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Crystal structures of *Plasmodium falciparum* cytosolic tryptophanyl-tRNA synthetase and its potential as a target for structure-guided drug design

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Abstract

Malaria, most commonly caused by the parasite *Plasmodium falciparum*, is a devastating disease that remains a large global health burden. Lack of vaccines and drug resistance necessitate the continual development of new drugs and exploration of new drug targets. Due to their essential role in protein synthesis, aminoacyl-tRNA synthetases are potential anti-malaria drug targets. Here we report the crystal structures of *P. falciparum* cytosolic tryptophanyl-tRNA synthetase (*Pf*-cTrpRS) in its ligand-free state and tryptophanyl-adenylate (WAMP)-bound state at 2.34 Å and 2.40 Å resolutions, respectively. Large conformational changes are observed when the ligand-free protein is bound to WAMP. Multiple residues, completely surrounding the active site pocket, collapsed onto WAMP. Comparison of the structures to those of human cytosolic TrpRS (*Hs*-cTrpRS) provides information about the possibility of targeting *Pf*-cTrpRS for inhibitor development. There is a high degree of similarity between *Pf*-cTrpRS and *Hs*-cTrpRS within the active site. However, the large motion that *Pf*-cTrpRS undergoes during transitions between different functional states avails an opportunity to arrive at compounds which selectively perturb the motion, and may provide a starting point for the development of new anti-malaria therapeutics.

Keywords

Plasmodium falciparum; malaria; tryptophanyl-tRNA synthetase; crystal structure; conformational changes; drug design

1. Introduction

Malaria is one of the most serious diseases in the world, causing around 1.2 million of deaths in the year 2010 [1]. The most common and serious human malaria parasite is *Plasmodium falciparum*. Currently, the near term prospects of arriving at a highly effective vaccines appear to be challenging [2], while drug resistant parasites remain one of the

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Coordinates and structure factors for *Pf*-cTrpRS F- and P-states are deposited in the Protein Data Bank under accession codes 4J76 and 4J75, respectively.

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biggest threats in malaria control [3]. Therefore, it is important that new drugs are developed to fill the pipeline of anti-plasmodium therapeutics.

The aminoacyl tRNA synthetases (aaRS) form a group of ubiquitous enzymes that perform the essential function of charging amino acids to their cognate tRNAs during protein synthesis [4]. In general, the reaction is completed in two steps:

1. amino acid + ATP $\rightarrow$ aminoacyl-AMP + PP_i
2. aminoacyl-AMP + tRNA $\rightarrow$ aminoacyl-tRNA + AMP

During this reaction, large conformational changes in aaRSs are often needed. Different conformations of the enzyme are required for binding of substrates, arranging the active site for catalysis and releasing products. Depending on the substrate bound, several structural states are observed in crystal structures of tRNA synthetases. Focusing here on tryptophanyl-tRNA synthetases (TrpRSs), extensive structural data are available on both the bacterial and the eukaryotic (cytoplasmic) TrpRS enzymes [5–10]. For bacterial TrpRS, at least three conformational states have been reported. In the open, ligand-free state (F-state), either Trp or ATP can bind to the enzyme without major changes in conformation. However, the simultaneous binding of Trp and ATP in the pre-transition state requires a compact active site where a conserved KMSKS loop closes onto the active site to interact with ATP, and its anticodon binding C-terminal domain moves towards the active site-containing Rossmann-fold domain. After the intermediate tryptophanyl-adenylate (WAMP) is formed, both the KMSKS loop and the C-terminal domain move slightly away from the catalytic core to allow turnover in the partially closed product-state (P-state) [5–7]. In contrast, in eukaryotic (cytoplasmic) TrpRS, the binding of Trp is accompanied by a change from an open F-state to a closed to Trp-bound state. Only the closed conformation has a Trp-binding pocket that complements the amino acid well. Structural elements that ‘close’ onto the active site are mainly the N-terminus and a conserved AIDQ motif, but the domain motion observed in bacterial TrpRS does not occur. Subtle movements in the KMSKS loop subsequently assist ATP binding, amino acid activation and product release, but the overall closed conformation is maintained in the P-state [8]. The N-terminus then needs to be displaced while the rest of the enzyme stays in the closed conformation to allow the acceptor arm of the tRNA^{Trp} to access the active site, resulting in another conformational state [9]. Hence TrpRS, like many other aaRSs, undergo a series of complex conformational changes to perform their function.

