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FAD/Folate-Dependent tRNA Methyltransferase: Flavin as a New Methyl-Transfer Agent

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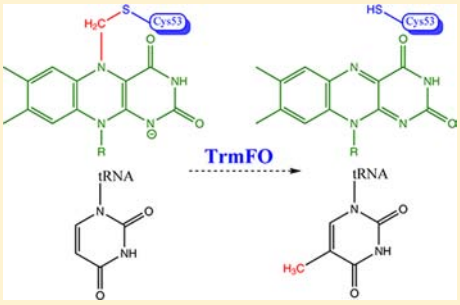
Supporting Information

ABSTRACT: RNAs contain structurally and functionally important modified nucleosides. Methylation, the most frequent RNA modification in all living organisms, mostly relies on SAM (*S*-adenosylmethionine)-dependent methyltransferases. TrmFO was recently discovered as a unique tRNA methyltransferase using instead methylenetetrahydrofolate and reduced flavin adenine dinucleotide (FAD) as essential cofactors, but its mechanism has remained elusive. Here, we report that TrmFO carries an active tRNA-methylating agent and characterize it as an original enzyme-methylene-FAD covalent adduct by mass spectrometry and a combination of spectroscopic and biochemical methods. Our data support a novel tRNA methylating mechanism.



INTRODUCTION

Methylation is the most frequent type of posttranscriptional modification found in RNAs. In tRNAs, methylated nucleotides not only stabilize the ternary L-shaped structure but also modulate the molecular interactions in which the tRNA is engaged.[1] For instance, the evolutionary conserved 5-methyluridine at position 54 (m^5U_{54}) is located in the strategic T-loop and interacts with another posttranscriptionally modified nucleotide, m^1A_{58}, thus contributing to tRNA structure stabilization. Methylation of U_{54} is generally catalyzed by a *S*-adenosyl-L-methionine (AdoMet or SAM)-dependent methyltransferase.[2] However, there are exceptions because, in certain Gram-positive bacteria, biosynthesis of m^5U_{54} was found to rely on the cellular pool of folates but not on SAM.[3] Studies on this methylating enzyme named TrmFO demonstrated that the carbon unit loaded onto m^5U_{54} derives from methylenetetrahydrofolate (CH_2THF) as in the prototypic thymidylate synthase (thyA) and that it contains an essential flavin adenine dinucleotide (FAD) cofactor.[4] Because CH_2THF donates a methylene rather than a methyl group, an additional reductive step is required, with electrons provided by a noncovalently bound reduced flavin. Accordingly, the three-dimensional structure of TrmFO from *Thermus thermophilus* ($TrmFO_{TT}$) in complex with tetrahydrofolate, one product of the reaction, shows that its pterin ring is located in close vicinity to the isoalloxazine ring of FAD.[5] Using TrmFO from *Bacillus subtilis* ($TrmFO_{BS}$) and a mini-RNA substrate containing a fluorine atom at C5 of U_{54}, we provided evidence for a reaction intermediate containing a covalent thioether linkage between Cys226 and the C6 atom of the targeted base.[6]

Surprisingly, we recently reported that as-isolated $TrmFO_{BS}$, despite containing only FAD and no CH2THF, was able to efficiently methylate a tRNA transcript in vitro (with rates 1 order of magnitude faster than the previously reported rates) in the absence of exogenous CH_2THF and NAD(P)H as reductant.[7,8] This then raised the intriguing question of the nature of the protein-bound methylating agent. In the present work, we have identified this species as an active TrmFO-methylene-flavin adduct by mass spectrometry and a variety of spectroscopic and biochemical methods, and we propose a novel tRNA methylation mechanism, in which the FAD cofactor is functioning as the methyl transfer agent.

METHODS

Protein Preparations. Recombinant $TrmFO_{BS}$ was expressed and purified as reported.[7] The C53A variant was prepared by site-directed mutagenesis as previously described.[6] The proteins were concentrated to ~500−800 μM in 50 mM sodium phosphate pH 8, 150 mM NaCl, 10% (v/v) glycerol and stored at −80 °C.

Optical Spectroscopy. All UV−visible absorption spectra were recorded from 250 to 750 nm on a Cary 50 spectrophotometer (Varian) at room temperature. $TrmFO_{BS}$ was denatured in the presence of 50 mM sodium phosphate pH 7, 4 M guanidinium-chloride. To remove the nonbound flavin from $TrmFO_{BS}$, the unfolded protein

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was subjected to several cycles of ultrafiltration on a Vivaspin 20 with a 30 kDa cutoff (Sartorius Stedim Biotech). The absorbance and fluorescence spectra of the filtrated and retained fractions were recorded after each cycle. Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation and emission slit widths of 5 nm. When the flavin fluorescence was excited at 350 and 450 nm, emission was monitored from 380 to 700 nm or from 465 to 700 nm, respectively. The excitation spectra were recorded by setting the λ_{em} at the maximum peak observed in the emission spectrum.

