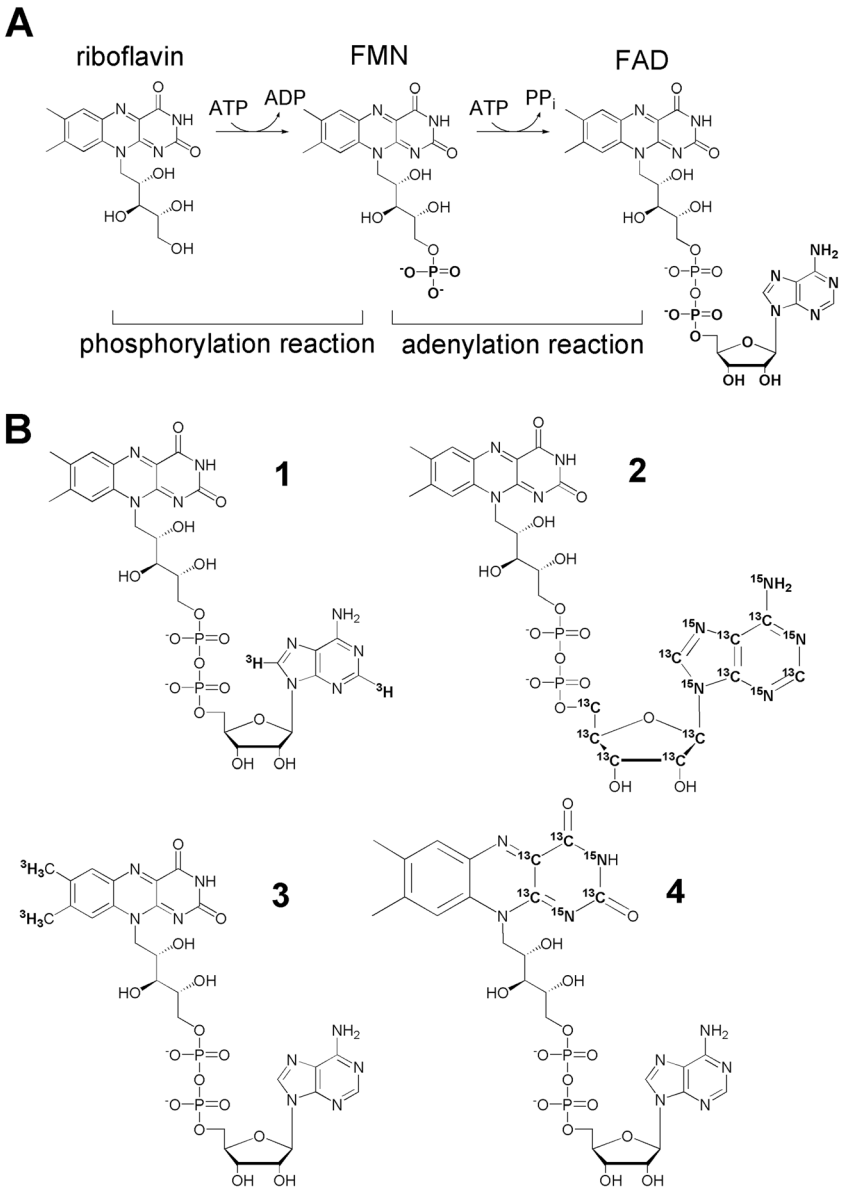

Figure 2. (A) *C. ammoniagenes* FAD synthetase-catalyzed synthesis of flavin nucleotides from riboflavin, employed in current work. (B) Structures of the isotopically labeled FAD molecules synthesized in this study.

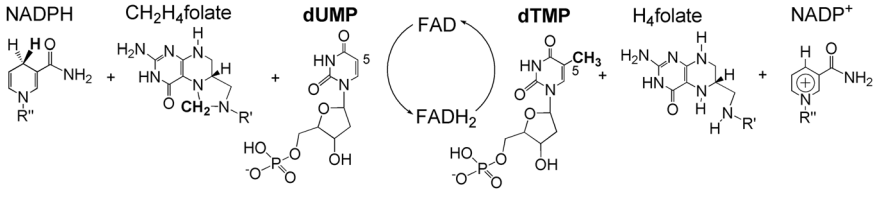

Figure 3. Reaction catalyzed by flavin-dependent thymidylate synthase. NADP(H), nicotinamide adenine dinucleotide phosphate; CH_2H_4folate, N^5,N^{10}-methylenetetrahydrofolate; H_4folate, tetrahydrofolate. R′ = (*p*-aminobenzoyl)glutamate and R″ = adenosine-5′-pyrophosphate-ribityl.

