Formation of catalytically active cross-species heterodimers of thymidylate synthase from *Plasmodium falciparum* and *Plasmodium vivax*

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**Abstract** Thymidylate synthase (TS) of *Plasmodium* dihydrofolate reductase-thymidylate synthase (DHFR-TS) functions as a homodimeric enzyme with two active sites located near the subunit interface. The dimerization is essential for catalysis, since the active site of each subunit contains amino acid residues contributed from the other TS domain. In *P. falciparum* DHFR-TS, it has been shown that the active sites require Cys-490 from one domain and Arg-470 from the other domain. Mutants of these two series can complement one another giving rise to active enzyme. Here, the potential to form cross-species heterodimers between *P. falciparum* and *P. vivax* TS has been explored. Formation of cross-species heterodimer was tested by co-transformation of TS-inactive Cys-490 mutants of *P. falciparum* or *P. vivax* with corresponding TS-inactive Arg-486 mutants of *P. vivax* or *P. falciparum* into thymidine-requiring *Escherichia coli*. Active heterodimers were detected by subunit complementation and 6-[3H]-FdUMP binding assays. All combinations of the mutants tested, except for (Pf)R470A+(Pv)C506Y, were able to form catalytically active cross-species heterodimers. The single active site formed by (Pf)R470D+(Pv)C506Y and (Pv)R486D+(Pf)C490A pairs of cross-species heterodimers has *k*~cat~ and *K*~m~ values similar to those of intra-species heterodimers of *P. falciparum* and *P. vivax*. This is the first report to demonstrate that the TS subunit interface between *Plasmodium* species is sufficiently conserved to allow formation of fully active cross-species heterodimer.

**Keywords** Malaria · *Plasmodium falciparum* · *Plasmodium vivax* · Thymidylate synthase · Dihydrofolate reductase · Subunit complementation

**Introduction**

Human malaria is caused mainly by the *Plasmodium falciparum* parasite, while *Plasmodium vivax* is a malarial pathogen in Asia and South America [1–3]. Folate metabolism offers several targets for antimalarial chemotherapy [4–7], in which the principal drug target for many years has been dihydrofolate reductase (DHFR) [4–7]. This enzyme catalyzes the reduction of 7,8-dihydrofolate (H<sub>2</sub>folate) in the presence of NADPH to 5,6,7,8-tetrahydrofolate (H<sub>4</sub>folate), which is then converted to 5,10-methylenetetrahydrofolate (CH<sub>2</sub>H<sub>4</sub>folate) [8]. The effectiveness of drugs targeting DHFR is compromised by drug-resistant *P. falciparum* and *P. vivax* parasites [4, 9]. Thymidylate synthase (TS) plays a key role in the de novo synthesis of deoxythymidine 5'-monophosphate (dTMP) by catalyzing...
the conversion of deoxyuridine 5'-monophosphate (dUMP) and CH₂H₂folate cofactor to dTMP and H₂folate [8]. Chemical inhibition of DHFR or TS disrupts dTMP synthesis and results in thymineless cell death [10–12]; hence, parasite TS is a potential drug target in addition to DHFR. Compounds which specifically inhibit *P. falciparum* TS have been described [13–16], although they have not yet been developed as drugs. It will be obviously useful to investigate the parasite TS enzyme further in the search for novel inhibitors.

Based on amino acid sequence alignment and crystal structures, TS is one of the most conserved enzymes with a highly conserved structural core [17–22]. The dimer interface of TS is composed primarily of a six-stranded β-sheet which forms part of the conserved core. Although at the superficial level TS function is the same between parasites and the human host, as in other biochemical processes such as snRNA modification [23], the TS enzymes of protozoan parasites, such as *P. falciparum*, *P. vivax*, *Leishmania major*, *Trypanosoma cruzi*, *Toxoplasma gondii* and *Cryptosporidium hominis*, are unusual in that they exist as a bifunctional enzyme with TS and DHFR activities [17, 24–28]. The DHFR-TS enzyme subunits of *P. falciparum* and *P. vivax* are similar in size (608 and 624 amino acids respectively) [17, 24]. Each DHFR-TS subunit is composed of two domains, with DHFR at the amino terminus and TS at the carboxy terminus, linked by a long junction region (JR) (91 and 98 amino acids in length for *P. falciparum* and *P. vivax* respectively). Plasmodial DHFR can function as a monofunctional protein separate from the TS and JR domains [24, 29]. In contrast, despite its highly conserved sequence, the TS domain requires the presence of the divergent DHFR and JR domains as a prerequisite for its biological activity [30, 31]. The precise role of the divergent DHFR and JR domains in TS activity is not known, since the structural data are incomplete for these regions [17].

