Molecular Characterization and Mutational Analysis of Recombinant Diadenosine 5′,5″-P^1,P^4-Tetraphosphate Hydrolase from *Plasmodium falciparum*

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Asymmetrical diadenosine 5′,5″-P^1,P^4-tetraphosphate hydrolase (EC 3.6.1.17) from human malaria parasite *Plasmodium falciparum* was expressed in *Escherichia coli*, purified to homogeneity, and characterized for the first time as a biological target for chemotherapeutic agents against malaria. *Plasmodium falciparum* Ap$_4$A (PfAp$_4$A) hydrolase not only catalyzes diadenosine 5′,5″-P^1,P^4-tetraphosphate (Ap$_4$A) to ATP and AMP, but also diadenosine 5′,5″-P^1,P^5-pentaphosphate (Ap$_5$A) to ATP and ADP. Marked enzyme heat stability corresponding to the highest level of activity was observed at 60°C. The recombinant enzyme showed maximal activity in the presence of 5 mM Mg^{2+} ions. Kinetic analysis revealed the values of K_m and K_{cat} as 0.6 μM and 2.5 min^{-1}, respectively. Comparative protein modeling indicated an additional space in the substrate binding site of the parasitic enzyme compared with that of humans. Mutagenic analysis of the amino acid residue (Pro133) forming the additional space revealed a 5-fold increase in the wild-type K_m value when replaced by a smaller (Ala) residue. Furthermore, catalytic activity was markedly affected by introducing a larger residue (Phe), thus creating the potential to develop a specific inhibitor of PfAp$_4$A hydrolase.

Key words malaria; *Plasmodium*; Ap$_4$A hydrolase; diadenosine tetraphosphate; mutational analysis; chemotherapeutic

Malaria is a tenacious and constantly evolving pandemic that kills nearly 800000 people annually. There are about 106 malaria-endemic countries, and it is estimated that the number of malaria cases peaked at 225 million in 2009. Moreover, antimalarial drug resistance is a major public health problem that hinders the control of malaria.[1] Thus, the continuous increase in drug resistance necessitates the discovery of new antimalarial drugs.

Enzymes are excellent and common targets for drug discovery and pharmacological intervention due to their essential roles in biological and pathophysiological processes.

Diadenosine 5′,5″-P^1,P^4-tetraphosphate (Ap$_4$A) is an unavoidable by-product of the reaction catalyzed by aminoacyl-tRNA synthetase and other ligases.[2] It is found at intracellular concentrations in the submicromolar to micromolar range.[3] In eukaryotes, Ap$_4$A plays an important role in several functions including regulation of ATP-sensitive K^+ channels,[4] activation of gene expression,[5] signal function for the initiation of DNA replication,[6,7] cellular responses to metabolic stress and DNA damage,[8] initiation of apoptosis,[9,10] and modulation of Fhit tumor suppressor protein activity.[11] Furthermore, the enhanced ability of asymmetrical Ap$_4$A hydrolase in some invasive bacteria is a characteristic feature that increases their chances of survival.[12] On the other hand, Ap$_4$A concentration needs to be kept below the potential toxic level that might otherwise inhibit kinases and other ATP-dependent enzymes.[13] It is conceivable that both interpretations are correct: synthesis and degradation of Ap$_4$A must be tightly controlled.

Ap$_4$A hydrolase is the enzyme responsible for controlling the intracellular level of Ap$_4$A ("housecleaning").[14] Asymmetrical Ap$_4$A hydrolase belongs to the Nudix hydrolase superfamily of Mg^{2+}-requiring enzymes. This family is recognized by the highly conserved 23-residue sequence motif or Nudix box (GX5EX7REUXEEXGU) where U is a bulky hydrophobic residue and X is any amino acid. It occurs in a variety of animals, bacteria, plants, yeasts, and proteobacteria.[15]

When considering Ap$_4$A hydrolase as a target for antimalarial agents, it is essential to be aware of the features that distinguish *Plasmodium* enzyme from human enzyme for the design of selective inhibitors. Therefore, differential characterization and mutational analysis may reveal a potential therapy against malaria.

