An enzymatic activation of formaldehyde for nucleotide methylation

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Folate enzyme cofactors and their derivatives have the unique ability to provide a single carbon unit at different oxidation levels for the de novo synthesis of amino-acids, purines, or thymidylate, an essential DNA nucleotide. How these cofactors mediate methylene transfer is not fully settled yet, particularly with regard to how the methylene is transferred to the methylene acceptor. Here, we uncovered that the bacterial thymidylate synthase ThyX, which relies on both folate and flavin for activity, can also use a formaldehyde-shunt to directly synthesize thymidylate. Combining biochemical, spectroscopic and anaerobic crystallographic analyses, we showed that formaldehyde reacts with the reduced flavin coenzyme to form a carbinolamine intermediate used by ThyX for dUMP methylation. The crystallographic structure of this intermediate reveals how ThyX activates formaldehyde and uses it, with the assistance of active site residues, to methylate dUMP. Our results reveal that carbinolamine species promote methylene transfer and suggest that the use of a CH2O-shunt may be relevant in several other important folate-dependent reactions.

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One-carbon transfer reactions are essential for the biosynthesis of many important metabolites including amino acids and nucleotides. Folate and methanopterin derivatives serve as central biological cofactors due to their ability to provide a one-carbon unit at various oxidation levels. Folate-dependent methylene transfer reactions are thought to proceed through the attack of an electrophilic iminium intermediate, derived from folate (Fig. 1) by a nucleophile, and thus yielding a methylene bridge between the cofactor and the carbon acceptor. However, the mechanism of methylene transfer mediated by these folate cofactors is not fully understood, but remained difficult to demonstrate (Fig. 1).

The reductive methylation of deoxyuridylate (dUMP) into deoxythymidylate (dTMP) is a critical step in DNA biosynthesis. In prokaryotes and some archaea, the homotetrameric flavin-dependent thymidylate synthase homotetrameric ThyX catalyzes dUMP methylation into dTMP, making ThyX mandatory for cell survival in the absence of external sources of thymidylate. ThyX relies on the flavin adenine dinucleotide (FAD) as well as N⁵,N¹⁰-methylenetetrahydrofolate (CH₂THF) as methylene donor for activity, while nicotinamide adenine dinucleotide phosphate (NADPH) acts as an electron donor. This enzyme forms a distinct class of thymidylate synthase that differs from the human thymidylate synthase TYMS or prokaryotic ThyA in terms of structure and mechanism. Indeed, the homodimeric ThyA uses CH₂THF for both the one-carbon methylene and the reducing hydride to form the C7 methyl of the dTMP product. Since ThyX is found nearly exclusively in prokaryotes (with Dictyostelium being the exception), especially in severe pathogens, it represents a promising antimicrobial target.

The mechanism of ThyX has been debated and revised many times since the enzyme’s discovery and is still unsettled. The carbon transfer reaction was initially thought to proceed via the direct transfer of a CH₃ from the folate to the dUMP substrate. However, such a mechanism became untenable after the folate binding site was identified in crystal structures, which showed that the FAD cofactor binds between the folate and dUMP in the ternary complex (Supplementary Fig. 1) and that the folate can not react directly with dUMP. An alternative mechanism suggested that the CH₃ is first transferred to an arginine residue present in the active site prior to its transfer to the dUMP. However, such a mechanism was discarded since the mutation of this arginine did not abolish ThyX’s activity. Recently, the detection of a reaction intermediate by chemical quenching, assigned to be derived from a covalent FAD-CH₃-dUMP adduct, suggested that the flavin acts as a methylene transfer agent between CH₂THF and the substrate, presumably through an iminium species. Here, we provide evidence for a flavin-carbinolamine species as the relevant methylene donor for ThyX catalysis. Combining mass spectrometry (MS), nuclear magnetic resonance (NMR), and kinetic analyses, we show that CH₃O can replace the natural methylene donor for ThyX-dependent dUMP methylation. Moreover, we uncover the molecular details of a flavin-carbinolamine species in ThyX active site by anaerobic cryocrystallography, expanding the repertoire of N₅-alkylated flavins used for biocatalysis.

