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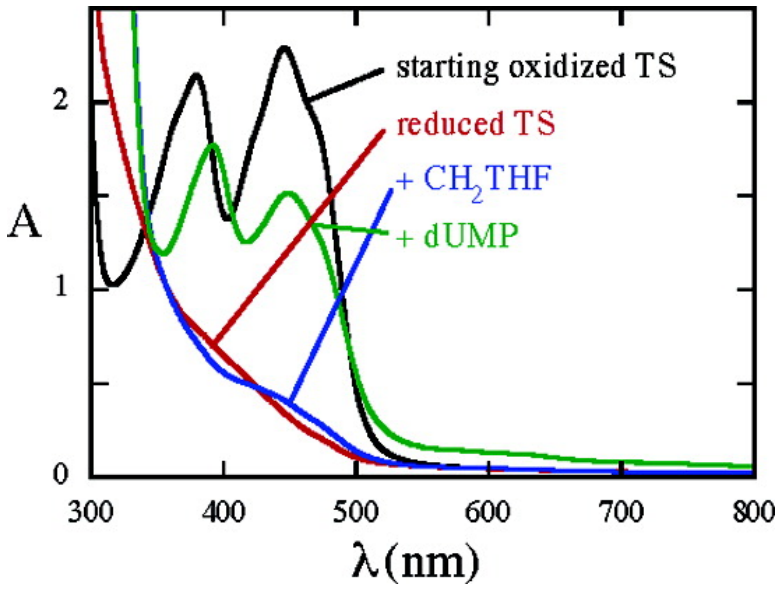

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Direct Observation of the Participation of Flavin in Product Formation by *thyX*-Encoded Thymidylate Synthase

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Thymine for DNA is synthesized by the enzyme thymidylate synthase (TS).[1] It has been shown recently that many pathogenic bacteria lack the well-known TS encoded by the *thyA* gene and instead synthesize thymidylate (dTMP) with a TS encoded by the *thyX* gene.[2] Some biochemical properties of TS encoded by the *thyX* gene have been reported, and the structure of the *Thermatoga maritima* enzyme has been determined.[3] This enzyme contains an FAD prosthetic group and requires a reducing substrate for activity,[2,3] indicating a departure from the chemistry of the TS encoded by *thyA*. The likely difference in mechanism from *thyA*-encoded TS and its occurrence in many pathogenic bacteria make *thyX*-encoded TS a promising drug target. Here we report studies on the oxidative half-reaction of the *thyX* gene product from *Campylobacter jejuni* that establish the stoichiometry of conversion of 2′-deoxyuridine monophosphate (dUMP) and 5,10-methylene-tetrahydrofolate (CH_2THF) into dTMP and tetrahydrofolate (THF), directly observe the participation of flavin adenine dinucleotide (FAD) in this reaction, and establish that the flavin is oxidized after dUMP reacts with CH_2THF.

All TSs catalyze the transfer of the methylene group of CH_2THF to the 5-position of dUMP and reduce this by two electrons to a methyl group. TS encoded by the *thyA* gene does this without the assistance of a prosthetic group or a third substrate, using folate as the source of reducing equivalents.[1] The likely role of the flavin in the reaction of the *thyX*-encoded TS is to provide reducing equivalents, derived from a reducing substrate, to produce dTMP and THF. We sought to directly observe the chemistry of the oxidative half-reaction by reacting the reduced enzyme with its substrates in the absence of oxygen. The flavin of TS[4] was reduced by titrating with one equivalent of sodium dithionite,[5] as monitored by the change in the flavin absorbance spectrum. The anaerobic addition of either CH_2THF (Figure 1) or dUMP (data not shown) to the free reduced enzyme caused a change in the absorbance spectrum, indicating ligand binding but *not* flavin oxidation. Upon adding the remaining substrate, the enzyme-bound flavin was oxidized quickly (less than a minute), demonstrating that reducing equivalents are transferred from the flavin to the substrates. Curiously, the flavin absorbance after the reaction was markedly shifted and notably lower than that of the starting enzyme. Neither exposure to oxygen nor passage through a gel filtration column altered the spectrum of this enzyme, indicating that the flavin is not in the reduced state. We do not yet know the origin of this unusual spectrum but speculate that a proportion of the oxidized flavin has formed an adduct with either the protein or the excess substrate.