MATERIALS AND METHODS

Materials Ap$_4$A, Ap$_5$A, and Ap$_3$A were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Talon metal affinity resin was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, U.S.A.). KOD-Plus Mutagenesis Kit was obtained from Toyobo (Osaka, Japan). ENLITEN® ATP Assay System was purchased from Promega (Madison, WI, U.S.A.).

Cloning and Construction of Expression Plasmid cDNA encoding the open reading frame of PfAp$_4$A hydrolase was amplified by reverse transcription-polymerase chain reaction (PCR) from *P. falciparum* (FCR-3 strain) mRNA. The gene encoding PfAp$_4$A hydrolase was obtained by PCR using a pair of specific primers (Table 1). The primers were designed to contain *Bam*HI and *Pst*I restriction sites. The PCR product was digested with *Bam*HI and *Pst*I restriction enzymes and checked for specificity by gel electrophoresis. Digested PCR product was extracted from the gel using a QIAquick Gel Extraction Kit and ligated to the His-Tag pQE-30 vector, which was previously purified and digested with the same enzymes. The resulting plasmid was termed pQE-PfAp$_4$AH. *Escherichia coli* JM109 competent cells were transformed with the plasmid pQE-PfAp$_4$AH. Grown colonies were picked from an LB

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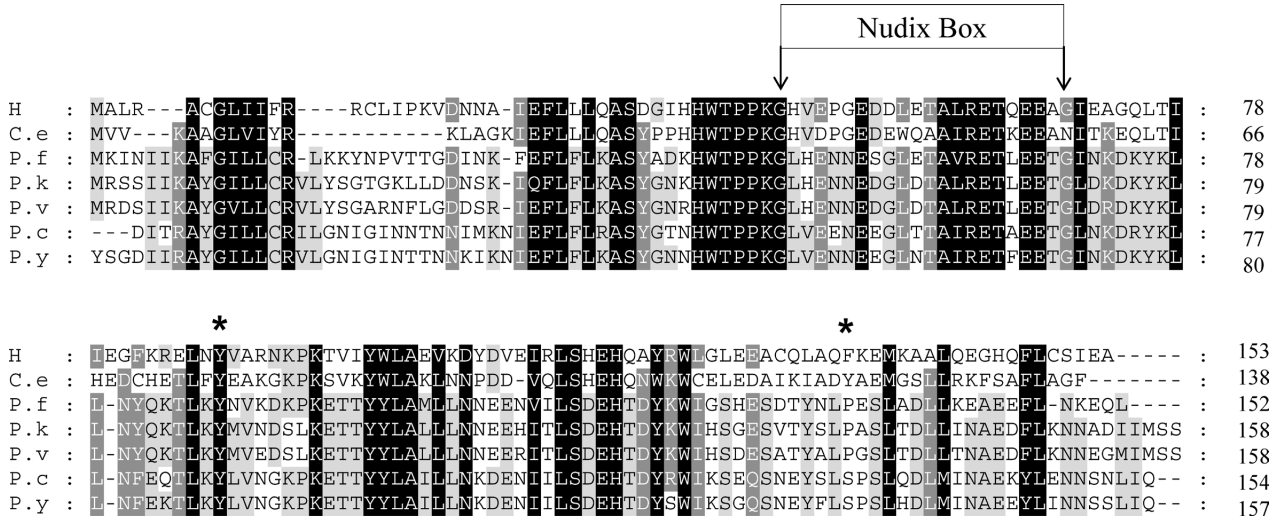

Fig. 1. Multiple Amino-Acid Sequence Alignment of Putative *P. falciparum* Ap_4A Hydrolase with Similar Animal and Worm Sequences

The protein accession numbers are as follows: *H* (*Homo sapiens*), P50583; *C.e* (*Caenorhabditis elegans*), CAB63351; *P.f* (*Plasmodium falciparum*), CAX63975; *P.k* (*Plasmodium knowlesi*), CAQ40378; *P.v* (*Plasmodium vivax*), EDL44024; *P.c* (*Plasmodium chabaudi*), CAH77553; and *P.y* (*Plasmodium yoelii*), EAA16360. Residues conserved in the seven protein sequences are shaded black, and those shared among more than four sequences are shaded grey. Adenosine binding residues are marked with asterisks.