Results

ThyX uses CH₃O as a direct methylene donor. We considered the possibility of using a CH₃O-shunt reaction in the catalytic cycle of ThyX. In such a scenario, FAD in its two-electron reduced FADH⁺ form could activate CH₃O leading to a flavin-carbinolamine species, which should naturally be in equilibrium with its iminium counterpart (Supplementary Fig. 2). This alternate route to generate a reactive enzyme intermediate is conceptually analogous to the hydrogen peroxide shunt employed to bypass the two-electron transfer and oxygen-binding steps in some artificial flavin, heme, and non-heme-dependent hydroxylases catalytic cycle. To substantiate this hypothesis, we tested under anaerobic conditions whether recombinant Thermotoga maritima ThyX methylates dUMP in the presence of NADPH and CH₃O, but in the absence of CH₂THF. Analysis of the products by mass spectrometry (MS) confirmed the formation of dTMP only in the simultaneous presence of ThyX, NADPH, and CH₃O (Supplementary Fig. 3). The analysis was repeated with ¹³C-labeled CH₃O and showed that the methylene incorporated in dTMP did not come from sources other than CH₃O since only [¹³C⁷]-dTMP was formed, as unambiguously detected by nuclear magnetic resonance (NMR) spectroscopy and MS analyses (Fig. 2 and Supplementary Figs. 3 and 4). Thus, ThyX is responsible for the CH₃O-dependent dUMP methylation.

Since NADPH was strictly required for dTMP formation (Supplementary Fig. 3), this suggested that an electron donor was needed consistent with a reductive methylation. To confirm this, we observed that the addition of CH₃O to the pre-reduced ThyX FADH⁻-dUMP complex, in anaerobic conditions and in the absence of NADPH, resulted in flavin oxidation and dTMP production (Supplementary Fig. 5). This establishes that the measured CH₃O-dependent ThyX activity is a reductive methylation. Indeed, stopped-flow experiments mixing ThyX FADH⁻-dUMP with CH₃O under anaerobic conditions revealed the transient formation of a flavin intermediate followed by a biphasic flavin oxidation process (Fig. 2b). The observed rate constant for the formation of this intermediate increases linearly with CH₃O concentration, consistent with a reversible bimolecular reaction ($k_{on} = 1.1 ± 0.01 M^{-1} s^{-1}$, $k_{off} = 0.022 ± 0.003 s^{-1}$, $K_D = 20 ± 3 mM$), while rate constants for flavin oxidation showed hyperbolic dependences ($K_D = 23 ± 8 mM$, $k_{ox} = 0.03 ± 0.001 s^{-1}$ and $K_D = 29 ± 10 mM$, $k_{ox} = 0.01 ± 0.001 s^{-1}$) (Fig. 2c). Taken together, these results confirmed that ThyX uses CH₃O as a direct methylene donor for dTMP synthesis and can therefore

![Fig. 1 Hydrolysis reaction of N⁵,N¹⁰-methylenetetrahydrofolate as a source of formaldehyde.](https://doi.org/10.1038/s41467-021-24756-8) The cyclic form of CH₂THF is activated by protonation leading to the iminium species (CH₂THF⁺°)). This iminium is potentially in equilibrium with its carbinolamine counterpart (HOCH₂THF⁻°). The carbinolamine intermediate can readily decompose into THF and formaldehyde (CH₂O) in a reversible reaction. CH₂O can then serve as a direct source of methylene.
substitute for CH$_2$THF by forming a flavin intermediate that could be a carbinolamine adduct. A flavin carbinolamine sustains ThyX activity. One way to firmly establish that activation of CH$_2$O by FADH$^-$ proceeds via a flavin carbinolamine would be to activate an apoprotein version of ThyX (apo-ThyX) with a synthetic flavin carbinolamine if the latter is stable enough to be used. Earlier studies reported that 1,5-dihydroflavum derivatives, used as chemical models of reduced flavin, readily react with CH$_2$O to form an N5 carbinolamine adduct easily identifiable by its light absorption features. However, this has never been tested directly with the natural coenzyme. We found that the addition of CH$_2$O to an anaerobic solution of FADH$^-$ produces a stable flavin adduct tentatively assigned to the synthetic flavin carbinolamine on the basis of its optical spectrum (Supplementary Fig. 6A). This synthetic flavin carbinolamine binds to apo-ThyX and is consumed upon anaerobic addition of dUMP leading to dTMP and oxidized FAD (Supplementary Fig. 6B), showing that flavin carbinolamine is a dUMP-methylating agent. This provides an example of a synthetic compound mimicking a potential key intermediate being active for dUMP methylation. We had previously used a similar strategy to activate a flavin- and folate-dependent transfer RNA (tRNA) methyltransferase, TrmFO, using a synthetic compound mimicking the tRNA-methylating agent, namely, a FAD-CH$_2$-cysteine adduct observed in the freshly purified enzyme.

