Involvement of δ-aminolaevulinate synthase encoded by the parasite gene in *de novo* haem synthesis by *Plasmodium falciparum*

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The malaria parasite can synthesize haem *de novo*. In the present study, the expression of the parasite gene for δ-aminolaevulinate synthase (Pf*ALAS*) has been studied by reverse transcriptase PCR analysis of the mRNA, protein expression using antibodies to the recombinant protein expressed in *Escherichia coli* and assay of ALAS enzyme activity in *Plasmodium falciparum* in culture. The gene is expressed through all stages of intra-erythrocytic parasite growth, with a small increase during the trophozoite stage. Antibodies to the erythrocyte ALAS do not cross-react with the parasite enzyme and vice versa. The recombinant enzyme activity is inhibited by ethanolamine and the latter inhibits haem synthesis in *P. falciparum* and growth in culture. The parasite ALAS is localized in the mitochondrion and its import into mitochondria in a cell-free import assay has been demonstrated. The import is blocked by haemin. On the basis of these results, the following conclusions are arrived at: PfALAS has distinct immunological identity and inhibitor specificity and is therefore a drug target. The malaria parasite synthesizes haem through the mitochondrion/cytosol partnership, and this assumes significance in light of the presence of apicoplasts in the parasite that may be capable of independent haem synthesis. The Pf*ALAS* gene is functional and vital for parasite haem synthesis and parasite survival.

Key words: ALAS expression, drug target, ethanolamine, mitochondria.

INTRODUCTION

Studies in this laboratory have shown that the malaria parasite is capable of haem biosynthesis *de novo* in the intra-erythrocytic stage, despite acquiring large amounts of haem through the degradation of red cell haemoglobin [1]. The enzyme activities of δ-aminolaevulinate synthase (ALAS), δ-aminolaevulinate dehydrase (ALAD) and ferrochelatase were detected in *Plasmodium falciparum* and *Plasmodium berghei*. Interestingly, the bulk of the parasite (*P. berghei*) ALAD was found to be of host origin, based on biochemical, immunological and immunoelectron microscopy studies. Antibodies to human red cell ALAD were found to react with the *P. berghei*/*P. falciparum* enzyme in Western blot analysis. However, antibodies to human red cell ALAS failed to cross-react with the parasite lysate, suggesting the possibility that the parasite may be making its own enzyme that is immunologically distinct from that of the host [2]. This notion was strengthened by the successful cloning of the ALAS gene from *P. falciparum* (Pf*ALAS*) by Wilson et al. [3]. Overall, the Pf*ALAS* gene has 52% similarity with the human gene. The gene has a putative mitochondrial targeting sequence with amino acid residues similar to a motif in the murine homologue, which has been associated with haem-regulated mitochondrial import [4]. However, Pf*ALAS* was poorly expressed and it was not clear how reliant the parasite is on the expression of this gene for its haem biosynthesis *de novo* [3].

In the present study, the full-length cDNA for PfALAS has been cloned, expressed in *Escherichia coli* and the recombinant protein used to raise antibodies. Expression of the Pf*ALAS* gene in the parasite in terms of mRNA, protein and enzyme activity has been studied. The consequence of inhibiting this enzyme on haem biosynthesis and parasite survival has been investigated. The localization of the enzyme in the parasite mitochondrion and its import into this organelle in an *in vitro* import assay have been studied. It is concluded that the mitochondrial ALAS is involved in parasite haem biosynthesis and the function of this enzyme is vital for parasite survival.

EXPERIMENTAL

Materials

The cDNA encoding *P. falciparum* Hsp60 (PfHsp60) was a kind gift from Dr Nirbhay Kumar, Johns Hopkins School of Public Health, Baltimore, MD, U.S.A. This cDNA was subcloned into the *E. coli* expression vector pRSETA (Invitrogen, Carlsbad, CA, U.S.A.) and was transformed into *E. coli* BL21(DE3)pLysS. The protein was expressed by isopropyl β-D-thiogalactoside (IPTG) induction and purified using Ni^{2+}-nitriloacetate affinity chromatography. Antibodies were raised against His-tagged PfHsp60 in rabbits. MitoTracker Red CM-H_2XRos, a mitochondrion-specific dye, was purchased from Molecular Probes (Eugene, OR, U.S.A.). RIG plasmid encoding three plasmodium tRNAs (Arg, Ile and Gly) was a kind gift from Dr Wim G. J. Hol (University of Washington, Seattle, WA, U.S.A.). Antisera to human red cell ALAS was a kind gift from Dr Gloria C. Ferreira (University of South Florida, Tampa, FL, U.S.A.). Peptide sequencing was carried out at Purdue University, West Lafayette, IN, U.S.A.