(Luria–Bertani) agar plate containing 50 μg/mL ampicillin and screened by colony PCR. Positive colonies were sequenced, confirming the desired recombinant plasmid-containing putative gene sequence.

Expression and Purification of Recombinant Plasmid *E. coli* cells containing recombinant plasmids were grown overnight in LB broth containing 50 μg/mL ampicillin at 37°C. Expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and cell growth was continued at 37°C for an additional 4 h. The harvested cells were lysed in an extraction buffer (25 mM Tris–HCl buffer, pH 7.5, containing 300 mM NaCl) and disintegrated by sonication on ice for 30 s (three cycles with 30 s intervals). The lysate was centrifuged and the supernatant (50 mL) was applied to a purification column containing 2 mL of Talon Metal Affinity Resin and purified according to the manufacturer's instructions. Protein concentration was determined by the Bradford method using Bio-Rad dye with bovine serum albumin as a standard.[16]

Site-Directed Mutagenesis Site-directed mutagenesis was performed to produce mutant enzymes containing a single amino-acid substitution. Pairs of complementary mutant primers (Table 1) were used to prime replication of the circular pQE-PfAp_4AH plasmid by KOD-Plus DNA Polymerase according to the manufacturer's instructions. The mutated plasmids were transformed to JM109 competent cells. The mutant enzymes were expressed and purified by the same procedures as those used for the wild-type.

Enzyme Assays The rate of Ap_4A degradation was determined by HPLC and luciferase-based bioluminescence assay. HPLC was used to determine enzyme activity by quantifying the amount of ATP produced. For optimal separation of the adsorbed nucleotides, a gradient separation was generated by mixing pure water (A) with buffered mobile phase (B) consisting of 150 mM NaH_2PO_4 (pH 6.0 with NaOH) and 10% (v/v) MeOH as follows: 0–3 min, 80% buffer B; 3–6 min, 90% buffer B; 6–9 min, 100% buffer B. The separation process was carried out at ambient temperature at a flow rate of 0.7 mL/min and detection at 254 nm. The reaction mixture (100 μL) consisted of 100 mM KH_2PO_4 (pH 7.5), 100 μM Ap_4A, 5 mM $MgCl_2$, and purified enzyme. This mixture was incubated at 37°C for 10 min, and the reaction was stopped by heating at 100°C for 5 min followed by centrifugation of the reaction solution at 15000 rpm for 3 min. The clear supernatant was analyzed using an Inertsil ODS-3 (5 μm, 4.6×150 mm) HPLC Column. The luciferase-based bioluminescence assay was used to identify the kinetic constants of wild-type and mutant enzymes by the detection of ATP. The 100 μL assay mixture consisted of 100 mM Tris–HCl buffer (pH 7.5) containing 10 μL of reconstituted rL/L Reagent, 5 mM $MgCl_2$, 10 μL of different concentrations of Ap_4A, and 10 ng of purified enzyme, and the reaction was carried out at room temperature. Light output was measured by a GloMax^{TM} 20/20 luminometer and converted into units of enzyme activity (μmol/min) using

Table 1. Wild and Mutagenic Primers Used for Site-Directed Mutagenesis of *P. falciparum* Ap_4A Hydrolase

Mutations	Primer name	Primer sequence (5′–3′)
Wild	Wild fw.	CGCGGATCCATGAAAATTAATATTATTAAAGC
	Wild rev.	GCGCTGCAGTTATAGTTGTTCTTTATTTAAAAATTC
P133A	P133A fw.	CGTATAACCTTGCTGAATCCTTAGCTG
	P133A rev.	CAGCTAAGGATTCAGCAAGGTTATACG
P133F	P133F fw.	GTATAACCTTTTTGAATCCTTAGCTGATTTAT
	P133F rev.	ATAAATCAGCTAAGGATTCAAAAAGGTTATAC

The mutated amino acids are underlined.

