Structural and functional attributes of malaria parasite diadenosine tetraphosphate hydrolase

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Malaria symptoms are driven by periodic multiplication cycles of Plasmodium parasites in human red blood corpuscles (RBCs). Malaria infection still accounts for ~600,000 annual deaths, and hence discovery of both new drug targets and drugs remains vital. In the present study, we have investigated the malaria parasite enzyme diadenosine tetraphosphate (Ap4A) hydrolase that regulates levels of signalling molecules like Ap4A by hydrolyzing them to ATP and AMP. We have tracked the spatial distribution of parasitic Ap4A hydrolase in infected RBCs, and reveal its unusual localization on the infected RBC membrane in subpopulation of infected cells. Interestingly, enzyme activity assays reveal an interaction between Ap4A hydrolase and the parasite growth inhibitor suramin. We also present a high resolution crystal structure of Ap4A hydrolase in apo- and sulphate-bound state, where the sulphate resides in the enzyme active site by mimicking the phosphate of substrates like Ap4A. The unexpected infected erythrocyte localization of the parasitic Ap4A hydrolase hints at a possible role of this enzyme in purinerigic signaling. In addition, atomic structure of Ap4A hydrolase provides insights for selective drug targeting.

Malaria causes >200 million infections and ~600,000 deaths annually\(^1\). This infection is caused by one of five members of Plasmodium in humans, where P. falciparum (Pf) causes the most severe form of malaria. Escalating resistance in parasite against known drugs in clinical use necessitates discovery of novel drug targets that can be used in future\(^2\). P. falciparum life cycle shuttles between the female Anopheles mosquito and human host by a series of complex progressions within varying cellular milieus of hepatocytes, erythrocytes and mosquito gut etc.\(^2,3\). These developments require a tight coordination of parasite's intracellular processes with changing environments that are primarily orchestrated by the multiple signalling pathways within malaria parasites\(^3\). Diadenosine tetraphosphate (Ap4A) is a ubiquitous signalling molecule present among eukaryotes, bacteria, archaea and viruses, and is well documented to participate in both intra- and extracellular signalling\(^4–6\). This molecule is a member of naturally occurring group of compounds, the dinucleoside 5',5'-p1,pn-polyphosphates; Np,nN's (where N and N' are 5'-O-nucleosides and n is the number of phosphate residues in the polyphosphate chain linking two 5'-esterified nucleosides). Diadenosine polyphosphates (Apn, n = 3–6) are predominantly synthesized as protein synthesis by-products by some of the members of aminoacyl-tRNA synthetase family (aaRSs), where Ap3A and Ap4A are the most prominent cellular forms\(^4–6\). Production of Ap4A by aaRSs is elevated during stress conditions and in mammals by a phosphorylation-induced nuclear migration of lysine-tRNA synthetase (KRS)\(^7–9\).

The Np,N, Ap4A, Ap5A and Gp4G levels within cells are primarily maintained by Nudix hydrolase superfamily member Ap4A hydrolase (Ap4AH from hereon). The Mg\(^{2+}\)-dependent Nudix hydrolase superfamily is recognized by a signature 23 amino acid Nudix motif G-x(5)-E-x(5)-[UA]-x-R-E-x(2)-E-E-x-G-U where U is an aliphatic, hydrophobic residue\(^8–10\). The consensus Nudix structural motif is located on a loop-helix and the signature Nudix fold has an α-β-α sandwich architecture\(^8–10\). Ap4AHs are phylogenetically classified into two distinct groups, animal-archael type and the plant-bacterial type enzymes. Based on sequence analysis the P. falciparum Ap4AH (PfAp4AH, EC 3.6.1.17) was predicted to be an animal-archael type which cleaves polyphosphate chain at the fourth phosphate from the tightly bound adenosine resulting in asymmetrical cleavage of Ap4A. This is distinct from some plant-bacterial type hydrolases\(^8,9,11\). Eukaryotic Ap4AHs are predominantly cytoplasmic or nuclear, while the bacterial Ap4AHs appear to be ribosome associated\(^12–14\). Levels of Ap4A in a cell are largely regulated by the synthesis and hydrolysis dynamics of KRS (which syntheses ~80% cellular Ap4A).
and Ap4A hydrolase\(^7,8,15\). Intracellular Ap4A levels can influence physiological processes such as DNA repair, DNA replication, apoptosis and ion channel regulation\(^7\). In contrast, the extracellular Ap4A act as a cytokine-like molecule and via purinergic signalling pathway it participate in modulation of various physiological processes such as neurotransmission, cardiac electrophysiology, vasodilation and cellular communications, most notably between blood cells\(^4\). Cumulative studies of pathogenic bacteria and viruses have suggested possible role for Ap4AH in invasion of human cells\(^8,16-18\). Ap4AH associations with epithelial cell or erythrocye invasion processes of *Bartonella bacilliformis*, *Escherichia coli* K1, *Actinobacillus actinomycetemcomitans*, *Salmonella enterica* and *Rickettsia prowazekii* are of particular interest for the present work\(^8,17,18\).