The nucleotides present after an oxidative half-reaction similar to that in Figure 1 were analyzed by HPLC.[6] The reaction (not shown) of 78 μM enzyme active sites (based on FAD absorbance), upon addition of 210 μM dUMP and 294 μM CH_2THF, produced 84 μM dTMP, indicating a 1:1 reaction stoichiometry. In separate experiments, the labile folates were analyzed by first converting

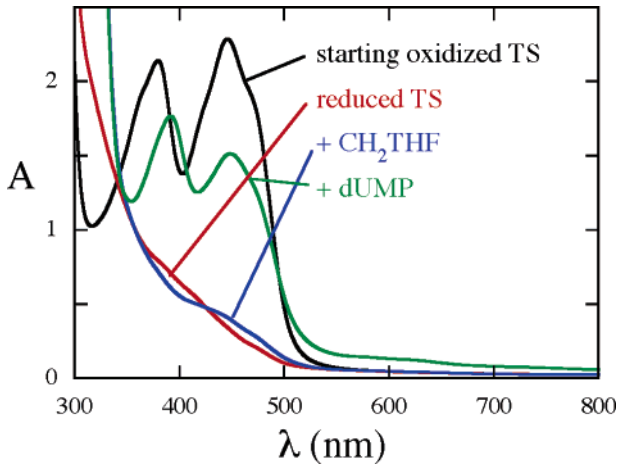

Figure 1. Oxidation of reduced enzyme by dUMP and CH_2THF. Anaerobic oxidized enzyme (209 μM) in 50 mM Tris-HCl, pH 8.0, 25 °C, was titrated with dithionite to complete reduction. The binding of CH_2THF (720 μM) was evident from its perturbation of the absorbance spectrum. The flavin was oxidized upon the addition of dUMP (840 μM).

unreacted CH_2THF to 5-methyltetrahydrofolate under anaerobic conditions,[7a] followed by treating the reaction mixture with formic acid,[7b] which converts THF (but not 5-methyltetrahydrofolate) into 5-methenyltetrahydrofolate. 5-Methenyltetrahydrofolate has a distinct absorbance peak at 350 nm,[7c] allowing its detection and quantification. The reaction of 78 μM active sites (based on FAD concentration) produced 76 μM THF, indicating a 1:1 stoichiometry for this reaction. These analytical data allow us to write a balanced equation for the oxidative half-reaction (eq 1).

$$TS_{red} + dUMP + CH_2THF \rightarrow TS_{OX} + dTMP + THF \quad (1)$$

We have also examined the inhibition of flavin-dependent TS by 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), a well-characterized inactivator of *thyA*-encoded TS.[8] Flavin dependent TS was reduced with one equivalent of dithionite and CH_2THF was added under anaerobic conditions, forming a complex (Figure 2). Anaerobic addition of FdUMP caused a small spectral change indicating binding but *not* oxidation of the flavin. After air-oxidation, the absorbance spectrum of the enzyme (Figure 2) was similar to the absorbance spectrum produced by oxidation with dUMP and CH_2THF (Figure 1). However, unlike enzyme that had been oxidized by dUMP and CH_2THF, enzyme that was treated with FdUMP as in Figure 2 was no longer able to bind dUMP after removal of small molecules by gel filtration (data not shown), indicating that FdUMP is bound to the inhibited enzyme either covalently or very tightly.

A number of mechanisms may be imagined for the involvement of FAD in producing dTMP and THF. Two chemical tasks must be accomplished by the enzyme—(1) the transfer of the one-carbon

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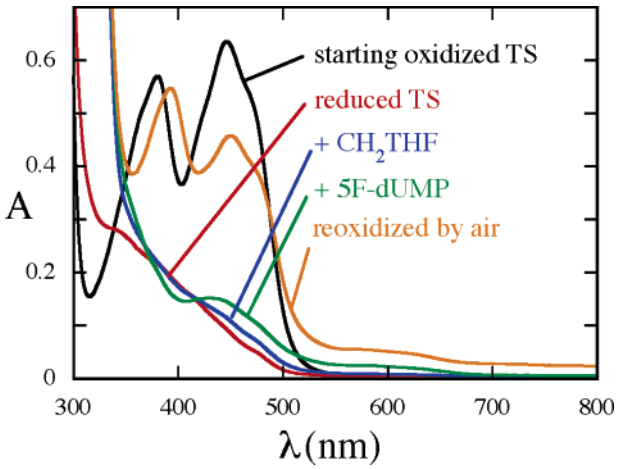
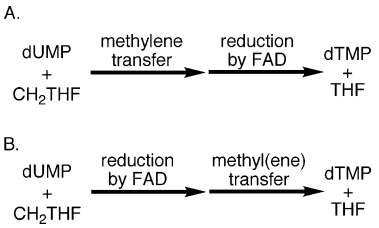