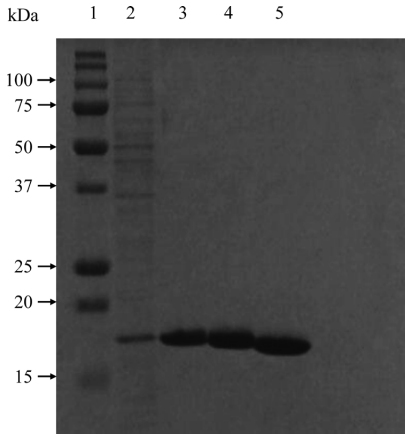

Fig. 2. Analysis of Expressed and Purified Recombinant $PfAp_4A$ Hydrolases by SDS-PAGE

Lane 1, protein marker; lane 2, whole cell proteins from *E. coli* expressing the putative protein (17 kDa); lane 3, purified wild-type $PfAp_4A$ hydrolase; lane 4, purified mutant P133A enzyme; lane 5, purified mutant P133F enzyme.

a calibration ATP curve.

RESULTS

Amino Acid Sequence Alignment $PfAp_4A$ hydrolase cDNA encodes an open reading frame of 152 amino acids. $PfAp_4A$ hydrolase possesses a Nudix motif, which is reported to be a characteristic feature of all asymmetrical Ap_4A hydrolases and is found at residues G49–G70. Amino acid sequence alignment was conducted between *P. falciparum* Ap_4A hydrolase and several Ap_4A hydrolases. Human and *Caenorhabditis elegans* (*C. elegans*) Ap_4A hydrolases shared 36% amino acid sequence identity with the parasitic enzyme. Ap_4A hydrolases of other *Plasmodium* species, *P. knowlesi*, *P. vivax*, *P. chabaudi*, and *P. yoelii*, showed 72%, 70%, 51%, and 66% identity, respectively (Fig. 1).

Table 3. Substrate Utilization by $PfAp_4A$ Hydrolase

Substrate	Major products[a)]	Relative activity (%)[b)]
Ap_4A	ATP+AMP	100%
Ap_5A	ATP+ADP	42%
Ap_3A	—	ND[c)]
ATP	—	ND[c)]
NADH	—	ND[c)]

a) Products were detected using HPLC. *b*) Related to the rate of Ap_4A hydrolysis in the presence of 100 μM Ap_4A. *c*) Not detected.

Expression and Purification Recombinant wild-type and mutant enzymes were generated as a histidine-tagged fusion protein from pQE-30 vector and expressed in JM109 *E. coli* cells. The fusion protein was bound to Co^{2+} ion of TALON Metal Affinity Resin. After binding and washing, the protein was eluted using 250 mM imidazole. The eluted wild-type protein was purified to 13.6-fold and a single band was observed at 17 kDa. Table 2 summarizes the results of wild-type enzyme purification. The size and purity of eluted wild-type and mutant proteins were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Brilliant Blue (Fig. 2).

Substrate Specificity $PfAp_4A$ hydrolase activity was screened by HPLC using various substrates (Ap_4A, Ap_5A, Ap_3A, ATP, and reduced nicotinamide adenine dinucleotide (NADH)) at a concentration of 100 μM in the presence of 5 mM $MgCl_2$, as described previously. $PfAp_4A$ hydrolase catalyzes asymmetric hydrolysis of Ap_4A to products ATP and AMP. *Plasmodium* enzyme showed significant activity towards Ap_5A, producing ATP, and ADP with relative activity reaching 42%. No enzyme activity was detected with diadenosine 5′,5″-P^1,P^3-triphosphate (Ap_3A), ATP, or NADH as substrates (Table 3).