The aaRSs are main source of Ap4A which are of particular interest for the present work\(^8,17,18\). We also show atypical expression and localization profile of *Pf*Ap4AH, which indicates post-translational modification of native enzyme and a cytoplasmic localization in blood stage parasites along with unusual presence on RBC membrane during the feeding (trophozoite) and multiplication (schizont) of parasite. We further show that suramin can weakly inhibit *Pf*Ap4AH with dissociation constant (K\(_d\)) value of \(\sim 18\) \(\mu\)M and that it binds *Pf*Ap4AH at an IC\(_{50}\) value of approximately \(\sim 11.8\) \(\mu\)M and that it binds *Pf*Ap4AH with dissociation constant (K\(_d\)) value of \(\sim 18\) \(\mu\)M. We also provide two crystal structures of *Pf*Ap4AH - in apo and sulphate-bound forms at atomic resolution. Finally, we provide a comprehensive comparison between human and parasite Ap4AHs and discuss key active site differences which can be used for structure based drug design.

**Results**

**P. falciparum possesses a diminished set of Nudix hydrolases.** Genes encoding Nudix hydrolases in two apicomplexan parasites *P. falciparum* and *Toxoplasma gondii* were searched and identified as described in methods section. Nudix hydrolases vary in number from 0 to 30 in organisms (human - 24, *E. coli* - 12), where parasitic organisms have been documented to possess either very less or no members of this family\(^4\). Our analysis shows that *P. falciparum* and *T. gondii* contain reduced and distinct sets of five Nudix hydrolases in their genome (Table 1). The localization predictions suggest different spatial distribution schemes for *Tg* and *Pf*Ap4AH, where *T. gondii* enzyme maybe dually located in mitochondria and apicoplast while the *P. falciparum* enzyme is nuclear (Table 1). The observed disparity in evolutionary terms indicates selective retention and deletion of Nudix hydrolases post evolutionary branching of apicomplexan members *P. falciparum* and *T. gondii*. Other Ap4AHs possess such ectonucleotide pyrophosphatase/phosphodiesterase family members were not found in *P. falciparum* suggesting that *Pf*Ap4AH could be the only enzyme responsible for Ap4A hydrolysis in parasite cell.

**PfAp4AH has unusual native expression and localization.** Full length *Pf*Ap4AH enzyme was expressed in *E. coli* and purified to homogeneity. Gel permeation chromatography results on a calibrated column suggested that the protein is a monomer of \(\sim 18\) kDa (Fig. 1B). Protein A affinity chromatography purified specific anti-*Pf*Ap4AH antibodies recognised recombinant protein, but did not cross-react with uninfected RBC proteins (Fig. 1C). We also did not observe any signal in our competitive western experiments where purified antibodies were pre-incubated with purified recombinant *Pf*Ap4AH protein in varying molar ratios and used to probe parasite lysate (1:1 ratio data shown) (Fig. 1C). In addition, pre-immune sera failed to detect any protein signal using parasite lysate, suggestive of specific antibody generation against *Pf*Ap4AH (Fig. 1C). However, when the

| Protein                         | Function                                      | PlasmoDB Gene ID   | Localization prediction | ToxoDB Gene ID    | Localization prediction |
|---------------------------------|-----------------------------------------------|--------------------|--------------------------|-------------------|-------------------------|
| Ap4A Hydrolase                  | Hydrolysis of Ap4A, Ap5A                       | PF3D7_0520600      | Nucleus                  | TGME49_214780     | Apicoplast, Mitochondria |
| mRNA decapping enzyme           | mRNA decapping                                 | PF3D7_1308900      | Nucleus                  | TGME49_227450     | Nucleus                 |
| Nucleoside diphosphate hydrolase| Hydrolysis of nucleoside diphosphates linked to other moieties | PF3D7_1349100      | Cytoplasm                | Absent            | –                       |
| mRNA cleavage factor-like protein | RNA3′ processing                                | PF3D7_0109200      | Nucleus                  | Absent            | –                       |
| ADP-ribose pyrophosphatase      | Hydrolysis of ADP-ribose, ADP-sugar conjugates | Absent             | –                        | TGME49_282190     | Cytoplasm               |
| ADP-ribose pyrophosphatase      | Hydrolysis of ADP-ribose, ADP-sugar conjugates | Absent             | –                        | TGME49_290900     | Mitochondria            |
| Conserved protein               | unknown                                        |                    |                          | TGME49_242270     | Endoplasmic reticulum   |

