Chapter 2. Biochemical and structural analysis of D-amino-acid deacylase (DTD)
2.1 INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs) transfer L-amino acids to their cognate tRNA and are essential in protein translation process. However, at times aaRS enzymes can also attach D-amino acids to tRNA and this mistake can be toxic to growing cells. Tyrosyl-tRNA synthetases from *Escherichia coli* and *Bacillus subtilis* (Calendar and Berg 1966), tyrosyl-tRNA synthetases from *Saccharomyces cerevisiae* (Soutourina et al. 2000) and tryptophanyl/aspartyl-tRNA synthetases from *Saccharomyces cerevisiae* can transfer D-forms of their cognate amino acids onto relevant tRNAs. In order to avoid introduction of D-amino acids in the translation machinery almost all cells have editing domains like D-tyrosyl-tRNA\textsubscript{Tyr} deacylase (DTD). This enzyme cleaves the bond between D-amino acids and tRNA (Fig. 1.1A) and is encoded by *dtd* gene in *Escherichia coli* (Soutourina et al. 1999) and by the *dtd1* gene in *Saccharomyces cerevisiae* (Soutourina et al. 2000). Homologs of *dtd/dtd1* genes are found in many genomes (*Saccharomyces cerevisiae*, *Caenorhabditis elegans*, mouse, human (Soutourina et al. 1999), but not in archaea (Soutourina et al. 2000). However, another *dtd2* (previously named *GEK1*) gene homologous to *dtd/dtd1* is found in archaea and plants (Ferri-Fioni et al. 2006; Wydau et al. 2007). DTD enzymes exhibit broad specificity toward various D-amino acids charged tRNAs (D-aa-tRNA) and is essentially inactive towards L-aa-tRNAs (Calendar and Berg 1966). Human DTD, also called DUE-B, has a long C-terminal extension and it seems to be involved in D-amino acid resistance by deacylating D-aa-tRNAs during tRNA export (Zheng et al. 2009).

*Plasmodium* parasites are causative agents of malaria which affects >500 million people and claims ~2 million lives annually (Snow et al. 2005). In the *Plasmodium falciparum* genome, there are no sequence homologs for D-amino acid oxidase and D-Ser racemase, but a single copy of the *dtd* gene is present. *PfDTD* may therefore be responsible for
detoxification of D-amino acids in this parasite. The molecular weight of PfDTD is ~20 kDa and the sequence identity between PfDTD and its human homolog (HsDUE-B) is ~35%.

Native DTD structures have been reported from *Aquifex aeolicus* (AaDTD - PDB code 2DBO), *Escherichia coli* (EcDTD - PDB code 1JKE (Ferri-Fioni et al. 2001)), *Haemophilus influenza* (HiDTD - PDB code 1J7G (Lim et al. 2003)), *Homo sapiens* (HsDTD - PDB code 2OKV (Kemp et al. 2007)) and *Leishmania major* (LmDTD - PDB code 1TC5). A catalytic mechanism has also been proposed on the basis of mutagenesis and tRNA modeling onto DTDs (Lim et al. 2003; Kemp et al. 2007). However, till date, there are no DTD-ligand complex structures known from any organism. Here, we report a whole set of crystal structures of DTD from *Plasmodium falciparum* complexed with adenosine and various D-amino acids. The crystal structures of native, ADP-bound and D-amino acid complexed PfDTDs provide key insights into the binding and recognition modes for various ligands. Based on the high resolution structures of PfDTD, We have also performed in *silico* inhibitor screening and present data for four compounds which inhibit parasite growth. We believe that our analysis not only provides a structural basis for the catalytic mechanism for this family of editing enzymes, but also highlights a possible new focus for development of specific anti-malarials.

### 2.2 METHODS

**Expression of PfDTD.** The *dtd* gene from *Plasmodium falciparum* was PCR amplified and cloned between *NcoI* and *KpnI* restriction sites in modified pET28a vector and PfDTD was expressed in fusion with histidine tag. *Escherichia coli* B834 (DE3) cells were transformed with pET28DTD plasmid and grown at 37°C in a culture medium (LB broth, USB). Culture was induced with isopropyl 1-thio-D-galactopyranoside (0.5 mM at OD of 0.6) and growth
was continued for 5 h at 37° C. Bacterial cell pellet was suspended in Ni-NTA buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 7.3) supplemented with lysozyme (100 µg ml⁻¹) and protease inhibitor cocktail. Cells were sonicated and centrifuged at 14,000 rpm. The cleared supernatant was passed through Ni-NTA beads (Qiagen) which were then washed with Ni-NTA buffer to remove impurities and protein was eluted with increasing concentration of imidazole (up to 500 mM). P/DTD protein was further purified via Superdex S-75 gel filtration chromatography (Amersham Pharmacia Biotech). Purified P/DTD was concentrated using a 10 kDa Centricon (Viva Biosciences) and was buffer exchanged into crystallization buffer (25 mM Tris-HCl pH 7.3, 100 mM NaCl and 0.02% NaN₃).