As in TS from most organisms, *Plasmodium* TS is a dimer of identical subunits, and the two TS active sites are located adjacent to the dimer interface [17]. The dimeric structure is required for enzyme activity since each of the two active sites contains amino acid residues contributed by the other subunit. For example, the positively charged side chains of Arg-470 and Arg-471 from one subunit of *P. falciparum* TS bind to the phosphate group of the substrate dUMP in the active site of the other subunit. Although the core TS dimer interface is highly conserved among Plasmodial species, it remains to be shown whether the divergent DHFR and JR domains are sufficiently conserved to allow formation of functional cross-species TS heterodimers.

The fact that TS is active only as a dimer allows for design of a simple test of whether *P. falciparum* and

**Fig. 1** Schematic representation of cross-species subunit complementation of TS in *P. falciparum* and *P. vivax* DHFR-TS. Inactive TS-R470 mutant of *P. falciparum* and inactive TS-C506 mutant of *P. vivax*, denoted in black, can combine to form a TS heterodimer with a single intact active site sharing *P. falciparum* and *P. vivax* contribution and a doubly defective active site. Similarly, inactive TS-R486 mutant of *P. vivax* and inactive TS-C600 mutant of *P. falciparum* can also combine to form a catalytically active TS heterodimer.

*P. vivax* TS enzymes can form cross-species heterodimers. It has been previously shown that catalytically active intraspecies heterodimers can be formed when certain types of TS-inactive mutant enzymes are combined using in vivo genetic complementation as reported first in *Lactobacillus casei* [32] and later in *P. falciparum* [33]. For *P. falciparum*, subunits of TS-inactive Arg-470 and Cys-490 mutant enzymes can combine to form an active heterodimeric enzyme with a single active site [33]. Similarly, if inactive subunits of *P. falciparum* and *P. vivax* TSs could dimerize to form a competent active site (Fig. 1), the presence of the cross-species heterodimer could also be monitored by the acquisition of catalytic activity using in vivo genetic complementation. The TS-inactive pairs of enzymes in this study were selected so that one member of the pair had an active site mutation at (Pr)Arg-470 [or (Pv)Arg-486], while the other had a mutation at (Pv)Cys-506 [or (Pr)Cys-490]. The homodimer of either mutant is inactive, but they have an intact Arg or Cys to donate to the other species' subunit (Fig. 1). In this paper, we demonstrate that cross-species TS subunit complementation of *P. falciparum* and *P. vivax* can be achieved from complementing pairs of TS-inactive Arg and Cys mutant enzymes.
Materials and methods

Materials

Restriction endonucleases, DNA ligases and other DNA modifying enzymes were obtained from New England BioLabs. Plasmid purification kits were purchased from Qiagen Inc. Other chemicals and reagents were of reagent grade. [6-3H]-FdUMP (5-fluoro-2'-deoxuryridine-5'-monophosphate) (15 Ci/mmol) was from Moravek Biochemicals. 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) P. falciparum DHFR-TS (pDHFR-TS) and ampicillin resistant genes, and CoE1 origin of replication was kindly provided by Jarnee Vanichthananukul and Sumalee Kamchonwongpaisan (BIOTEC, Thailand). The 4.9 kb pMC-DHFR-TS, a pACYC184-derived plasmid carrying a K1CB1 (C59R+S108N double mutant) pDHFR-TS gene, chloramphenicol resistant gene and p15A origin of replication, and TS-inactive mutants of pDHFR-TS at positions Arg-470 and Cys-490 were constructed as described previously [33]. The 5.1 kb pET-PvDHFR-TS, a pET-17b-derived plasmid carrying P. vivax DHFR-TS (pDHFR-TS) and ampicillin resistant genes, and CoE1 origin of replication was constructed as described [24]. Escherichia coli χ2913RecA (AthA572, recA560)(DE3) and the expression vector pET-17b, E. coli strains DH5α, BL21(DE3) pLysS, and BL21(DE3) were from Novagen.

