Insights into the role of the junctional region of Plasmodium falciparum dihydrofolate reductase-thymidylate synthase

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Abstract

Background: Plasmodium falciparum dihydrofolate reductase-thymidylate synthase (pfDHFR-TS) is a well-defined target of anti-malarial drug, such as pyrimethamine and cycloguanil. Emergence of malaria parasites resistant to these drugs has been shown to be associated with point mutations of the gene coding for the target enzymes. Although the 3D-structure of P. falciparum bifunctional pfDHFR-TS has been reported previously, relatively little is known about the interactions between the pfDHFR and pfTS domains and the roles of the junctional region that links the two domains together. Therefore, a thorough understanding of the interaction of the two domains and the role of the junctional region of this target is important as the knowledge could assist the development of new effective anti-malarial drugs aimed at overcoming drug-resistant malaria.

Methods: A system was developed to investigate the interaction between pfDHFR and pfTS domains and the role of the junctional region on the activity of the recombinant pfTS. Based on the ability of co-transformed plasmids coding for pfDHFR and pfTS with truncated junctional region to complement the growth of TS-deficient Escherichia coli strain χ2913recA(DE3) on minimum media without thymidine supplementation, active pfTS mutants with minimal length of junctional region were identified. Interactions between active pfDHFR and the pfTS domains were demonstrated by using a bacterial two-hybrid system.

Results: Using TS-deficient E. coli strain χ2913recA(DE3), the authors have shown for the first time that in P. falciparum a junctional region of at least 44 amino acids or longer was necessary for the pfTS domain to be active for the synthesis of thymidylate for the cells. Truncation of the junctional region of the bifunctional pfDHFR-TS further confirmed the above results, and suggested that a critical length of the junctional peptide of pfDHFR-TS would be essential for the activity of TS to catalyze the synthesis of thymidylate.

Conclusion: The present study demonstrated the interactions between the pfDHFR and pfTS domains of the bifunctional pfDHFR-TS, and revealed that the junctional region linking the two protein domains is essential for the expression of catalytically active pfTS domain. The findings could be useful since inhibition of the pfDHFR-TS domain-domain interaction could form a basis for the development of new anti-malarial drugs based on targeting the non-active site region of this important enzyme.

Keywords: Malaria, Plasmodium falciparum, DHFR-TS, Junctional region
Background

Malaria remains an important disease in many tropical and sub-tropical countries [1]. The disease has become a global health threat, with over one million deaths annually, mostly children in sub-Saharan Africa [2]. The emergence of malaria resistance to almost all the currently available anti-malarial drugs has highlighted an urgent need to identify new malarial targets, and develop new effective drugs to combat the drug-resistant parasites [3-8]. *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase (*pf*DHFR-TS) is a well-defined target of antifolate drugs such as pyrimethamine and cycloquinan. The enzyme is responsible for the production of folates as well as thymidylate (dTMP) required for DNA synthesis [9]. Unfortunately, the emergence of anti-folate resistance has compromised the utility of the drugs and presented an urgent need to discover new drug targets and to develop novel effective drugs to combat drug-resistant parasites.

Structural studies of *P. falciparum* DHFR-TS revealed that the native enzyme is a homodimeric protein comprising 231 residues of DHFR domain (~27 kDa) at the N-terminus, followed by a short junctional region of 89 residues (~11 kDa) and 288 residues (~34 kDa) of the TS domain at the C-terminus of the protein [10,11]. It has been postulated that such a bifunctional arrangement could have evolved as a mechanism for the tight coupling generation of reduced folates required for the synthesis of amino acids, purine, pyrimidine, and dTMP, a phenomenon called “substrate channelling”. Support for this hypothesis comes from the evidence of metabolic channelling of the H₂folate produced in the TS-catalyzed reaction, which was found to proceed at a faster rate than the diffusion rate [12]. Data from the bifunctional DHFR-TS of *Leishmania major* and *Toxoplasma gondii* also supported the substrate channelling hypothesis [12,13]. Nevertheless, the mechanism of substrate channelling for *pf*DHFR-TS remains unclear since, based on the structure of *pf*DHFR-TS, such a mechanism could not explain the delivery of H₂folate from the TS domain to the active site of *pf*DHFR, and an electrostatic channelling mechanism was proposed as a possible alternative method [14-16].

The junctional region (JR) linked between the DHFR and TS domains in parasitic protozoa reported thus far varies significantly in length depending upon the source [9,10,16,17]. The long JR found in *P. falciparum* (89 amino acids) provides a number of interactions which facilitate contacts with the DHFR domain of the opposite half of the DHFR-TS dimer, and brings the two DHFR domains closer together. Structural alignments of DHFR-TS enzymes from *Cryptosporidium hominis* and *P. falciparum* revealed that the JR played an important role in the orientation of the DHFR domain relative to TS [17]. Therefore, inhibition of the interaction between the JR, the DHFR and TS domains could be a possible approach for the development of novel effective anti-malarial drugs [18]. The present study therefore describes an approach towards understanding the interactions between DHFR and TS domains of the bifunctional *pf*DHFR-TS, and the important role of JR on the activity of the TS domain. This is the first successful expression of the catalytically active *pf*TS domain that has JR attached at its N-terminus, as all attempts in the past to express *pf*TS domain failed. Through deletion of the JR, it is demonstrated that a critical length of JR is required for proper folding of the *pf*TS domain leading to an active molecule of *pf*TS. The findings highlight the importance of the JR which links DHFR and TS domains and suggest a possible alternative in exploiting the non-active site region of this important enzyme in developing new anti-malarial drugs to overcome drug-resistant parasites.

Methods

Materials

Restriction endonucleases and T₄ DNA ligase were obtained from New England Biolabs, Life Technology, Inc. 2’-deoxuryridylate, 5-fluoro-2’- deoxyuridyline, and NADPH were from Sigma. MTX-sepharose CL-6B (~ 1 µmole/ml) [12], H₂folate [19], and CH₂H₄folate [20] were prepared as described. Custom primer syntheses and DNA sequencing were from BioDesign Co Ltd and Genome Institute. *Escherichia coli* strain χ2913 recA(DE3) was used for the genetic complementation studies to monitor the function of TS.