**Assay of D-Tyr-tRNATyr deacylase activity.** [³H]D- and [14C]L-Tyr-tRNA^{Tyr} were prepared as described previously (Wydau et al. 2007). D-Tyr-tRNA^{Tyr} hydrolysis was allowed for 5 min at 28° C, in 100 µl assays containing: 20 mM Tris-HCl (pH 7.8), 3 mM MgCl₂, 100 nM [³H] D-Tyr-tRNA and 50 µg ml⁻¹ BSA. Prior to its addition to the assay, the enzyme was diluted in 20 mM Tris-HCl (pH 7.8) containing 200 µg ml⁻¹ BSA in order to obtain 80-400 pM of P/DTD in the test. Reaction was quenched by addition of 340 µl ethanol, 14 µl sodium acetate 3M (pH 4.8) and 20 µl carrier RNA from yeast at 4 mg/ml. Samples were centrifuged (20 min, 20600 g, 4° C) and radioactivity in the supernatant was measured by scintillation counting. [14C]L-Tyr-tRNA^{Tyr} hydrolysis was measured in same conditions, except that enzyme concentration was increased up to 400 nM. Enzyme concentration was based on extinction coefficient of 25565 M⁻¹ cm⁻¹.

**Immunofluorescence assays.** These were done essentially as described (Winter et al. 2005) but with slight modifications. Asynchronous parasite pellet was washed twice in PBS, smeared onto a cover slip and air dried. The smears were fixed in 1% formaldehyde in PBS for 30 min at room temperature and then rinsed in wash buffer (0.5% fetal bovine serum, 0.5% normal goat serum, 0.05% saponin) followed by blocking in PBS solution containing
5% skimmed milk powder, 5% goat serum, and 0.1% saponin. After washing, the cover slip were incubated with a mixture of affinity-purified rabbit anti-\( PjNapL \) antibodies (1:1000 dilution) and affinity purified mouse anti-\( PjDTD \) antibody (1:1000 dilution) at room temperature with gentle shaking for 1 h. The cover slip was washed and incubated with a mixture of anti-rabbit antibody (1:500 dilution) conjugated to Alexa flour 594 (Molecular Probes) and anti-mouse antibody (1:500 dilution) conjugated to Alexa flour 488 (Molecular Probes) for 1 h at room temperature. Nuclei were stained by incubating the cover slip in PBS containing 3 \( \mu \)g/ml DAPI stain (Molecular Probes) for 5 min at room temperature, and after washing, the cover slip was mounted on slides with Antifade (Molecular Probes). Fluorescence microscopy was performed on a Nikon eclipse TE2000-U microscope.

Crystallization and data collection. Crystallization of \( PjDTD \) was carried out at room temperature using the hanging-drop vapour-diffusion method by mixing 1 \( \mu \)l of protein solution (5-10 mg ml\(^{-1}\)) with 1 \( \mu \)l reservoir solution and this was equilibrated against 200 \( \mu \)l well liquor. Plate shaped crystals were obtained using 0.1 M MES pH 6.2-6.8 and 25-30% PEG3350 or 0.1 M TRIS/HEPES pH 7.5-8.5 and 25% PEG3350. Pyramidal shaped crystals were also obtained from 25% PEG1500 as the precipitant solution. Crystals were transferred to a cryoprotectant solution (composed of a higher concentration of mother liquor) for a short period (10-30s) prior to freezing. Halide derivative of \( PjDTD \) crystals was prepared by soaking native crystals for 10-30s in cryoprotectant solution containing 0.2 to 1 M sodium iodide. \( PjDTD-ADP \) complex crystals were obtained by soaking the native crystals for 10 to 30 min in cryoprotectant solution containing 5 mM ATP. \( PjDTD-D\)-amino acid complex crystals were obtained by soaking crystals from 12 to 60 h in cryoprotectant solution containing 5 to 20 mM of various D-amino acids individually. Native, \( PjDTD\)-iodide and \( PjDTD-D\)-amino acid complex data sets were collected at 100 K using Cu K\( \alpha \) radiation (\( \lambda = 1.54 \) \( \AA \)) generated by a Rigaku Micro-Max007 rotating anode X-ray generator operated at 40
kV and 20 mA with VariMax HR mirrors. Diffraction images were recorded using a MAR345dtb image plate detector. *PfDTD*-ADP complex data were collected on a MARCCD detector at the beamline BM14 of the European Synchrotron Radiation Facility. Data were processed using HKL2000.

**Structure determination and refinement.** The crystal structure of *PfDTD* was determined using iodide-single wavelength anomalous diffraction phasing technique as implemented in PHENIX (Adams et al. 2002a). One refined *PfDTD*-iodide dimer was subsequently used to determine the native *PfDTD*, *PfDTD*-ADP and *PfDTD*-D-amino acid complex structures by molecular replacement method using programs Molrep (Vagin and Teplyakov) and Auto-Rickshaw (Panjikar et al. 2005). All model refinements were carried out using CNS (Brunger et al. 1998) and REFMAC (Murshudov et al. 1997). Visual inspection and model building were carried out using the graphics program COOT (Emsley and Cowtan 2004). Crystallographic refinement statistics are given in Supplementary Table S1. PROCHECK (Laskowski et al. 1993) was used to validate the stereo-chemical quality of the final models. Structural superimpositions, sequence alignments and figures were generated using CHIMERA (Pettersen et al. 2004) and PyMOL (available at www.pymol.org).

**In-silico screening of PfDTD inhibitors.** Molecular docking and *in-silico* screening were carried out for high resolution *PfDTD* dimer molecule using docking program FlexX in Tripos Sybyl 7.7 suite (Tripos, St. Louis, MO) and NCI diversity library which containing ~2000 compounds. Lead compounds were selected based on docking score, energy, and hydrogen bond interactions.