Construction of pACET coexpression plasmid

The DNA sequences between upstream of T7 promoter and downstream of T7 terminator of pET-17b plasmid vector was PCR amplified from pET-17b DNA using two primers as mentioned previously [33]. The reaction mixture and PCR condition, except for the use of pET-17b as DNA template, 25 cycles of amplification and extension step of 72°C for 1 min, were performed as described previously [33]. The derived fragment of 355 bp DNA sequence was purified, digested with BsrII/SalI, and ligated into the corresponding sites of vector pACYC184 (2.8 kb) to obtain pACET (3.2 kb). The pACET plasmid thus carries the same T7 promoter sequence as pET-17b, allowing protein coexpression from compatible plasmids. The pDHFR-TS gene was obtained from the digestion of pET-PvDHFR-TS DNA with NdeI and XhoI, then purified and ligated into the corresponding sites of pACET to obtain pACET-PvDHFR-TS (5.0 kb).

Preparation of two separate mutants at Arg-486 and Cys-506 of PvDHFR-TS

TS mutations of PvDHFR-TS at residues 486 and 506 were carried out individually using the QuikChange™ site-directed mutagenesis method (Stratagene). The Arg-486 mutants were prepared from pACET-PvDHFR-TS whereas the Cys-506 mutants were from pET-PvDHFR-TS. Two sets of oligonucleotide pairs, set I and set II, were designed for C506A/D/N/T and C506F/H/L/P/S/Y, respectively following the same rationale as for the corresponding mutation at Cys-490 of PpDHFR-TS [33]. For R486A/D/N/T and R486F/H/L/P/S/Y, two sets of oligonucleotide pairs, set I: 5'-AATGAACCAACAGGT(AG)(AC)TAGAAATATAATTTGTTGTGCTAGAATTTGCTCA(GT)ACTCTTCACTTTCTATTCTTCTCATT-3' and 5'-ATTCATGCGACAAATATTATCTCAGTACTTATGTTGGTCTATT-3', and set II: 5'-AATGAACCAACAGGT(AC)TAGAAATATAATTTGTTGTGCTAGAATTTGCTCA(GT)ACTCTTCACTTTCTATTCTTCTCATT-3' and 5'-ATTCATGCGACAAATATTATCTCAGTACTTATGTTGGTCTATT-3' were designed respectively. The mismatched bases are in bold for the mutation of the desired amino acid, and the insertion of the restriction site. The SnaBI restriction site for C506A/D/N/T and C490F/H/L/P/S/Y [33], and the SapI restriction site for R486A/D/N/T and R486F/H/L/P/S/Y are underlined. Each reaction mixture, PCR condition, and screening for mutants were performed as described previously [33].

Co-transformation of two compatible plasmids

Heterodimers of TS were obtained from co-transformation of two separate compatible plasmids of TS-inactive mutants. For intra-species heterodimer testing of PpDHFR-TS, pACYC-derived PpDHFR-TS(Arg-486) mutant was co-transformed with pET-17b-derived PpDHFR-TS(Cys-506) mutant into E. coli χ2913RecA(DE3). For cross-species heterodimer testing of PpDHFR-TS and PpDHFR-TS, pACYC-derived PpDHFR-TS(Arg-470) mutant was co-transformed with pET-17b-derived PpDHFR-TS(Cys-506) mutant into E. coli χ2913RecA(DE3), or vice versa. All co-transformants were plated on Luria–Bertani (LB) agar containing 50 μg/ml thymidine, 100 μg/ml ampicillin, and 34 μg/ml chloramphenicol. Chloramphenicol/ampicillin-resistant colonies were isolated and tested for genetic complementation in E. coli χ2913RecA(DE3) as described previously [33].