UV absorbance (at 264 and 450 nm). The concentration of riboflavin for the reactions was determined by the absorbance at 445 nm ($\varepsilon = 12\,200\ cm^{-1}\,M^{-1}$) and that of final purified FAD at 450 nm ($\varepsilon = 11\,300\ cm^{-1}\,M^{-1}$). The concentration of FDTS for rapid-quenching experiments was determined by the 454-nm absorbance of bound FAD ($\varepsilon = 11\,300\ cm^{-1}\,M^{-1}$). Electron-spray ionization mass spectrometry (ESI-MS) analysis was performed on a Waters Q-TOF mass spectrometer. Flavin samples were directly infused for mass analysis in a 1:1 water: acetonitrile solution. A −1 charge FAD ion was produced using collision energy of 4 eV. The expected shift in mass of ^{13}C,^{15}N-labeled flavins was confirmed by comparison to the mass of unlabeled commercial FAD standard, under identical analysis conditions.

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Purification of synthesized labeled flavins

The final synthesis mixture was passed through Amicon® Ultra centrifugal filter (10 000 MWCO) to remove proteins and applied to an HPLC semi-preparative reverse phase Supelco column (Discovery series 250 mm × 10 mm, 5 μm) at a flow rate of 3.2 mL/min. Elution of the flavins was monitored by 450-nm absorbance. Eluent containing FAD (~15 min) was collected, purged with argon to remove methanol, frozen and lyophilized to dryness. Care was taken to minimize exposure of the flavin to light.

ApoFDTS preparation and reconstitution with labeled FAD

Recombinant FDTS from *T. maritima* was prepared as described elsewhere.[31] The enzyme was purified with a tightly bound FAD. To remove this native FAD from the enzyme, sodium chloride solid was added to the solution of FDTS to a final concentration of 30% *w/v*. This enzyme solution was warmed to 40 °C and gently inverted to dissolve NaCl solid. The aggregated protein was pelleted by centrifugation at 13 000 rpm and 4 °C, and washed with 30% *w/v* NaCl solution until the pellet was visibly white, hence lacking bound FAD. This apoFDTS was re-suspended in 50 mM Tris buffer, pH 8.0, 1 mM EDTA and washed with Tris buffer by filtration to remove excess NaCl. ApoFDTS was then incubated with labeled FAD at 1:1 concentration ratio overnight. Binding of FAD to FDTS was corroborated by the shift of flavin λ_{max} from 450 nm (free FAD) to 454 nm (FDTS-bound FAD). This reconstituted FDTS was washed with Tris buffer to remove any unbound FAD, until the 280:454-nm absorbance ratio, indicative of protein and flavin content in the solution, was ~6. With radiolabeled FAD-FDTS, the amount of enzyme-bound ^{3}H-labeled FAD was determined by liquid-scintillation counting. The ability of the reconstituted enzyme to convert dUMP to dTMP was always tested prior to quenching experiments, and no loss in this thymidylate synthase activity was ever observed upon reconstitution.

Base-quenching of labeled FAD-FDTS reactions

Rapid-quenching experiments with flavin-labeled FDTS were carried out according to the published procedure,[30] except with 1 M NaOH as the reaction quencher. Reaction time points containing maximal accumulation of the base-trapped intermediate were analyzed by HPLC-LSC (radioactive samples) or LCMS (stable-isotope labeled samples). In the experiments with ^{3}H-labeled FAD, [2-^{14}C]dUMP substrate was used to track the base-trapped intermediate in HPLC analysis.

Results and discussion

Synthesis of labeled flavin nucleotides

In our excursion into the synthesis of labeled flavins, we were mostly interested in labeled FAD, with purpose of employing it in mechanistic studies of flavin-dependent thymidylate synthase. Adenyl- and isoalloxazine-labeled FAD molecules were each synthesized from the labeled ATP and riboflavin, respectively. The incorporation of the isotopic labels was verified by MS for ^{13}C,^{15}N-labeled flavins (Figure 4), or scintillation counting for tritiated compounds. The purity of flavins was confirmed by direct infusion into MS (for stable isotopic labelling) or re-injection on HPLC with FSA detection (for radiolabeled flavins), and the identity of synthesized compounds as FAD was corroborated by their uptake by apoFDTS to produce an active enzyme. Inclusion of ADP → ATP recycling system (phosphocreatine/creatine phosphate kinase) significantly improved enzymatic FAD yields, reaching >95% conversion of riboflavin to FAD after 24-h reactions, as