**Parasite growth inhibition assays.** In order to assess the effect of selected inhibitors on malaria parasite growth *in vitro*, sorbitol synchronized 3D7 strain *Plasmodium falciparum* parasites were cultured in a 96-well plate with varying amounts of different inhibitors for 48
h starting from the ring stage. The culture medium was then changed every day maintaining
an appropriate concentration of inhibitor. Parasitemia was counted by fluorescence assay as
described earlier (Smilkstein et al. 2004). After 48 h of growth, 100 μl of SYBR Green I in
lysis buffer (0.2 μl of SYBR Green I ml⁻¹ of lysis buffer) was added to each well and the
contents were mixed until no visible erythrocyte sediment remained. After 1 h of incubation
in the dark at room temperature, fluorescence was measured with a Cytofluor II fluorescence
multi-well plate reader from PerSeptive Biosystems (Framingham, Mass) with excitation and
emission wavelength bands centered at 485 and 530 nm, respectively, and a gain setting of
50. By using the accompanying Cytofluor software, background reading for an empty well
was subtracted to yield fluorescence counts for analysis. Inhibitory concentrations of all the
lead compounds was then compared and subtracted with chloroquine IC₅₀ in the parasite
cultures. To assess the effect of D-amino acids on malaria parasite growth, synchronized 3D7
strain *Plasmodium falciparum* parasites were cultured in the presence of varying
concentration (2 mM, 5 mM and 10 mM) of D-amino acids. The parasite cultures were also
carried out with and without inhibitor compounds at ring stage. Giemsa stained smears of
malaria parasite were made after 48 h of growth and counted under light microscope. Each
experiment was repeated thrice.
2.3 RESULTS

2.3.1 Deacylase assay with *Plasmodium falciparum* DTD

The over-expressed and purified *Pf*DTD enzyme catalyzed D-Tyr-tRNA\(^{\text{Tyr}}\) hydrolysis at an initial rate of 0.11±0.01 s\(^{-1}\). When L-Tyr-tRNA\(^{\text{Tyr}}\) was assayed as substrate in identical conditions no hydrolysis could be observed. To detect L-Tyr-tRNA\(^{\text{Tyr}}\) hydrolysis, the enzyme concentration in the assay had to be increased up to 400 nM. At this concentration, the [E] << [S] condition was no more fulfilled. However, we verified that the extent of substrate hydrolysis remained proportional to the enzyme concentration in the assay. A rate of hydrolysis (v = [P]/[E]) of the order of 0.2±0.1 x 10\(^{-3}\) s\(^{-1}\) could be derived from the experiments with L-Tyr-tRNA\(^{\text{Tyr}}\). Altogether, these results clearly indicate that *Pf*DTD specifically catalyzes the hydrolysis of D-Tyr-tRNA\(^{\text{Tyr}}\) (Fig. 2.1B). The specific activity of wild type *Pf*DTD was determined and compared with single mutant of S87A (remained active) or double mutants of S87A and T90A (inactive). The double mutant (S87A, T90A) of *Pf*DTD was significantly less active than wild type (data not shown), thus implicating Thr90 as the reactive residue for catalysis in *Pf*DTD, in line with previous reports suggesting the same (Lim et al. 2003; Kemp et al. 2007).

2.3.2 Sub-cellular localization of *Pf*DTD

To test localization of *Pf*DTD, antibodies were raised in mice against the purified protein. Antibodies were used along with anti-*Pf*NapL antibodies in parasitized red blood cells. The fixed cells were probed with anti-*Pf*DTD and anti-*Pf*NapL antibodies along with DAPI staining (Fig. 2.1C). Results clearly show the co-localization of *Pf*DTD with *Pf*NapL at all the asexual life stages of parasite, suggesting *Pf*DTD to be cytoplasmic in nature - like for *Pf*NapL (Chandra et al. 2005). A signal for *Pf*DTD was also seen at nuclear periphery with
slight overlap with DAPI, in agreement with previously reported peri-nuclear localization of human DTD (Zheng et al. 2009).

2.3.3 Description of *PfDTD*

Triclinic unit cell of *PfDTD* crystals contains three independent dimeric *PfDTDs*. *PfDTD* folds into a compact domain of α/β class of proteins and consists of twisted five-stranded mixed β-sheets covered by two parallel α-helices, three-stranded anti-parallel β-sheets and two long loops (loop L1, residues 62-81 and loop L2, residues 88-109) (Fig. 2.2A). *PfDTD* is a two-fold related dimer and displays extensive dimeric contacts with a buried interface area of ~1200 Å². The dimeric interactions are mainly between strand β7 residues, and result in formation of continuous six-stranded anti-parallel β-sheets. Part of the long loop L2 is also involved in dimeric interactions, which extends from one subunit to other subunit. Apart from the interactions between strands β7. There are several key hydrogen bonds at the dimer interface- i) side chain atoms of Gln88 interact with main chain atoms of Pro150 and Thr152, ii) side chain atom of Thr90 interacts with main-chain atoms of Thr152, iii) main chain atoms of Arg63 interact with main chain atoms of Asn99, and iv) side chain atoms of Gln6 interact with their two-fold related counterpart. The conformations of the three dimeric *PfDTDs* in the unit cell are identical except for small yet key differences, including those in the loop regions. The two long loops L1 and L2 show high mobility and these protrude out of the protein core. Two of six molecules (monomers) in the unit cell have higher B-values (indicative of mobility) than others. Interestingly, *Plasmodium falciparum* proteins are often characterized by low-complexity loop insertions when compared to their homologs (Singh et al. 2004).
Figure 2.1