Protein expression and purification

Expressions of individual DHFR-TS proteins of P. falciparum and P. vivax were performed in two different E. coli strains. The TS-inactive (Pp)Cys-490 and (Pv)Cys-506 mutants were expressed in E. coli BL21(DE3)pLysS using
LB medium with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The TS-inactive (Pf)Arg-470 and (Pv)Arg-486 mutants were expressed in E. coli χ2913RecA (DE3) using LB medium supplemented with 50 μg/ml thymidine and 34 μg/ml chloramphenicol. The active intra-species and cross-species heterodimer plasmids from the combination of each pair of inactive Arg and inactive Cys mutants were expressed in E. coli χ2913RecA(DE3) using LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for small scale expression, whereas for large scale purification, expression was carried out in E. coli BL21(DE3). Briefly, a single colony of E. coli cell was grown in liquid medium with shaking at 37°C overnight. Using 1.5% inoculum from an overnight culture, each E. coli culture was grown in LB medium containing appropriate drugs to the mid-log phase (OD660 ~ 0.8) at 37°C and then induced with 0.4 mM IPTG for 20 h at 16°C. Cells were collected by centrifugation at 10,500g for 8 min. Protein purification was carried out employing methotrexate- Sepharose and anion exchange chromatographic columns as described previously [24, 34]. Total protein concentration was determined by Bradford protein assay using BSA as a standard [35].

Enzyme assays and kinetic studies

The DHFR and TS activities were determined spectrophotometrically at 340 nm wavelength by monitoring the rates of NADPH decline and H₂folate formation respectively [36]. The activity assay was performed in a Beckman Coulter UV–VIS spectrophotometer (DU800) using a single acquisition. The Kₚ values for the substrate and cofactor of TS enzyme were calculated from the Michaelis–Menten equation and determined from an initial rate plot of a pseudo-first-order kinetics for a single substrate (within 30 s) using a non-linear least squares fitting module of Kaleidagraph™ software (Synergy Software) [37].

6-[³H]-FdUMP thymidylate synthase binding assay

TS labeling using 6-[³H]-FdUMP was carried out according to the procedure described previously [33, 34]. The concentration of the purified TS-active heterodimer was determined on the basis that the TS-active protein can interact and form a covalent complex with FdUMP in the presence of CH₂H₂folate. Although truncated label products are present, they are not considered in the calculation of TS activity since only full-length enzyme is active in vivo [30]. The calibration curve was first constructed from the radioactive signals of 6-[³H]-FdUMP–protein complexes at 72 kDa on X-ray film of the purified PvDHFR-TS homodimer at varying concentrations. The active signal at 72 kDa at each concentration was defined by the integrated intensity using AlphaEase™ software (Alpha Innotech Corporation). The values of concentration dependent signals were then plotted versus their corresponding PVDHFR-TS homodimer concentrations determined by Bradford assay. The validity of the calibration curve for the deduction of heterodimer concentration was indicated by a good fit with the correlation coefficient of 0.99. The concentration of the heterodimer was then determined from the curve based solely on the value of integrated intensity of the heterodimer band at 72 kDa.