Parasite maintenance and isolation

P. falciparum culture was maintained on human O^+ red blood cells by the candle jar method [5]. Synchronization of the culture was achieved using sorbitol [6]. The cells were isolated at the desired stages and the parasites released by treatment with

Abbreviations used: ALA, δ-aminolaevulinate; ALAD, δ-aminolaevulinate dehydrase; ALAS, δ-aminolaevulinate synthase; IPTG, isopropyl β-D-thiogalactoside; Pf, *Plasmodium falciparum*; RT-PCR, reverse transcriptase PCR.

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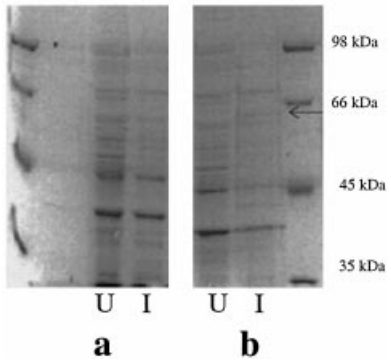

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121 kndhsfqind ntiqknniiy kyinsldeyk lfknncnnnl kdllnklytd kryriftiln
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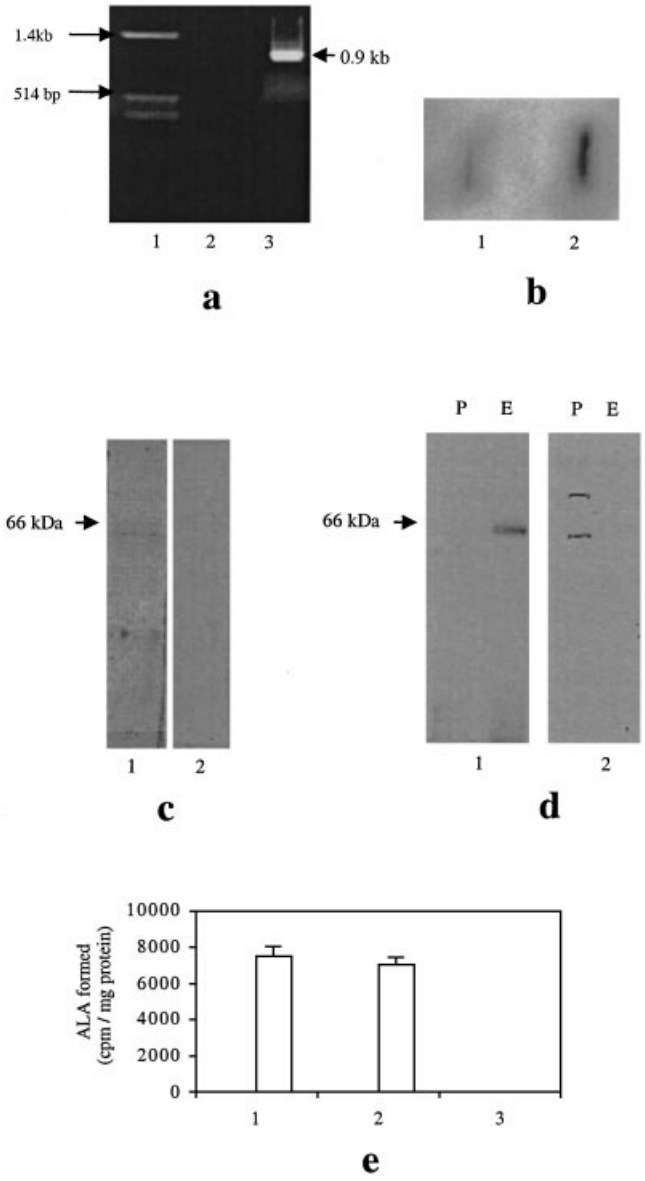
541 hnnshivvlm insaekckqi cddllk**eyni yiqpinyptv** pmgmeririt pspfhtdeqi
601 fklvnslytl fkkyqvnmfd kknkhtlmkl

c

Figure 1 Expression of PfALAS cDNA in pRSETB in the presence and absence of the RIG plasmid

U, uninduced; I, IPTG (1 mM) induced. Protein profile of the expression clone in the absence (**a**) and presence (**b**) of the RIG plasmid. Arrow indicates the induced band around ≈ 63 kDa. (**c**) The amino acid sequence derived from the cDNA sequence. The two peptides sequenced from the ≈ 63 kDa protein digest obtained from the gel piece are highlighted in bold.



0.15 % (w/v) saponin [7]. The parasite pellet was washed at least three times with PBS and then used for different purposes.

Cloning of PfALAS cDNA

Based on the reported sequence of Pf*ALAS* gene [3], primers were designed for amplifying different regions of the gene from a genomic DNA template. The primers used were: GP1, 5′-AGGATCCGATGGGGAAAAAAAGAACTTTGA-3′; GP1b, 5′-AAAACGGTTGTGTGGTGT-3′; GPi1, 5′-GCGCCATGG-GAATGGAAAGAATTCGCATA-3′; GP2, 5′-GCTCCTACA-TTAGCAATATAA-3′; GP2b, 5′-GGAAGCTTATATAGAG-AGGCAGTGG-3′; GPi2, 5′-GCGCCATGGGAACGGTCG-GGTAATTT-3′.