**Structural capture of flavin carbinolamine in ThyX active site.** We used crystallography under anaerobic conditions to provide further molecular insights into CH$_2$O activation by ThyX, taking advantage of the fact that flavin carbinolamine is a relatively long-lived compound under anaerobic conditions. Well-diffracting crystals of the apo-ThyX/synthetic flavin carbinolamine complex
were obtained in a glove box. In addition, we attempted to study the reaction of reduced ThyX with CH₂O in crystalllo. For that purpose, we crystallized reduced ThyX in a glove box and then soaked the resulting crystals with CH₂O in the absence of dUMP under anaerobic conditions (FADH⁻•CH₂O). Under these conditions, we found that CH₂O diffused within ThyX active site and reacted with FADH⁻ in crystalllo, thus forming a flavin carbinolamine as ascertained by the optical spectrum of (FADH⁻•CH₂O) crystal recorded on a micro-spectrophotometer directly coupled at the X-ray beamline at the synchrotron (Supplementary Fig. 7). Structures of apo-ThyX/synthetic flavin carbinolamine and FADH⁻•CH₂O were determined at 2.8 and 2.Å resolution, respectively (Supplementary Table 1). The structures show that the homotetrameric enzyme does not undergo significant conformational changes with an overall root-mean-square deviation of 0.2 Å over 167 residues (Supplementary Fig. 8).

A clear additional density on FAD was observed in both structures, specifically in three out of four FAD molecules present in apo-ThyX/synthetic flavin carbinolamine and in two FAD molecules in FADH⁻•CH₂O (Fig. 3a, b and Supplementary Figs. 9–11). This density fits with a CH₂OH group attached to the N5 atom of the isoalloxazine ring and is thus attributed to the N5-C5a carbinolamine FAD adduct in both structures: in the first case, it is the synthetic compound, while in the second it is the product of the reaction between FADH⁻ and CH₂O in crystalllo. The carbinolamine adduct adopts a butterfly conformation, significantly bent along its C10a–C4a axis with a dihedral angle ~12° and its N5 is pyramidal, consistent with an sp³ hybridized nitrogen and alkylation of N5. In both structures, the carbinolamine groups adopt a similar axial position lying toward the surface of FAD and interact with a water molecule. In the case of the FADH⁻•CH₂O structure, an inorganic phosphate occupies the cUMP-binding site and makes an additional interaction with the carbinolamine moiety (Fig. 3a).

Flavin carbinolamine as a bona fide methylene donor. Our structures are perfectly superimposable with that of the ThyX/dUMP/folate ternary complex previously reported, with the phosphate ligand placed at a position similar to that of the phosphate group of dUMP. This allows us to provide a model of a catalytically relevant species in which the flavin carbinolamine is sandwiched between dUMP and folate (Fig. 3c). In this model structure, the methylene group of the carbinolamine adduct is located only 2.4 Å from C5-dUMP and is ideally poised for SN2 attack by the activated nucleobase to generate an intermolecular C–C bond, producing the FAD-CH₂-dUMP adduct (Supplementary Fig. 12). However, a prior reorientation of the β-hydroxyl leaving group would be mandatory to facilitate its departure during catalysis. For example, a simple clockwise or anti-clockwise rotation of this hydroxyl group would be sufficient to orient it optimally for the SN2 attack (Fig. 3d). The structure shows that the strictly conserved Tyr91 and Ser88 (Fig. 3d and Supplementary Fig. 13), previously shown to be critical for

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**Fig. 3 Activation of CH₂O by T. maritima ThyX and structural capture of the carbinolamine flavin intermediate.** a Section of the 2mFo-DFc electron density contoured at 1σ around the flavin carbinolamine of ThyX obtained by the anaerobic reaction of CH₂O with FADH⁻ (FADH⁻•CH₂O). Waters and a phosphate molecule are labeled w and P, respectively. R174 participates in dUMP activation and interacts with an active site water molecule. b Chemical structure of the N5 flavin-carbinolamine adduct. c Model of ThyX in complex with the flavin carbinolamine, dUMP, and folate. The model was obtained by superimposing the crystal structure of FADH⁻•CH₂O complex with the structure of ThyX in complex with dUMP and folate (pdb, 4gt9). d Structural model of ThyX in complex with the flavin carbinolamine, dUMP, and folate, in which the carbinolamine is properly oriented for attack by the activated C5-dUMP. This SN2 is likely assisted by two residues, S88 and Y91, which are conserved among ThyX enzymes. This model shows the different orientations adopted by these residues in two different structures of ThyX and they may assist the SN2 and departure of the β-hydroxyl leaving group of the flavin carbinolamine. The structures used for this model are: (i) ThyX FADH⁻ soaked with 20 mM CH₂O vs (ii) pdb 4gt9.
activity by site-directed mutagenesis\textsuperscript{24}, could stabilize such a conformation through hydrogen bonds (Fig. 3d). Both residues are dynamic and can adopt different conformations according to the presence of the carbinolamine adduct (Fig. 3d).