Results

In vivo subunit complementation

Sequence alignment of the PvDHFR-TS and PfDHFR-TS protein sequences revealed that the cognate TS active site residues Arg-470 and Cys-506 showed to be essential in PfDHFR-TS [33] are Arg-486 and Cys-506 in PvDHFR-TS. Two compatible plasmids, pET-17b and pACET, were used for construction of PvDHFR-TS Arg-486 and Cys-506 mutants. The mutants were constructed by QuickChange™ site-directed mutagenesis of pET-PvDHFR-TS and pACET-PvDHFR-TS using oligonucleotides specifically designed for mutation at residues 486 and 506. Ten mutants of Arg-486 (A, D, F, H, L, N, P, S, T and Y) and five mutants of Cys-506 (A, D, F, P and Y) and were obtained. These mutant residues at both positions represented a wide range of size, charge, and hydrophobicity in an attempt to explore the tolerance to mutation at these sites. The wild-type PvDHFR-TS, and Arg-486 and Cys-506 mutants were examined by genetic complementation of TS deficient E. coli χ2913RecA(DE3) on minimal agar in the absence of exogenous thymidine. Wild-type PvDHFR-TS could express sufficient TS activity to support the growth of TS-deficient cells. All five Cys-506 mutants (C506A, C506D, C506F, C506P and C506Y), and three of the Arg-486 mutants (R486D, R486L and R486Y) could not support growth, indicating that the in vivo enzyme activities were lacking (Table S1). In other words, these mutants were TS-inactive.

In contrast to the intra-species homodimer of PvDHFR-TS, cells carrying intra-species heterodimers of PvDHFR-TS, i.e. pairs of TS-inactive Arg-486 and Cys-506 mutants, were able to complement the growth of the Thy⁻ phenotype χ2913RecA(DE3) (Table S1). Similar results were observed for cross-species pairings of TS-inactive Arg-470 mutant of PfDHFR-TS and TS-inactive Cys-506 mutant of PvDHFR-TS, or vice versa, except for (Pf)R470A+(Pv)C506Y (Table S1). The results indicate that stable TS-active intra-species heterodimer and cross-species
heterodimer were formed in each case and the heterodimers provide adequate TS activity for cell growth.

6-[^3]H]-FdUMP binding assay

When TS is incubated with 6-[^3]H]-FdUMP in the presence of cofactor CH₂H₄folate, a covalent ternary complex is formed, which is stable during SDS-PAGE [38]. To verify that the in vivo subunit complementation tests reflected an actual TS activity, crude extracts of the single transformants and co-transformants were assayed for TS activity using 6-[^3]H]-FdUMP binding assay (Fig. 2). Under the conditions used, the inactive *P. vivax* or *P. falciparum* mutant enzymes did not form significant amounts of covalent ternary complex (data not shown). Like *P. falciparum* [33], intra-species heterodimers of *P. vivax* ([Pf]R486D+(Pv)C506A, (Pv)R486D+(Pv)C506D, (Pv)R486D+(Pv)C506F and (Pv)R486D+(Pv)C506Y) formed radioactive 6-[^3]H]-FdUMP-protein complexes, visible at 72 kDa (Fig. 2, Panel I). A radioactive signal of the ternary complex at 72 kDa was also detected for different pairs of cross-species heterodimers of *P. falciparum* and *P. vivax* ([Pf]R470D+(Pv)C506D, (Pf)R470D+(Pv)C506Y, (Pf)R470N+(Pv)C506D, (Pf)R470N+(Pv)C506Y, (Pf)R470A+(Pv)C506D, (Pf)R486D+(Pv)C490D, (Pf)R486D+(Pv)C490A, (Pf)R486D+(Pv)C490N and (Pf)R486D+(Pv)C490T) (Fig. 2, Panel II–III). The finding by 6-[^3]H]-FdUMP incorporation assay demonstrated that the functional TS of intra-species heterodimers and cross-species heterodimers of *P. falciparum* and *P. vivax* was formed, which is in good agreement with in vivo genetic complementation assay. Truncated bands at about 35 and 40 kDa, presumably representing truncated DHF-TR, were also readily detected by autoradiography, indicating that the TS active site was seemingly intact. In addition, the TS activities of purified proteins of wild-type PfDHF-TR and PvdHF-TR, (Pf)R470D, (Pf)R486D, (Pf)C490A and (Pv)C506Y mutant homodimers and heterodimers of (Pv)R486D+(Pv)C506Y, (Pf)R470D+(Pv)C506Y and (Pf)R486D+(Pv)C490A were determined by 6-[^3]H]-FdUMP binding assay. Only the wild-type enzymes, and the intra-species and cross-species heterodimers formed detectable signals of covalent complexes with 6-[^3]H]-FdUMP in the presence of CH₂H₄folate cofactor as seen from autoradiography in Fig. 2, Panel IV. In contrast, the mutant homodimers showed no signal.