Construction and transformation of recombinant plasmids

The gene coding for *P. falciparum* DHFR (amino acids 1–228) was amplified using a template DNA from pET*pf*DHFR-TS [21]. The *pf*IRTS mutants were constructed by the PCR mutagenesis method. The PCR reaction (100 µl) is composed of 50 ng pET*pf*DHFR-TS as a DNA template, 25 µmole of each primer (Table 1), 200 µM of dNTPs, 1.5 mM MgCl₂, and 2.5 units of *Taq* DNA polymerase in 1 x reaction buffer. The PCR conditions were as follows: 1 cycle of 94°C for 3 min, then 25 cycles of 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 5 min. Deletion mutants of bifunctional *pf*DHFR-TS were constructed using the whole plasmid amplification PCR approach [22] employing recombinant plasmid pET15b carrying the *P. falciparum* DHFR-TS(3D7) gene as a template and sets of primers as shown in Table 2. The PCR products were analysed by agarose electrophoresis, and were further purified using a Qiagen Gel Extraction kit.

The amplified product was cloned into pAC28 expression plasmid [23] to express catalytically active pACpfDHFR. Likewise, sequences coding for truncated JR with the *pf*TS domain attached were amplified using the primers as listed in Table 1, and cloned into pET-15b
Table 1 Primers used for the construction of truncated pJRTS mutants

| Name | Length (bases) | Sequence (5' → 3') | Utilities and description |
|------|---------------|---------------------|--------------------------|
| JRTSΔ232-235 | 28 | AAAGAATCCGATGGAACAAAATGTTATA | Sense strand PCR primer for the construction of pET-pJRTSΔ232-235. Bold-face letters represent Met introduced in front of Glu266. Underlined sequence is the restriction site for NcoI. |
| JRTSΔ232-251 | 28 | AAAGAATCCGATGGAAGAAAGAATGTAG | Sense strand PCR primer for the construction of pET-pJRTSΔ232-251. Bold-face letters represent Met-Glu introduced in front of Lys235. Underlined sequence is the restriction site for NcoI. |
| JRTSΔ232-265 | 28 | AAAGAATCCGATGGAATTTCACAAAAT | Sense strand PCR primer for the construction of pET-pJRTSΔ232-265. Bold-face letters represent Met introduced in front of Glu266. Underlined sequence is the restriction site for NcoI. |
| JRTSΔ232-271 | 28 | AAAGAATCCGATGGAACAAATATTTAATT | Sense strand PCR primer for the construction of pET-pJRTSΔ232-271. Bold-face letters represent Met introduced in front of Glu266. Underlined sequence is the restriction site for NcoI. |
| JRTSΔ232-274 | 57 | CCCCTCTAGAATAATTTTTACTTAAAGAATTTTCATTCAGGATACATACCCAGGAAATT | Sense strand PCR primer for the construction of pET-pJRTSΔ232-274. Bold-face letters represent Met introduced in front of Lys251. Underlined sequence is the restriction site for XbaI. |
| JRTSΔ232-276 | 57 | CCCCTCTAGAATAATTTTTACTTAAAAGAATTTTCATTCAGGATACATACCCAGGAAATT | Sense strand PCR primer for the construction of pET-pJRTSΔ232-276. Bold-face letters represent Met introduced in front of Asn277. Underlined sequence is the restriction site for XbaI. |
| JRTSΔ232-277 | 57 | CCCCTCTAGAATAATTTTTACTTAAAAGAATTTTCATTCAGGATACATACCCAGGAAATT | Sense strand PCR primer to prepare construct pET-pJRTSΔ232-277. Bold-face letters represent Met introduced in front of Tyr275. Underlined sequence is the restriction site for XbaI. |
| JRTSΔ232-299 | 28 | AAAGAATCCGATGGAAGAAAGAATTTAATT | Sense strand PCR primer to prepare construct pET-pJRTSΔ232-299. Bold-face letters represent Met introduced in front of Glu266. Underlined sequence is the restriction site for NcoI. |
| N3TS | 22 | ACTCATGATCCCCGAGCC | Antisense strand PCR primer for the construction of all pET-pJRTS mutants. Underlined sequence is the restriction site for BamHI. |