Figure 2. Lack of oxidation of reduced enzyme by CH_2THF and FdUMP. Anaerobic ligand-free oxidized enzyme (58.5 μM, marked "starting oxidized TS") in 50 mM Tris-HCl, pH 8.0, 25 °C, was titrated with dithionite to complete reduction ("reduced TS"). CH_2THF (356 μM) was added anaerobically forming a complex ("+CH_2THF"), evident from its perturbation of the absorbance spectrum. Anaerobic addition of FdUMP (529 μM) caused a perturbation of the flavin spectrum but did not result in flavin oxidation, even after 12 h. The enzyme could be reoxidized by exposure to air ("reoxidized by air").

Scheme 1. Possible Sequence of One-Carbon Transfer and Redox Reactions



fragment from the folate to the nucleotide and (2) its reduction. This could conceivably occur in either order (Scheme 1), allowing many mechanisms to be written for the reaction. FdUMP inactivates *thyA*-encoded TS because deprotonation of the 5-position is necessary for scission of an intermediate folate−nucleotide adduct. By blocking this step in the *thyX*-encoded TS, flavin oxidation has been prevented. This finding limits the number of mechanisms that are possible to those with methylene transfer occurring before redox chemistry, represented in sequence A in Scheme 1. The recent suggestion that the flavin reduces an exocyclic enone nucleotide−TS adduct formed after methylene transfer[2d] is consistent with our findings, although alternative mechanisms may also be imagined.

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(4) The *thyX* gene from *C. jejuni*[2b] was expressed in *Escherichia coli* grown on M9 medium. Enzyme synthesis was induced by the addition of IPTG (0.1 g L^{-1}) overnight at 25 °C, and bacteria were harvested by centrifugation. The cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0, with 1 mM EDTA and 0.1 mM PMSF and disrupted by sonication. After removing cell debris by centrifugation, solid ammonium sulfate was added to 35% saturation. The precipitate was removed by centrifugation, and the enzyme was precipitated with 60% saturated ammonium sulfate. The protein was dissolved in buffer and dialyzed against 5 L of buffer for 2 h. The enzyme was applied to a DEAE-Sepharose column using a 500 mL 50−200 mM linear NaCl gradient. Enzyme-containing fractions were pooled, dialyzed against 40 mM potassium phosphate, pH 7.0, 1 mM EDTA, and further purified on a Q-Sepharose column using the same NaCl gradient at pH 7.0. The enzyme was concentrated and stored as an ammonium sulfate precipitate. The extinction coefficient of enzyme-bound FAD was determined by recording the absorbance spectrum of the native enzyme in 50 mM Tris-HCl, pH 8.0, before the addition of SDS (0.1% final concentration) and after heating to 50 °C to release the flavin. The ratio of absorbances of free and enzyme-bound FAD allowed a value of 10.9 mM^{-1} cm^{-1} at 446 nm to be calculated for the enzyme.

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(6) Nucleotides were analyzed with a 4.6 mm × 150 mm C18 column using isocratic elution (0.1 M potassium phosphate, pH 5.5, 2% acetonitrile) at 1.0 mL/min with absorbance detection at 260 and 295 nm.

(7) (a) After the oxidation of enzyme-bound FAD was complete, unreacted CH_2THF was converted to methyltetrahydrofolate in situ using methylene tetrahydrofolate reductase (17 μM) from *E. coli*. A catalytic amount of NADH (2 μM) was regenerated by the action of glucose-6-phosphate dehydrogenase and 1 mM glucose-6-phosphate. (b) THF was detected by its reaction with formic acid (3.1 M) and HCl (1 M). Precipitated protein was removed from the mixture by centrifugation, and THF was converted to methenyltetrahydrofolate by heating at 80 °C for 10 min. (c) Drummond, J. T.; Jarrett, J.; González, J.; Huang, S.; Matthews, R. G. *Anal. Biochem.* **1995**, *228*, 323−329.

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