Table 1. Distribution of putative Nudix hydrolases among *P. falciparum* and *T. gondii*. Divergent Nudix hydrolase sets present among apicomplexans *P. falciparum* and *T. gondii* are shown. Proteins were identified using hmmmsearch tool in the HMMR web server (http://hmmer.janelia.org/) and by protein blast of Pfam annotated Nudix members (id: PF00293) in PfPlasmoDB- http://plasmodb.org/plasmo/ and Tg (ToxoDB- http://toxodb.org/toxo/) sequence databases.
protein was probed in parasite lysate using these antibodies a high migrating band was observed, possibly indicating post-translational modification(s) (Fig. 1C). In order to test the predicted nuclear localization of PfAp4AH, we performed the confocal microscopy experiments (Fig. 2). We observed that PfAp4AH is constitutively expressed during all blood stages of parasites and is non-nuclear (Fig. 2A). Competitive confocal immunofluorescence assays, where antibodies were pre-incubated with PfAp4AH at varying molar concentrations, failed to produce fluorescence, thus validating the specificity of anti-PfAp4AH antibodies (1:5 ratio data shown) (Fig. 2B). To assess if PfAp4AH is mitochondrial (as has been reported in some organisms) we tested localization in presence of mitochondrial marker but failed to observe co-localization (Fig 2B). In these experiments, D-tyrosyl-tRNATyr deacylase (DTD) was used as cytoplasmic marker 27. During these investigations, we noted that ~50% cells displayed PfAp4AH localization on the infected RBC membrane (Fig. 2C). This localization was confirmed by using anti-varC antibodies, (varC is cytoplasmic domain of Pf erythrocyte membrane protein 1) as markers for RBC membrane (Fig. 2C)28. The protein signal was not a result of cross reactivity with an RBC membrane protein as we did not observe signal in uninfected RBCs (Fig. 2D). Interestingly, although conditional, membrane localization has been observed for human Ap4AH in mast cells12.

**PfAp4AH is weakly inhibited by suramin.** Suramin is a symmetric polysulfonated naphtylurea that inhibits *P. falciparum* growth (IC\(_{50}\) ~ 10\(\mu\)M), invasion of RBCs (IC\(_{50}\) ~ 60\(\mu\)M), HepB cells (IC\(_{50}\) ~ 50\(\mu\)M) and was used as remedy for trypanosomiasis and African river blindness (Fig. 3A)29. Also, suramin was earlier reported to inhibit rat Ap4AH competitively30. We studied the thermal stability profile of PfAp4AH in the presence of suramin and found that suramin decreased the melting point (Tm) of PfAp4AH by ~ −2.3 °C (50\(\mu\)M) and ~ −6 °C (500\(\mu\)M) in a concentration-dependent manner (Fig. 3B). The negative shifts indicate suramin binding and stabilization of a partially unfolded PfAp4AH state31. We performed PfAp4AH enzyme assays to access activity of recombinant enzyme (Fig. 3C), which displayed kinetic parameters similar to the earlier reports (data not shown)11. Enzyme assays in the presence of suramin suggested inhibition with an IC\(_{50}\) value of ~11.8\(\mu\)M (Fig. 3D). Isothermal titration calorimetry (ITC) was performed to determine the binding affinity. Favourable hydrogen bonding (\(\Delta S\) ~ 8817 cal/mol) and hydrophobic interactions (\(\Delta S\) ~ 7.4 cal/mol) with a binding affinity of ~18\(\mu\)M and stoichiometry of 1 were observed for suramin and recombinant PfAp4AH (Fig. 3E) (Table 2).