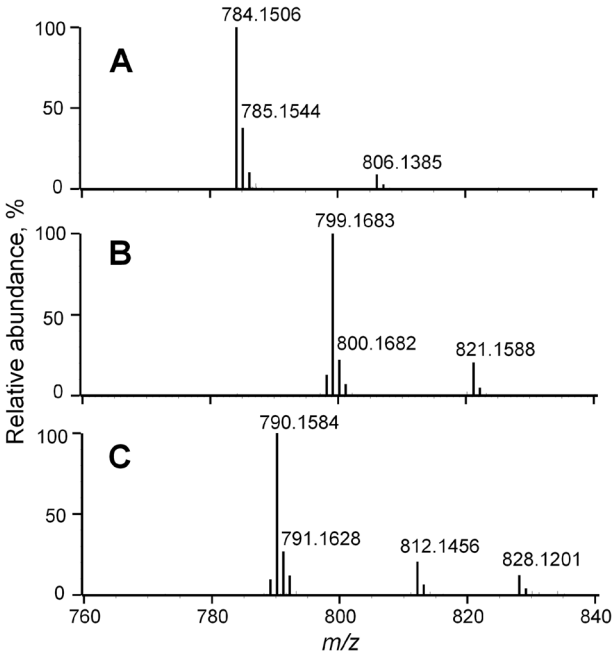

Figure 4. Negative-ion ESI-MS spectra of unlabeled FAD standard (A), purified **2** (B) and **4** (C). Note the expected mass shift of +15 for **2** and +6 for **4**. Ions in *m/z* 800 region are because of formation of sodium and potassium adducts.

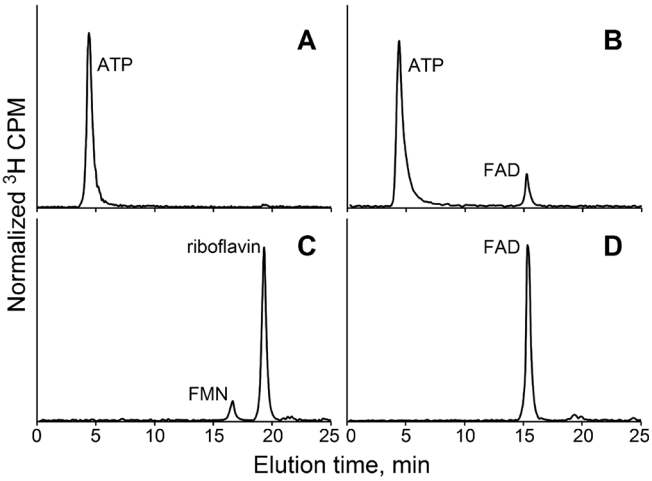

Figure 5. HPLC radiograms of reaction mixtures for the synthesis of ^{3}H-labeled FAD: **1** (*top panel*) and **3** (*bottom panel*). (A, C) Synthetic mixtures at t-zero. Note rapid formation of FMN intermediate in (C). (B, D) Reaction mixtures after 24-h incubation.

evidenced by the radiogram in Figure 5D for instance (the tritiated peak at ~19 min can be attributed to unreacted riboflavin). Large excess of ATP over riboflavin (at least 20-fold) was a key to complete conversion to FAD product. Amounts of ATP stoichiometric to riboflavin, on the other hand, halted reaction at FMN stage—conditions that may be used in the synthesis of labeled FMN. FADs with all four isotopic-labeling patterns illustrated in Figure 2B were synthesized and HPLC purified, as described in Experimental section. The final isolated FAD yields after HPLC purification were as follows: 40% for compound **1** (radiochemical yield ~3% because of large dilution of ^{3}H-ATP with unlabeled ATP, see Experimental section); 47% for compound **2**; 66% for compound **3**

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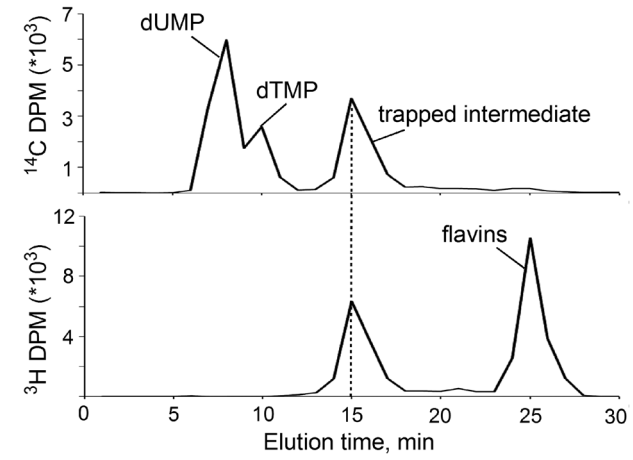

Figure 6. HPLC radiogram for the reaction of **3**-reconstituted FDTS with [2-^{14}C] dUMP quenched at 1 s with 1 M NaOH. Radioactive counts from ^{14}C (*top*) and ^{3}H (*bottom*) were determined by liquid-scintillation counting, which better separates the β-spectra of these isotopes but results in a lower elution-time resolution relative to FSA (Figure 5). The base-trapped intermediate clearly contains the ^{3}H-labeled isoalloxazine portion of FAD.