The effect of tRNA on the absorption spectrum of alkylated $TrmFO_{BS}$ was followed spectrophotometrically in a 1 cm path length quartz cuvette after the mixing of 37.5 μM as-isolated $TrmFO_{BS}$ in an oxygenated buffer (50 mM *N*-[2-hydroxyethyl] piperazine-*N*-[2-ethanesulfonic acid]−Na buffer (HEPES−Na, Sigma), pH 7.5, 100 mM ammonium sulfate, 0.1 mM EDTA, 25 mM mercaptoethanol (Promega), and 20% glycerol) with 7.5 μM of an in vitro yeast $tRNA^{Phe}$ transcript at room temperature. After the addition of tRNA, a spectrum was recorded every 5 min.

Mass Spectrometry. *Direct ESI−MS and MS/MS.* Mass spectrometry measurements were performed with an electrospray Q/TOF mass spectrometer (Q/TOF Premier, Waters) equipped with the Nanomate device (Advion). The HD_A_384 chip (5 μm I.D. nozzle chip, flow rate range 100−500 nL/min) was calibrated before use. For ESI−MS measurements, the Q/TOF instrument was operated in RF quadrupole mode with the TOF data being collected between m/z 300−2000 for whole proteins and between m/z 50−1000 for FAD derivatives. Collision energy was set to 6 eV, and argon was used as collision gas. The Mass Lynx 4.1 software was used for acquisition and data processing. Deconvolution of multiply charged ions was performed by applying the MaxEnt1 algorithm. The protein average masses are annotated in the spectra, and the estimated mass accuracy is ±2 Da. For MS/MS measurements, ions were automatically selected, fragmented with a 10−50 V collision ramp, and scans were accumulated for 5 min. External calibration was performed with NaI (2 μg/μL, isopropanol/H_2O 50/50, Waters) in the m/z 472−1971 mass range.

LC−MS/MS. Enzymatic protein digestion was performed with Lys-C endoprotease (Roche, 10 ng/μL in 25 mM NH_4HCO_3) overnight at room temperature. The enzyme/substrate ratio was 1/20 (w/w). Lys-C generated peptide mixtures were dried under vacuum, resuspended in aqueous solution (0.1% HCOOH), and finally analyzed with the Q/TOF Premier mass spectrometer (Waters) coupled to the nanoRSLC chromatography (Dionex) equipped with a trap column (Acclaim PepMap100C18, 75 μm i.d. × 2 cm, 3 μm) and an analytical column (Acclaim PepMapRSLCC18, 75 μm i.d. × 15 cm, 2 μm, 100 Å). The loading buffer was H_2O/CH_3CN/TFA (98%/2%/0.05%), and buffers A and B were H_2O/HCOOH (0.1%) and CH_3CN/HCOOH (0.1%), respectively. A 2−50% B gradient was set for 60 min with a flow rate of 0.3 μL/min. Data-dependent scanning was applied to generate MS/MS spectra with collision energies optimized according to ion masses and charges. Standard MS/MS acquisitions were performed on the three most intense parent ions of the previous MS scan. Raw data were processed with ProteinLynx Global Server (Waters). Standard peptide identification was achieved using the Mascot software with the following parameters: data bank recombinant wild-type and C53A-TrmFO proteins; peptide tolerance 15 ppm; fragment tolerance 0.1 Da; digest reagent Lys-C (cleavage at the C-terminal of lysine) and variable modification oxidation of methionine. The FAD-C covalently modified peptide was identified manually by searching for the FAD marker ions in the MS/MS spectra. The Maxent3 and PepSeq software (Waters) were used for data processing and peptide sequence analyses.

Methylation Activity. The tRNA m^5U54 methyltransferase activity of $TrmFO_{BS}$ was determined, as described previously,[7] using an *E. coli* [a-^{32}P]UTP-labeled $tRNA^{Ala1}$ transcript. The effects of NaCl and $MgCl_2$ on the methylation activity were determined with a final concentration of 0.2 μM of $TrmFO_{BS}$.