Based on the above data, we propose a revised mechanism for ThyX-dependent methylation of dUMP that uses a flavin carbinolamine as the key methylene donor as opposed to a flavin-iminium intermediate (Fig. 4). The reaction between the reduced flavin cofactor and CH$_2$THF results in the flavin carbinolamine and tetrahydrofolate (THF). The carbinolamine geometry favors an SN$_2$-like attack by dUMP, which is activated as a nucleophile by deprotonation of N3 through electrostatic interaction with R174, enhancing the enamine character of the pyrimidine moiety\textsuperscript{33}. Nucleophilic attack leads to the formation of a covalent FAD-CH$_2$dUMP adduct, previously proposed based upon the fragments obtained from rapid-quenching by a base\textsuperscript{27}, and the displacement of a water molecule. The two conserved residues, Ser88 and Tyr91, at hydrogen-bonding distance with the carbinolamine hydroxyl group may assist the SN$_2$ reaction by acting as acids and promoting the displacement of the $\beta$-hydroxyl leaving group (Fig. 3d). It is worth noting that the phenolic oxygen of Tyr91 is within hydrogen-bonding distance to a guanidinium nitrogen of Arg 90 (also conserved; Supplementary Fig. 13); the proximity of such a (presumably) positive charge could enhance the acidity of Tyr91, which might be the general acid catalyst that assists the displacement of the leaving water. This rationalizes our previous kinetic observations that \textit{T. maritima} ThyX S88A, R90A, or Y91A mutants induced a large decrease (by more than two orders of magnitude for Y91A) in the rate constant for the consumption of the flavin carbinolamine triggered by dUMP\textsuperscript{24}.

Lastly, elimination of FADH$^-$ results in the formation of a nucleotide with an exocyclic methylene group, which is subsequently reduced by FADH$^-$ into thymidine. The cycle closes with the reduction of FAD by NADPH.

**Discussion**

Our study presents the first example of the direct use of CH$_2$O as methylene donor instead of CH$_2$THF during an enzymatic methylation reaction, namely, thymidylate formation catalyzed by ThyX. The tighter binding of ThyX to CH$_2$THF ($K_D \sim 4 \mu$M\textsuperscript{34}) compared to CH$_2$O ($K_D \sim 20$ mM) confirms that methylene tetrahydrofolate acts as the biological carbon donor for ThyX, serving as a CH$_2$O carrier/transporter and thus avoiding genotoxic effects\textsuperscript{35–37}. This CH$_2$O shunt allowed us to isolate a catalytically active FAD derivative and to structurally characterize it as an N5 carbinolamine adduct bound in the active site of ThyX. This expands the known structural examples of an in crystallo capture of N5-alkylated flavin as a reactive enzyme species following the recently reported flavin-isopentenyl adduct found in a class of flavin-dependent decarboxylases and the galactopyranose linked to the FAD through a covalent bond between the anomeric C of galactopyranose and N5 of the FAD in UDP-galactopyranose mutase\textsuperscript{38–40}.

We propose here a revised mechanism for ThyX in which this flavin-carbinolamine species is the methylene donor via an acid-catalyzed S$_2$2 process that releases water and forms methylene-dUMP (Fig. 4). As this carbinolamine is theoretically in equilibrium with the corresponding flavin-iminium species (Supplementary Fig. 2), the latter could, as previously proposed, conceivably be the actual electrophile. However, this scenario is unlikely for the following reasons. First, the flavin-iminium is highly unstable and has