**Structure determination of PfAp4AH.** Two different crystal structures of PfAp4AH were obtained by hanging-drop vapour-diffusion method. Our attempts to solve structure using molecular replacement (MR) methods failed, and we used heavy atom soaking method to solve the phase problem. Iodine derivatives were produced by soaking native crystals for 1 min in cryoprotectant solution containing 100 mM NaI. Iodide-SAD
data was collected to 3 Å resolution at home source and the anomalous signal was significant only to 4.2 Å resolution. Heavy atom sites were located using SHELXD32 and the sites were used for likelihood-based SAD phasing in PHASER for experimental phasing33. Initially, 17 iodide sites were located with AutoSol in PHENIX34 with a low FOM of 0.34 and these sites were used for phasing. The obtained partial model was fed into AutoBuild for iterative model building and refinement. A total of 534 residues (of the total 608) for 4 molecules in the asymmetric unit were built automatically with Rwork and Rfree values of 32 and 39% respectively. The phased map quality is shown in Fig. 4A and relevant statistics are summarized in Table 3.

PfAp4AH apoenzyme (PfAp4AH-apo from hereon) and suphate bound PfAp4AH (PfAp4AH-SO4 from hereon) structures were solved using PHASER MR35 and one chain of iodide-SAD structure was used as template. Initially, the models were built using AutoBuild in PHENIX. Subsequently, the model was rebuilt manually using COOT36 and refined using phenix.refine in PHENIX35. There are four molecules in asymmetric unit for PfAp4AH-apo and designated as A, B, C and D. The atomic resolution structure of PfAp4AH-SO4 has three SO4 ions and a PEG molecule which arise from crystallization buffer. The quality of the electron density map is shown in Fig. 4A.

Figure 2. Spatial distribution of P. falciparum Ap4AH during erythrocytic schizogony. Shown are DAPI staining of nucleus in blue and PfAp4AH stained with Alexa 488 in green. (A) Confocal microscopy-data based spatial distribution of PfAp4AH in infected RBCs. PfAp4AH is non-nuclear in blood stages of the parasite and resides in its cytoplasm. (B) Non-mitochondrial localization with various controls is shown. Upper panel shows pre-immune serum (Pre-Imm Sera) control which does not stain the parasite or RBCs. Middle panel shows competitive binding of anti-PfAp4AH antibody to infected cells, where anti-PfAp4AH antibodies were incubated with recombinant PfAp4AH protein in 1:5 ratio. PfD-tyrosyl-tRNATyr37 deacylase (DTD) is a cytoplasmic marker. Lower panel shows non-mitochondrial localization where mitochondria are stained in red. (C) RBC membrane localization of PfAp4AH during trophozoite and schizont stages of parasite. VarC is a marker for infected RBC membrane localization. (D) A field view of anti-PfAp4AH antibody staining of infected RBCs. Significant fraction of cells (~50%) showed membrane localization of PfAp4AH - here cell is marked with white arrow. Uninfected RBCs (without DAPI and PfDTD staining here) are unstained. White scale bar in confocal figures is of 5 μm.
SO₄ binding induces conformational changes. Global structural differences between apo- and SO₄-bound PfAp4AH were apparent upon superimposition (Fig. 4C). Three major variable regions arising from SO₄ binding and different space group packing of PfAp4AH were identified in loop regions L2, L3 and L5 and

Table 2. Isothermal titration calorimetry data showing binding of suramin to PfAp4AH.
Figure 4. Crystal structure of PfAp4AH. Residues are denoted by three letter code and adjoining number indicates position in the polypeptide chain. (A) First panel shows view of a protein segment showing quality of the model. The experimental electron density is contoured at 5σ for bound iodide ion (in purple) and at 1.4σ for protein respectively (3 Å resolution Iodide-SAD data). Second panel is a segment of final model superimposed on 2Fo–2Fc electron density map contoured at 2.5σ level (B) Overall architecture of PfAp4AH structure along with marked secondary structure elements. The Nudix box region (Nudix Motif) is highlighted in orange. (C) Superimposition of PfAp4AH-apo (yellow) and PfAp4AH-SO₄ (blue) structures with significant displacement regions circled. Three bound sulphate ions are marked as SO₄ 1, SO₄ 2 (alternative conformer SO₄ 2') and SO₄ 3. (D) SO₄ 1 and SO₄ 2 binding residues and their different rotameric forms are shown. SO₄ 1 engages the residues (Tyr 87 and Lys 94) present on loop 5 (L5) and His43 on loop 2 (L2). Tyr 87 bound to SO₄ 1 also makes a hydrogen bond with a water molecule. Lys48 is also at hydrogen bonding distance from SO₄ 1. SO₄ 2 and SO₄ 2' bind Trp44, water molecule and Lys 36 in PfAp4AH-SO₄. The interactions are marked by dotted lines and arrows show direction of sidechain flipping upon SO₄ binding. (E) Interactions of SO₄ 3 ion. (F) Left panel shows superposition of loop L3 in PfAp4AH-apo (yellow) and PfAp4AH-SO₄ (blue). Middle and right panels show hydrogen bonding interactions.
Table 3. Data collection and refinement settings. aValues in parentheses are for the highest resolution shell. b$R_{merge} = \sum |I_{hkl}(j) - I_{hkl}(j)|/\sum I_{hkl}(j)$ where $I_{hkl}(j)$ is the observed intensity and $I_{hkl}$ is the final average intensity value. c$R_{pk} = \sum |F_{obs} - F_{calc}|/\sum F_{calc}$ and $R_{free} = \sum |F_{obs} - F_{calc}|/\sum F_{calc}$, where all reflections belong to a test set of 5 or 10% randomly selected data. dRoot-mean-square-deviation from ideal value.