RESULTS

An Active Methylating Agent Is Sequestered in the Freshly Purified TrmFO. As shown in Figure S1A of the Supporting Information and in agreement with our previous results, freshly purified $TrmFO_{BS}$ was able to methylate tRNA almost quantitatively in an assay using [α-^{32}P]-labeled $tRNA^{Ala1}$ as the substrate and an excess of enzyme, thus under single-turnover conditions.[7,8] Figure S1B shows the saturation behavior of the activity with regard to enzyme concentration, indicating the formation of an active TrmFO−tRNA complex. This is consistent with the observed inhibitory effect of NaCl and $MgCl_2$, both known to modulate the stability of a protein/RNA complex (Figure S1C and S1D). These results thus demonstrate the existence of an active methylating agent within this preparation. Because no protein bound CH_2THF could be detected,[8] we investigate here whether the carbon source was fixed to FAD or to a specific amino acid of the enzyme, as in the case of RlmN and Cfr, two SAM-dependent RNA methyltransferases.[9−11]

Identification and Characterization of an Alkylated Flavin in TrmFO by UV−Visible and Fluorescence Spectroscopies. As previously reported, the UV−visible spectrum (Figure 1A) of freshly purified $TrmFO_{BS}$ contains: (i) a broad band at around 600 nm, corresponding to a neutral FAD radical;[8] (ii) a band at 444 nm assigned to the oxidized FAD cofactor; and (iii) an intense and broad band centered at 370 nm of unknown origin. Upon denaturation with 4 M guanidinium chloride, the first band disappeared with a red-shift during the course of the reaction, while the second one shifted to 450 nm, indicating radical decay and release of FAD, respectively. The 370 nm band shifted only slightly to 366 nm, without loss of absorbance. This indicated that the species responsible for the band at 370 nm is stable under denaturing conditions.

To determine whether this chromophore is free or covalently attached to $TrmFO_{BS}$, the denatured enzyme was subjected to several cycles of ultrafiltration. The UV−visible spectra of the filtrate and retained material are shown in Figure 1B. The former, with two maxima at 378 and 450 nm, is consistent with fully oxidized FAD released from the protein, whereas the latter displayed the broad band at 360 nm, indicating that the species responsible for that spectrum is a chromophore covalently attached to the polypeptide chain. This spectrum is reminiscent of that of an alkylated flavin, suggesting that the chromophore is derived from FAD. Indeed, alkylated flavins are usually characterized by absorption maxima between 325 and 365 nm and exhibit strong fluorescence emission between 440 and 490 nm.[12−16] Comparable features were observed in the fluorescence spectra of the retentate fraction of $TrmFO_{BS}$, as obtained above (Figure 1C). When excited at 350 nm, the protein displayed a strong fluorescence with a maximum at 460 nm. The excitation spectrum (λ_{em}= 460 nm) presents three transitions at 292, 350, and 362 nm with obvious similarities with the light-absorption spectrum. These fluorescence properties are fully consistent with the presence of an alkylated flavin covalently attached to $TrmFO_{BS}$.

Determination of the Chemical Structure of the Alkylated Flavin in TrmFO by Mass Spectrometry. To confirm this finding and identify the chemical structure of this adduct, denatured $TrmFO_{BS}$ was further characterized by direct nano electrospray−ionization (ESI) mass spectrometry in positive ion mode. Besides a peak with a mass of 49 473 Da

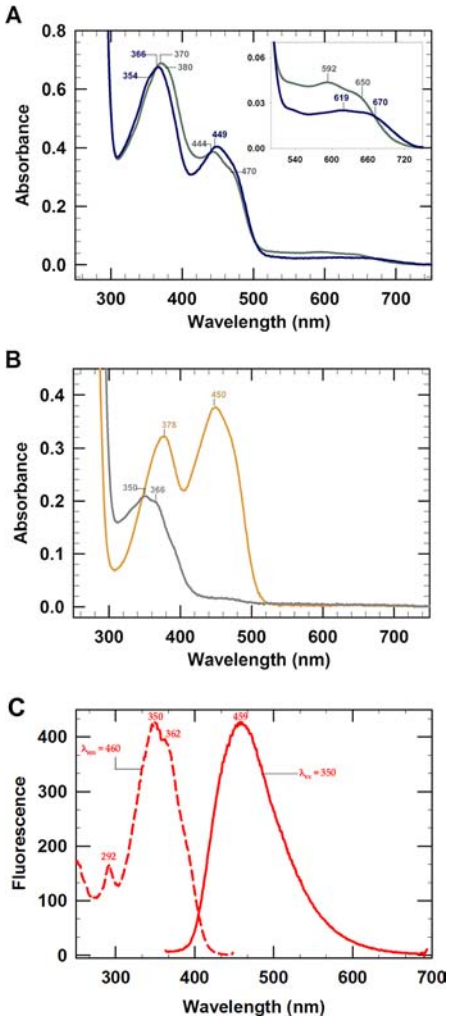

Figure 1. (A) Absorbance spectra of $TrmFO_{BS}$ in the absence (green line) or presence of 4 M guanidinium-chloride pH 7 (blue line). The inset represents an enlargement of the 480−750 nm region corresponding to the absorption band of the air-stable FAD radical. (B) Absorbance spectra of the filtrated fraction (orange) and retained fraction (gray) after ultrafiltration on a 30 kDa membrane of $TrmFO_{BS}$ denatured by 4 M guanidinium-chloride pH 7. (C) Fluorescence excitation (dashed line) and emission (solid line) spectra of the flavin−$TrmFO_{BS}$ covalent complex isolated by ultrafiltration. The excitation and emission spectra of the alkylated flavin were recorded at λ_{em} = 460 and λ_{ex} = 350 nm.