Expression and activity of *Pj*DTD. (A) Schematic representation of deacylation process. (B) Enzyme activity and selectivity of *Pj*DTD. Rate of L-Tyr-tRNA deacylation was significantly lower than of D-Tyr-tRNA hydrolysis. (C) Localization of *Pj*DTD in different intra-erythrocytic stages of *Plasmodium falciparum* by immunofluorescence staining, (R) Ring stage, (T) trophozoite stage and (S) schizont stage. In each panel (i) image of cell stained with DAPI (blue); (ii) anti-*Pj*DTD antibodies (iii) anti-*Pj*NapL antibodies (iv) merged image (v) merge with phase contrast.
Figure 2.2

Structure of PjDTD. (A) Two-fold related dimeric molecules A and B are colored in blue and purple, respectively. Each monomer contains eight β-strands (B1-B8), two α-helices (A1 and A2) and three long loops (IL, L1 and L2). The low complexity insertion loop IL residues 17-26, loop L1 residues 55-65 and loop L2 residues 90-110 are highlighted in orange, yellow and brown color respectively. N- and C-termini are marked. (B) Ordered insertion loop (IL) in PjDTD-D-Lys complex structure. Difference Fourier (Fo-Fc) and final 2Fo-Fc maps are contoured at 2.5 and 1.0σ level, respectively. (C) Orthogonal views showing electrostatic potential at the molecular surface of PjDTD dimer. Orientation of the dimer is similar to the one shown in Fig. 2.2A. Electrostatic surface is displayed as a color gradient in red (electronegative, ≤ -12 kTe⁻¹) and blue (electropositive, ≥ 12 kTe⁻¹). (D) View of reduced thiols in PjDTD. The final 2Fo-Fc map is contoured at 2.5σ level.
In the case of PfDTD, the low complexity insertion loop IL (residues 17-26) was not evident in the electron density map in most monomers, again suggesting high degree of flexibility. However, this loop IL was well ordered in few PfDTD monomers within the unit cell of native and D-amino acid bound structures (Fig. 2.2B). The IL loop forms an extended β-hairpin structure and makes no contacts with other parts of the PfDTD structure. It is ordered due to lattice contacts only, and we believe that IL which has the sequence KENIGENEKE represents one of the few views of low complexity insertions that are in abundance in Plasmodium falciparum proteins (Singh et al. 2004).

PfDTD surface has positive charge character around the active site region (Fig. 2.2C). Residues Cys39 and Cys59 are proximal but the electron density maps clearly show that the sulfur atoms do not form a disulfide linkage (Fig. 2.2D). The distance between S-S is ~3.5-3.8 Å and these distances are comparable with reduced thiols (3.7 Å). These two Cys residues are located in the buried hydrophobic core part of PfDTD. In most DTDs, these two Cys positions are not conserved and are replaced by hydrophobic residues Val/Ile (Fig. 2.3A).
Sequence conservation in DTDs. A. Structure based sequence alignment of DTDs. Proteins are: *Plasmodium falciparum*, *Aquificae aeolicus*, *Escherichia coli*, *Haemophilus influenzae* and *Homo sapiens* (also known as DNA-unwinding element binding protein, DUEB). Identical/well conserved residues, conserved residues and semi-conserved residues are marked with asterisks, semicolons and dots, respectively. The two conserved active site motifs (blue), tRNA recognition residues (green), 3’-end of tRNA and D-amino acid binding residues (red) are highlighted. B. Superimposition of crystal structures of *Pj*DTD (cyan), HsDTD (purple), AaDTD (yellow), EcDTD (pink) and HiDTD (green). C. Residue conservation in DTDs is shown as cartoon diagram. Identical, well-conserved, semi-conserved and likely conserved residues are rendered in red, pink, grey and blue colour, respectively.
2.3.4 Comparison with known DTDs: dimer interfaces and active sites

PfDTD fold is very similar to its counterparts (AaDTD, EcDTD, HiDTD and LmDTD, Dali score range of 17-24 with 16-42% sequence identity, rmsd ~1.1-1.9 Å, Fig. 2.3A and Fig. 2.3B). PfDTD has high Dali score of ~25 with HsDTD (rmsd of 0.9 Å for 148 Cα pairs; 45% sequence identity) and of ~15 with the N-terminal editing domain of Thr-tRNA synthetase (called Pab-NTD (Dwivedi et al. 2005; Hussain et al. 2006)) (rmsd 1.9 Å for 114 Cα pairs; 16% sequence identity). Amongst ~50 residues located at dimer interface of DTDs ~50% are conserved (Fig. 2.3A and Fig. 2.3C). From structure-based sequence alignment, it is clear that conservation amongst DTDs is highest for dimeric interface residues - possibly linking function with dimeric assembly. In DTDs, the active site is believed to be formed by two highly conserved motifs: residues 87-91 (SQFTL) (Ser87 is replaced by Pro at times) and 147-152 [NXGP(V/F)T]. The motif SQFTL is at the protein surface, forms an accessible cavity, and links to NDGPVT motif from the other monomer. The later motif is mainly involved in dimeric interactions. The DTD active site cavity is surrounded with hydrophobic residues including Phe89, Phe103 and Phe137. The conserved, positively charged residues Arg/Lys100, Lys97, Arg7*, Arg/Lys58* and Arg/Lys63* (two-fold related residues are shown with *) are proximal to the active site and these may contribute in making electrostatic interactions with tRNA. Core part of the DTD dimer interface is formed in part due to direct or water mediated interactions between two fold related Gln6 from each monomer.
2.3.5 *Pf*DTD active site with bound adenosine

