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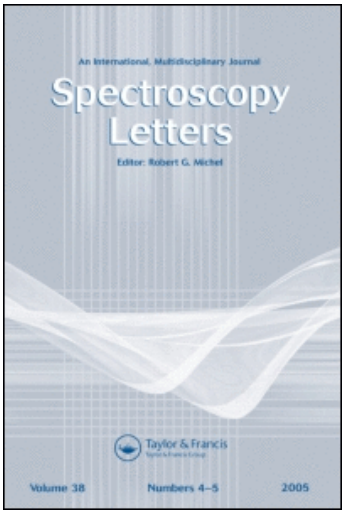

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Spectroscopic Analysis of Folate Binding to Thymidylate Synthase Active Site

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Spectroscopic Analysis of Folate Binding to Thymidylate Synthase Active Site

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ABSTRACT The fluorescence properties of folate binding to thymidylate synthase (TS) were analyzed. Two antifolates with different binding modes to the TS active site were the ligands. Intrinsic tryptophan fluorescence was used to evaluate the binding of both antifolates to the wild-type TS and a mutant *Escherichia coli* TS (K48Q) that is impaired in folate binding. During titration of wild-type TS with PDDF, tryptophan fluorescence was quenched at 330 nm, which was accompanied by an increase in emission at 379 nm, suggesting an energy transfer process from a tryptophan in the TS active site to the folate analogue. Energy transfer was not observed with the mutant TS, as expected. Tryptophan emission is a very useful tool to test for substrate-like inhibitors with biological activity.

KEYWORDS antifolate binding, mutant K48Q, protein fluorescence, thymidylate synthase

INTRODUCTION

Thymidylate synthase (TS; E.C. 2.1.1.45) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) using 5,10-methylene-5,6,7,8-tetrahydrofolate (MTHF) as cofactor. In many organisms, this is the only *de novo* source for dTMP required for DNA synthesis[1] and a reason for designing nucleotide and folate analogues as anticancer drugs.

Escherichia coli TS has two tryptophan residues (Trp80 and Trp83 in Fig. 1) in its active site, and both residues are in van der Waals contact with the cofactor. Trp80 is an invariant residue that contacts MTHF or folate analogues.[2–4] Protein fluorescence quenching has been used to monitor the binding of nucleotide and folate ligands to the TS active site, taking advantage of those Trp residues.[5–8] Moreover, fluorescence resonance energy transfer (FRET) in the fluorescence spectra of TS is important to understand folate biophysics and biochemistry. Similar to TS, DNA photolyases, a family of enzymes implicated in DNA damage repair of pyrimidine dimer formation, bind MTHF. However, folate functions in photolyases as a light-harvesting cofactor, which in turn transfers its energy to the catalytic flavin cofactor. Recently, ThyX (a new type of TS) was discovered in some bacteria and viruses, in which a flavin mediates hydride transfer from NADPH, and MTHF serves only as a carbon donor.[9,10]

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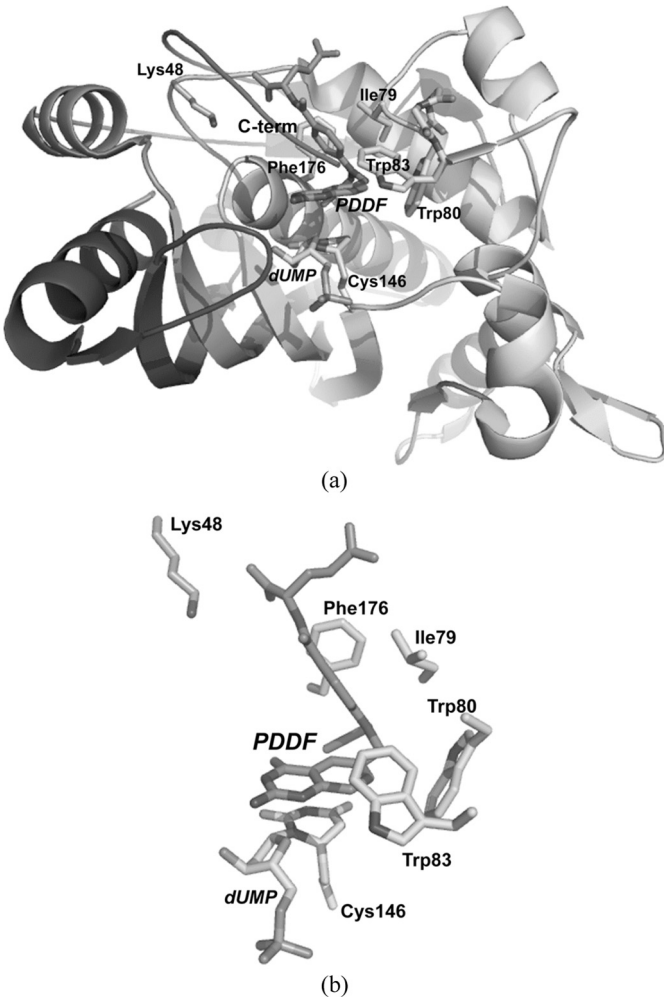