| Data set | Pf/ Ap4AH_JOD | Pf/ Ap4AH-apo | Pf/ Ap4A-SO4 |
|----------|---------------|---------------|--------------|
| PDB code | 5CFI          | 5CFI          | 5CFI         |
| Space group | C121         | C121          | P2₁,2₁,2₁ |
| Unit cell dimensions (Å, °) |
| a = 164.14, b = 64.87, c = 61.46, α = 90, β = 99.53, γ = 90 | a = 163.31, b = 64.25, c = 61.41, α = 90, β = 100, γ = 90 | a = 31.49, b = 44.34, c = 94.67, α = 90 |
| Molecules in ASU | 4 | 4 | 1 |
| Resolution range (Å) |
| 50.00–3.04 (3.04–2.99) | 30.00–2.60 (2.64–2.60) | 50.00–1.15 (1.17–1.15) |
| Unique reflections | 13007 (579) | 19340 (953) | 47576 (2332) |
| Completeness (%) | 99.4 | 98.3 (97.9) | 99.1 (98.6) |
| I/Io (I) | 449/70.3 (70.3/13.6) 6.38 (5.1) | 403/29.0 (21.7/14.1) 13.8 (1.5) | 684/18.9 (22.5/10.3) 36.2 (2.2) |
| Redundancy | 7.4 (5.5) | 5.0 (5.0) | 5.3 (5.1) |
| Corresponding solvent content | 47.19% | 47.01% | 35.54% |
| Ramachandran plot |
| Ramachandran favored (%) | 95.3 | 100.0 |
| Ramachandran outliers (%) | 0.5 | 0 |

analyzed further. In the catalytically important loops L2 and L5, a SO₄ molecule (SO₄, 1) was found to bind in the P1 position. (Fig. 4C,D). SO₄ I makes contact with four amino acids in the active site and induces a flip in His43 and Tyr87 side-chains (Fig. 4D). Tyr87 binding to SO₄ I predisposes it to an adenine ring stacking conformation. Other residues involved in hydrogen bonding to SO₄ I are Lys94 (one conformer of the two alternative conformations) and Lys48. His43 binding to SO₄ I leads to changes in loop orientation (L2) of Pf/ Ap4AH-SO₄ structure. SO₄ 2 was observed in alternative confor- mations, where SO₄ 2 engages mainly the Trp44 and the alternative conformer SO₄ 2’ engages Lys36 and a water molecule (Fig. 4D). The SO₄ 3 is coordinated to a water molecule and a conserved Arg15 (Fig. 4E). Binding positions of SO₄ 2, 2’ and 3 do not comply with the earlier reported phosphate binding sites elsewhere⁴⁷, and hence may not be relevant for hydrolysis and substrate binding functions of the enzyme. In another major displacement between two structures, the backbone hydrogen bonding keeps the loop L3 in a specific orientation (Fig. 4F). In case of Pf/ Ap4AH-SO₄, His51 forms a hydrogen bond with one of the water molecule in a nearby water network linked to Ser56 (Fig. 4F). A movie showing overall conformational changes and alterations in interacting residues (within 5 Å distance) of Pf/ Ap4AH upon various ligand bindings is part of supplementary material.