*Pf*DTD crystals were soaked in 5mM ATP for 10-30 minutes and then diffraction data were collected on these crystals. Strong and clear electron density (for ADP) was observed close to the active site Phe89, and the electron density maps allowed us to unequivocally model ADP in various *Pf*DTD monomers in *Pf*DTD-ATP complex I ([Fig. 2.4](#)), in *Pf*DTD-ATP complex II and in *Pf*DTD-ATP complex III structures. Binding of ADP appears to be stabilized by a number of interactions with *Pf*DTD. The active site Phe89 is positioned adjacent to adenine ring with a stacking distance of ~3.5 - 4 Å. The other side of adenine ring is partially in the hydrophobic pocket created by Ala106 and Pro109. The ribose hydroxyl groups form hydrogen bonds with side chains of Thr71 and Trp72. Amino group N6 of adenine forms a hydrogen bond with main-chain O atom of Lys107. The phosphate tail of ADP protrudes from the adenine-binding pocket onto the protein surface and adopts variable orientations. The α- and γ-phosphate groups are contacted by a number of direct or water-mediated hydrogen bonds with residues including His104, Arg16, Asn69 and Asp68. In native and ADP bound *Pf*DTDs, up to five waters can be found in the active site including the catalytic water Wa1. Waters are also found between Phe89 and Phe137, in the active site upper rim region (residues 135-141) and near Tyr116.
Figure 2.4

Omit electron density map and surface representation of bound ADP in PjDTO-ATP complex I. A. Omit map is contoured at 1.5σ level near the active site residues in PjDTO-ATP complex I structure. B. Surface representation of PjDTO active site and bound ADP molecules is shown as ball-and-stick model. The right side panel represents 3'-end of tRNA. Active site residues Phe89, Thr90 and Met141 are shown in orange, blue and red color, respectively.
In molecule B of *PjDTD*-ATP complex II structure, we found partially occupied adenine along with five additional waters in the active site region. Thus, a total of ten waters (including the catalytic Wa1) participate in an elaborate hydrogen bonding network, which strikingly differs between ADP-bound and native *PjDTD*. Finally, in molecule B of *PjDTD*-ATP complex II, we found the catalytic water Wa1 displaced by ~4 Å from the active site along with an alternate conformation of the active site pocket residue Gln88. These observations imply crucial roles for waters in ligand bound and unbound states of *PjDTD* enzyme, and have implications in the proposed catalytic mechanism detailed later.

### 2.3.6 D-amino acid subsites in *PjDTD*

D-amino acid specificity in *PjDTDs* was investigated based on crystals structures of *PjDTD* complexed with various D-amino acids. Native *PjDTD* crystals were soaked in D-amino acids and then diffraction data were collected on these crystals. For each complex, difference Fourier electron density and omit maps revealed docking of D-amino acids near the active site pocket. We soaked in D-Arg, D-Lys, D-Glu, D-Ser, and D-His in these studies, and we observed clear electron density for these D-amino acids (Fig. 2.5A). In the case of D-His complex, one of the bound D-His is located near active site Phe89 and its side chain is involved in edge-to-face $\pi \ldots \pi$ interaction with active site Phe89. The other D-His is located near the upper rim of the active site loop where the potentially enantio-selective Met141 residue sits (see below). The amino group of this latter D-His interacts with main-chain carbonyl oxygen atoms of Gly138 and Asn139. The bound D-Ser is found at two different places within the active site cavity. Finally, the negatively charged D-Glu and the positively charged D-Arg and D-Lys were found near the active Thr90. The carboxyl oxygen atoms of D-Glu form one hydrogen bond with Asn66 and make a salt-bridge interaction with Arg7. D-Arg and D-Lys make similar interactions with protein atoms by means of their $\alpha$-amino and carboxyl groups.
Figure 2.5

Omit electron density map near active site in PfDTD-D-Arg complex and subsite classifications of bound D-amino acids. A. Omit map is contoured at 1.5 $\sigma$ level around the active site residues. Hydrogen bonds and N-H...$\pi$ are marked with black and red dashed lines, respectively. B. Bound D-these sites as transition subsites (T1 and T2), active subsite A and exit subsite E.
Figure 2.6
Surface representation of bound D-amino acids in various subsites. D-amino acids (ball-and-stick) located in different subsites (T1, T2, A and E) within the active site pocket of $Pf$DTD. Active site residues are colored same as in Fig. 2.5.
Carboxyl group electropositive α-amino group is stabilized by N-H...π interaction with benzene ring of Trp72 and O$^{51}$ of Asn66. The carboxyl group of D-Arg and D-Lys also interacts with N$^{62}$ of Asn66. As shown in Fig. 2.5B and Fig. 2.6, the bound D-amino acids cluster at four different subsites within the PfDTD active site pocket and we have termed these sites as transition subsites (T1 and T2), active subsite (A) and exit subsite (E). T1 and T2 are the transition subsites where initial recognition and positioning of D-amino acids take place. Chiral selection most likely takes place at T2 subsite by the residue Met141. The breakdown of ester bond between D-amino acid and tRNA likely occurs at active subsite A where Thr90 resides. The subsite E likely represents post-transfer state of the deacylation reaction where cleaved D-residues are dumped for subsequent diffusion away from PfDTD.