DNA was isolated from the parasite pellet by standard procedures using phenol/chloroform extraction. The 5′ 1 kb region, the middle 1 kb region and the 3′ 0.2 kb region were amplified using primer sets GP1/GP2, GP1b/GPi2 and GPi1/GP2b respectively and cloned into the pUC19 vector. The two 1 kb fragments were used to carry out an overlap PCR using primers GP1 and GPi2 to obtain a 1.7 kb fragment which was cloned into pUC19 vector as a blunt-ended fragment. The 3′ 0.2 kb fragment was released from the pUC clone as an *Nco*I–*Hin*dIII fragment and ligated into the *Nco*I/*Hin*dIII-digested 1.7 kb pUC19 clone. The full-length 1.9 kb fragment was released by *Bam*HI/*Hin*dIII digestion. The DNA sequence of the fragment matched the reported gene sequence [3] without the intron.

Figure 2 Expression of ALAS mRNA and protein in *P. falciparum*

(**a**) RT-PCR analysis of RNA from the parasite with primers to amplify a 0.9 kb region. Lane 1, marker; lane 2, without reverse transcriptase; lane 3, with reverse transcriptase. (**b**) Slot-blot analysis of RNA from mouse liver (lane 1) and the parasite (lane 2) probed with labelled 0.9 kb fragment; 20 μg of total RNA was loaded on to the filter. (**c**) Western blot analysis of parasite lysate probed with PfALAS (≈ 63 kDa) antiserum (lane 1) and preimmune sera (lane 2). (**d**) Western blot analysis with antibodies to human erythroid ALAS (panel 1) and PfALAS (≈ 63 kDa; panel 2). P, parasite lysate; E, human erythrocyte lysate. The identity of the higher-molecular-mass band in P lane of panel 2 is not known. (**e**) Effect of antibodies to PfALAS (≈ 63 kDa) on recombinant ALAS enzyme activity. 1, Control (untreated); 2, preimmune serum; 3, PfALAS antiserum; 100 μg of *E. coli* lysate was used as the source of recombinant protein.

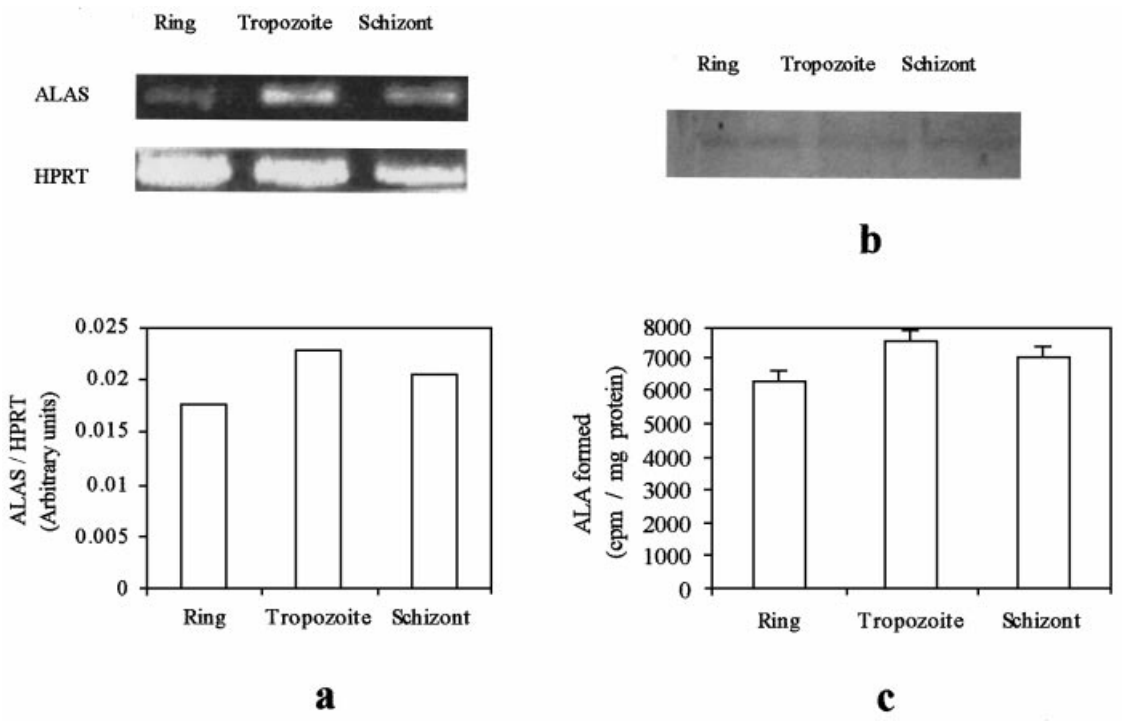


Figure 3 Expression of ALAS mRNA, protein and enzyme activity in synchronized cultures of *P. falciparum*

The cultures were synchronized twice using sorbitol. (**a**) RT-PCR analysis of ALAS (top panel) and HPRT (middle panel), and ALAS/HPRT ratio (bottom panel) in three stages of parasite growth (Ring, 5 h; Trophozoite, 24 h; Schizonts, 36 h). (**b**) Western blot analysis of parasite lysates; 50 μg of protein was used in each case. (**c**) ALAS enzyme activity in parasite lysates; 250 μg of protein was used in each case.