Owing to its vital role in protein synthesis, this group of enzymes is in principle an important target for the development of anti-parasitic agents [11]. Recently, there have been an increasing number of reports targeting aaRSs to treat infectious diseases [12–18]. Inhibitors of aaRSs typically bind to the active site or the editing site of these enzymes, preventing the binding or release of amino acid, ATP or tRNA. In order to be effective, these substrate binding site inhibitors need to have a high affinity for the pathogen but not for the homologous enzymes of the human host. High resolution three-dimensional structures should be helpful in identifying differences in the substrate binding sites and also for exploring the nature of conformational changes. Such changes might be prevented by small molecules, ultimately leading to new drugs.

As part of our ongoing efforts to explore aaRSs as anti-parasitic drug targets [14, 19–25], we have determined the crystal structure of one of the two tryptophanyl-tRNA synthetases from *P. falciparum*. Like many other higher eukaryotes, *P. falciparum* has two genes encoding for TrpRS. The two gene products are predicted to be localized in the cytosol (PlasmoDB PF3D7_1336900) and in the apicoplast (PlasmoDB PF3D7_1251700) [26]. Here we name the two proteins *Pf*-cTrpRS and *Pf*-aTrpRS, respectively, to denote their likely localization compartments. The structures reported here are of *Pf*-cTrpRS. Based on sequence

comparison, *Pf*-cTrpRS is more closely related to human cytosolic TrpRS (*Hs*-cTrpRS, ~ 44 % identity) than to human mitochondrial TrpRS (~ 16 % identity). In addition to the Rossmann-fold and the C-terminal anticodon-binding α -helical domains common to all Class I aaRS, *Pf*-cTrpRS has an N-terminal extension of 300 residues. The first 228 residues appear to be apicomplexan-specific, with weak homology to the editing domain of archaeal and eukaryotic AlaRS, although the function of these residues in *P. falciparum* is unknown. The next 72 residues of the N-terminal extension can be aligned with the eukaryote-specific extension (ESE) of *Hs*-cTrpRS (Fig. 1).

We crystallized *Pf*-cTrpRS in its F-state and solved its structure to 2.34 Å resolution. Subsequently, tryptophan (Trp), ATP and Mg^{2+} were soaked into crystals which resulted in the 2.40 Å structure of the WAMP-bound P-state of this malaria parasite enzyme. Based on these two structures, which reveal considerable conformational differences, we examine the potential of this enzyme as a drug target.

2. Materials and methods

2.1. Protein expression and purification

Attempts to express soluble protein from the full-length *Pf*-cTrpRS sequence (PlasmoDB PF3D7_1336900) were unsuccessful. However, soluble expression of a protein from a construct representing residues 229 – 632 of the protein was achieved. The sequence represents the complete ESE, Rossmann-fold domain and C-terminal helical domain (Fig. 1A). While not tested for *Pf*-cTrpRS, the deletion of the first part of N-terminal extension did not affect the aminoacylation activity of the homologous apicomplexan *Cryptosporidium parvum* TrpRS [25]. The construct was cloned into the AVA0421 vector for expression in *E. coli* [27]. Protein was purified by a Ni-NTA affinity column followed by overnight cleavage of the N-terminal hexa-histidine tag using N-terminally histidine tagged 3C protease at 4°C. Cleaved protein was purified by a second Ni-NTA step and then size-exclusion chromatography on a Superdex 75 column (Amersham Pharmacia Biotech) using a buffer containing 25 mM HEPES, 500 mM NaCl, 2 mM DTT, 5% glycerol, and 0.025% NaN_3 at pH 7.0. Purified protein retained five residues of the 3C protease cleavage site (GPGSM) at the N-terminus.