Table 2 Primers used for the construction of truncated bifunctional pDHFR-TS

| Name | Length (bases) | Sequence (5' → 3') | Utilities and description |
|------|---------------|---------------------|--------------------------|
| Δ229-277 Sense | 60 | ACAACATTGGATTTTATCATTTAATAAGAAAATAT | Sense strand PCR primer for the construction of pDHFR-TSΔ229-277. Lys236 (italic) was followed by Tyr277 (bold). |
| Δ229-277 Antisense | 60 | TTCTCTTCTCATATCATATCTATCATTATCTTCTAATAAGAAAATAT | Antisense strand PCR primer for the construction of pDHFR-TSΔ229-277. |
| Δ229-276 Sense | 60 | ACAACATTGGATTTTATCATTTAATAAGAAAATAT | Sense strand PCR primer for the construction of pDHFR-TSΔ229-276. Lys236 (italic) was followed by Asn277 (bold). |
| Δ229-276 Antisense | 60 | TTCTCTTCTCATATCATATCTATCATTATCTTCTAATAAGAAAATAT | Sense strand PCR primer to amplify pDHFR-TSΔ229-276. |
| Δ229-275 Sense | 60 | ACAACATTGGATTTTATCATTTAATAAGAAAATAT | Sense strand PCR primer for the construction of pDHFR-TSΔ229-275. Lys236 (italic) was followed by Ile251 (bold). |
| Δ229-275 Antisense | 60 | TTCTCTTCTCATATCATATCTATCATTATCTTCTAATAAGAAAATAT | Antisense strand PCR primer to amplify pDHFR-TSΔ229-275. |
| Δ229-274 Sense | 60 | ACAACATTGGATTTTATCATTTAATAAGAAAATAT | Sense strand PCR primer for the construction of pDHFR-TSΔ229-274. Lys236 (italic) was followed by Lys268 (bold). |
| Δ229-274 Antisense | 60 | TTCTCTTCTCATATCATATCTATCATTATCTTCTAATAAGAAAATAT | Antisense strand PCR primer to amplify pDHFR-TSΔ229-274. |
| Δ229-271 Sense | 60 | ACAACATTGGATTTTATCATTTAATAAGAAAATAT | Sense strand PCR primer for the construction of pDHFR-TSΔ229-271. Lys236 (italic) was followed by Asp267 (bold). |
| Δ229-271 Antisense | 60 | TTCTCTTCTCATATCATATCTATCATTATCTTCTAATAAGAAAATAT | Antisense strand PCR primer to amplify pDHFR-TSΔ229-271. |
| Δ229-265 Sense | 60 | ACAACATTGGATTTTATCATTTAATAAGAAAATAT | Sense strand PCR primer for the construction of pDHFR-TSΔ229-265. Lys236 (italic) was followed by Glu268 (bold). |
| Δ229-265 Antisense | 60 | TTCTCTTCTCATATCATATCTATCATTATCTTCTAATAAGAAAATAT | Antisense strand PCR primer to amplify pDHFR-TSΔ229-265. |
to yield plasmids pET-pfJRTS\texttextsuperscript{△}232-235, pET-pfJRTS\texttextsuperscript{△}232-251, pET-pfJRTS\texttextsuperscript{△}232-265, pET-pfJRTS\texttextsuperscript{△}232-271, pET-pfJRTS\texttextsuperscript{△}232-274, pET-pfJRTS\texttextsuperscript{△}232-276, pET-pfJRTS\texttextsuperscript{△}232-277, and pET-pfJRTS\texttextsuperscript{△}232-299. These mutant pET-pfJRTS plasmids were transformed into electro-competent \textit{E. coli} strain \( \chi \text{2913} \) cells by electroporation using pulses set at \( 1.8 \text{ kV}, 400 \Omega, 25 \mu \text{F} \) and a pulse length of \( \sim 10 \text{ min} \). After centrifugation at \( 6,500 \text{ g} \) for \( 4^\circ \text{C} \) for 10 min and resuspending the cell pellets in \( 2 \text{ ml} \) of \( 10\% \) glycerol, a second electroporation was performed to transform \( 100 \mu l \) of the first transformed cells with \( \sim 50 \text{ ng} \) of \( \text{pAC-pfDHFR} \).

After recovering the cells by addition of \( 0.9 \text{ ml} \) of LB broth followed by vigorous shaking for 1 hr at \( 37^\circ \text{C} \), the cells were plated onto LB agar containing \( 100 \mu g/\text{ml} \) ampicillin and \( 30 \mu g/\text{ml} \) kanamycin. Colonies appeared on the plates at \( 37^\circ \text{C} \) for 48 hours. Colonies appearing on this plate were selected for subsequent characterization at \( 37^\circ \text{C} \) for 48 hours. Colonies appearing on this plate were selected for subsequent characterization at \( 37^\circ \text{C} \) for 48 hours.

Truncation of JR of the bifunctional \( \text{pfDHFR-TS} \) was performed by PCR amplification using the whole recombinant plasmid pET15b carrying gene coding for the bifunctional \textit{P. falciparum} DHFR-TS (3D7) as a template, and the primers as listed in Table 2. The resulting truncated mutants, i.e., \( \text{pfDHFR-TS} \Delta \text{229-265}, \text{pfDHFR-TS} \Delta \text{229-271}, \text{pfDHFR-TS} \Delta \text{229-274}, \text{pfDHFR-TS} \Delta \text{229-276}, \text{pfDHFR-TS} \Delta \text{232-274}, \text{pfDHFR-TS} \Delta \text{232-276}, \) and \( \text{pfDHFR-TS} \Delta \text{232-277}, \) were used to co-transform \( \text{pAC-28 plasmid} \) in the complementation study.

**Genetic complementation studies**

Overnight culture of the co-transformants was streaked on a minimal agar plate [24] containing \( 100 \mu g/\text{ml} \) ampicillin, \( 30 \mu g/\text{ml} \) kanamycin, and \( 0.025 \text{ mM} \) IPTG without thymidine supplementation. The plates were incubated at \( 37^\circ \text{C} \) for 48 hours. Colonies appearing on this plate were considered to have positive genetic complementation, and were selected for subsequent characterization and verification of the expressed enzymes.

**Expression and purification of the expressed enzymes**

An overnight culture of \textit{E. coli} \( \chi \text{2913} \) harbouring two plasmids, i.e., \( \text{pAC-pfDHFR} \) and truncated pET-pfJRTS with different lengths of truncated JR, was inoculated at 1\% inoculum in LB containing \( 100 \mu g/\text{ml} \) of ampicillin, \( 30 \mu g/\text{ml} \) kanamycin. The bacteria were grown at \( 37^\circ \text{C} \) until the OD\texttextsubscript{600} of the cell suspension reached \(-0.5-0.6\), and isopropyl \textpi-D-thiogalactopyranoside (IPTG) was added at a final concentration of \( 0.025 \text{ mM} \). The culture was allowed to grow with shaking at \( 20^\circ \text{C} \) for 20 hours. The cells were harvested by centrifugation at \( 6,500 \text{ g} \) for 10 min at \( 4^\circ \text{C} \), washed once with 250 ml cold phosphate buffered saline, \( \text{pH} 7.4 \), resuspended in buffer A (20 mM potassium phosphate buffer, \( \text{pH} 7.0 \), 0.1 mM EDTA, 10 mM DTT, 20% glycerol) containing 0.2 M KCl, and passed through a French Pressure Cell (American Instruments Co Inc, USA) at 12,000 psi three times. After centrifugation at \( 20,000 \text{ g} \) for 1 hour at \( 4^\circ \text{C} \), the clear supernatant of the crude sample was circulated at a flow rate of \( \sim 0.5 \text{ ml/min} \) in a methotrexate-sepharose CL-6B column (1.5 \times 5.0 cm) pre-equilibrated with buffer A containing \( 0.2 \text{ M KCl} \). After overnight circulation, the column was washed with 30 ml of buffer A containing \( 0.75 \text{ M KCl} \), followed by 20 ml of buffer A containing \( 0.2 \text{ M KCl} \). The column was then washed with 30 ml of elution buffer (50 mM TES \text{pH} 7.8, 0.1 mM EDTA, 10 mM DTT, 20% glycerol, 50 mM KCl) containing 4 mM \( \text{H}_2\text{folate} \) to elute DHFR. Fractions of 1 ml were collected. Active fractions with DHFR activity were pooled, concentrated, and \( \text{H}_2\text{folate} \) in the pooled fraction was removed by passing the pooled fraction through a pre-packed NAP-25 column (Pharmacia) pre-equilibrated with buffer A.