Expression of ALAS

The cDNA was cloned as a *Bam*HI–*Hin*dIII fragment in *E. coli* expression vectors, but no protein expression was seen. Finally, expression could be achieved when *E. coli* BL21(DE3) cells were co-transformed with Pf*ALAS* in pRSETB vector and RIG plasmid [8]. One of the clones in pRSETB, expressing a band $\approx$ 63 kDa after induction with IPTG, in the absence and presence of RIG plasmid, is depicted in Figures 1(a) and 1(b). The identity of the $\approx$ 63 kDa band as PfALAS was confirmed by the sequencing of two internal peptides (Figure 1c). The gel piece containing the $\approx$ 63 kDa protein was crushed and injected into mice to raise antibodies.

Assay of ALAS

Two different methods were used to assay the enzyme. In the colorimetric method [9] the assay mixture in a total volume of 100 μl contained glycine (100 mM), Tris/HCl, pH 7.5 (50 mM), sodium succinate (10 mM), CoA (500 μM), $MgCl_2$ (5 mM), pyridoxal phosphate (200 μM), partially purified *E. coli* succinyl-CoA synthase (20 μg) and ALAS enzyme extract. For the enzyme extract, *P. falciparum* cells were lysed in 10 vol. of 10 mM Tris/HCl (pH 7.5) in the presence of 1 mM PMSF, leupeptin and aprotinin for 30 min on ice and given brief sonication. In experiments where recombinant *E. coli* was used as the enzyme source, cells were lysed by lysosyme treatment and sonication and spun at 12000 ***g*** for 15 min at 4 °C. The supernatant was used as the crude enzyme extract. The mixture was incubated at 37 °C for 1 h. The product formed, δ-aminolaevulinate (ALA), was converted into porphobilinogen by the addition of purified ALAD and the porphobilinogen formed was assayed using modified Ehrlich reagent [10].

In the radiometric assay [11], 10 μCi of [2-^{14}C]glycine (4.8 mCi/mmol) was included along with 2 mM succinylacetone in the primary incubation mixture to prevent any possible conversion of ALA into porphobilinogen. The reaction was stopped with trichloroacetic acid. Radioactive ALA formed was separated from unutilized [2-^{14}C]glycine by ion-exchange chromatography on Dowex 50W X-8 resin (0.5 ml bed volume, 200–400 mesh). After equilibrating the column with 0.05 M sodium acetate, pH 3.9, the sample was loaded and the resin was washed extensively with 50 mM sodium acetate buffer, pH 3.9, and radioactive ALA was eluted with 0.25 M phosphate buffer, pH 7.0. An aliquot was used to measure radioactivity. The recovery of ALA was around 75 %.

Localization of ALAS in the parasite

Localization of the enzyme was studied by two different methods. In one approach, co-localization of ALAS with MitoTracker Red CM-H2XRos, a mitochondrion-specific dye [12], was studied. For this purpose, 1.5 ml of parasite culture was incubated with the dye (250 nM) for 30 min. The cells were then washed with complete RPMI and a smear was prepared on coverslips, fixed and processed for detection of PfALAS localization as described below.

In another approach, co-localization of PfALAS with PfHsp60, shown to be localized in *P. falciparum* mitochondria [13], was studied. The coverslips with the parasitized cells were fixed in a 70:30 mixture of ice-cold acetone/methanol. The coverslips were incubated with blocking buffer (2 % BSA in PBS)

and then with primary antisera (anti-PfALAS in mice and anti-PfHsp60 antibodies in rabbit, 1:50 dilution) for 2 h. The coverslips were then washed with blocking buffer and incubated with anti-mouse antibodies conjugated to tetramethylrhodamine β-isothiocyanate (TRITC) and anti-rabbit antibodies conjugated to FITC. The coverslips were once again washed with blocking buffer, nuclei stained with Hoechst stain (1 μg/ml) and mounted on to glass slides in 90% glycerol in PBS. Analysis was carried out with a confocal microscope (Leica, Brussels, Belgium).