corresponding to the apoprotein, a second peak with a mass of 50 272 Da was observed (Figure 2A). The mass difference of 799 Da can be assigned to the presence of one FAD (theoretical mass of m/z 785.17) and one methylene group (CH_2, M = 14 Da) attached to the protein, which would correspond to a mass of 800 in positive ion mode. The low mass region of the spectrum (400 < m/z < 815, Figure 2B) shows a peak at m/z 786.15 consistent with the presence of FAD and a peak at m/z 800.17 corresponding to a FAD species with an additional methylene group. This spectrum also displays two additional ions at lower m/z values that differed by 14 Da at m/z 439.10 and 453.12 (Figure 2B).

These low mass ions were selected, fragmented by collision-induced dissociation (CID), and analyzed in positive ion mode (Figure 2C−F). As expected, the m/z 786.15 fragmentation spectrum (Figure 2C) was identical to that of commercially available FAD (Figure S2), with three major fragment ions corresponding to adenine (m/z 136.07, mass accuracy = 73 ppm), adenosine monophosphate (m/z 348.07, mass accuracy = 29 ppm), and ribityl monophosphate isoalloxazine (m/z 439.10, mass accuracy = 46 ppm). The fragmentation pattern of the m/z 800.17 was similar to that of FAD except for the ribityl monophosphate isoalloxazine ion, which had a mass increased by 14 units (m/z 453.11, Figure 2D), and therefore bore a methylene group. Finally, the fragmentation spectrum of the m/z 439.10 ion displayed a peak assigned to the isoalloxazine ring (m/z 243.09, mass accuracy = 7 ppm) (Figure 2E), while that of the 453.12 ion showed a peak at m/z 257.11 (mass accuracy = 7 ppm) (Figure 2F), thus 14 units greater. All of these results unambiguously show that after denaturation, $TrmFO_{BS}$ is a mixture of apoprotein and a protein fraction covalently modified with a FAD derivative containing a methylene group (FAD-C) localized on the isoalloxazine ring.

To identify the amino acid bearing the methylene-FAD derivative, the freshly purified $TrmFO_{BS}$ protein was digested with the Lys-C endoprotease, and the peptide mixtures were analyzed by liquid chromatography−electrospray-ionization−tandem mass spectrometry (LC−ESI−MS/MS) (Figure S3A and S3D). Only the Lys-C-generated $TrmFO_{48-71}$ peptide contained FAD, as shown from the presence of FAD marker ions (m/z 136.06, 348.07, and 439.09, Figure S2) in the MS/MS peptide spectra (Figure S3A). Because of the presence of the apoprotein, the nonmodified $TRMFO_{48-71}$ peptide was also found in the LC−ESI−MS/MS analysis (Figure S3B). The FAD-C modified peptide eluted later during chromatography (Figure S3C).

Accurate analysis of the MS/MS spectra revealed the presence, in addition to the standard y_{18-22} ion series, of a new unexpected y^*_{18-22} ion series with a mass increased by 12 Da (Figure S3A and S3D) observed exclusively in the spectrum of the FAD-C $TRMFO_{48-71}$ peptide. This mass shift of 12 Da is an indication of the exocyclic carbon of FAD-C left on the peptide during MS/MS fragmentation (Figure S3E). Because the +12 modification has been observed on y_{18-22} but not y_{17} (Figure S3A and S3D), we conclude that FAD-C is covalently attached to the protein through a bond between the methylene moiety and Cys53. More detailed annotation of the MS/MS spectra can be found in Figure S4.

The three-dimensional structure of $TrmFO_{TT}$ shows that Cys51, a strictly conserved residue, is less than 3 Å from both the C4α and the N5 atoms of the isoalloxazine ring of FAD (Figure S5).[5] The equivalent cysteine in $TrmFO_{BS}$, Cys53, the last residue of the $TRMFO_{48-53}$ sequence, constitutes therefore a good candidate for being the site of alkylation. In some flavoproteins, a cysteine sulfur atom forms a bond with the C4α of the flavin cofactor during catalysis.[17−19] However, these adducts are not fluorescent, and we thus exclude such a structure for the $TrmFO_{BS}$ adduct studied here. Cys53 has been replaced by an alanine in $TrmFO_{BS}$, and the resulting mutants were found to be as stable as the wild type enzyme and correctly folded, but totally inactive.[6] Interestingly, the optical and fluorescence spectra of the C53A mutant revealed the presence of oxidized FAD exclusively, and upon denaturation by either SDS or urea, FAD was instantaneously released (Figure S6). LC−MS/MS analysis of the peptides derived from the digestion of the C53A-$TrmFO_{BS}$ variant protein was performed (Figure S7). The MS/MS spectrum of C53A-$TrmFO_{48-71}$ peptide was found to lack both the FAD marker ions and the y^*_{18-22} ion series. All of these results are fully consistent with Cys53 being the site of alkylation by FAD-C.