Optimum pH and Temperature The activity of $PfAp_4A$ hydrolase was observed over a wide pH range (5–11) with

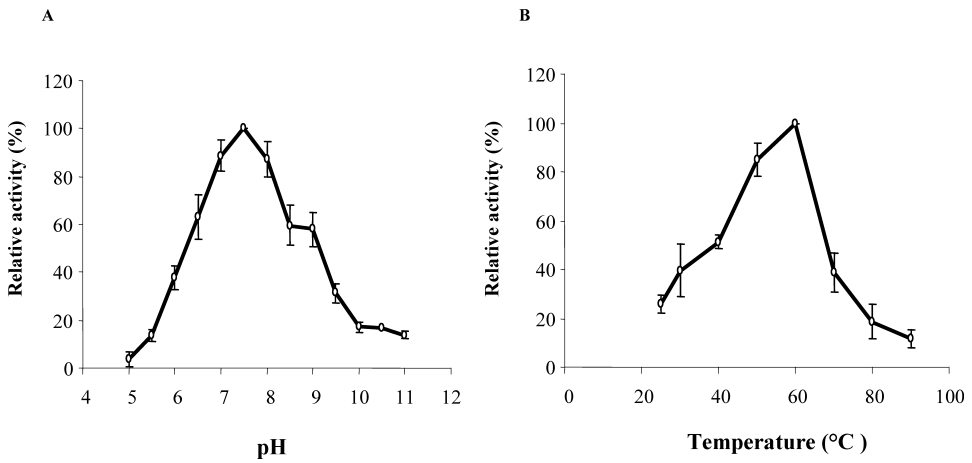

Fig. 3. The Effect of pH (A) and Temperature (B) on *Plasmodium falciparum* Ap_4A Hydrolase Enzyme Activity

Table 2. Purification Summary of *P. falciparum* Ap_4A Hydrolase

Fraction	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purity (fold)
Cell extract	61.0	146.4	2.4	1.0
Metal affinity	1.7	55.7	32.8	13.6

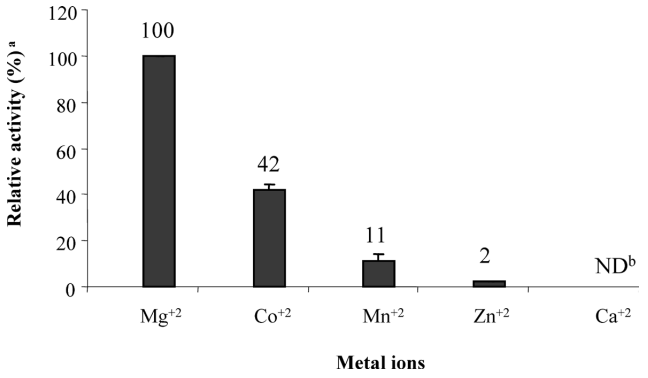

Fig. 4. The Effects of Divalent Metal Ions (5 mM) on Enzymatic Activity

[a] Relative to the rate of hydrolysis of 100 μM Ap_4A (considered 100% in the presence of Mg^{2+}); [b] ND, not detected.

optimum activity noted at pH 7.5 (Fig. 3A). The enzyme showed marked heat stability at 60°C at which the most efficient activity was detected, while the enzyme maintained 39% of its activity when incubated at 70°C for 10 min (Fig. 3B).

Metal Ion Requirements $PfAp_4A$ hydrolase showed maximum activity in the presence of 5 mM Mg^{2+}. Furthermore, enzyme activity was observed in the presence of 5 mM Co^{2+} (relative activity 42%), 5 mM Mn^{2+} (relative activity 11%), and 5 mM Zn^{2+} (relative activity 2%) (Fig. 4). Enzyme activity was not detected in the presence of Ca^{2+}, and relative activity decreased by 70% in the presence of 5 mM of both Mg^{2+} and Ca^{2+}.