2.2. Protein crystallization and treatment

Crystals of *Pf*-cTrpRS were obtained by vapor diffusion using sitting drops equilibrated at room temperature against 50 μ L of a reservoir containing 0.2 M sodium citrate and 20% PEG 3350. The drop consisted of 0.15 μ L protein at 21.8 mg/mL plus 0.15 μ L of the reservoir solution. Crystals grew in 1–2 days at room temperature. Crystals were cryo-protected with 20% of glycerol before freezing under liquid nitrogen for data collection. In order to determine the intermediate product WAMP-bound structure, the crystals were soaked in the cryo-protecting solution in the presence of 5 mM L-Trp, ATP and $MgCl_2$ for 5 min before freezing.

2.3. Data collection and structure determination

Data for the ligand-free structure was collected from a single crystal using a wavelength of 0.98 Å at synchrotron beamline 5.0.2 of the Advanced Light Source in Berkeley, CA. Data was processed with HKL2000 [28] (Table 1). The coordinates of TrpRS from *C. parvum* were used as search model for phase determination by molecular replacement using the program Phaser [29]. The solution was fed to the model building software ARP/wARP [30] for automatic model rebuilding. Subsequent iterated manual building/rebuilding and refinement of models were performed using Coot [31] and Refmac5 [32], respectively. In the final cycles of refinement, protein structures were refined with translational/libration/

screw (TLS) groups identified by the TLS motion determination server [33] before restrained refinement in Refmac5. The structure validation server MolProbity [34] was used throughout the process to monitor the progress of structure determination. The final crystallographic refinement statistics are given in the Table 1. Figures were created and rendered with Pymol [35]. Superposition of structures are carried out with Pymol, which first aligns proteins by sequence followed by structural alignment using five cycles of refinement to improve the fit by discarding pairs with high relative variability [35].

Data for the WAMP-bound structure were collected from a single crystal soaked for 5 min with 5 mM of L-Trp, ATP and MgCl₂ using a MicroMax-007 HF rotating anode (Rigaku) equipped with VariMax HF (Osmic) monochromator and a Saturn 994 (Rigaku) CCD detector at a wavelength of 1.54 Å. Data were integrated using XDS [36] and scaled with Scala [37]. Coordinates of the ligand-free structure of *Pf*-cTrpRS were used as search model for molecular replacement in Phaser [29]. Subsequent model building and validation process are the same as described above for the ligand-free structure.

3. Results and Discussions

3.1. Overall structure and domain architecture

The construct used for the structure determination contains the ESE (Ser229 to His300), the Rossmann-fold (Lys301 to Thr520 & Thr611 to Met632) and the C-terminal helical domain (Asp521 to Leu610) (Fig. 1). *Pf*-cTrpRS crystallized in space group P2₁2₁2 with a dimer in the asymmetric unit (Fig. 2A). The structure of the ligand-free F-state was refined to 2.34 Å with good statistics (Table 1). Part of the ESE (Ser229 to Glu243), along with an insertion loop near the KMSKS motif (KMSST in *Pf*-cTrpRS), are disordered in both subunits. In addition, residues Gly538 to Gly550 are disordered in subunit B only. Subunits A and B superimpose with an rmsd of 0.23 Å for 299 C_α atoms and are essentially identical in structure. A search on the DALI server [38] showed that *Pf*-cTrpRS is structurally closest to *Hs*-cTrpRS (PDB: 2QUK) [8]. The dimer of *Pf*-cTrpRS superimposes onto *Hs*-cTrpRS with an rmsd of 1.11 Å for 586 equivalent C_α atoms. Soaking of the crystals in Trp, ATP and Mg²⁺ resulted in a 2.40 Å structure with clear and strong density of WAMP in both active sites representing the P-state of the *Pf*-cTrpRS dimer (Fig. 2B). In their P-state, subunit A can be superimposed onto subunit B with an rmsd of 0.25 Å for 310 C_α atoms. Since in both the F structure and the P structure the two subunits A and B per asymmetric unit are essentially identical to each other, the analysis below will focus on subunits A only.