Kinetic characterization of heterodimeric TS

Bacterial TS complementation assay showed that TS activity could be restored by subunit complementation of two separate mutants at active site Arg and Cys residues as intra-species and cross-species heterodimers. The 6-[^3]H]-FdUMP binding assays further support the notion that TS complementation in vivo is wholly dependent on formation of catalytically active heterodimers. As further proof that the complementing TS activity occurs through the formation of one functional TS active site in the heterodimer, it is necessary to measure the TS kinetic activity of the heterodimer. In addition to the cross-species heterodimer of *P. falciparum* and *P. vivax*, the intra-species TS heterodimer of *P. vivax* was also characterized for comparison. The intra-species *P. falciparum* heterodimer has been characterized previously [33]. The value of the catalytic rate constant (*k₅₅₅*) determines the activity of the protein, which reflects the number of active sites by comparison with the wild-type protein. In order to calculate the *k₅₅₅* of the reaction, measurement of the absolute concentration of the particular enzyme species in question is required. However, it is not trivial to determine the concentration of TS-active heterodimer since a homodimer of each mutant can form in addition to the heterodimer (Fig. 1). Based on the assumption that only the active form of TS enzyme can bind 6-[^3]H]-FdUMP, the concentration of the purified TS-active heterodimer was therefore inferred from its signal intensity in relation to a calibration obtained from wild-type active homodimer.

The catalytic constants of intra-species and inter-species heterodimers were compared and shown in Table 1. It is noted that these values for the TS of the purified intra-species (Pv)R486D+(Pv)C506Y heterodimer, and inter-species (Pf)R470D+(Pv)C506Y and (Pv)R486D+(Pf)C490A heterodimers were reduced by a factor of approximately two compared with the wild-type enzyme homodimer, indicating that only one TS active site existed. The fact that the *k₅₅₅* per active site and *Kₚₘ* values of the cross-species heterodimers were similar to those of the component wild-type enzymes and the intra-species heterodimers (Table 1) indicates that the heterodimer active site is fully functional, and the mutations of both subunits did not perturb the dimerization significantly.