Import of ALAS into mitochondria

Import of the enzyme was carried out using a cell-free mitochondria import assay [14]. Coupled transcription/translation was carried out in a cell-free rabbit reticulocyte lysate kit (Promega, Madison, WI, U.S.A.) containing T7 RNA polymerase. The reaction was carried out as per the manufacturer's protocol in the presence of 10 μCi of [^{35}S]methionine. To the translated products, BSA buffer (3% fatty acid free BSA, 25 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$ and 40 mM Mops) containing 25 mM NADH and 5 mM methionine was added. Reaction was started by the addition of rat liver mitochondria (5 mg/ml). In some experiments haemin (25 μM) was added to study its effect on ALAS import. Incubation was carried out for 25 min at 25 °C. Mitochondria were re-isolated by centrifugation at 12000 ***g*** for 20 min, resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA and 20 mM Mops, pH 7.4) and washed with the same buffer. The final mitochondrial suspension was divided into two aliquots. One aliquot (20 μl) was treated with proteinase K (20 μg) and another aliquot was lysed with 0.1% SDS and then treated with proteinase K in ice for 20 min. Proteinase K was then inactivated by the addition of PMSF (1 mM). The mitochondria were suspended in SDS loading dye and analysed by SDS/PAGE (15% polyacrylamide). In another set of experiments, after incubation with mitochondria, mitoplasts were prepared by osmotic shock [15] and then subjected to SDS/PAGE.

PfHsp60 import into mitochondria was also studied. But, the cDNA clone was not efficiently transcribed/translated in the reticulocyte lysate system. Therefore, experiments were carried out by adding 5 μg of recombinant protein purified form *E. coli* to the mitochondrial import assay mixture and the imported protein analysed by Western blot analysis.

Other analytical methods

PfALAS mRNA in the synchronized cultures of *P. falciparum* was quantified using reverse transcriptase PCR (RT-PCR) and slot-blot analysis. PfALAS protein in the parasite was analysed by Western blot analysis using antibodies generated in mice. [2-^{14}C]Glycine incorporation into haem in parasite lysate was measured as described earlier [2].

RESULTS

Detection of PfALAS mRNA and protein in parasite lysates

A primer set to amplify a 0.9 kb (GP1b/GP2b) fragment was used to carry out RT-PCR on total RNA isolated from the parasite pellet. The results presented in Figure 2(a) reveal that the expected fragment could be obtained from the PCR reaction. In addition, the labelled RT-PCR fragment was also used to carry out slot-blot analysis. Signal could be seen with parasite RNA, whereas it was poor with mouse liver RNA (Figure 2b). Similarly, the antisera prepared against the recombinant PfALAS

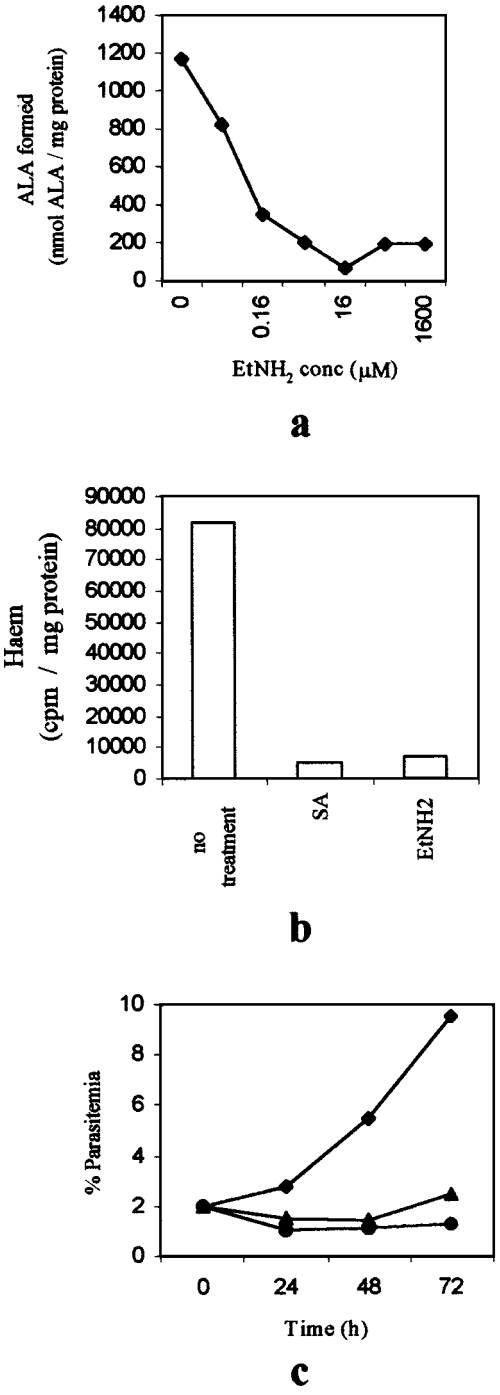


Figure 4 Effect of ethanolamine on recombinant PfALAS enzyme activity, haem synthesis and growth in *P. falciparum*

(**a**) Effect of ethanolamine on recombinant PfALAS enzyme activity measured using the colorimetric procedure. The 30–60% $(NH_4)_2SO_4$ fraction of *E. coli* extracts containing the induced enzyme was used; 250 μg of protein was used for the assay. (**b**) Effect of ethanolamine on [2-^{14}C]glycine incorporation into haem in *P. falciparum* lysates (300 μg of total protein); SA, succinylacetone (2 mM); $EtNH_2$, ethanolamine (2 mM). (**c**) Effect of ethanolamine on growth of *P. falciparum* in culture. Parasitaemia was assessed after examining several fields in slides stained with Giemsa and counting parasitized cells using microscopy: ◆, control; ▲, ethanolamine; ●, succinylacetone.