3.2. Motions in *Pf*-cTrpRS during state transitions

Comparing the P-state with the F-state of *Pf*-cTrpRS reveals large structural differences of the protein (Fig. 3A). Subunit As of the F- and P-states superimpose with an rmsd of 0.42 Å based on 283 out of 370 C_α atoms. A total of 87 residues exhibit considerable changes in conformation. Many of these residues are located in secondary structure elements spread over two domains, the Rossmann-fold and the ESE, and completely surrounding the active site. This contributes to an overall ‘collapse’ of the active site pocket in the P-state (Fig. 3A). On average, the shift of the C_α atoms of the 87 residues is 1.9 Å. The overall volume of the active site decreases from 1658 Å³ to 838 Å³ as calculated using the CASTp server [39].

Conformational changes of both bacterial and eukaryotic (cytoplasmic) TrpRS enzymes upon ligand binding are well documented as described above. Interestingly, structural features involved in the transitions are different. Bacterial TrpRS is postulated to move from an open, F-state to the closed, P-state via a closed, pre-transition state. The ‘closing’ of the active site pocket in this case is mainly due to an inter-domain twisting motion of the Rossmann-fold relative to the C-terminal domain, instead of an intra-domain collapsing of the active site pocket as observed in *Pf*-cTrpRS. This collapsing of the active site pocket of

Pf-cTrpRS is replicated, albeit not completely, in eukaryote cytosolic TrpRS, specifically *Hs*-cTrpRS, as discussed below.

The most complete model of *Hs*-cTrpRS available (PDB: 1R6T) [40] contains in the asymmetric unit one subunit in the F-state and another subunit in the P-state. Superpositions of the F-state on the P-state of *Hs*-cTrpRS results in an RMSD of 0.56 Å for 323 C α atoms, with only 39 residues (out of 362) differing significantly in position (Fig. 3B). These structures are used for comparison with *Pf*-cTrpRS. Many of these shifts in the human and the parasite enzyme are similar:

- (A) the C-terminus of β -strand β 6, containing the conserved AIDQ motif, continuing into 3_{10} -helix η 4 and α -helix α 1 closes onto the Trp moiety;
- (B) the C-terminus of β 7 continuing into the KMSKS motif-containing loop closes onto the adenine ring;
- (C) the first part of the KMSST (KMSAS in *Hs*-cTrpRS) motif containing loop, also closes onto the adenine ring;
- (D) the entire helix α 1, as second shell residues, collapses onto the first shell residues in specified in (A) and (B) above; and
- (E) the tip of a loop between β 3 and α 5, immediately preceding the HLGH (HVGH in *Hs*-TrpRS) motif changes position;

However, major additional shifts which do not occur in the human enzyme are observed in *Pf*-cTrpRS upon WAMP binding (compare Fig 3A and Fig 3B):

- (F) α 6 continuing into α 7 closes onto the Trp moiety of the bound WAMP;
- (G) α 9, continuing into α 10, as second shell residues, move into the space created by shifts described in (A) and (F) above; and
- (H) residues of the N-terminus of α 1 and residues in β 2, as second shell residues, move into space created by shifts described in (D) above.

Another difference between WAMP binding by the parasite and the human enzyme is that two features remain disordered up WAMP binding by the parasite enzyme while these become ordered in the human enzyme [8, 40–42]:

- (I) a β -hairpin structure in the ESE covering the ATP binding site; and
- (J) the KMSST motif-containing loop.

Pf-cTrpRS has a unique insertion of 15 residues in the loop after the KMSST motif (Fig. 1), which is equivalent to the KMSAS motif in the human enzyme, and this insertion may contribute to the flexibility of the KMSST motif-containing loop in *Pf*-cTrpRS, in both the F- and P-states.

The key difference in the conformational changes of ligand-free parasite and human cTrpRS upon WAMP binding is that in the *Pf*-cTrpRS the active site is completely surrounded by shifting residues, while in *Hs*-cTrpRS this is only partially so (Fig. 3A vs. Fig. 3B). The motions of *Hs*-cTrpRS upon WAMP binding are less extensive in that movements listed above in (F), (G) and (H) for *Pf*-cTrpRS, do not occur in *Hs*-cTrpRS.

