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# Subunit complementation of thymidylate synthase in *Plasmodium falciparum* bifunctional dihydrofolate reductase-thymidylate synthase

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**Abstract**

Thymidylate synthase of *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase (*Pf*DHFR-TS) functions as a dimeric enzyme with extensive contact between the two TS domains. Structural data of *Pf*DHFR-TS shows that the formation of the two TS active sites involves contribution of the amino acid residues from both TS domains. Arg-470 donated from the adjoining domain is shown to hydrogen-bond to dUMP, while Cys-490 is a key nucleophile for TS catalysis by attacking C-6 of dUMP. However, mutants of the two series could complement one another, giving rise to active enzyme. By means of subunit complementation assay using Arg-470 and Cys-490 mutants, it is shown that co-transformants of both TS-inactive Arg-470 and Cys-490 mutants can complement the growth of thymidine auxotroph χ2913RecA(DE3) by formation of a functional TS heterodimer contributing from both Arg-470 and Cys-490 mutant subunits. 6-[$^3$H]-FdUMP thymidylate synthase activity assay further elaborate the essence of restoration of TS activity. The TS $k_{cat}$ value of the R470D + C490A heterodimer is decreased by half from that of the wild-type *Pf*DHFR-TS. However, the $K_m$ values for dUMP and $CH_2H_4$folate of the R470D + C490A heterodimer are similar to those of wild-type enzyme, indicating that the catalytic efficiency of the functional TS from the R470D + C490A heterodimer is similar to the wild-type TS enzyme in *P. falciparum* DHFR-TS.


*Keywords:* Malaria; *Plasmodium falciparum*; Thymidylate synthase; Dihydrofolate reductase; Subunit complementation

## 1. Introduction

Thymidylate synthase (TS), a key enzyme in the metabolic cycle for deoxythymidine 5′-monophosphate (dTMP) de novo synthesis in malarial parasites, catalyzes the methylation of deoxyuridine 5′-monophosphate (dUMP) to dTMP by transferring a methylene group from a 5,10-methylenetetrahydrofolate ($CH_2H_4$folate) cofactor [1]. Dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate ($H_2$folate) in presence of NADPH to 5,6,7,8-tetrahydrofolate ($H_4$folate), which is then converted to methylenetetrahydrofolate ($CH_2H_4$folate) [1]. The inhibition of DHFR or TS disrupts dTMP synthesis and results in thymineless death of cells [2–4]. TS and DHFR exist as distinct monofunctional proteins in bacteriophages, bacteria, mammalian cells, viruses, yeast and vertebrates [1,5]. All known TS enzymes, except flavin-dependent TS, are functional as homodimers of identical subunits whereas

*Abbreviations:* *Pf*, *Plasmodium falciparum*; *Lc*, *Lactobacillus casei*; DHFR, dihydrofolate reductase; TS, thymidylate synthase; DHFR-TS, dihydrofolate reductase-thymidylate synthase; dUMP, deoxyuridine 5′-monophosphate; dTMP, deoxythymidine 5′-monophosphate; $CH_2H_4$folate, 5,10-methylenetetrahydrofolate; $H_2$folate, 7,8-dihydrofolate; ON, oligonucleotide; PCR, polymerase chain reaction; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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the DHFR is a monomer [1,6,7]. TS of protozoan parasites exists as a bifunctional enzyme which codes for TS and DHFR activity [8–11]. One of these protozoa, *Plasmodium falciparum*, causes acute malaria which is one of the major infectious diseases in the world, complicated by the emergence of highly drug-resistant parasites [12–14]. In malaria, the chemotherapy targeting DHFR initially proved to be highly effective [14–18], but resistance problems soon emerged to reduce the effectiveness. Drugs targeted to TS also have potentials [19–21], although they are still in experimental stages. It will be obviously useful to probe the TS active site in the search for potential inhibitors.

*P. falciparum* DHFR-TS (*Pf*DHFR-TS, 608 amino acids in length) is composed of two subunits of identical size (ca. 70 kDa) [9,22,23]. Each DHFR-TS subunit consists of two domains, with DHFR at the amino terminus and TS at the carboxy terminus, linked by a long (ca. 89 amino acids) junction peptide. Although the DHFR sequence exhibits low homology ranging from 20 to 35% among different organisms, *Pf*DHFR can effectively function without the need of the TS domain or the junction region (JR) [24]. In contrast, despite its highly conserved sequence, the *Pf*TS domain requires the presence of *Pf*DHFR domain and the JR as a prerequisite for its biological activity [25,26].