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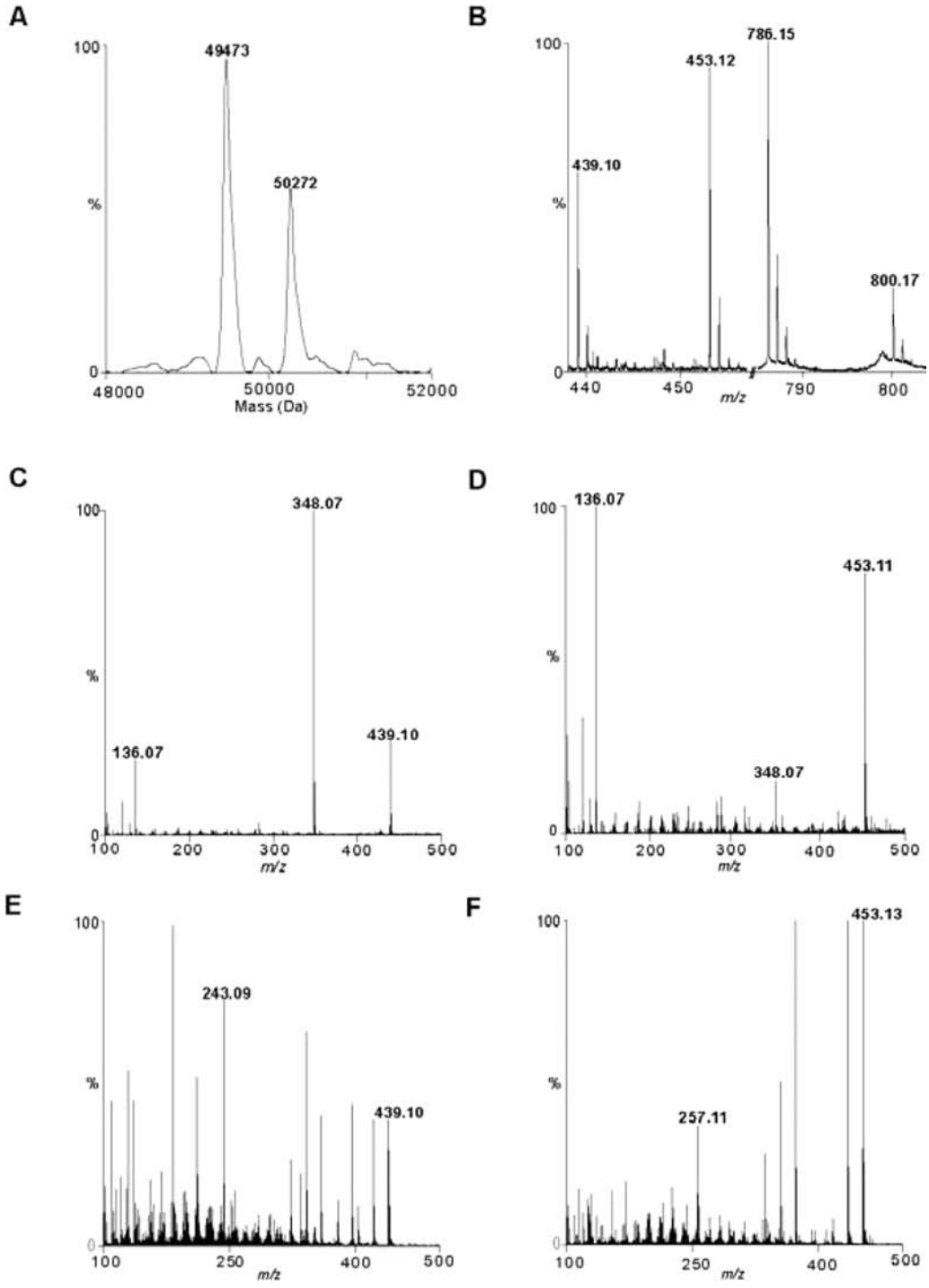