**Enzyme assays and protein analysis**

The activity of DHFR was determined spectrophotometrically by monitoring the rate of decrease in absorbance at \( 340 \text{ nm} \) [12,25]. The standard DHFR assay (1 ml) in a 1-cm path-length cuvette was composed of \( 100 \mu \text{M} \) \( \text{H}_2\text{folate} \), \( 100 \mu \text{M NADPH} \), 50 mM TES, \( \text{pH} 7.0 \), 75 mM \( \beta \)-mercaptopethanol, 1 mg/ml bovine serum albumin, and \( \sim 0.01 \text{ units} \) of enzyme. The reaction was initiated with \( \text{H}_2\text{folate} \). One unit of DHFR activity is defined as the amount of enzyme that produces 1 \( \text{nmole} \) of product per minute at \( 25^\circ \text{C} \).

The activity of TS was determined by monitoring the increase in absorbance at \( 340 \text{ nm} \) due to the formation of \( \text{dUMP} \) at \( 25^\circ \text{C} \) [26]. The reaction (1 ml) in 1-cm path-length cuvette was composed of 50 mM TES, \( \text{pH} 7.4 \), 25 mM MgCl\text{\textsubscript{2}}, 1 mM EDTA, 6.5 mM HCHO, 75 mM \( \beta \)-mercaptopethanol, 100 \( \mu \text{M} \) (6R) \( \text{CH}_2\text{H}_2\text{folate} \), 125 \( \mu \text{M} \) dUMP and the enzyme. The reaction components, except for dUMP, were incubated at \( 25^\circ \text{C} \) for at least 5 min to obtain the baseline prior to initiating the reaction with dUMP. One unit of TS activity is defined as the amount of enzyme that produces 1 \( \text{nmole} \) of product per minute at \( 25^\circ \text{C} \). The activities of the expressed enzymes were reported as mean ± standard deviation.

**\( [\text{H}]\text{-FdUMP binding assay} **

The ability of the pfJRTS mutants with truncated JR to form covalent complex with \( [\text{H}]\text{-FdUMP} \) and \( \text{CH}_2\text{H}_4\text{folate} \) was investigated by incubating the mutant proteins with \( 0.5 \mu \text{M} [\text{H}]\text{-FdUMP} (19.3 \text{ Ci/mmol}), 0.1 \text{ mM CH}_2\text{H}_4\text{folate}, \) and 6.5 mM formaldehyde in 50 mM TES \( \text{pH} 7.4, 25 \text{ mM MgCl}_2, 1 \text{ mM EDTA}, \) and 75 mM \( \beta \)-mercaptopethanol for 15 min at room temperature. The reaction was then electrophoresed on 12.5% SDS-PAGE. After Coomassie-Blue staining and destaining, the gel was soaked with autoradiography enhancer (\text{EN3HANCE™}, New England
Nuclear) with gentle shaking for 30 min at room temperature. This was followed by washing the gel with cold water and drying the gel on a piece of filter paper under vacuum at 80°C for 30 min. The dried gel was then exposed on AGFA X-ray film at ~80°C with intensifying screens for three to five days before development of the X-ray film.

Bacterial two-hybrid system
Interactions between pfDHFR domain and pfTS domain including those pfTS mutants with truncated JR were investigated using an E. coli two-hybrid system (Stratagene Inc) [27,28]. The sequence coding for pfDHFR domain was cloned into pBT (bait) vector to yield pBT-pfDHFR, whereas the pfJRTS was cloned into pTRG (target) vector to yield the pTRG-pfJRTS plasmid. The two plasmids were co-transformed into BacterioMatch two-hybrid system reporter strain E. coli XL1-blue MRF. Positive interaction is indicated by the ability of the bacteria to grow on LB agar plate supplemented with 250 μg/ml carbenicillin, 15 μg/ml tetracycline, 34 μg/ml chloramphenical, and 50 μg/ml kanamycine (LB-CTCK).

Results
Construction of pfJRTS and pfDHFR-TS mutants and growth complementation studies
Unlike the DHFR domain of the bifunctional pfDHFR-TS, which can be heterologously expressed to yield the catalytically active form of enzyme [29-32], all attempts to express the catalytically active domain of pfTS have failed. Evidence from deletion of pfDHFR-TS suggested that the amino terminus of the pfDHFR domain is important for the function of the pfTS domain, and interactions between the pfDHFR and pfTS domains are important [18]. To address the important function of JR, mutant constructs of the pfJRTS domain with various lengths of JR sequences were constructed and co-transformed with plasmid expressing the catalytically active pfDHFR domain (pAC-pfDHFR) in TS-deficient E. coli strain χ2913 recA(DE3) transformed with pET-pfJRTS and pAC-28 could not grow on a minimum agar plate (Figure 4B, lane 7). Therefore, pfDHFR-TS mutants with a longer JR sequence, i.e. pET-pfDHFR-TS Δ229-235, pET-pfDHFR-TS Δ229-271, pET-pfDHFR-TS Δ229-274, pET-pfDHFR-TS Δ229-275, pET-pfDHFR-TS Δ229-276, and pET-pfDHFR-TS Δ229-277 (Figure 2), Figure 3 shows the 89 amino acids of P. falciparum JR sequence and indicates the positions of amino acids that were chosen to prepare truncated and deletion mutants. Results from growth complementation monitored after incubation of the plates overnight at 37°C revealed that pET-pfJRTS Δ223-235, pET-pfJRTS Δ232-251, pET-pfJRTS Δ232-265, pET-pfJRTS Δ232-271, pET-pfJRTS Δ232-274, and pET-pfJRTS Δ232-276 with corresponding JR length of 85, 69, 55, 49, 46, 44 amino acid residues, respectively, could grow on minimum media (Figure 4A, lanes 2–7), while pET-pfJRTS Δ232-277 and pET-pfJRTS Δ232-299 which had the JR length of 43 and 21 residues, respectively, did not show growth complementation (Figure 4A, lanes 8–9). The results suggest that the length of JR was important for the proper folding of the TS domain and hence affected its function. The data revealed that at least 44 residues (the mutant pET-pfJRTS Δ232-276) were required in the case of JR of P. falciparum in order for the pfTS domain to function properly.