Homology Modeling A comparison of the three dimensional structures of human (as observed in the NMR structure)[17] and *P. falciparum* (as predicted from protein modeling) Ap_4A hydrolases indicated an overall similarity between both enzymes (data not shown), with an interesting difference at the adenosine binding site. In the human enzyme, the adenosine moiety is stacked between two phenyl rings of Tyr87 and Phe133 leaving a small space, while the adenosine moiety of the enzyme of *P. falciparum* lies between the phenyl ring of Tyr87 on one side and Pro133 on other side, leaving a larger space (Fig. 5). Therefore, the *Plasmodium* Pro133 residue should have an important role in substrate binding, thus the effects of replacing the residue with Ala (small size) or Phe (large size) were investigated.

Table 4. Kinetic Constants of Wild and Mutant *P. falciparum* Ap_4A Hydrolases

Mutations	K_m (μM)	K_{cat} (min^{-1})
Wild	0.6±0.05	2.5±0.09
P133A	3.0±0.26	4.8±1.07
P133F	0.8±0.12	0.8±0.15

Mutational Analysis Mutational analysis was conducted at the adenosine binding site by site-directed mutagenesis. K_m and K_{cat} values were identified for the mutant enzymes to determine the effects of each mutation on substrate binding and catalytic efficiency. The kinetic constants of both the wild-type and mutant enzymes were measured using a luciferase-based bioluminescence assay (Table 4). The wild-type K_m and K_{cat} values were 0.6 μM and 2.5 min^{-1}, respectively, in common with animal Ap_4A hydrolases. As expected, P133A showed a 5-fold increase in K_m compared with wild-type, while the K_{cat} value did not differ significantly. The K_m value of P133F was not significantly affected while the K_{cat} value showed a 3-fold decrease.

DISCUSSION

In the development of antimalarial drugs, it may be appropriate to select targets from parasitic pathways that are absent in humans. Nevertheless, even if a target is common to both parasite and host, slight structural differences can enhance the optimization of a new drug.[18] A difference in only one amino acid residue between humans and *Plasmodium S*-adenosyl-l-homocysteine hydrolase has led to the discovery of a highly selective inhibitor for the malaria parasite.[19] To aid the development of antimalarial agents targeting Ap_4A hydrolase, characterization of the parasitic enzyme has been conducted and compared with other forms of Ap_4A hydrolase, particularly that of humans. Ap_4A hydrolases are classified into two subgroups according to their primary structure: animals including archaea, and plants with proteobacteria.[20] Amino acid sequence analysis confirmed that $PfAp_4A$ hydrolase belongs to the animal subgroup. The properties of *P. falciparum* Ap_4A hydrolase are in good agreement with those of animal Ap_4A hydrolases previously described. The substrates utilized

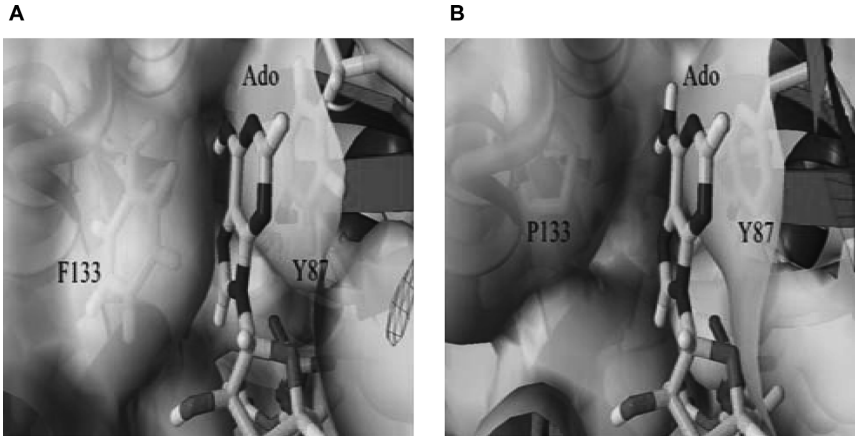

Fig. 5. A Comparison of the Surface Models of the Ado (Adenosine) Binding Site in *H. sapiens* (A) and *Plasmodium falciparum* (B) Ap_4A Hydrolases