was able to pick up a protein of $\approx$ 63 kDa in the Western blot analysis. Often a doublet was seen with the parasite lysate, with the bottom band being of stronger intensity. These bands did not

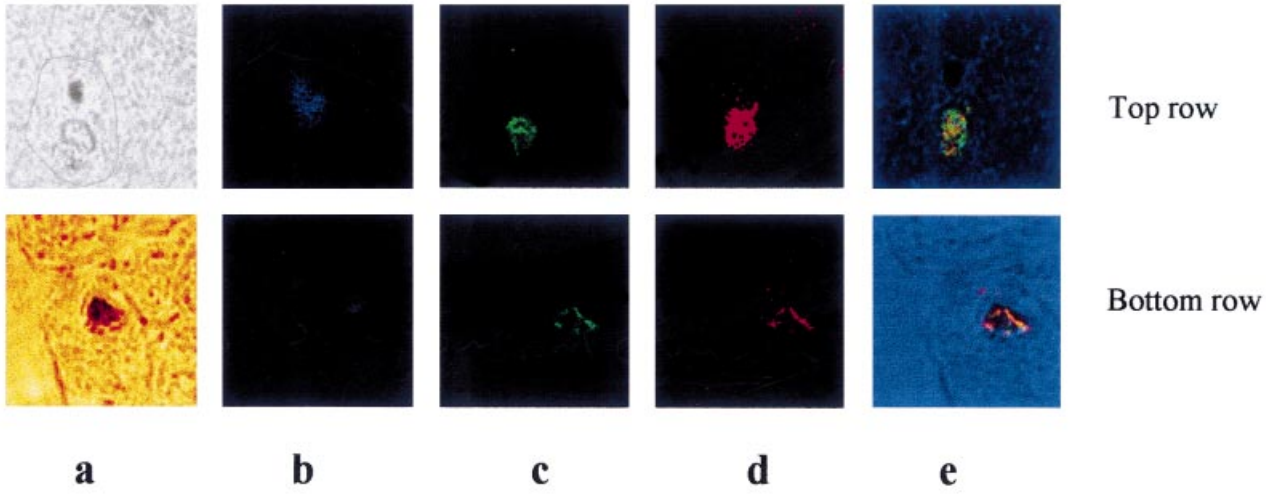


Figure 5 Co-localization of ALAS with MitoTracker dye and Hsp60 in *P. falciparum*

The experimental details are given in text. Top row: (**a**) bright field (the contour of the infected cell is indicated since it is not clearly visible); (**b**) Hoechst stain; (**c**) MitoTracker fluorescence in parasite; (**d**) ALAS immunofluorescence with PfALAS antibody and FITC-conjugated secondary antibody; (**e**) co-localization of (**c**) and (**d**). Bottom row: (**a**) bright field; (**b**) Hoescht stain; (**c**) Hsp60 immunofluorescence with Hsp60 antibody in rabbits and tetramethylrhodamine β-isothiocyanate (TRITC)-conjugated anti-rabbit antibody in goat; (**d**) ALAS immunofluorescence with ALAS antibody in mice and FITC-conjugated anti-mouse antibody in goat; (**e**) c-localization of (**c**) and (**d**).

light up with preimmune sera (Figure 2c). In addition, antisera to PfALAS did not react with red cell lysate, and antisera to human red cell ALAS did not cross-react with PfALAS (Figure 2d). The PfALAS antibodies were also able to inhibit the enzyme activity of the recombinant enzyme in *E. coli* lysate (Figure 2e).

It was, therefore, of interest to study the expression of Pf*ALAS* gene in the parasite at different stages. The results on PfALAS mRNA, protein and enzyme activities are presented in Figure 3. The results indicate that Pf*ALAS* gene was expressed at all stages with a slight increase at the trophozoite stage, which is not surprising considering that it is the metabolically most active stage.

Properties of PfALAS

A major single band in SDS/PAGE (≈ 63 kDa) could be obtained with one-step purification using Ni^{2+}-nitriloacetate beads and elution with imidazole. But the enzyme had very little activity. Analysis of the preparation on a Superdex-200 gel-filtration column revealed that the protein eluted predominantly as a peak of 67 kDa and a minor peak of 158 kDa. Low but detectable enzyme activity was seen only in fractions under the 158 kDa minor peak, but not under the 67 kDa major peak. In addition, when the crude enzyme extract from the recombinant *E. coli* was fractionated on the same column, there was loss of enzyme activity, but again no enzyme activity could be seen in fractions eluting around 67 kDa, but low activity could be detected in fractions around 158 kDa (results not shown). It is known that ALAS acts as a dimer [16] and it appears that the purified recombinant enzyme is unable to dimerize. This aspect needs further study.