FIGURE 1 **Interaction between TS active site residues and ligands. (a) The ribbon diagram shows the protein fold. (b) A zoom-in view for specific residues interacting with the ligands.**

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In *E. coli* TS, the mutation of Lys48 to Gln (K48Q) impairs enzyme activity by destabilizing folate binding.[11] Therefore, this mutant TS is useful to compare the fluorescence properties of folate binding to wild-type TS. In this letter, we describe the fluorescence properties of two folate analogues on binding to wild-type TS and a mutant TS from *E. coli*.

MATERIALS AND METHODS

L-Trp, dUMP, and *para*-aminobenzoic acid (PABA) were purchased from Sigma (Jona, Switzerland). MTHF was purchased from Schircks Laboratories (St. Louis MO, USA). The antifolates 10-propargyl-5,8-dideazafolate (PDDF) and BW1843U89 (U89) were a gift from Dr. William Montfort of the University of Arizona. The chemical structures of dUMP, MTHF, and antifolates used are represented in Fig. 2. TS wild-type and K48Q mutant were provided by Dr. Frank Maley.

Steady-state fluorescence was monitored using a PTI QM-2003 fluorometer (Photon Technology Int. Birmingham, NJ, USA) with a xenon lamp as a light source. The excitation wavelengths were 295 or 333 nm, and emission data were collected from 300 to 500 nm or 340 to 500 nm, respectively. Measurements of Trp fluorescence were performed using 2 μM L-Trp in 20 mM potassium phosphate buffer, pH 7.5, and 10 μM folate or PABA. TS fluorescence titration experiments were done with a preformed binary complex of 1 μM TS (wild-type or mutant) and 10 μM dUMP in the same buffer. Each point for titration was prepared independently, and baseline emission from the free ligand was subtracted to obtain the final emission spectrum. The final concentration of the antifolate for titration was threefold molar excess. Quenching data were analyzed with Stern–Volmer (Eq. 1) and modified Stern–Volmer (Eq. 2) plots after correcting for inner filter effects (Eq. 3)[12] and dissociation constants (K_d) were estimated from these plots.[13]

$$\frac{F_0}{F} = 1 + Kc \tag{1}$$

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a c} + \frac{1}{f_a} \tag{2}$$

$$F_{corr} = F_{abs}\,\text{anti}\log\left(\frac{OD_{ex} + OD_{em}}{2}\right) \tag{3}$$

where F and F_0 are the fluorescence intensity in the absence and presence of the quencher, respectively, c corresponds to the concentration of the quencher, and K is a constant. ΔF is equal to $F_0 - F$, f_a is the

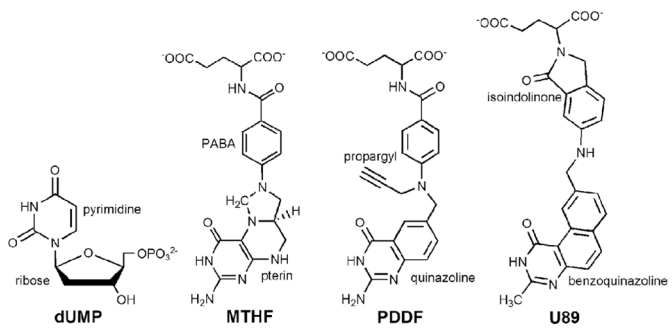

FIGURE 2 **Chemical structure of cofactor and antifolates. Note that although all three folates are glutamylated, U89 has an isoindolinone instead of a PABA ring compared with MTHF and PDDF.**

fraction of accessible fluorophores, and K_a is the association constant. In Eq. (3), F_{corr} is the fluorescence corrected for inner filter effects, F_{obs} is the observed fluorescence, and OD_{ex} and OD_{em} are the absorbance at the excitation and emission wavelengths, respectively.

RESULTS AND DISCUSSION

Ligand binding at the TS active site leads to Trp fluorescence quenching. The crystal structure of *E. coli* TS in the presence of antifolates indicates that there are two Trp residues in the active site.[1] Therefore, Trp fluorescence is a useful technique for structure–function studies.