In the five PfDTD-D-amino acid complex structures studied, we found two (D-His and D-Ser) in subsite T1 and D-His in T2. We could also locate another two (D-Glu and D-Ser) proximal to the active subsite A, and last two (D-Arg and D-Lys) were identified at subsite E (Fig. 2.6).

2.3.7 Active site comparison of PfDTD with Pab-NTD and subsite classifications.
PfDTD and Pab-NTD have similar folds but these two enzymes differ in amino acid enantioselectivity (Dwivedi et al. 2005). DTD is specific to D-aa-tRNA and not to L-aa-tRNA, whereas Pab-NTD is specific for L-ser-tRNA$^{Thr}$ amongst others. We have superimposed the crystal structure of Pab-NTD complex with pre-transfer (seryl-3'-aminoadenosine, A3S) analog on to ADP bound PfDTD (Fig. 2.7A).
Figure 2.7

Views of PfDTD-ADP structure with substrate analog of Pab-NTD along with PfDTD subsites. A. Superimposition of pre-transfer substrate analog (seryl-3'-aminoadenosine, A3S, yellow) complex structure of Pab-NTD onto ADP bound PfDTD. B. Bound adenine ring in PfDTD and in Pab-NTD complexes mimics entry point of tRNA. We termed these subsites as transition sites B1 and B2, respectively. C. The classification of various subsites in the active site pocket of DTDs. The active site residues are colored same as in Fig. 2.5.
These structural comparisons have been very revealing. The bound adenine ring in PfDTD and Pab-NTD complex structures do not coincide, as the ADP in PfDTD is located near the entry point of the active channel. In contrast, in Pab-NTD the adenine ring of A3S analogs is found deeper in the active site cleft - a region where we found five water molecules in molecule B of PfDTD-ATP complex II structure. The invariant residues Val45, Ala94 and Phe117 of Pab-NTD in the adenine-binding pocket are replaced by corresponding conserved residues Ile/Val43, Ala112 and Phe137 in other DTDs. However, the two key DTD active site motifs [SQFTL and NXGP(V/F)T] are not conserved in Pab-NTD and therefore several important differences are to be expected in adenine recognition. In PfDTD, Ser87 (of motif SQFTL) interacts with N3 of the bound adenine. When Pab-NTD is superimposed on PfDTD, it is evident that the L-Ser of A3S is resident in our defined subsite T2, close to the chiral checkpoint residue Met141 of PfDTD. This placement is in proximity to the crucial Met141 which potentially serves as the enantiomeric selectivity checkpoint, based on mutagenesis studies(Hussain et al. 2006). In these previous experiments, the D/L-amino acid binding affinities were studied based on fluorescence binding assays for wild type and single mutant of EcDTD (Met129 to Lys) and Pab-NTD (Lys121 to Met). DTDs are specific for D-amino acids (D-aa-tRNA), while Pab-NTD is specific for only L-Ser-tRNAThr. However, M129K mutant of EcDTD was shown to bind L-amino acids while K121M mutant of Pab-NTD showed deacylation activity thus assigning enantioselectivity role to the conserved methionine in DTDs(Hussain et al. 2006). Based on bound ADP molecules in PfDTD and substrate analogues in Pab-NTD, we propose that recognition and docking of the terminal adenine of D-aa-tRNA involves two spatial regions - termed as transition subsites B1 and B2 (Fig. 2.7B). In PfDTD-D-amino acid complex structures, we found various D-amino acids at different positions of the active site (Fig. 2.6).
Figure 2.8

Surface representation of open and close conformations of Phe89 at adenosine docking site. A. Open conformation of active site Phe89 in native DTDs, in D-amino acid bound and ADP bound structures. Stick model of Phe89 is shown in right side panel. Color codes of active site residues are same as in Fig. 2.5. B. Closed conformation of Phe89 is observed in HiDTD, molecule D of EcDTD and molecule C of HsDTD.
Taken together, we have classified the active site pockets within PfDTD as B1, B2 for adenosine positions and as T1, T2, A and E for the various D-amino acid positions. These spatial sectors within PfDTD allow a complete explanation of the catalytic cycle (Fig. 2.7C). The conserved Met141 side chain points towards the bottom of A subsite. Interestingly, in all representations of DTDs, and specifically in the multiple copies of PfDTDs we have solved here, the conserved Met141 always adopts an invariant conformation. This observation suggests that this key residue may provide chiral selectivity property to DTDs (26, 27). Further, the buried Met141 is chaperoned by highly conserved hydrophobic residues - Pro150, Val86, Phe40, Val15, Leu13 and Gly135. Additionally, conserved residues Phe137 and Tyr116 are located at bottom of subsite T2. When we compared all DTDs, We observed flipping of the main chain carbonyl groups of Phe137 and Gly135. We also noticed movement of Ser87, Gln88, Phe137 and Tyr116 side chains. These small perturbations possibly indicate flexibility at the bottom of the active site region and may reflect closing and opening of the active site lid. The active site Thr90 is shielded from solvent by Phe89, which adopts similar conformations in most DTD structures - We believe these represent tRNA-bound open conformations of Phe89 in the active site (subsites B1 and B2) (Fig. 2.8A). In contrast, closed conformations are represented by some structures (HiDTD, one case of EcDTD-molecule D and by HsDTD-molecule C, Fig. 2.8B). In these closed DTDs, Phe89 (or its equivalent) adopts shut conformations thereby disallowing docking of terminal adenine of charged tRNA onto DTD.