As with the result for the construct pfJRTS TSA229-277, TS-deficient E. coli strain χ2913 recA(DE3) transformed with pET-pfDHFR-TS Δ229-277 and pAC-28 could not grow on a minimum agar plate (Figure 4B, lane 7). Therefore, pfDHFR-TS mutants with a longer JR sequence, i.e. pET-pfDHFR-TS Δ229-265, pET-pfDHFR-TS Δ229-271, pET-pfDHFR-TS Δ229-274, pET-pfDHFR-TS Δ229-275, and pET-pfDHFR-TS Δ229-276, were constructed and tested for growth complementation. Complementation studies revealed that the constructs pET-pfDHFR-TS Δ229-265, pET-pfDHFR-TS Δ229-271, and pET-pfDHFR-TS Δ229-274 were able to grow on both LB agar and minimum agar supplemented with 0.025 mM IPTG (Figure 4B, lanes 2–4), whereas the construct pET-pfDHFR-TS Δ229-275 failed to show complementation (Figure 4B, lanes 5–6). The results using deletion mutants of bifunctional pfDHFR-TS are in good agreement with those from co-transformation of plasmids encoding pfJRTS with varying lengths of JR and plasmid expressing catalytically active pfDHFR.

[1H]-FdUMP binding studies of pfJRTS and pfDHFR-TS mutant constructs expressing pfTS
[1H]-FdUMP binding studies were carried out to monitor the expression of catalytically active pfTS from truncated pfJRTS mutants and the pfDHFR-TS deletion mutants found to show growth complementation on minimum media. Crude extracts from E. coli χ2913 co-transformed with plasmid expressing pfDHFR and mutant pfJRTSs with varying length of JR were subjected to SDS-PAGE (Figure 5A(I) and B(I)) and the gel was exposed to X-ray...
film (Figure 5A(II) and B(II)). In agreement with the results from genetic complementation results, co-transformation of pAC-pfDHFR and pET-pfJRTS$^{\Delta}$232-235, pET-pfJRTS$^{\Delta}$232-251, pET-pfJRTS$^{\Delta}$232-265, pET-pfJRTS$^{\Delta}$232-271, pET-pfJRTS$^{\Delta}$232-274 and pET-pfJRTS$^{\Delta}$232-276 formed [$^{3}$H]-FdUMP-enzyme complexes which could be visualized on X-ray film as a band according to the size predicted from that of pfJRTS (Figure 5B, lanes 2–7). Co-transformation of pAC-pfDHFR and pfJRTS$^{\Delta}$232-277 and pfJRTS$^{\Delta}$232-299, however, showed relatively weak signal from [$^{3}$H]-FdUMP-enzyme complexes (Figure 5B, lanes 8–9).

To investigate the role of JR in bifunctional pfDHFR-TS, formation of [$^{3}$H]-FdUMP-enzyme complexes were monitored from the extracts of E. coli cells co-transformed with pAC28 plasmid and pET-pfDHFR-TS$^{\Delta}$229-265, pET-pfDHFR-TS$^{\Delta}$229-271, pET-pfDHFR-TS$^{\Delta}$229-274, pET-pfDHFR-TS$^{\Delta}$229-275, and pET-pfDHFR-TS$^{\Delta}$229-276 (Figure 5C and 5D). The results showed that both crude extracts and partially purified proteins of these constructs could form complexes with [$^{3}$H]-FdUMP, though growth complementation could not be observed from pET-pfDHFR-TS$^{\Delta}$229-275, pET-pfDHFR-TS$^{\Delta}$229-276, and pET-pfDHFR-TS$^{\Delta}$229-277 (Figure 5D, lanes 5–7).

Purification and characterization of the expressed enzymes
Crude extracts of the transformed TS-deficient E. coli that were both positive and negative from the growth complementation studies were assayed for DHFR and TS activities. Extracts that were positive were passed through a MTX-affinity column for purification of the pfDHFR domain [32]. The results from enzyme assays

![Figure 1](http://www.malariajournal.com/content/12/1/91)

**Figure 1** Schematic diagram representing full-length pfDHFR-TS, pfDHFR domain, and pTS domains with truncated junction region (JR). Gene encoding amino acid residues 1–228 of pfDHFR domain was cloned into pAC28 expression vector, whereas gene fragments encoding the pTS domain with different lengths of JR that are attached to the N-terminus (amino acid residue 320) of pTS were cloned into the pET15b expression vector. The numbers of amino acid are shown above the gene and the names of the mutants are indicated at the right.

![Figure 2](http://www.malariajournal.com/content/12/1/91)

**Figure 2** Schematic diagram representing full-length and truncated pfDHFR-TS. Constructs of pfDHFR domain containing amino acids 1–228 followed by truncated JR sequences of different lengths linked to the N-terminus of the pTS domain were cloned into the pET15b expression vector. The numbers of amino acid are shown above the gene and the names of the mutants are indicated.
revealed that the specific activity of pfDHFR from all the co-transformants were not significantly different among the crude extracts (average 36.2 ± 3.1 nmole/min/mg), but the activity was only about half of that observed from the crude extract of the bifunctional pfDHFR-TS (Table 3). The specific activities of the partially purified pfDHFR upon affinity purification were 1,371 ± 208 nmole/min/mg (Table 3), about 3 times lower than that obtained from the purified pfDHFR-TS. Interestingly, the TS activities of the purified enzymes from the truncated pfJRTS mutants are...
in most cases 1–2 times higher than that from the purified pfDHFR-TS. The activity of pfTS was found to co-elute with that of pfDHFR, suggesting that the expressed pfTS domain was somehow associated with the pfDHFR domain. As indicated in Table 3, the TS activity of the pfJRTS mutants was found to decrease upon shortening the length of JR in the construct.