The crystal structure of *Pf*DHFR-TS has shown that the functional TS is a homodimer of two TS subunits, and the two TS active sites are related by C2 symmetry and located adjacent to the dimer interface [27]. Each TS active site contains amino acid residues from its neighboring monomers. Among those, Arg-470 and Arg-471 are probably involved in catalysis: the positively charged side chains of the Arg-470 and Arg-471 of the adjoining domain extend into the neighboring TS active site and forms hydrogen bonding to the phosphate group of the dUMP. From the crystal structures, Arg-470 of the *Pf*DHFR-TS [27] equivalent with Arg-178 of *Lactobacillus casei* TS (*Lc*TS) [28], is more protruded and is H-bonded to the phosphate oxygen at C′-6 of the sugar ring of the neighboring dUMP. Therefore, mutation at Arg-470 may perturb the orientation of dUMP, thereby disrupting normal catalysis. Cys-490, equivalent with Cys-198 of *Lc*TS, is important for TS catalysis by acting as a nucleophile attacking at C-6 of the uridine ring of the dUMP. Therefore, mutation at Cys-490 abolishes the biological function of TS enzyme. In *Lc*TS, it has been clearly shown that the Arg-178 and the Cys-198 mutants, each forming an inactive homodimer, can yet become biologically active by generation of active heterodimers through contribution of functional Cys-198 and Arg-178 residues provided from each mutant [29]. These active heterodimers can be detected by the use of genetic complementation selected for catalytically active TS in $Thy^-$ bacteria. Even though *Pf*TS has bifunctionality with the adjoining DHFR, the structure at TS dimer interface is quite conserved [27]. Likewise, formation of a TS-active heterodimer from the two separate TS-inactive *Pf*DHFR-TS of Arg-470 and Cys-490 mutants is hence possible. In this paper, we demonstrate that TS-subunit complementation can be achieved from the two TS-inactive Arg-470 and Cys-490 mutants derived from *Pf*DHFR-TS.

## 2. Materials and methods

### 2.1. Materials

Restriction endonucleases, DNA ligases and other DNA modifying enzymes were obtained from New England Biolabs and Promega. Plasmid isolation and purification kit was purchased from Qiagen Inc. Other chemicals and reagents used were of reagent grade. Oligonucleotide synthesis and DNA sequencing were performed at the Bioservice Unit, BIOTEC, Thailand. The 5.1 kb pJU-DHFR-TS, a pET-17b-derived plasmid carrying a K1CB1 (C59R + S108N double mutant) *Pf*DHFR-TS and ampicillin resistant genes, and ColE1 origin of replication was obtained from Jarunee Vanichtanankul and Sumalee Kamchonwongpaisan (BIOTEC, National Science and Technology Development Agency, Thailand). *Escherichia coli* χ2913RecA (Δ*thyA572*, *recA56*) (DE3) and the vector pACYC184 containing chloramphenicol and tetracycline resistance genes, and p15A origin of replication were kindly provided by Daniel V. Santi (Kosan Biosciences Company, CA) and Patricia J. Greene (University of California, San Francisco). The expression vector pET-17b, *E. coli* DH5α and *E. coli* BL21(DE3)pLysS were from Novagen.

### 2.2. Construction of *Pf*DHFR-TS gene into pACYC184 plasmid

*Pf*DHFR-TS gene was PCR amplified from pJU-DHFR-TS DNA using two oligonucleotide primers, ON1 and ON2. ON1 (5′-GCGTTGATCAAGATCTC-GATCCCGCGAA-ATTAAT-3′) contains *Bcl*I site (underlined) followed by 20 bases complementary to the direct sequence upstream of the T7 promoter, and 4 bases complementary to the T7 promoter sequence of pET-17b. ON2 (5′-ACGCGTCGAC-TTCAGCAAAAAACCCCTCAAGAACC-3′) contains *Sal*I site (underlined) followed by 5 bases complementary to the direct sequence downstream of the T7 terminator, and 19 bases complementary to the T7 terminator sequence. A reaction mixture (50 μl) contained 30 ng of pJU-DHFR-TS DNA, 50 pmol of each of the two oligonucleotide primers (ON1 and ON2), all four dNTPs at 200 μM each, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 5 units of *Taq* DNA polymerase. The amplification was carried out at 94 °C for 2 min followed by 30 cycles of 94 °C for 0.5 min, 50 °C for 0.5 min, and 72 °C for 4 min and finally an additional 72 °C for 5 min. The derived fragment of 2.1 kb complete *Pf*DHFR-TS gene was purified, digested with *Bcl*I/*Sal*I, and ligated into the corresponding sites