Sequence alignment and comparison with human structures. Hs/ Ap4A hydrolase has sequence identity of ~36% with the Pf/ Ap4AH. Alignment show conservation of key residues implicated in catalysis and binding of substrate (Fig. 5A). Overall 3D architecture of both these proteins is similar with overall root mean square deviation (r.m.s.d.) of 0.88 Å for 110 Cα-atoms (Fig. 5B). Pf/ Ap4AH contains an insertion of 13 residues in loop region L1 (Fig. 5B) compared to the 10 and 2 amino acid insertions in human and C. elegans respectively⁴⁷,⁴⁸. The SO₄ bound Pf/ Ap4AH-SO₄ atomic structure is similar to that of sulphate-bound Hs/ Ap4AH structure where a SO₄ ion is also located in P1 binding site (Hs/ Ap4AH; PDB id 3U53)⁴⁷. We were able to directly compare the active site residues involved in engaging sulphates (or P1 by analogy). Active site-bound SO₄ is coordinated by analogous residues (Pf/ Hs/ Hs/ Ap4AH; His43/His32, Lys48/Lys42 and Tyr87/Tyr82, but unlike Pf/ Lys94 analogous Hs/ Lys89 does not engage sulphate (Fig. 5C). Structural comparison of Pf/ Ap4AH with known structures of ATP-bound human counterpart⁴⁷ and AMP bound C. elegans Ap4AH display a common scheme of substrate engagement and hydrolysis by these enzymes (Fig. 5D). The adenine ring of substrate is stabilized by π–π stacking interactions with a
conserved Tyr on loop 5 and another Tyr/Phe (Fig. 5D). In PfAp4A hydrolase structure, these two positions are occupied by Tyr87 and Pro133. Ap4A substrate is generally accommodated in a negative charge zone with help of magnesium ions and hydrolysis occurs at 3rd phosphate (P3) by a conserved glutamic acid (Fig. 5D). Presence of Pro133 and Ser 135 in PfAp4A instead of larger Phe 128 and Glu 130 provides extra space in substrate binding pocket that can be used to design inhibitory compounds that selectively bind PfAp4AH (Fig. 5E).

Discussion
The Nudix hydrolase enzyme set present in an organism is often dictated by host metabolic complexity and adaptability. Most intra- and extracellular parasites, including apicomplexans, have either diminished number of hydrolases or none (e.g. mycoplasmas). Intriguingly, the diverse Nudix enzyme sets in P. falciparum and T. gondii reported in this study suggest their selective retention post-evolutionary branching (Table 1). Amongst Nudix hydrolases, Ap4AH is a key mediator of invasion and virulence for many bacterial and viral pathogens, especially as Ap4AHs play central roles in bacterial invasion of human RBCs. Ap4A and Ap5A molecules,
chief substrates of Ap4AH, are key mediators of cellular communication and function through purinergic receptors\textsuperscript{9,10,11}. Hence, signalling mediated by these molecules within RBCs is of special interest in malaria\textsuperscript{8,10,11}. Purinergic signalling has been shown to play role in parasite invasion\textsuperscript{11}. Absence of additional domains and presence of \textit{Pf}Ap4AH on infected RBC membrane (Figs 1A and 2) implies that \textit{Pf}Ap4AH has the potential to modulate RBC purinergic signalling and invasion. Intriguingly, we found the \textit{Pf}Ap4AH thermal melting profile to be unusually high (Fig. 3B), a fact that is consistent with the earlier reported high activity of this enzyme at elevated temperatures\textsuperscript{11,25–27}. It has been reported that erythrocytes, which can synthesize Ap4A on their own, elevate the intracellular levels of Ap4A ~10 fold during heat shock or high temperatures (as occur in blood stage infection of human malaria). Additionally, Ap4A molecule has been shown to regulate haemoglobin functioning\textsuperscript{8,42}. These observations link with our data that show (a) \textit{Pf}Ap4AH localization on the infected RBC membrane (Fig. 2C,D), and (b) \textit{Pf}Ap4AH's high thermostability and thermoactivity (Fig. 3B). Hence, it is feasible that \textit{Pf}Ap4AH can access host cell synthesized intracellular as well as extracellular Ap4A and Ap5A molecules, and lower their concentrations - with even higher enzymatic activity during fever conditions (to perhaps tackle higher levels of RBC concentrations - with even higher enzymatic activity during fever conditions (to perhaps tackle higher levels of RBC