The importance of the length of the JR sequence on the activity of pfTS was also investigated using the bifunctional pfDHFR-TS. Site-directed mutagenesis of the bifunctional pfDHFR-TS was performed to yield mutants containing the same lengths of JR as for the pfJRTS mutant constructs described above. These mutants include pET-pfDHFR-TSΔ229-265, pET-pfDHFR-TSΔ229-271, pET-pfDHFR-TSΔ229-274, pET-pfDHFR-TSΔ229-275, pET-pfDHFR-TSΔ229-276, and pET-pfDHFR-TSΔ229-277. Table 4 summarizes the pfDHFR and pfTS activities of the bifunctional DHFR-TS and the bifunctional mutants from the crude extracts.

Figure 5 [3H]-FdUMP binding of crude extracts and partially purified proteins. Crude extracts and partially purified proteins from plasmids encoding pfDHFR co-transformed with pfJRTS mutants with truncated JR (panels A and B, respectively), and plasmids encoding pAC28 co-transformed with pfDHFR-TS with truncated JR (panel C and D, respectively) were labeled with [3H]-FdUMP. For panels A and B, lane 1, pfDHFR-TS (3D7); lane 2, pfJRTSΔ232-235 co-expressed with pAC28-pfDHFR; lane 3, pfJRTSΔ232-251 co-expressed with pAC28-pfDHFR; lane 4, pfJRTSΔ232-265 co-expressed with pAC28-pfDHFR; lane 5, pfJRTSΔ232-271 co-expressed with pAC28-pfDHFR; lane 6, pfJRTSΔ232-274 co-expressed with pAC28-pfDHFR; lane 7, pfJRTSΔ232-276 co-expressed with pAC28-pfDHFR; lane 8, pfJRTSΔ232-277 co-expressed with pAC28-pfDHFR; lane 9, pfJRTSΔ232-279 co-expressed with pAC28-pfDHFR. For panels C and D, lane 1, pfDHFR-TS (3D7); lane 2, pfDHFR-TSΔ229-265; lane 3, pfDHFR-TSΔ229-271; lane 4, pfDHFR-TSΔ229-274; lane 5, pfDHFR-TSΔ229-275; lane 6, pfDHFR-TSΔ229-276; lane 7, pfDHFR-TSΔ229-277; lane 8, pfDHFR-TSΔ229-277993. M is the molecular weight standard markers. (I) Coomassie blue stained SDS-PAGE, (II) autoradiogram of the SDS gel.
and upon partial purification. In agreement with the growth complementation experiments, no pfTS activity was detected from the crude extracts and partial purification of pET-pfDHFR-TSΔ229-275, pET-pfDHFR-TSΔ229-276, and pET-pfDHFR-TSΔ229-277 mutants. However, the activities of the pfDHFR domain of these mutants remained active, though they were found to gradually decrease upon shortening the length of the JR sequence.

Demonstration of pfDHFR-TS domain-domain interaction by *Escherichia coli* two-hybrid system

The interaction between the pfDHFR domain and pfJRTS was demonstrated using *E. coli* BacterioMatch™ two-hybrid system (Strategene). The pfDHFR domain was cloned into NotI-BamHI sites of a pBT bait plasmid, resulting in a pBT-pfDHFR plasmid, which contains the pfdhfr gene fused at the end of the bacteriophage λcl gene. The pfJRTS domain was cloned into BamHI-XhoI sites of the pTRG target plasmid, resulting in constructs that express truncated pfJRTS domains fused with the α-subunit of RNA polymerase. If the pfJRTS domain could interact with the pfDHFR domain, then this would stabilize the binding of RNA polymerase located close to the promoter and activate the transcription of the AmpR reporter gene. As a consequence, the transformed reporter strain (*E. coli* XL1-blue MRF') showed growth upon partial purification. In agreement with the growth complementation experiments, no pfTS activity was detected from the crude extracts and partial purification of pET-pfDHFR-TSΔ229-275, pET-pfDHFR-TSΔ229-276, and pET-pfDHFR-TSΔ229-277 mutants. However, the activities of the pfDHFR domain of these mutants remained active, though they were found to gradually decrease upon shortening the length of the JR sequence.

**Table 3** DHFR and TS activities in crude extracts and purified enzymes from TS-deficient *Escherichia coli χ2913* harbouring pAC-pfDHFR and pET-pfJRTS with different lengths of JR

| Specific activity | Plasmid                              | Crude extract* (nmole/min/mg) | Purified enzyme** (nmole/min/mg) |
|------------------|--------------------------------------|-------------------------------|---------------------------------|
|                  |                                      | DHFR                          | TS                              |
|                  |                                      | 1. pET-pfDHFR-TS (3D7)        | 70.3 ± 3.6                     | 3.7 ± 0.4                       |
|                  |                                      | 2. pAC-pfDHFR+pET-pfJRTSΔ229-235 | 39.5 ± 5.8                     | 2.6 ± 0.2                       |
|                  |                                      | 3. pAC-pfDHFR+pET-pfJRTSΔ229-251 | 34.2 ± 5.8                     | 1.1 ± 0.4                       |
|                  |                                      | 4. pAC-pfDHFR+pET-pfJRTSΔ229-265 | 37.8 ± 5.3                     | 3.8 ± 0.5                       |
|                  |                                      | 5. pAC-pfDHFR+pET-pfJRTSΔ229-271 | 39.9 ± 7.3                     | 3.9 ± 0.7                       |
|                  |                                      | 6. pAC-pfDHFR+pET-pfJRTSΔ229-274 | 38.3 ± 5.6                     | 1.5 ± 0.3                       |
|                  |                                      | 7. pAC-pfDHFR+pET-pfJRTSΔ229-276 | 34.2 ± 9.1                     | 1.1 ± 0.1                       |
|                  |                                      | 8. pAC-pfDHFR+pET-pfJRTSΔ229-277 | 31.0 ± 8.1                     | ND                              |
|                  |                                      | 9. pAC-pfDHFR+pET-pfJRTSΔ232-279 | 34.9 ± 7.2                     | ND                              |
|                  |                                      | 10. pAC-28+pET-15b             | 7.7 ± 0.4                      | ND                              |

* Average values from three independent experiments.
** Average values from two independent experiments.
ND: not detectable.

and upon partial purification. In agreement with the growth complementation experiments, no pfTS activity was detected from the crude extracts and partial purification of pET-pfDHFR-TSΔ229-275, pET-pfDHFR-TSΔ229-276, and pET-pfDHFR-TSΔ229-277 mutants. However, the activities of the pfDHFR domain of these mutants remained active, though they were found to gradually decrease upon shortening the length of the JR sequence.