3.3. Comparison of active site residues in *Pf*-cTrpRS and *Hs*-cTrpRS

Residues that are directly in contact with WAMP are highly conserved between *Pf*-cTrpRS and *Hs*-cTrpRS. Based on a 4.5 Å radius around WAMP in *Pf*-cTrpRS P-state structure, 28 contact residues can be identified (Fig. 3C). Twenty one of these 28 residues are identical in

Pf and *Hs*-cTrpRS. The remaining 7 residues are mostly conserved substitutions (Fig. 1B). Overall, the positions of contact residues of the adenine binding site appear to be slightly more variable between *Pf*-cTrpRS and *Hs*-cTrpRS but the size of the differences is limited. In several cases contact *atoms* are conserved even though there is a difference in the identity of the *residues* (e.g. Gln452 *vs.* Cys309, Val481 *vs.* Thr338). There are a few subtle differences, however, likely to decrease the size of the pocket in *Pf*-cTrpRS (Fig. 3C). Examples are: (i) the phenolic oxygen of Tyr425 has no equivalent in human (Phe280 is at this position); (ii) the positions of the side chain amino groups of *Pf*-cTrpRS Lys347 and equivalent *Hs*-cTrpRS Lys200 differ by 3.5 Å, but this difference is quite far from the ligand; and, (iii) Phe482 in *Pf*-cTrpRS is ~ 1.0 Å closer to the adenine ring of WAMP than Phe339 in *Hs*-cTrpRS. Overall, no striking features can be readily identified and exploited for the design of selective inhibitors based solely on the structure of the active site pockets in human and parasite enzymes.

4. Conclusions

When looking at the ligand binding region only, it seems that there are limited opportunities to arrive at selective active site inhibitors for *Pf*-cTrpRS. However, instead of targeting the active site, conformational changes occurring during the transition from F- to P-state as observed in the current structures (Fig. 3A,B) could well be a basis of selective inhibition by small molecules using an alternative mode of action. As discussed above, there are major differences in the transitions of states in *Pf*-cTrpRS and *Hs*-cTrpRS. The need for the conformational changes during the aminoacylation reaction could make *Pf*-cTrpRS susceptible to inhibitors that prevent or perturb the motion. Specific inhibitors could possibly be developed to target residues that are involved in facilitating the motions of the protein while performing its function. Away from the substrate binding sites, the conservation of residues and conformations decreases and hence the chance of specificity increases. In this case, *Pf*-cTrpRS and *Hs*-cTrpRS have 75 % identity regarding WAMP-contacting residues, but the identity decreases to 55 % among residues involved in the motion of state transitions. More importantly, as mentioned earlier, many shifts in residues located in *Pf*-cTrpRS are not observed in *Hs*-cTrpRS, forming a patch of surface that could be targeted to arrive at specific inhibitors (Fig. 3B). Admittedly, it could be challenging to design such inhibitors *de novo*. However, high-throughput screening of small molecule libraries or fragment-based screening using *Pf*-cTrpRS are powerful tools to discover inhibitory small molecules which prevent conformational changes, discoveries which could be followed up by structure-based approaches as in the case of HIV reverse transcriptase [43, 44], protein tyrosine phosphatase 1B [45] and fructose 1,6-bisphosphatase [46]. The enzyme•ligand structures reported here reveal differences in conformational changes upon ligand binding by parasite and human enzyme which might inspire screening compound libraries to arrive at hits for further development as anti-malaria agents.

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Highlights

- Two crystal structures of *Plasmodium falciparum* cytosolic TrpRS are reported.
- The ligand-free protein undergoes conformational changes upon product formation.
- Multiple residues collapse onto the active site when the product is bound.
- The conformational changes are not completely replicated in human cytosolic TrpRS.
- Molecules perturbing these changes could be useful for developing an anti-malaria drug.

Figure 1. The domain organization and primary structure of *Pf*-cTrpRS

(A) The domain organization of *Hs*-cTrpRS and *Pf*-cTrpRS. VSE: vertebrate-specific N-extension; ESE: eukaryote-specific N-terminal extension; ASE: apicomplexan-specific N-terminal extension. A 15-residues insertion in the KMSST-containing loop specific to *Pf*-cTrpRS is also indicated.