Conventional methods of purification [$(NH_4)_2SO_4$ fractionation, ion-exchange and molecular-sieve chromatography] also led to loss of enzyme activity at every step. Therefore, the $(NH_4)_2SO_4$ fraction of the recombinant *E. coli* extract, which retained enzyme activity, was used to determine the K_m value, which was estimated to be ≈ 50 mM for glycine and ≈ 100 μM for succinyl-CoA. This range is not very different from the values for ALAS from mammalian sources [16]. Several potential inhibitors for the enzyme based on an early report on *Rhodopseudomonas spheroides* and avian erythrocyte preparations [17] were tested. Interestingly, aminomalonate, which was reported to be the best inhibitor (K_i of 22.5 μM), was found to be a poor inhibitor of recombinant PfALAS enzyme activity (results not shown). However, ethanolamine, which was reported to inhibit ALAS by 50 % at a 6-fold higher concentration than aminomalonate [17], was found to do better with the PfALAS enzyme (Figure 4a). The recombinant enzyme was inhibited by 50 % at 0.16 μM even at the high concentration of the glycine substrate that was used. The results presented in Figure 4(b) indicate that ethanolamine inhibits haem synthesis strikingly when added to parasite lysates and is as effective as succinylacetone, a known inhibitor of ALAD, the second enzyme of the pathway. Finally, when ethanolamine was added to *P. falciparum* culture, growth inhibition could be clearly seen (Figure 4c).

Next, it was of interest to study the localization of ALAS in *P. falciparum*, since the mature enzyme is mitochondrial in mammalian systems [18]. This was examined by studying ALAS localization with respect to MitoTracker, a mitochondrial marker dye, and PfHsp60, which was reported to be localized in the parasite mitochondrion [13]. Immunofluorescence studies clearly reveal that PfALAS co-localized with MitoTracker (Figure 5, top row) and PfHsp60 (Figure 5, bottom row).

It was then of interest to demonstrate the translocation of PfALAS into mitochondria in an *in vitro* import assay [14]. The results presented in Figures 6(a) and 6(b) reveal that PfALAS cDNA could be transcribed and translated and the product identified by immunoprecipitation. Import studies using rat liver mitochondria clearly reveal import and processing of the precursor protein, where the precursor and mature forms could be detected after proteinase K treatment of the re-isolated mitochondria. Under identical conditions luciferase was not imported into mitochondria. There was also enrichment of the two bands of ALAS in the mitoplasts (Figure 6c), indicating translocation into inner mitochondria, although it is not clear at this stage