revealed that the enzyme is active toward dinucleoside polyphosphates containing four or five phosphate residues, in common with animal Ap_4A hydrolases. There was no evidence that the enzyme could catalyze the symmetrical hydrolysis of Ap_4A to ADP as in the case of *E. coli*.[21] $PfAp_4A$ hydrolase is inhibited by Ca^{2+}, unlike the human placental enzyme which shows limited activity (relative activity 20%) when Ca^{2+} is the only divalent ion in the incubation mixture.[22] A possible mechanism for inhibition of parasitic enzyme by Ca^{2+} is the competitive binding between Ca^{2+} and Mg^{2+}, and hence displacement of Mg^{2+} from the active site. Despite the ability of Ca^{2+} to bind to the same active sites of Mg^{2+}, it fails to activate Mg^{2+}-activated enzymes, was first demonstrated with pyruvate kinase.[23] It may be ascribed to the larger ionic radius of Ca^{2+} compared to Mg^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+}, which disturbs its proper positioning for catalysis. The low K_m value of wild-type enzyme indicated that the *Plasmodium* enzyme has high affinity for Ap_4A; this high substrate affinity was previously observed for Ap_4A hydrolase isolated from both human erythrocytes (K_m 0.7 μM) and leukocytes (K_m 1.5 μM).[24]

Several studies of Nudix hydrolases have referred to the importance of catalytic residues inside and adjacent to the catalytic motif. The principles of this catalytic mechanism appear to be well conserved among the Nudix hydrolases, including those of humans. However, less attention has been focused on residues involved in substrate binding.[25] The aromatic residues, which stack one substrate adenosine moiety between them, are highly conserved in structure-based amino acid sequence alignments of animal and plant Ap_4A hydrolases,[17] suggesting that both residues have important effects on substrate binding. The importance of stacking residues for substrate binding in *C. elegans* has been previously confirmed by replacing Tyr121 by Ala, which led to an 8-fold increase in wild-type K_m value.[25] Only one aromatic residue (Tyr87) is conserved in the *Plasmodium* enzyme and the other stacking equivalent residue is smaller Pro133, which creates additional space in the *Plasmodium* adenosine binding site compared with the human enzyme. An increase in the size of the adenosine binding cavity of the *Plasmodium* enzyme (by replacing Pro133 by Ala) led to a 5-fold increase in K_m value due to loose stacking of the adenosine ring. While extensive stacking of the adenosine ring (replacing Pro133 by Phe) resulted in a 3-fold decrease in enzyme catalysis, suggests that the introducing of a larger residue to the active site precludes the free movement of domain which is expected to occur during catalysis, with keeping the complete substrate ability to bind to active site effectively. These findings confirmed the importance of the adenosine binding pocket in enzyme binding and catalysis. The additional space in the binding site of the *Plasmodium* enzyme compared with the human enzyme offers an opportunity to design potent and selective $PfAp_4A$ hydrolase inhibitors.

We conclude that the properties of *P. falciparum* Ap_4A hydrolase showed differences compared with human Ap_4A hydrolase. $PfAp_4A$ hydrolase (a) exhibited high temperature stability, (b) did not recruit Ca^{2+} ions as cofactors for enzyme activity, and (c) showed a marked divergence in the adenosine ring binding site compared with the human Ap_4A hydrolase. Our findings could aid the discovery of a *Plasmodium* Ap_4A hydrolase chemotherapeutic agent. Analysis of the crystal structures of this enzyme in free and binary complex forms will confirm this possibility.

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