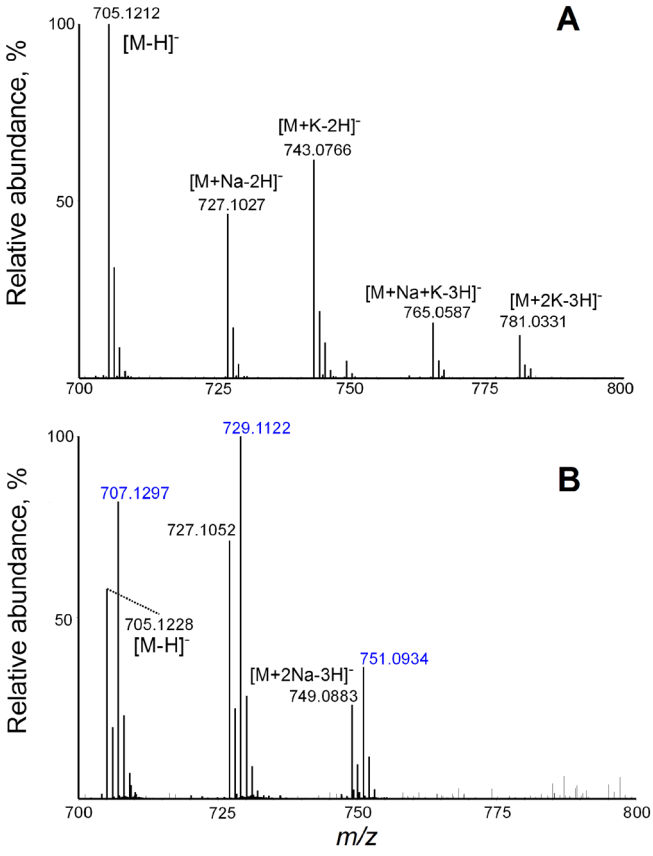

Figure 7. Negative-ion ESI-MS spectra of the trapped intermediate in the reactions with unlabeled FAD-FDTS (A) and **4**-reconstituted FDTS (B). Note the +2 Da shift in the mass of the trapped species and its sodium adducts upon labeling of the enzyme-bound flavin cofactor (B). Potassium adducts observed in panel A are because of trace amounts of potassium in the buffer used in purification of the trapped intermediate (not used in purification of the compound in panel B).

(radiochemical yield 40%) and 67% for compound **4**. Specific activities of the tritiated FAD were 0.245 and 0.9 Ci/mmol, for compounds **1** and **3** respectively.

Demonstration of the utility of labeled FAD cofactors in mechanistic studies of flavin-dependent thymidylate synthase

Our rapid acid-quench of FDTS reactions produced a trapped intermediate derivative, 5-hydroxymethyl-dUMP.[30] In a base-quenching experiment, on the other hand, a completely different species is trapped. To test whether the base-trapped intermediate contained any component of the enzyme-bound FAD, we reconstituted the enzyme with isotopically labeled FAD, using all four labeling patterns presented in Figure 2B. No shift in mass of the trapped intermediate was observed with compound **2**, and no radioactivity was found on the trapped intermediate when using compound **1**. However, FDTS reconstituted with **3** produced a tritiated trapped-intermediate species (Figure 6), and with **4** a trapped species 2 Da heavier than with unlabeled enzyme (Figure 7). These findings indicate that rings A and B of FAD cofactor (Figure 1), but not the rest of the isoalloxazine nucleus or the adenyl tail, are included in the base-trapped intermediate. This is highly unusual and of high potential impact on our understanding of FDTS mechanism, because no covalent involvement of the flavin has ever been proposed for FDTS.[28,30] The efforts are underway to elucidate the full structure of this base-trapped intermediate.

Conclusions

In light of their utility, efficient synthetic routes to the isotopically labeled flavins and analogues are of great interest. We report the successful synthesis of four different isotopically labeled FAD cofactors. Our synthetic route utilizes a bifunctional FAD synthetase, which offers control over the final flavin nucleotide product (FMN vs. FAD). The synthetic procedure described here can be easily modified to convert riboflavin analogues into their nucleotide forms.

To demonstrate their applicability to mechanistic problems, we incorporated labeled FADs into flavin-dependent thymidylate synthase and followed the integration of isotopes into the unidentified base-trapped intermediate in the FDTS-catalyzed reaction. The synthesized FAD cofactors provided critical information about the FDTS mechanism which otherwise would be challenging to obtain, i.e. that only two rings of the isoalloxazine are included in the base-trapped intermediate, while the rest of the isoalloxazine and adenyl portions are not.

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Conflict of interest

The authors did not report any conflict of interest.

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