Figure 2. Mass spectrometry characterization of 20 μM freshly purified $TrmFO_{BS}$ covalently modified with a methylene-FAD derivative after denaturation for 5 min (by 50% acetonitrile, 1% formic acid). (A) Deconvoluted ESI mass spectrum of $TrmFO_{BS}$. (B) Low mass region of the ESI mass spectrum ($400 < m/z < 815$). The monoisotopic most intense peaks are annotated. Peaks at m/z 800.17 and 786.15 correspond to methylene-FAD and FAD, respectively. Peaks at m/z 453.12 and 439.10 correspond to methylene-ribityl monophosphate isoalloxazine and ribityl monophosphate isoalloxazine, respectively. (C) MS/MS spectrum of the FAD peak (m/z 786.15). Peaks at m/z 136.07, 348.07, and 439.10 correspond to adenine, adenosine monophosphate, and ribityl monophosphate isoalloxazine, respectively. (D) MS/MS spectrum of the methylene-FAD peak (m/z 800.17). Two out of the three detected ions are identical to those observed in the FAD MS/MS spectrum (m/z 136.07 and 348.07), whereas the third ion corresponds to methylene-ribityl monophosphate isoalloxazine (m/z 453.11). (E) MS/MS spectrum of the FAD ribityl monophosphate isoalloxazine peak (m/z 439.10). The peak at m/z 243.09 corresponds to the isoalloxazine ring. (F) MS/MS spectrum of the methylene-FAD ribityl monophosphate isoalloxazine peak (m/z 453.11). The peak at m/z 257.11 corresponds to the methylene-isoalloxazine.

The Alkylated Flavin Intermediate Is Consumed in the Presence of a tRNA Substrate. Indication for this species being the tRNA methylating species was provided by the following experiment. The $tRNA^{Phe}$ substrate was incubated aerobically in the presence of the as-isolated $TrmFO_{BS}$ in excess, and the reaction was monitored by UV−visible spectroscopy

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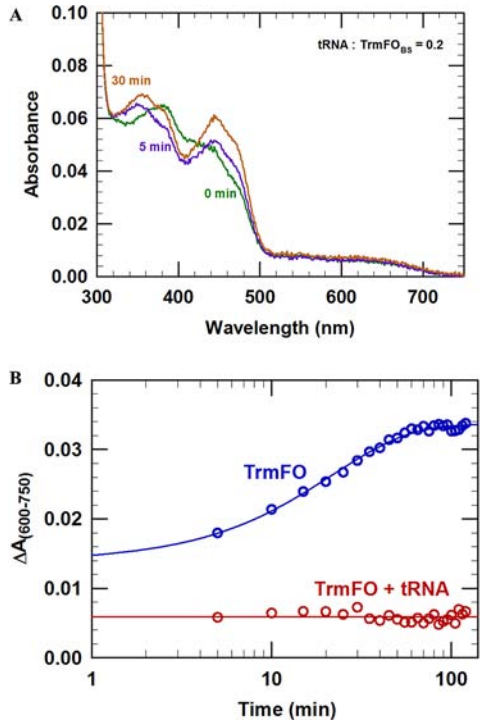

Figure 3. Effect of the presence of the tRNA^{Phe} transcript on the absorption spectrum of freshly purified TrmFO$_{BS}$ under aerobic conditions. (A) The reaction was performed in 50 mM HEPES pH 8, 150 mM ammonium acetate, 25 mM β-mercaptoethanol, 15% (v/v) glycerol, containing TrmFO$_{BS}$ and tRNA at 37.5 and 7.5 μM, respectively. The spectra shown are those recorded immediately after addition of tRNA (green), after 5 min (purple), and after 30 min (orange), respectively. (B) Kinetics of TrmFO-methylene-FAD radical formation in the presence (red) or absence (blue) of tRNA. $\Delta A_{(600-750)}$ is used to monitor the radical species. The traces show that the presence of tRNA prevents reduced TrmFO-methylene-FAD from oxidation.

(Figure 3A). Remarkably, the absorption band at 370 nm, characteristic of the TrmFO$_{BS}$-methylene-FAD adduct, disappeared rapidly, and the final spectrum was mostly that of oxidized FAD. Figure 3A and B shows that the radical species absorbing at 600 nm was not affected by the presence of tRNA, whereas, in its absence, the alkylated flavin radical accumulated as the reaction proceeded, reflecting an oxidation of the reduced FAD moiety (Figure 4A, compounds **5** and **5′**). This experiment clearly shows that the RNA substrate protects the covalent adduct from uncoupled oxidation, which yields the radical.

DISCUSSION

Our data indicate that the TrmFO$_{BS}$ preparation, which efficiently methylates tRNA in the absence of the carbon donor CH_2THF and the reducing agent NADPH,[8] is a mixture of four species: the apoprotein, the TrmFO-methylene-FAD adduct, the protein with noncovalently bound oxidized FAD cofactor, and the protein containing a neutral flavin radical. The methylation activity of such preparation is exclusively due to the methylene-FAD-TrmFO$_{BS}$ adduct, consistent with a reduced flavin being absolutely required for the reaction. All of its spectroscopic characteristics together with the mass spectrometry favor structure **5**, in which a methylene group bridges the sulfur atom of Cys53 and the isoalloxazine ring (Figure 4A). Four observations support this assignment of the structure of species **5**: First, this species absorbs light at 350−370 nm and exhibits strong fluorescence emission at 460 nm consistent with an alkylated flavin structure. Second, the chromophore is still attached to the protein under denaturing conditions. Third, detailed mass spectrometry analysis of TrmFO$_{BS}$ indicates the presence of an additional carbon on the isoalloxazine ring of FAD and demonstrates that the FAD derivative is attached to Cys53 of the protein. Fourth, mutation of Cysteine 53 into alanine results in a protein lacking both the 370 nm chromophore and the activity.