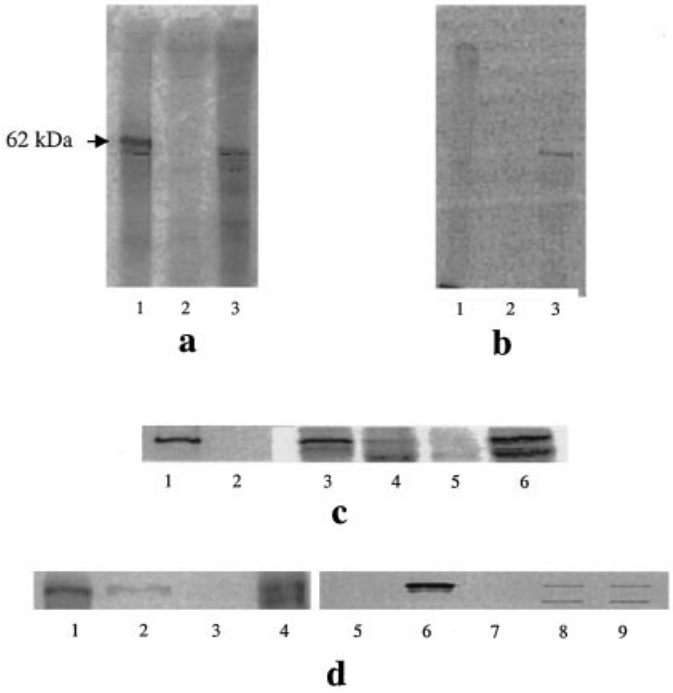


Figure 6 Translocation of PfALAS into mitochondria in import assays *in vitro*

The PfALAS cDNA (in CMX vector) was transcribed and translated in reticulocyte lysate/T7 polymerase assay kit in the presence of [^{35}S]methionine and import of the translated protein into rat liver mitochondria assessed as described in the text. An aliquot of the translated product was used as a marker. (**a**) Coupled transcription/translation of cDNAs and (**b**) immunoprecipitation of the ^{35}S-labelled protein products with PfALAS antisera. Lanes 1, luciferase cDNA (part of the kit); lanes 2, no DNA added; lanes 3, CMX-ALAS. (**c**) Import of ^{35}S-labelled proteins synthesized in the cell-free system into rat liver mitochondria. After import assay, the mitochondria were re-isolated, treated with proteinase K, washed and then subjected to SDS/PAGE. In another set, the re-isolated mitochondria were first lysed with 0.1% SDS and then treated with proteinase K followed by SDS/PAGE and autoradiography. Lanes 1 and 2, luciferase marker and mitochondria lysate after import. Lanes 3–6, PfALAS: lane 3, marker (cell-free product); lane 4, proteinase K-treated intact mitochondria; lane 5, proteinase K-treated lysed mitochondria; lane 6, mitoplasts. (**d**) Effect of haemin addition on protein import into mitochondria. Lanes 1–4, PfALAS. Lane 1, marker; lane 2, [^{35}S]methionine-labelled protein from *P. falciparum* lysate, immunoprecipitated with PfALAS antisera. Labelling was carried out for 6 h with 100 μCi of [^{35}S]methionine/1.5 ml of culture. Parasite was isolated and lysed in RIPA buffer (150 mM NaCl, 500 mM Tris/HCl, pH 8.0, 0.1% SDS, 1% Triton X-100 and 0.5% deoxycholate). Immunoprecipitation was carried out using standard protocols. It appears that under these conditions the precursor form is predominantly labelled. Lanes 3 and 4, proteinase K-treated intact mitochondria re-isolated after import in the presence and absence of haemin (25 μM) respectively. Lanes 5–9, PfHsp60 signal using Western blot analysis: lane 5, control (mitochondria lysate); lane 6, marker (purified recombinant PfHsp60); lane 7, proteinase K-treated lysed mitochondria; lanes 8 and 9, proteinase K-treated intact mitochondria with and without haemin (25 μM) treatment, respectively. The two bands seen in re-isolated mitochondria with PfALAS and PfHsp60 are presumed to be the precursor and mature forms.

whether the final destination was the inner membrane or the matrix. Import of PfALAS into mitochondria was inhibited by haemin and the specificity of the process is indicated by the fact that, under similar conditions, PfHsp60 import into mitochondria was not affected (Figure 6d).

DISCUSSION

The question of whether the Pf*ALAS* gene is functional and participates in the haem biosynthetic pathway of the malaria parasite [3,19] has been answered in this study. With the use of RT-PCR analysis, ALAS protein Western blot analysis using antibodies to the recombinant protein and ALAS enzyme assay in the parasite lysates it has been shown that the Pf*ALAS* gene is expressed at all stages of intra-erythrocytic growth of the parasite. The parasite enzyme is immunologically distinct from the host enzyme. Its activity manifests high sensitivity to ethanolamine, a compound that inhibits parasite haem synthesis and growth. Therefore, PfALAS is a potential drug target. The loss in enzyme activity with purification appears to be associated with its inability to form dimers. This aspect needs further study and may also provide a handle with which to exploit PfALAS as a drug target.

PfALAS is localized in the parasite mitochondrion and its import into mitochondria could be demonstrated in a cell-free import assay. Haemin specifically inhibits this import process. The mitochondrial localization of functional PfALAS, together with data already published from this laboratory on the cytosolic enzymes [2,20], provides evidence for the operation of the mitochondrion/cytosol pathway of haem biosynthesis in the parasite, as is the case in liver [21]. *P. falciparum* genome analysis indicates the presence of parasite-encoded genes for all the enzymes of the haem-biosynthetic pathway [22]. The derived sequence of ALAD, the second enzyme of the pathway, indicates the enzyme to be plant-like and may be targeted to the apicoplast [23]. At the same time, studies in this laboratory have indicated that the bulk of ALAD from *P. falciparum* and *P. berghei* is of host origin and that the enzyme is essential for parasite haem synthesis and survival [2,20]. Therefore, several questions arise. To start with, the *de novo* biosynthesis of haem by the parasite was surprising [1], since the parasite acquires large amounts of haem from host haemoglobin. It now turns out that the parasite may be making haem in more than one compartment. One could involve the classical mitochondrial/cytosolic pathway [21], where ALAS and the terminal enzymes of the pathway are mitochondrial, with ALAD and subsequent enzymes up to coproporphyrinogen formation being cytosolic. Suggestions were made by this laboratory that, in addition to ALAD, these cytosolic enzymes could also be of host origin [2]. The second pathway would be localized in the apicoplast, containing enzymes coded for by the parasite genome. If this is the case, questions would arise as to the source of ALA for the apicoplast, since chloroplasts do not have ALAS, but make ALAS by the C5 pathway involving glutamate and glutamyl tRNA [21]. Although earlier studies [1] could not detect incorporation of [1-^{14}C]glutamate into parasite haem, these studies need to be re-evaluated, since the pool of apicoplast haem could be very small compared with that derived from the glycine/succinyl-CoA pathway. The other option is that ALA diffuses from mitochondria into the cytoplasm as well as the apicoplast for the subsequent steps to operate. In this context, the close proximity of mitochondrion/apicoplast structures during intra-erythrocytic growth is of significance [24]. Several features of the haem biosynthetic pathway of the malaria parasite are unique and appear amenable for intervention to design new anti-malarial drugs.

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