A systematic evaluation of folate binding to TS led to identify an emission peak at 379 nm that correlated with Trp quenching (Fig. 3a). This effect did not occur for the K48Q mutant (Fig. 3b), nor for U89 with wild-type TS (Fig. 3c) or mutant TS (Fig. 3d). To determine whether TS-Trp emission was able to excite PDDF, MTHF, U89, or PABA, these compounds were excited at 333 nm (TS-Trp emission wavelength) and the emission spectra were collected. Only PDDF had an emission peak at 400 nm (Fig. 4a, dotted line). To simulate the energy transfer process in solution, a mixture of PDDF and L-Trp (instead of the enzyme) was excited at 295 nm, and the emission spectrum was collected. As expected, the Trp emission was detected at 350 nm, and a small shoulder was detected at 400 nm,

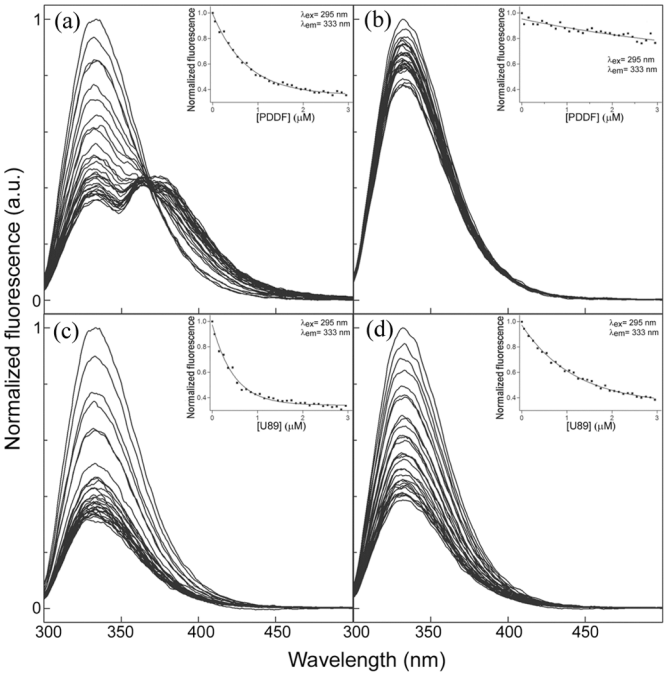

FIGURE 3 **Fluorescence spectra of wild-type and K48Q mutant TS in the presence of 0.01 mM dUMP and antifolates in the concentration range of zero to threefold molar excess. (a) Wild-type TS titration with PDDF (b) K48Q TS titration with PDDF (c) wild-type TS titration with U89, and (d) K48Q titration with U89. [enzyme] = 1 μM, λ_{exc} = 295 nm, pH = 7.5 (20 mM potassium phosphate buffer).**

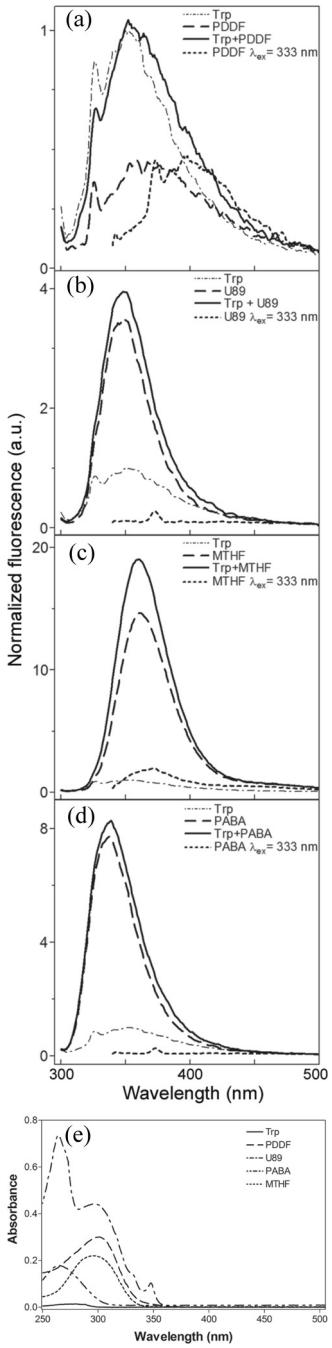

FIGURE 4 **Fluorescence intensity for solutions of L-Trp and folates. (a) L-Trp plus PDDF (b) L-Trp plus U89 (c) L-Trp plus MTHF (d) L-Trp plus PABA. The spectra are unitary at the maximum intensity of Trp emission. (e) Absorption spectra of L-Trp, PDDF, U89, MTHF, and PABA.**

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the postulated energy transfer peak from PDDF (Fig. 4a, solid line). For neither U89 (Fig. 4c) nor PABA (Fig. 4d) was this effect detected. These results suggest a specific interaction between PDDF and the TS Trp residues in the active site.