A species with a methylene group bridging the isoalloxazine ring of a flavin and a protein-derived sulfur atom has been previously observed during irradiation of the FMN-containing LOV (light oxygen and voltage sensitive) domain of a phototropin (Phot) photoreceptor mutant.[19−23] Flavin alkylation generally occurs either at N5 or at C4α of the isoalloxazine ring.[12−16,24] In the case of the Phot-LOV photoadduct, crystallographic studies showed that alkylation occurred at N5,[22] whereas a C4α adduct was reported for isopentenyl diphosphate isomerase.[16] The site of alkylation is most likely controlled in part by the relative orientations of the flavin cofactor and the alkylating agent. Even though at this stage it is difficult to conclude whether the bridging methylene moiety in TrmFO$_{BS}$ is attached to N5 or C4α, the first hypothesis is favored. Indeed, the neutral flavin radical present in TrmFO$_{BS}$, previously studied by high-field EPR and ENDOR spectroscopy and assigned as a neutral FAD radical,[8] displays unusual g values with a g_{iso} value of 2.00388, significantly higher than those reported for most of other flavoprotein systems.[25,26] It is very interesting to note that a comparably unusual g_{iso} value of 2.0039 has been found for the neutral flavin radical of the oxidized form of the Phot-LOV photoadduct, which was shown by EPR and ENDOR spectroscopies to be a protein bound N(5)-alkylated flavin species similar to structure **5′** in Figure 4A.[22,27] We thus propose that the radical in TrmFO$_{BS}$ is derived from compound **5** by a one electron oxidation and is best described as compound **5′**, a TrmFO-methylene-N(5)FAD radical with the methylene group at N5 of FAD and the radical density localized at C4α (Figure 4A). It has been suggested that the high g_{iso} value of such species originates from spin−orbital coupling due to the heavy sulfur atom interacting with the unpaired electron.[27]

Our data allow us to propose a novel mechanism for tRNA methylation by the CH_2THF- and FAD-dependent enzyme TrmFO, in which compound **5** and the corresponding methylene-iminium FAD derivative (compound **4**) play a central role (Figure 4). In the first half of the reaction, a nucleophilic attack of N5 of FAD hydroquinone onto the activated methylene of CH_2THF leads to the formation of compound **3** (Figure 4A). Nucleophilic reactivity of N5 in reduced flavin has precedents.[28−30] For example, an N5-alkylated flavin was observed in UDP-galactopyranose mutase, suggesting that a FAD-substrate adduct is a reaction intermediate. Generation of this intermediate is proposed to occur via nucleophilic attack of N5 from FAD hydroquinone onto the sugar anomeric C1 carbon as part of a typical S_N2-type substitution.[31] Next, displacement of THF generates a methylene-iminium FAD derivative (compound **4**), which is stabilized in the form of compound **5** by the attack of Cys53, thus creating a covalent bond between the protein and FAD. Compound **5** can undergo one-electron oxidation generating the stable radical **5′**.

Figure 4B shows how compound **5** transfers a methyl group to U_{54} of tRNA. Compound **5**, as a source of the reactive methylene-iminium FAD derivative (compound **4**), is attacked