Discussion

In protozoan parasites such as *P. falciparum* and *P. vivax*, even though TS is expressed as a bifunctional enzyme with adjoining DHFR and JR, the enzyme exists as a dimer of identical subunit, and forms active sites near the dimer interface [17]. Moreover, TS can exert its activity in the presence of DHFR and JR domains provided in trans [30]. In this study, we sought to determine whether TS dimerization can tolerate differences in the TS subunit interface, DHFR and JR domains, as tested by formation of *P. falciparum*–*P. vivax* cross-species TS heterodimers. The full-length DHFR-TR of *P. falciparum* and *P. vivax* was needed for the
Fig. 2 6-[14]F-dUMP thymidylate synthase activity assay of crude extracts and purified intra-species and cross-species TS heterodimers: (A) Coomassie-blue stained SDS-PAGE, and (B) autoradiograph of (A), Panel I, crude extracts of intra-species heterodimers of P. vivax in E. coli y2913RecA(DE3), lane 1, purified PrDHFR-TS; lane 2, vector pACET; lane 3, vector pET-17b; lane 4, (Pr)R486D+(Pr)C506A; lane 5, (Pr)R486D+(Pr)C490D; lane 6, (Pr)R486D+(Pr)C506F; lane 7, (Pr)R486D+(Pr)C506P; lane 8, (Pr)R486D+(Pr)C506Y. Panel II, crude extracts of cross-species heterodimers of PrDHFR-TS(Arg-470) and PrDHFR-TS(Cys-506) mutants in E. coli y2913RecA(DE3), lane 1, vector pET-17b; lane 2, pACYC184/pDHFR-TS; lane 3, (Pr)R470D+(Pr)C506D; lane 4, (Pr)R470D+(Pr)C506Y; lane 5, (Pr)R470N+(Pr)C506D; lane 6, (Pr)R470N+(Pr)C506Y; lane 7, (Pr)R470A+(Pr)C506D. Panel III, crude extracts of cross-species heterodimers of PrDHFR-TS(Arg-486) and PrDHFR-TS(Cys-490) mutants in E. coli y2913RecA(DE3), lane 1, pET-17b; lane 2, pACYC184/pDHFR-TS; lane 3, pACET/pDHFR-TS; lane 4, (Pr)R486D+(Pr)C490D; lane 5, (Pr)R486D+(Pr)C490A; lane 6, (Pr)R486D+(Pr)C490N; lane 7, (Pr)R486D+(Pr)C490T. Panel IV, purified proteins, lane 1, PrDHFR-TS; lane 2, PrDHFR-TS; lane 3, (Pr)R470D; lane 4, (Pr)R486D; lane 5, (Pr)C490A; lane 6, (Pr)C506Y; lane 7, (Pr)R470D+(Pr)C506Y; lane 8, (Pr)R486D+(Pr)C490A; lane 9, (Pr)R486D+(Pr)C506Y. Truncated DHFR-TS indicated is the N-terminal part of the DHFR-TS protein.
study of TS activity. The strategy was to co-transform a TS-deficient host with an inactive mutant from one species together with a complementing inactive mutant from the other species. This complementation approach has been demonstrated previously for intra-species subunit complementation of *L. casei* [32] and *P. falciparum* [33]. If a cross-species TS heterodimer of *P. falciparum* and *P. vivax* formed, it was hypothesized to have one catalytically functional active site and one catalytically incompetent active site (Fig. 1).

In this report, several mutants of (Pv)Arg-486 and (P)Arg-470 were constructed. Mutants at this residue (Arg donor) are typically inactive because hydrogen bonding to dUMP is disrupted [8]. It was observed that when *P. vivax* Arg-486 was mutated to Ala, Asp or Asn, only the Asp mutant was TS-inactive. By contrast, all mutants of the corresponding *P. falciparum* Arg-470 residue are TS-inactive [33]. This implied that Arg-486 of *P. vivax* was more tolerant to mutagenesis than the corresponding position of *P. falciparum*. Another series of inactive mutants, at residues (P)Cys-490 and (Pv)Cys-506, were constructed which are all inactive since they do not possess the essential active site nucleophile (Cys donor) [8]. Although the residues replacing both of these conserved Arg and Cys donors in the mutants showed large differences in size, charge and hydrophobicity, all of inactive (P)Cys-490 and (Pv)Cys-506 mutant enzymes could form active dimers with an inactive Arg donor mutant partner from the same species. Most importantly, with the exception of (P)R470A+(Pv)C506Y, all defined pairs of TS-inactive mutants (Arg and Cys donor mutants) formed active *P. falciparum-P. vivax* hybrid heterodimers. In the case of (P)R470A+(Pv)C506Y, although the TS subunits of (P)R470A and (Pv)C506Y could not complement each other to form an active cross-species heterodimer, each of purified TS-inactive homodimers was intact as judged by SDS-PAGE and DHFR activity (data not shown). The absence of TS activity in this combination suggests that the C506Y and/or the R470A mutation of the respective *P. vivax* or *P. falciparum* subunit preclude adaptation of the correct conformation for activity in the cross-species heterodimer, although there are no structural data to test this hypothesis.