The gene encoding \textit{Pf}Ap4AH (PF3D7_0520600) was cloned into pETM11 vector and expressed in \textit{E. coli} B834 (DE3). For expression, \textit{E. coli} culture was induced at 0.6 OD with 1 mM IPTG and harvested after growth at 18°C for 20 h post induction. Cells were resuspended in lysis buffer (20 mM Tris pH 8.0, 100 mM NaCl, 5% glycerol, 15 mM imidazole and 2 mM beta-mercaptoethanol (\textit{β}-Me) and lysed by sonication. Supernatant was separated by centrifugation at 16,000 g for 1 h and loaded onto Ni-NTA beads. Protein was eluted using imidazole gradient and purity of fractions was checked on gel. Pure fractions were pooled and His-tag was removed by adding 1 mM DTT, 0.5 mM EDTA and TEV protease (1:50) and incubation for 16 h at 20°C. Cleaved protein was buffer exchanged overnight to 20 mM Tris (pH 8.0), 40 mM NaCl and 10 mM \textit{β}-Me. Protein was loaded once again to Ni-NTA column to remove uncut protein and TEV protease (which contains non-cleavable N-terminal His tag). Pure protein was collected in flow through. Protein was further purified using gel permeation chromatography (GPC) using a GE HiLoad 10/300 Superdex 75 column in 20 mM Tris pH 8.0, 40 mM NaCl and 10 mM \textit{β}-Me buffer system. Purity was checked once again on SDS PAGE and pure fractions were pooled. Protein was concentrated to 9.5 mg ml\textsuperscript{-1} (A\textsubscript{280}, extinction coefficient – 24410 M\textsuperscript{-1} cm\textsuperscript{-1}) and stored in –80°C for further use. Pure recombinant protein was provided to Merck (Merck Millipore) for generation of specific protein A affinity chromatography purified anti-\textit{Pf}Ap4AH antibodies in rabbits. These specific antibodies were used for all western and immunofluorescence studies. Recombinant \textit{Pf}Ap4AH (10 ng) was probed in western blot using 1:5000 antibody dilution. Same concentration of pre-immune sera was used in control.

**Methods**

**Identification and annotation of Nudix hydrolases in \textit{P. falciparum} and \textit{T. gondii}**. NUDIX hydrolases were probed using HMM-search tool in the HMMPR web server (http://hmmer.janelia.org/) by restricting the taxonomy against \textit{T. gondii} and \textit{P. falciparum} and an E-value cut-off of 0.01. Additionally, independent searches for each available Nudix family member annotated in Pfam (id: PF00293) were performed by protein blast in \textit{P. falciparum} (PlasmoDB- http://plasmodb.org/plasmo/) and \textit{T. gondii} (ToxoDB- http://toxodb.org/toxo/) sequence databases. Domains were annotated using SMART\textsuperscript{46}, CD - search\textsuperscript{47}, superfamily servers\textsuperscript{48} and also by visual inspection of sequence alignments. Localizations were predicted using online servers, MitoProt (mitochondrial localization- http://ihg.gsf.de/ihg/mitoprot.html), WoLF PSORT (nuclear localization- http://wolfpsort.org/) and PATS (for apicoplast localization).

**Cloning, expression, purification and antibody generation.** The gene encoding \textit{Pf}Ap4AH (PF3D7_0520600) was cloned into pETM11 vector and expressed in \textit{E. coli} B834 (DE3). For expression, \textit{E. coli} culture was induced at 0.6 OD with 1 mM IPTG and harvested after growth at 18°C for 20 h post induction. Cells were resuspended in lysis buffer (20 mM Tris pH 8.0, 100 mM NaCl, 5% glycerol, 15 mM imidazole and 2 mM beta-mercaptoethanol (\textit{β}-Me) and lysed by sonication. Supernatant was separated by centrifugation at 16,000 g for 1 h and loaded onto Ni-NTA beads. Protein was eluted using imidazole gradient and purity of fractions was checked on gel. Pure fractions were pooled and His-tag was removed by adding 1 mM DTT, 0.5 mM EDTA and TEV protease (1:50) and incubation for 16 h at 20°C. Cleaved protein was buffer exchanged overnight to 20 mM Tris (pH 8.0), 40 mM NaCl and 10 mM \textit{β}-Me. Protein was loaded once again to Ni-NTA column to remove uncut protein and TEV protease (which contains non-cleavable N-terminal His tag). Pure protein was collected in flow through. Protein was further purified using gel permeation chromatography (GPC) using a GE HiLoad 10/300 Superdex 75 column in 20 mM Tris pH 8.0, 40 mM NaCl and 10 mM \textit{β}-Me buffer system. Purity was checked once again on SDS PAGE and pure fractions were pooled. Protein was concentrated to 9.5 mg ml\textsuperscript{-1} (A\textsubscript{280}, extinction coefficient – 24410 M\textsuperscript{-1} cm\textsuperscript{-1}) and stored in –80°C for further use. Pure recombinant protein was provided to Merck (Merck Millipore) for generation of specific protein A affinity chromatography purified anti-\textit{Pf}Ap4AH antibodies in rabbits. These specific antibodies were used for all western and immunofluorescence studies. Recombinant \textit{Pf}Ap4AH (10 ng) was probed in western blot using 1:5000 antibody dilution. Same concentration of pre-immune sera was used in control.