From the chemical structure of PDDF, two chromophores, quinazoline or PABA rings, may be excited by FRET. Although we were not able to obtain quinazoline compounds to analyze their interaction with L-Trp, it was observed that PABA did not fluoresce when excited at 333 nm, thus it appears that PABA is not the fluorophore excited by FRET from Trp. Therefore, the quinazoline group from PDDF has to be the FRET emissor. Furthermore, quinazoline is in van der Waals contact with the Trp residues in the TS active site, as observed in diverse crystallographic structures. In photolyases, it was observed that the orientation of folate in relation to flavin greatly increases the fluorescence quantum yield for FRET,[14,15] as we observed in TS binding.

MTHF, PDDF, and U89 are compounds that bind in the active site of TS. U89 has a distinct chemical structure that leads to distortion in the active site in the crystal structure. The results obtained in this work indicate that such a change in geometry allowed for the fluorescence quenching by FRET to occur, as the absorption spectrum of U89 overlaps with the emission spectrum of TS (Fig. 4e).

Calculation of dissociation constants is important during the screening of ligands as potential inhibitors or leads in drug design. We applied Stern–Volmer plots to analyze the fluorescence quenching of wild-type TS and K48Q mutant TS by PDDF and U89. For wild-type TS, nonlinear Stern–Volmer plots were obtained, indicating that two types of Trp fluorophores are involved, one type being accessible to the quencher (i.e., Trp80 and Trp83) and a second type that is buried or inaccessible.[12] In this case, it can be postulated that PDDF and U89 interact in solution with both Trp80 and Trp83 (Fig. 5a). To calculate the dissociation constants (Table 1), the modified Stern–Volmer plots for the wild-type quenching were used. After this modification, the data showed linearity as expected for static quenching caused by ligand binding.

However, K48Q mutant TS presented linear Stern–Volmer plots that are indicative of only one type of Trp fluorophore or a dynamic quenching process (Fig. 5b). Interestingly, the mutation site is ~10 Å

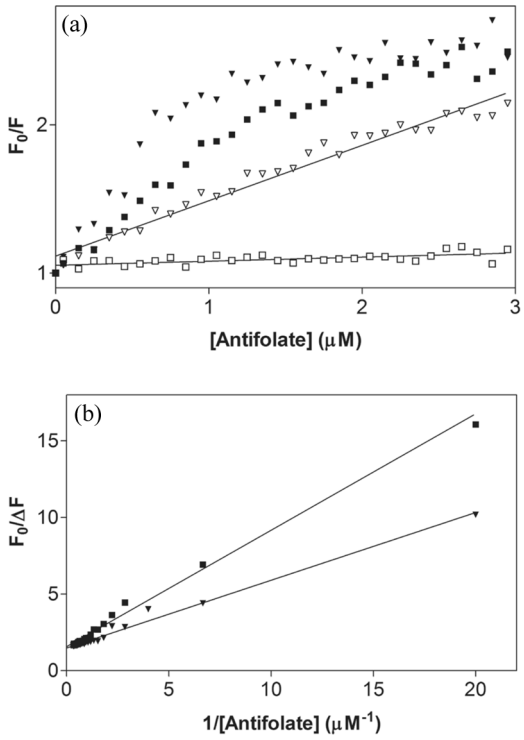

FIGURE 5 **Stern–Volmer plots for the quenching of wild-type and K48Q mutant TS. (a) F_0/F plots for the titration of wild-type TS with PDDF (■) and U89 (▼) deviate from linearity, typical for protein fluorescence quenching by binding. The K48Q mutant TS presents linear F_0/F plots in the titration with PDDF (□) and U89 (▽) (b) Modified Stern–Volmer plots for wild-type TS titration data.**

distant to the Trp sites, but modifies the binding mode of folates with respect to both Trp residues, hence altering quenching. Furthermore, the mutant TS lost the energy transfer emission peak at 379 nm when it was bound to PDDF, which is another evidence that the Trp–ligand interaction is altered in the mutant active site.[11] Binding constants were estimated from linear Stern–Volmer plots and were very similar to the dissociation constants obtained directly from isothermal titration calorimetry (Table 1).

In conclusion, fluorescence provided important information in solution about folate binding to the

TABLE 1 **Dissociation Constants for the Binding of Antifolates to Thymidylate Synthase**

		K_d (μM)	
	Antifolate	Quenching	ITC*
WT-dUMP	PDDF	0.48	0.33
K48Q-dUMP	PDDF	36.41	42.70
WT-dUMP	U89	0.29	0.05
K48Q-dUMP	U89	2.68	5.0

*K_d determined by Isothermal Titration Calorimetry.[11]

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TS active site. Screening of molecules or libraries in search of inhibitors or drug leads using Trp fluorescence quenching is feasible due to the availability of microplate instruments with diode array instrumentation. Furthermore, fluorescence stands as a key technique for the biophysicist to understand protein–ligand interactions in solution, complementing structural information such as protein crystallography or molecular modeling.

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