2.3.8 Docking of D-Tyr-tRNA into PfDTD active site

Based on the above insights, we built a model for 3’end of D-Tyr-tRNA substrate analog (tyrosyl-3’-aminoadenosine, A3Y, Fig. 2.7A) at the PfDTD active site. We mutated (In silico) the L-ser of A3S (Pab-NTD) to D-Tyr and considered its placement in subsite A of PfDTD.
This modeling made it immediately apparent that the D-Tyr was now ideally placed for chirality selection near Met141 (subsite T2) at a distance of 2.5-3.0 Å and without steric clashes. This resulted in a distance of ~5 Å between D-Tyr carboxyl carbon atom and the active site Thr90 hydroxyl group. Based on observed PfDTD conformations in our structures, we then modeled side chain-conformations of Gln88, Phe89 and of the active residue Thr90 towards the D-Tyr carbonyl carbon atom. The above described minor adjustments allow the D-Tyr carbonyl carbon to point directly towards the hydroxyl group of active site residue Thr90, ready for catalysis.

2.3.9 A model for catalytic mechanism

A comprehensive scheme for catalytic mechanism of DTDs, which involve cleavage of ester bond between D-Tyr/tRNA and of D-Tyr/DTO, can now be proposed based on our studies (Fig. 2.9). In this family of enzymes, catalysis is a substrate-assisted two step process. In the first step, side chain O' of Thr90 attacks the D-amino acid carbonyl carbon (C') in a nucleophilic manner and also deprotonates the α-amino group of D-amino acid (Fig. 2.9A). This leads to formation of tetrahedral transition state which is stabilized by the oxyanion hole formed by main-chain N atoms of Phe89 and Thr90 as well as by the side chain Nε of Gln88. This is followed by cleavage of the D-amino acid-tRNA ester bond. The uncharged tRNA then moves out of the active site for diffusion into the solution while the D-amino acid remains bound to the DTD enzyme. Subsequently, the ester bond between DTD and the D-amino acid is cleaved by catalytic water molecule Wa1, leading to removal of the cleaved free D-amino acid from subsite A and its placement into the exit subsite E (Fig. 2.9B and Fig. 2.9C). These events thus complete catalytic cycle of deacylation and lead to generation of both free tRNA and D-amino acids.
Figure 2.9

Proposed catalytic mechanism. A. Hydroxyl group of Thr90 attacks carbonyl carbon of A3Y in a nucleophilic manner, followed by cleavage of ester bond and formation of acyl enzyme (D-amino acid bound to DTD). B. Movement of catalytic water Wa1 close to carbonyl carbon of D-amino acid. C. Cleavage of ester bond between D-amino acid and DTD. Thus, free D-amino acid may migrate to subsite E from where it could diffuse.
Figure 2.10

Parasite inhibition assays. A. Growth of cultures of Plasmodium falciparum after 48 h in the presence of four different inhibitors at varying concentrations ranging from 0.01 to 100 μM. B. Parasites growth at varying concentrations of D-isoleucine in presence or absence of 0.1 μM concentration of compound 2 in the media. Structure of compound 2 is shown.
2.3.10 *In-silico screening and parasite growth inhibition assays*

Based on the docking scores, energy values and hydrogen bonding interactions, several *P. falciparum*-TD-inhibitor complex structures (*in-silico*) were short listed and top 40 compounds were selected for testing in parasite growth inhibition assays. Synchronized parasite cultures at ring stages were incubated with inhibitors at varying concentrations ranging from 10 nM to 100 μM. Out of 40 selected compounds, four showed inhibition of parasite growth (Fig. 2.10A). At 10 μM concentration compound 2 [\(N,N'\)-bis(4-amino-2-methyl-6-quinolinyl)urea] should more than 90% inhibition of parasite growth. Further, parasites were grown in varying concentrations of D-isoleucine either in the presence or absence of 0.1 μM concentration of compound 2. The data suggest that addition of compound 2 likely makes parasite sensitive to higher concentrations of D-isoleucine (Fig. 2.10B), suggesting inhibition of *P. falciparum*-TD by compound 2 - possibly by disabling detoxification of D-isoleucine by the parasite.

2.4 DISCUSSION

*P. falciparum*-TD was over-expressed in *Escherichia coli* and the recombinant purified protein was crystallized in three different crystal growth solutions. We determined *P. falciparum*-TD structure using iodide-SAD technique, and we subsequently solved three different *P. falciparum*-TD-ADP structures along with several *P. falciparum*-TD-D-amino acid complex structures. The *P. falciparum*-TD crystals belong to triclinic unit cell with three dimers each in the asymmetric unit. This presented *P. falciparum*-TD-ligand structures with three independent atomic views of the dimers. Further, the ligand-soaked crystals diffracted to higher resolution than native crystals facilitating better quality atomic dissection of *P. falciparum*-TD-ligand complexes.