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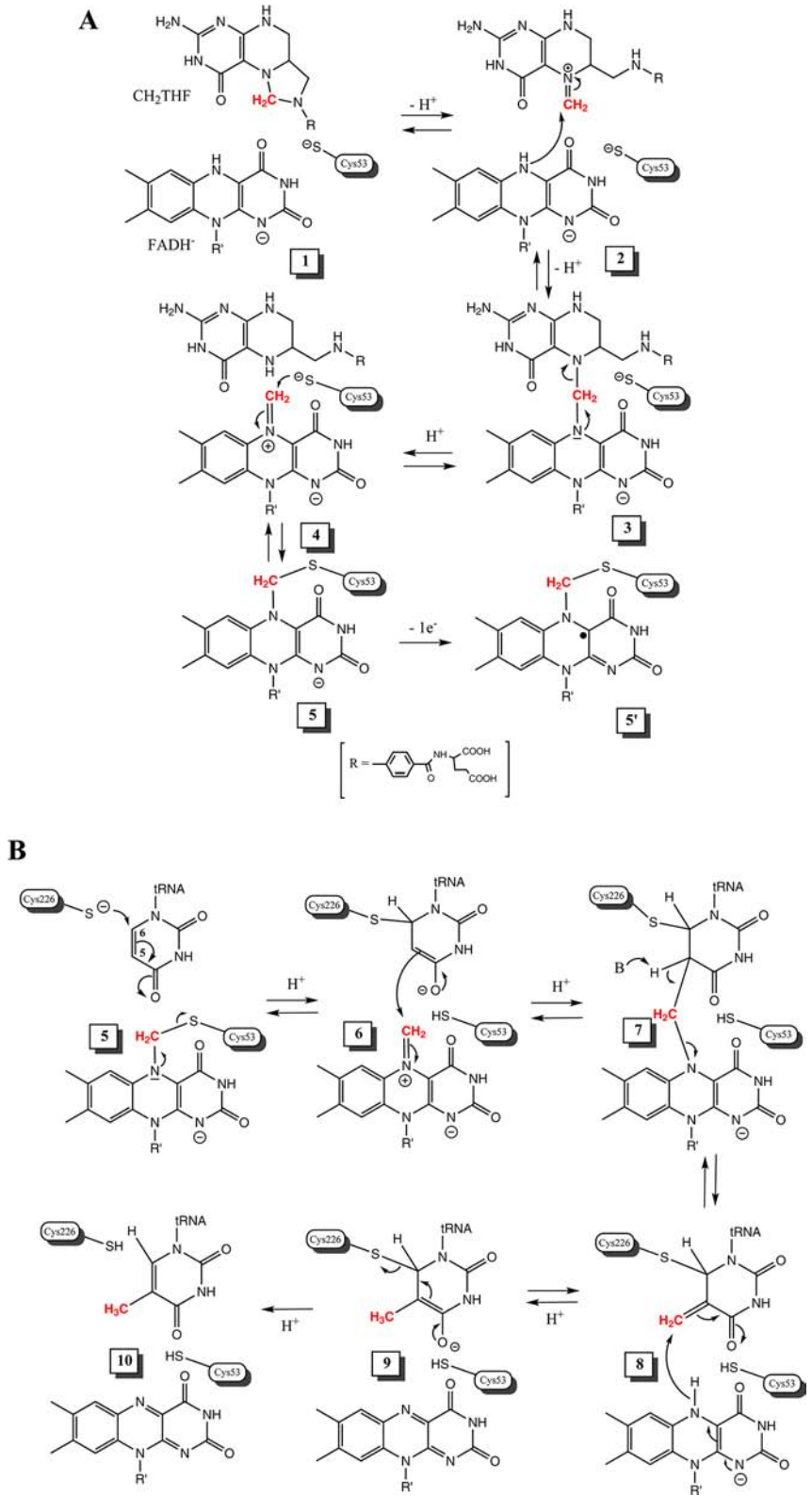

Figure 4. Postulated mechanism of TrmFO. (A) Formation of the TrmFO-methylene-FAD adduct **5**. The first step is the formation of a FAD-methylenetetrahydrofolate adduct **3**, followed by elimination of tetrahydrofolate and formation of a methylene-iminium FAD derivative **4**. Attack of Cys53 on the latter yields the TrmFO-methylene-FAD adduct **5**. The FAD radical observed in the absorption spectrum of $TrmFO_{BS}$ might be generated by the loss of one electron from **5** to lead to radical **5′**. (B) Methylation of the tRNA substrate by the TrmFO-methylene-FAD adduct. Compound **5** decomposes into the methylene-iminium FAD derivative, which is subsequently attacked by C5 of uridine, as the result of addition of Cys226 on C6 of uridine of the tRNA substrate. The product of the reaction is compound **7** with a methylene group bridging uridine and FAD. Deprotonation at C5 favors formation of an exocyclic methylene group on U54 (**8**), which is then converted to methyl (**9**) by hydride transfer from reduced FAD.

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by the negative charge at C5, resulting from the addition of Cys226 to C6 of the uridine substrate.[6] This converts a covalent bond between FAD and the protein to a covalent bond between FAD and the tRNA substrate (compound **7**). Next, as in thymidylate synthase, a base is proposed to abstract a proton at C5 of U54 leading to an exocyclic methylene (compound **8**) on the nucleic base and a two-electron reduced FAD. Hydride transfer from the latter converts the methylene into the final methyl group (compound **10**). Altogether, the mechanism in Figure 4 takes into account the existence of the partially retained species **5** during purification of TrmFO and explains its tRNA methylation activity.

This work adds a new facet to the versatile chemistry of flavins. We show here that besides functioning as electron, hydride, and oxo transfer agents,[30,32] flavins can also behave as methyl transfer cofactors in enzymes. Further investigation of this fascinating mechanism should focus on finding in vitro experimental conditions under which TrmFO can regenerate the methylating species **5** and function catalytically.

■ ASSOCIATED CONTENT

Supporting Information

Methylation activities, mass spectrometry characterization, crystal structure, and optical spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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