(B) Sequence alignment of *Pf*-cTrpRS and *Hs*-cTrpRS. Identical residues are highlighted in red, similar residues are boxed in blue. Secondary structures are indicated for *Pf*-cTrpRS. Residues in direct contact with WAMP are indicated with blue upward arrows. Residues with high variability excluded during the superpositions of *Pf*-cTrpRS F- and P-states are indicated with green bars. Panel (B) is rendered with the ESPript server [47].

Figure 2. The dimer of *Pf*-cTrpRS

(A) The F-state of *Pf*-cTrpRS dimer. One subunit is colored based on their separate domains while the other subunit is in grey. The buried solvent accessible surface area of the interface upon dimer formation is 1459.5 Å² according to PDBePISA server [48].

(B) The active site of the P-state of *Pf*-cTrpRS dimer. σ A-weighted Fo-Fc electron density maps around WAMP, calculated after WAMP molecules are omitted for subunit A and subunit B. Maps are contoured at ± 4 sigma. Green is positive density, red is negative density.

Figure 3. Comparisons of *Pf*-cTrpRS and *Hs*-cTrpRS

(A) Superpositions of the *Pf*-cTrpRS F- and P-states reveal significant shifts in residues in the catalytic domain and the ESE. These residues are colored blue and red in the F-state and P-state, respectively. These residues completely surround the active site pocket and collapse onto the active site during the transition from F- to P-state, as indicated by yellow arrows.

(B) Superpositions of *Hs*-cTrpRS F-state and P-state (PDB: 1R6T) [40] show that fewer residues are involved in shifts compared to *Pf*-cTrpRS. These residues are colored purple and green in the F-state and P-state, respectively. These residues, unlike in (A), are concentrated mainly in the lower part of the enzyme.

(C) Stereo views of the WAMP contacting residues in superposed *Pf*-cTrpRS and *Hs*-cTrpRS P-states. All residues within a 4.5 Å radius of WAMP are shown. Identical residues

are colored grey. Residues in *Pf*-cTrpRS and *Hs*-cTrpRS that are different are colored and labeled red and green, respectively. Two contacting residues, K347 and F339 in *Pf*-cTrpRS, although identical, have different conformations. They are colored orange and purple in *Pf*-cTrpRS and *Hs*-cTrpRS, respectively.

Table 1

Crystallographic data collection and refinement statistics.

Data collection	Ligand-free (F-state)	WAMP-bound (P-state)
Beamline	ALS 5.0.2	Rotating anode, Saturn 944 CCD detector
Wavelength (Å)	0.977	1.54
Space group	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Unit cell parameters [a, b, c (Å); $\alpha=\beta=\gamma$ (°)]	117.3, 190.7, 51.7; 90	117.0, 189.5, 51.5; 90
Resolution (Å)	50 – 2.34	38.6 – 2.40
Unique reflections	49735 (2412) *	45354 (4348)
Completeness (%)	100 (100)	99.2 (98.2)
R_{merge}	0.088 (0.363)	0.159 (0.957)
R_{pim}	0.047 (0.204)	0.093 (0.559)
Mean $I/\sigma(I)$	22.8 (5.2)	12.5 (2.2)
Redundancy	7.3 (6.5)	7.3 (7.4)
Refinement	Ligand-free (F-state)	WAMP-bound (P-state)
Resolution (Å)	30 – 2.34	30 – 2.40
R_{work}	0.179	0.193
R_{free}	0.209	0.222
No. reflections; refinement/test set	46998/2506	43003/2309
RMSD bonds (Å)	0.007	0.007
RMSD angles (°)	1.08	1.17
No. atoms (protein/substrate/glycerol/water)	5989/0/24/324	6073/74/12/213
Residues in favoured regions (%) [#]	99.3	99.6
Residues in allowed regions (%)	0.7	0.4
Residues in disallowed regions (%)	0	0
Average B factors for atoms (protein/substrate/glycerol/water) (Å ²)	46.0/-/61.7/41.5	44.4/29.5/60.0/34.1
PDB entry	4J76	4J75

* Values in parenthesis are for the highest resolution shell

[#] Ramachandran Plot statistics are as reported by the Molprobit server [31]