The dimer interface of TS is mainly formed by contact between the surfaces of a six-stranded β-sheet from each monomer [39]. Based on the crystal structure and structure-based alignment of TS from *P. falciparum*, *P. vivax* and *L. casei* [17] including known amino acids in the subunit contact of *L. casei* TS dimer [18], amino acids whose side chains are involved in intersubunit contacts were determined (Table S2). The overall sequence conservation of the β-sheet of *P. falciparum* and *P. vivax* is approximately 87%: 21 of 24 residues whose side chains are in close contact across the interface are identical (Table S2). In addition, the region of TS domain in *Plasmodium* DHFR-TS, which is attached to the DHFR domain on the opposite side of the dimer, is conserved [17]. Because the JR of PfDHFR-TS crystal structure is not clearly resolved, it is still unclear how extensive and which part of JR makes the interaction with its TS counterpart. The JR also exhibits low homology (identity of 25%) between *P. falciparum* and *P. vivax*. By means of in vivo genetic complementation assay using TS-inactive Arg and Cys mutants, the cross-species heterodimer of *P. falciparum* and *P. vivax* was formed with activity comparable to the intra-species heterodimer of each *Plasmodium* species. This result indicated that the difference between *P. falciparum* and *P. vivax* in the JR is not functionally significant. The results are consistent with the recent finding that mutations in the JR do not affect catalytic rate or domain-domain communication in PfDHFR-TS [40].
The comparison of TS enzyme activities of wild-type homodimer with intra-species and cross-species heterodimers showed that the specific activity of heterodimers was about half that of wild-type homodimer. This finding is consistent with the expected activity if the heterodimer has one functional active site compared with the two functional sites in wild-type homodimer. However, three dimer forms are present in the co-expressed TS mutant preparations (Arg-inactive homodimer, active heterodimer and Cys-inactive homodimer) in a binomial distribution of 1:2:1 ratio respectively, assuming that the TS inactive mutants are expressed at the same concentration and assort into dimers randomly. Therefore, the heterodimer amounts to only half of the enzyme and the total activity will be a quarter of the wild-type enzyme. Kinetic parameters were measured for (P)R470D+(P)C506Y and (P)R486D-(P)C490A pairs in order to determine the competency of the active site formed in cross-species heterodimers. The $k_{cat}$ and $K_m$ values for dUMP and CH$_3$H$_2$folate of these heterodimers are, within experimental error, identical to the $P$TS and $P$VT$S$ wild-type values and to the values for the intra-species pairs. The similar kinetic constants indicate that the cross-species active site is catalytically very similar to the single species active site. The comparable catalytic activities of the cross-species heterodimer and the intra-species heterodimer indicate that the structural conservation between $P$. falciparum and $P$. vivax is sufficient for strong dimerization between these two different TSs. On the other hand, cross-species heterodimers of $P$. falciparum TS with the more distantly related protozoan T. cruzi TS are inactive (data not shown), suggestive of functional differences among Protozoa with respect to TS dimerization. From the evolutionary tree of DHFR-TS sequences, $P$. falciparum belongs to the "long linker" family, whereas T. cruzi belongs to the "short linker" family [41]. Based on available crystal structures of protozoan DHFR-TS, the DHFR and TS active sites are on the same side of the monomer in TcDHFR-TS, while they are on opposite sides of the monomer in P/DHFR-TS [42]. The sequence conservation of the side chains making contact at the subunit interface between PTS and TcTS is approximately 58% in the $\beta$-sheet when compared to 87% between PTS and PVT$S$ (Table S2). The differences provide a framework for an analysis of subunit interface directed at defining requirements for subunit recognition.

In conclusion, this work has used an in vivo genetic complementation and biochemical approach for study of cross-species heterodimer formation of PTS and PVT$S$ enzymes. To our knowledge, this is the first report to demonstrate that the $P$. falciparum and $P$. vivax TS enzymes, which are adjoined with JR and DHFR domains as bifunctional enzymes, can complement to form a catalytically active cross-species TS heterodimer with an intact functional active site. This approach can be used as a tool for further examination of cross-species interaction of the TS interface. The divergent domains outside of the core TS domain, such as DHFR and JR in protozoa have roles in TS function which are still largely unexplored; better understanding of these domains through cross-species heterodimer assays could lead to the design of non-active site drugs targeting the TS interface for chemotherapy against both $P$. falciparum and $P$. vivax infections.

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