**Demonstration of pfDHFR-TS domain-domain interaction by *Escherichia coli* two-hybrid system**

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**Table 4** DHFR and TS activities in crude extracts and purified enzymes from TS-deficient *Escherichia coli χ2913* harbouring pET-pfDHFR-TS and pET-pfDHFR-TS constructs with different lengths of JR

| Specific activity | Plasmid                          | Crude extract* (nmole/min/mg) | Purified enzyme** (nmole/min/mg) |
|------------------|----------------------------------|-------------------------------|---------------------------------|
|                  |                                  | DHFR                          | TS                              |
|                  |                                  | 1. pET-pfDHFR-TS (3D7)        | 70.3 ± 3.6                     | 3.7 ± 0.4                       |
|                  |                                  | 2. pET-pfDHFR-TSΔ229-265      | 64.7 ± 5.8                     | 1.1 ± 0.3                       |
|                  |                                  | 3. pET-pfDHFR-TSΔ229-271      | 75.5 ± 12.4                    | 1.0 ± 0.3                       |
|                  |                                  | 4. pET-pfDHFR-TSΔ229-274      | 74.4 ± 18.5                    | 0.9 ± 0.2                       |
|                  |                                  | 5. pET-pfDHFR-TSΔ229-275      | 66.7 ± 7.3                     | ND                              |
|                  |                                  | 6. pET-pfDHFR-TSΔ229-276      | 83.9 ± 1.0                     | ND                              |
|                  |                                  | 7. pET-pfDHFR-TSΔ229-277      | 82.1 ± 3.0                     | ND                              |
|                  |                                  | 8. pET-15b                    | 3.1 ± 0.1                      | ND                              |

* Average values from three independent experiments.
** Average values from two independent experiments.
ND: not detectable.
on Luria-Bertani agar containing carbenicillin, tetracycline, chloramphenicol and kanamycin (LB-CTCK).

The interaction between the DHFR monomer and the donated helix within the JR sequence has earlier been noted from the structures of the bifunctional DHFR-TS enzymes of *C. hominis* and *P. falciparum* [17,33]. An interaction of the *pf*DHFR and *pf*JRTS domains was demonstrated using the *E. coli* two-hybrid system [27,28,34]. The *E. coli* reporter strain co-transformed with plasmids pBT-*pf*DHFR and pTRG-*pf*JRTS (with full-length JR sequence) can grow on a LB-CTCK agar plate (Figure 6). The data is in agreement with the complementation results and results from [3H]-FdUMP binding studies supporting the importance of JR on the folding of the pTS domain.

**Discussion**

The JR represents a junctional region linked between the DHFR and the TS domain of parasitic protozoa. This region has been proposed as potential target for drug development in many parasitic protozoa. Indeed, this region in *C. hominis* from one monomer was reported to make extension contacts with the DHFR active site of the other monomer [17]. In *P. falciparum*, the amino acid residues Asp<sup>283</sup>-Asn<sup>290</sup> of JR are strongly predicted to be involved in domain-domain interaction. Unfortunately, the major portions of JR (Lys<sup>232</sup>-Asn<sup>280</sup>) were not seen in the crystal structure previously reported [33]. Therefore, it remains unclear whether each DHFR domain of *P. falciparum* is linked to the TS domain as seen in *C. hominis* or there is a domain swapping assembly. The role of JR was characterized with respect to interdomain interaction.

A series of truncated mutants of *pf*JRTS were constructed containing varying lengths of JR and their interactions with the active *pf*DHFR domain were examined. By employing TS-deficient *E. coli* χ2913 and monitoring its growth complementation in minimum media without thymidine supplementation, the study showed that the *pf*JRTS construct with the shortest length of JR that could still show growth complementation in TS-deficient *E. coli* χ2913 was *pf*JRTS<sup>232-276</sup>. The specific activity of DHFR determined for the crude extract of the monofunctional *pf*DHFR upon co-expression with *pf*JRTS<sup>232-276</sup> was 34.2 ± 9.1 nmole/min/mg, a value which was about half that obtained from the bifunctional *pf*DHFR-TS (70.3 ± 3.6 nmole/min/mg), but was about the same level for all truncated *pf*JRTS mutants. However, the specificity of TS of this truncated construct was only 1.1 ± 0.1 nmole/min/mg (Table 3), which is about 29% of the wild-type enzyme (3.7 ± 0.4 nmole/min/mg). It is noteworthy that the DHFR specific activities among the co-transformants investigated were comparable whereas the TS specific activities were dramatically reduced upon shortening of the JR sequence. The *pf*DHFR and *pf*JRTS domains could be co-purified by using a MTX-affinity column, suggesting that the *pf*DHFR and *pf*TS domains interacted with each other because methotrexate binds only to the *pf*DHFR domain. For the co-transformants that lose the *pf*TS activity including *pf*JRTS<sup>232-277</sup> and *pf*JRTS<sup>232-299</sup>, the constructs still expressed active *pf*DHFR (Table 3). The expression of catalytically active pTS upon cotransformation with *pf*DHFR plasmid was further confirmed by showing a positive autoradiogram of covalent complex formed as a result of [3H]-FdUMP bound to the expressed pTS from the *pf*JRTS<sup>232-276</sup> mutant construct.

These results agree well with the previous studies, suggesting that the *pf*DHFR domain is essential for pTS to be active [18,35]. However, one interesting piece of data from this laboratory showed that co-expression of
the death mutant pfDHFR (Ser\textsuperscript{108} to Trp\textsuperscript{108} mutation) [36] and pfJRTS\textsuperscript{232-235} or P. vivax DHFR with pfJRTS\textsuperscript{232-235} could not confer expression of catalytically active pfTS (data not shown). This finding suggests that not only minimal length of JR is required but active pfDHFR domain is also important for the pfTS domain to be active. Although endogenous DHFR from an E. coli host exists, the enzyme could not promote the growth of TS-deficient E. coli. It is possible that amino acids within the pfDHFR domain are critically necessary to stabilize the DHFR tertiary structure which could indirectly influence the correct folding and hence the activity of the pfTS domain.