**Confocal microscopy and expression studies.** \textit{P. falciparum} 3D7 strain was cultured using human erythrocytes (4% hematocrit) in RPMI-1640 supplemented with 0.5% AlbumaxII (Invitrogen) as previously
Cells were centrifuged and supernatant containing protease inhibitors cocktail. Parasite lysate was formed by incubating purified antibodies with pure protein in molar ratios of 1:1, 1:2 and 1:5 (antibody:protein). Same dilutions of pre-immune sera were used in each case as western controls. Competitive western was performed using horseradish peroxidase conjugated antibodies (1:1500 dilutions). Bands were visualized using ECL detection kit.

Enzyme activity and inhibition assays. PfAp4AH activity assays and inhibition were performed by detecting ATP (catalysis product) in a luciferase-based bioluminescence assay (E/NLITEN ATP Assay kit, Promega) as reported elsewhere. Briefly, a 100 μl reaction volume was used for each reaction in assay buffer containing 50 mM Tris (pH 7.5), 20 mM NaCl and 5 mM MgCl2, with 0.2 nM enzyme at room temperature. Varying substrate concentrations in assay buffer were used to determine kinetics. Ap4A at a concentration of 2 μM was heated from 20° to 96 °C at a rate of 1 °C min⁻¹. Fluorescence signals were monitored by StepOnePlus quantitative real-time PCR system (Life Technologies). Each curve was an average of three measurements and was analysed on Thermal shift software (Life Technologies) for ΔTm and Tm calculations. Suramin alone in assay buffer was taken as no protein controls and flat line was observed for fluorescence readings at all temperatures. Melt profiles were plotted by instrument software using derivative curve method.

Isothermal titration calorimetry. ITC experiments were conducted at 30 °C in a MicroCal ITC-200 apparatus (GE Healthcare) and results were analysed using Microcal origin software. PfAp4AH was prepared in PBS (phosphate-buffered saline) pH 7.4 and suramin was solubilized in PBS buffer. Suramin at a concentration of 1.5 mM was titrated into 100 μM PfAp4AH. Titrations consisted of a 0.4 μl injection followed by 39 × 1 μl injections with a 120 s interval between injections. Data analyses and peak integration were carried out using Origin 7 software. Titration of suramin in buffer alone was performed to determine the change in enthalpy caused by dilution of the ligand and subtracted as background from actual ligand-binding experiments.

Crystallization and preparation of iodine derivatives. Crystallization was carried out at 20 °C using hanging drop vapour diffusion method. Crystals were obtained in two conditions: i. 1 μl of 0.2 M lithium sulphate, 0.1 M sodium acetate, 3% ethylene glycol, 50% PEG400 and 1 μl of protein (9.5 mg ml⁻¹, PfAp4AH-SO4) and ii. 1 μl of 20% PEG, 0.3 M potassium nitrate, 0.4 M sodium bromide and 1 μl of protein (9.5 mg ml⁻¹, PfAp4AH-apo). Single crystals were added to cryoprotectant (20% glycerol + mother liquor) for one minute before flash freeze in cooled nitrogen gas at 100 K. For phasing crystals were soaked into cryoprotectant solution supplement with 100 mM NaI for 1 min before flash freeze.

Data collection and processing. Data set for phasing were collected using Cu Kα radiation (λ = 1.54 Å) at 100 K on MAR345 image-plate detector attached on a Rigaku MicroMax-007 rotating-anode X-ray generator operated at 40 kV and 20 mA. A total of 360 images were collected in 1° oscillation steps with 300 s exposure per
frame. Diffraction data for crystals of two different conditions (PfAp4AH-apo and PfAp4AH-SO₄) were collected on MARCCD detector at BM14 beam line of European Synchrotron Radiation Facility (ESRF) at Grenoble, France. The diffraction images were processed and scaled with HKL2000 suite programme. Phasing, model building and refinement. Iodine SAD data was analysed using SHELEX 34 and SHELXD 35 in HKL2MAP 55. Model was obtained using AutoSol and AutoBuild modules in PHENIX 34. The models were built manually in COOT 36 and refined using phenix.refine 33. The quality of all models was checked using PROCHECK 56 and MolProbity 57. Structure was analysed and figures were prepared using Chimera 58 and PyMOL (http://www.pymol.org). References
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Author Contributions
A.S. and A.S. designed the study and wrote the paper. A.S. performed all the experiments. M.Y. helped in X-ray data collection and structure solution. All authors analysed the results and approved the final version of the manuscript.

Additional Information
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