**Fig. 4 Proposed chemical mechanism for ThyX.** FAD is first reduced by NADPH. Then, the reduced flavin, FADH$^-$, reacts with the CH$_2$THF to form a carbinolamine flavin, which acts as the genuine methylene donor. The flavin carbinolamine can be obtained directly via a CH$_2$O-shunt reaction consisting of a reaction of FADH$^-$ with free CH$_2$O. Methylene transfer from FAD to dUMP is initiated by an SN$_2$ reaction of activated dUMP and the flavin carbinolamine, leading to water elimination and formation of a transient FAD-CH$_2$-dUMP adduct.
been shown to react with water rapidly to form the corresponding flavin carbinolamine.\(^6\) Second, efficient attack of nucleophiles on the π-system of carbonyls or imines occurs along the so-called Bürgi–Dunitz trajectory,\(^2\) with the nucleophile attacking the unsaturated carbon with an obtuse angle of ~107° with respect to the C–X bond (X being the leaving group)\(^43\). In contrast, the protonated carbon with an obtuse angle of ~107° with respect to the C

Methods

**Protein expression and purification.** ThyX from *T. maritima* was expressed in a pET11d transformed in BL21(DE3) using LB medium following induction with 1 mM Tris-HCl (pH 8) at 25 °C using a TgS Scientific SF-61DX2 KinetAssyst stopped-flow instrument that had been previously equilibrated with a glucose/glucose oxidase solution to make the internal components of the system anaerobic. A solution containing ThyX (4 µM tetramer) and dUMP was made anaerobic in a glass tonometer by cycling with vacuum and argon. The ThyX-dUMP complex was stoichiometrically reduced by titration with dithionite, loaded onto the instrument, and mixed with buffer containing formaldehyde that had been bubbled with argon to make the solution anaerobic. Reaction traces were monitored at 450 nm and were fit to sums of exponentials using KaleidaGraph (Synergy Software) to obtain observed rate constants. The plot for \(k_{	ext{obs}}\) against CH\(_2\)O concentration was fit to a line to determine \(k_{\text{cat}}\) and \(k_{\text{m}}\). The plots for \(k_{	ext{obs},\text{H}}\) and \(k_{	ext{obs},\text{D}}\) were each fit to a square hyperbola to determine the apparent \(K_{\text{m}}\) and \(k_{\text{cat}}\) for these two phases.

**Crystallography of ThyX.** All crystals were obtained by vapor diffusion in a glove box. For crystallization of apo-ThyX/synthetic 4′, the apoenzyme was freshly reconstituted with 6′ in anaerobic conditions and concentrated up to 10 mg/mL. Oligomeric ThyX was collected from the holoenzyme in 50 mM Tris-HCl pH 7.5 and 150 mM NaCl. Sodium formate was mixed with 1 µL of reservoir composed of 40% polyethylene glycol 200 (PEG 200). Colorless crystals were obtained overnight and directly frozen in liquid propane. For crystallization of 5′CH\(_2\)O, freshly reconstituted ThyX with FAD was reduced with 2 molar equivalent of dithionite and further purified by a desalting PD10 column in 50 mM Tris-HCl pH 7.5 and 150 mM NaCl. One microliter of 7 mg/mL reduced ThyX was mixed with 1 µL of reservoir solution composed of 40% PEG 200. Colorless crystals grew overnight and were soaked for 30 min in 20 mM formaldehyde in 40% PEG 200 before flash freezing in liquid propane. All diffraction data were collected on single crystals at the micro-focused PROXIMA-2 beamline at the SOLEIL synchrotron (Saint-Aubin, France) at 100 K using an Eiger X-9M. Data were indexed, processed, and scaled using XDS.\(^4\) Both structures were solved by molecular replacement using a monomer from PDB 4g9t as a search template and further refined with autoBUSTER.\(^4\) A clear density for the FAD moieties was observed in both structures with additional density on the N5 modeled as CH\(_2\)OH adduct. Chemical restraints for the carbinolamine flavin adduct were generated using ILi-gand and grade.\(^4\) The UV–visible absorption spectrum of a crystal of ThyX in complex with the flavin carbinolamine compound was recorded at 100 K at the iCOS Lab located at the ESRF in Grenoble.\(^4\)

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The raw kinetic traces used in this study are provided in the Source data file. The atomic coordinates and structure factor amplitudes for the crystal structures of ThyX-FADH–soaked with CH\(_2\)O and apo-ThyX/synthetic compound 4′ have been deposited in the Protein Data Bank (https://www.rcsb.org) under accession codes 7NDW and 7NDZ, respectively. Source data are provided with this paper.

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