High resolution *P. falciparum*-TD-ligand structures allowed us to map transition/movement of each of the chemical groups involved in the deacylation reaction – including those involving
protein side chains, the adenine ring and the ribose pucker. The bound adenine ring stacks with active site Phe89 in PfDTD and this binding marks the transition site B1 which participates in recognition of 3’ end of charged tRNA. We show that PfDTD bound D-amino acids cluster at three different subsites, termed transition, active and exit, around and near the active site Thr90 residue which initiates catalysis. Our PfDTD co-crystal structures provide glimpses of several reaction intermediates. An in-depth analysis of substrates, products and water networks has therefore allowed us to make an elaboration of PfDTD active site into multiple spatial components. Previously, catalytic mechanisms for DTDs have been proposed based on modeling studies (Lim et al. 2003; Kemp et al. 2007). Our experimental data on PfDTD substrate interactions support the proposed reaction schemes and provide the first structural basis for these mechanisms. We propose several sequential steps that eventually catalyze deacylation of D-aa-tRNAs by DTD. These steps are: i) electrostatic interactions along with minor conformational changes in PfDTD facilitate docking of 3’-end of tRNA on to PfDTD, ii) insertion of water W1 at the dimeric interface and side-chain movements of Asn147 and water W2 then create space between PfDTD monomers. This is followed by tethering of tRNA terminal adenine to entry point/transition site B1 of the active site Phe89. It also allows for entry of covalently bound D-amino acid into the amino acid transition subsite T1, iii) due to these steps, the pre-bound waters in the active region escape, making way for authentic substrates to reposition themselves. For example, exit of the pre-bound waters (found in our PfDTD complexes) likely facilitate flipping of carbonyl groups belonging to residues 135/137 and also allow movement of Ser87 (Fig. 2.7). These minor conformational adjustments structurally and spatially rebuild the PfDTD active site, iv) the above structural alterations then translate into side-chain movements of Tyr116, Phe137, Phe89 and Gln88. Also, crucially there is a 120° rotation of active site Thr90 side-chain. These then trigger traversal of D-amino acid charged tRNA from transition site B1 to B2.
Subsequently, the D-amino acid is presented in the enantiomer selectivity pocket (subsite T2). The task of enantionemeric selectivity is then potentially done by the completely invariant, conformationally restrained and biochemically implicated Met141 (Dwivedi et al. 2005; Hussain et al. 2006). Once the amino acid chirality is acceptable, its carbonyl carbon is placed proximal to the catalytic Thr90, vi) in the subsequent catalytic step, side chain O' of Thr90 attacks the D-amino acid carbonyl carbon (C') in a nucleophilic manner and the deprotonated amino group of D-amino acid accepts hydroxyl proton from Thr90. Thus, the tetrahedral transition state is formed and cleavage of D-aa-tRNA ester bond can take place. The uncharged tRNA can thus exit the active site while D-amino acid remains bound to DTD enzyme, vii) in the last step, catalytic water Wa1 moves into the active site and attacks the ester bond between DTD and D-amino acid resulting in its cleavage. The cleaved free D-amino acid subsequently enters the exit subsite E, which we have annotated in Pf/DTD. These steps represent the catalytic cycle and therefore allow generation of free DTD, free tRNA and free D-amino acid. In summary, we present direct experimental evidence for presence of several subsites within DTD which together enable docking, cleavage and egress of the recognized D-amino acid - thus leaving uncharged tRNA for another round of the aminoacylation reaction. The co-crystal structures of five D-amino acids with Pf/DTD also reveal inherent plasticity in the D-amino acid recognition sites. Latter is significant because Pf/DTD is required to accommodate and catalyze reactions despite considerable differences in shape, size and charge of D-amino acids.

Using structure-based in-silico screening routines, we selected 40 compounds for testing in parasite growth inhibition assays. Out of these, four compounds should significant inhibition of parasite growth and compound 2 (N,N'-bis(4-amino-2-methyl-6-quinolinyl)urea, Fig. 2.10B) should more than 90% inhibition. Compound 2 was found to dock at B1 and B2 subsites of Pf/DTD active site pocket, where the terminal adenosine of D-amino acid charged
tRNA docks during deacylation - this observation implies that compound 2 might be competing with \( P/\text{DTD} \) substrate. These data provide initial evidence that inhibitors like compound 2 may be useful as novel inhibitors against the malaria parasite given that only a single copy of \( \text{dtd} \) gene is present in the malaria genome. We also observed that higher concentrations of D-isoleucine (L-isoleucine is absent in hemoglobin and therefore presumably it is important for the parasite to import this) in the RPMI media do not affect growth of malaria parasite. Amongst others, there could be two possible reasons for this - either D-isoleucine is poorly absorbed by the parasite or the parasite encoded DTD enzyme neutralizes expected toxicity of D-isoleucine. Our data indicate that the presence of inhibitory compound 2 in the media makes parasite growth vulnerable to higher D-isoleucine concentrations (Fig. 2.10B). This observation suggests that active \( P/\text{DTD} \) may be important for the malaria parasite in terms of overcoming D-amino acid induced toxicity. Interestingly, compound 2 has been previously tested on \( \text{J774A.1} \) cells (macrophage cell line) at up to 100 \( \mu \text{M} \) concentration, and it should negligible cytotoxicity(Panchal et al. 2004).

In conclusion, we present a comprehensive mechanism for the deacylation reaction by DTD class of proof reading enzymes. Our structural studies suggest how D-amino acid charged tRNA may be recognized and processed by \( P/\text{DTD} \). These studies also provide direct comparisons with the previously characterized \( \text{Pab-NTD} \) enzyme family. The description of multiple subsites within DTD active site pocket provides a first view of the structural architecture required by these enzymes to perform their editing functions. Our results also suggest that specific inhibitors against \( P/\text{DTD} \) may hinder parasite growth in the presence of D-amino acids. DTDs from pathogenic organisms like \( \text{Plasmodium falciparum} \) can therefore be explored further for their value as novel and unique targets for focus of anti-malarials.