The domain-domain interaction of the bifunctional pfDHFR\textsuperscript{-}TS was further demonstrated using an E. coli two hybrid system and the results obtained were consistent with other experiments. The pBT\textunderscore pfDHFR and pTRG\textunderscore pfJRTS co-transformant showed interaction and turned on the Amp\textsuperscript{R} gene, allowing the growth of E. coli XL1-blue MRF\textsuperscript{c}. The results suggest that the minimal length of JR which could allow expression of the catalytically active pfTS was 44 amino acids, approximately half the full length of JR of the P. falciparum DHFR\textunderscore TS.

The importance of the length of JR was investigated by the ability to complement the growth of TS-deficient E. coli in the absence of thymine of co-transformed plasmids harbouring the genes for pfDHFR and truncated pfJRTS compared to plasmid harbouring the gene for the bifunctional pfDHFR\textunderscore TS. Complementation experiments support the conclusion that the length of JR sequence is crucial. The finding that positive complementation was still observed from the construct of truncated bifunctional pfDHFR\textunderscore TS\textsuperscript{229-277} which possesses two more amino acid residues than the case of monofunctional pfJRTS\textsuperscript{232-276} could be due to the more complex structure of the bifunctional protein as compared to the monofunctional enzymes resulting from the interaction, or associated with the sensitivity of the complementation assay system. To further address this discrepancy, a more sensitive \([3H]\)-FdUMP binding assay method was used to confirm that pfDHFR\textunderscore TS\textsuperscript{229-276} is the construct with the shortest length of JR that could still express active pfTS. The result was in good agreement with the results from complementation studies using monofunctional pfJRTS\textsuperscript{229-276}. The fact that the bifunctional mutant failed to show complementation in TS-deficient E. coli but could not detect the activity of pfTS by \textit{in vitro} enzymatic assay could be due to the insufficient expression of protein in combination with the sensitivity of the complementation method, since a minimum TS activity of \~1-5 nmole/min/mg is necessary for complementation to be observed in E. coli J913 [37]. It should also be noted that the low expression observed in the truncated bifunctional pfDHFR\textunderscore TS was consistent with a previous mutation study which proposed that the significant intracellular proteolysis activity associated with the \(\alpha\)-helix structure in this region could be attributed to the low expression of the mutant proteins [38].

The present results clearly demonstrated that shortening the JR sequence in the pfDHFR\textunderscore TS could affect the activity of both pfTS and pfDHFR. Contrary to that observed in T. gondii and C. hominis which have longer JR sequences, mutations of the JR sequence were reported to affect only the activity of DHFR but not the activity of TS [39,40]. This and other data support the conclusion that the length of JR sequence is necessary for the activity of both pfDHFR and pfTS domains, and hence highlights the importance of the JR sequence in \textit{P. falciparum} bifunctional DHFR\textunderscore TS.

It was not so surprising that the constructs pfJRTS\textsuperscript{232-277} and pfDHFR\textunderscore TS\textsuperscript{229-277} showed no expression of catalytically active pfTS, despite the fact that the constructs contain a sequence that encompasses Asp\textsuperscript{283} - Asn\textsuperscript{296}, the region previously predicted to form \(\alpha\)-helix and responsible for electrostatic interaction between the negatively charge residues and positive charge of the pfDHFR and pfTS from different domains [33]. A number of possible explanations could be as follows: (i) the length of JR in the constructs is too short, and this could affect the formation of a stable helix and consequently disturb the interaction with pfDHFR and interfere with the proper formation of pfTS conformation; (ii) too short a JR sequence could affect the structure which could result in a steric constraint or functional infringement of one domain upon the other; (iii) the existence of translational autoregulation could block translation of mRNA coding for bifunctional enzyme from binding to the DHFR domain of the protein [41]; and, (iv) the mutation could cause exposure of some parts of the enzyme which are sensitive to proteolysis [38]. Therefore, too short a JR sequence could neither support interaction between pfDHFR and pfTS domains nor contribute to proper folding of the pfDHFR\textunderscore TS bifunctional protein.

**Conclusions**

The data presented in this study show that: a) the presence of an active pfDHFR domain and the appropriate length of JR are critical for pfTS to fold correctly to be catalytically active; and, b) deletion of JR affects both the activities of the pfTS and the pfDHFR domain of the bifunctional pfDHFR\textunderscore TS. From this study, the JR of \textit{P. falciparum} has a unique role which is different from other long linker parasitic protozoa. The data described here could be useful for the development of compounds that bind to the JR and interfere with the dimerization of the pfTS subdomains. These studies could potentially lead to novel means of development of inhibitors targeting the JR sequence of the parasite.
Abbreviations

DHFTR-TS: Dihydrofolate reductase-thymidylate synthase; SHMT: Serine hydroxymethyltransferase; dTMP; 2'-deoxythymidine 5'-monophosphate; [3H]-FdUMP; Tritiated-5-fluoro-2'-deoxyuridine 5'-monophosphate; JR: Junctional region; MM: Minimal media; MTX: Methotrexate.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

NC participated in performing experiments and drafted the manuscript. RS helped to perform the experiments and discussion. WS conceived the study, participated in its design, data analysis and critical revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to Drs. Michael A. Allen, Alfredo Villarreal and Alejandro Sáiz for critical review of the manuscript. This work was supported in part by the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative to WS. NC is a recipient of a grant under the programme Strategic Scholarships for Frontier Research Network for the Joint PhD Program Thai Doctoral degree from the Commission on Higher Education, Thailand.

Received: 19 December 2012 Accepted: 9 March 2013
Published: 12 March 2013

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doi:10.1186/1475-2875-12-91
Cite this article as: Chaianantakul et al: Insights into the role of the junctional region of Plasmodium falciparum dihydrofolate reductase-thymidylate synthase. Malaria Journal 2013 12:91.