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Short communication

Mitochondrial localization of functional ferrochelatase from *Plasmodium falciparum*

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ABSTRACT

In the malarial parasite, enzymes of heme-biosynthetic pathway are distributed in different cellular compartments. The site of localization of ferrochelatase in the malarial parasite is crucial, since it will decide the ultimate site of heme synthesis. Earlier results have differed in terms of localization, being the mitochondrion or apicoplast and the functional enzyme has not been cloned, expressed and characterized. The present study reveals that *Plasmodium falciparum* ferrochelatase (*PfFC*) gene encodes multiple transcripts of which the one encoding the full length functional protein (PfFC) has been cloned and the recombinant protein over-expressed and purified from *E. coli* cells. The enzyme shows maximum activity with iron, while zinc is a poor substrate. Immunofluorescence studies with antibodies to functional ferrochelatase reveal that the native enzyme is localized to the mitochondrion of the parasite indicating that this organelle is the ultimate site of heme synthesis.

The widespread development of resistance to frontline antimalarials with the artemisinin derivatives as the only reliable effective drugs in the field, has led to intense investigation of several metabolic pathways of the malarial parasite as possible drug targets. Studies in this laboratory had shown that the malarial parasite (*Plasmodium falciparum*, *Plasmodium berghei*) is capable of de novo heme biosynthesis, despite the acquisition of heme from the host red cell hemoglobin in the intraerythrocytic stage and that the pathway is a drug target [1,2]. Subsequent studies in many laboratories have led to the formulation of a unique hybrid pathway involving the localization of the enzymes in three different compartments, namely apicoplast, cytosol and mitochondrion [3–6]. The present picture available regarding the localization of the enzymes of the pathway is based on bioinformatics-based predictions using identification of the presence or absence of N-terminal extensions of the proteins, suggesting apicoplast, mitochondrial or cytosolic localization as well as available experimental evidence using reporter localization in parasites transfected with 5′-upstream-GFP (green fluorescent protein) fusion constructs and/or localization of native enzymes in the parasite using immunofluorescence/immunoelectron microscopy. Thus, there is agreement between predictions and experimental evidences for localization sites in the cases of δ-aminolevulinate synthetase (PfALAS) as mitochondrial [4,7,8] and δ-aminolevulinate dehydratase (PfALAD), porphobilinogen deaminase (PfPBGD) and uroporphyrinogen decarboxylase (PfUROD) as apicoplast [4,9–11]. It has been shown that PfPBGD is a bifunctional enzyme, also manifesting the activity of the next enzyme, uroporphyrinogen III synthase [10]. Coproporphyrinogen III oxidase (PfCPO) and protoporphyrinogen IX oxidase (PfPPO) do not carry a bipartite upstream signal to target them to the apicoplast and no experimental evidence is as yet available on their sites of localization. It has, however, been suggested that PfCPO could localize to the parasite cytosol or mitochondrion and PfPPO to the mitochondrion [4,6].

The site of localization of ferrochelatase (PfFC), the terminal enzyme of the pathway is very crucial, since it would ultimately decide on the site of heme synthesis in the parasite. It was suggested that PfFC could localize to the mitochondrion, since it does not carry the N-terminal bipartite signal to target it to the apicoplast but has short extra N-terminal extension, although the N-terminal-GFP fusion transfectants did not give clear cut results [4]. However, studies in this laboratory indicated that the native FC of the parasite is localized to the apicoplast [12]. But, van Dooren et al. [6] have alluded to unpublished results that in *P. falciparum* transfected with full length cDNA sequence of PfFC fused to a short epitope tag, the fusion protein clearly co-localized with a mitochondrial marker in immunofluorescence assays. It is of importance to reconcile the differences in the results obtained.

There is an unusual level of complexity in the transcripts derived from the PfFC gene containing 4 or 5 exons (two versions are available in PlasmoDB database 5.4, Fig. S1A), as evident from earlier studies and data obtained in the present study. This complexity has not been observed with any of the other genes of the heme-biosynthetic pathway so far studied in the parasite. Thus, Sato and

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Wilson [13] obtained two cDNAs with RT-PCR carried out on a cDNA library of *P. falciparum*, one (cDNA 1) with 1050 bp (350 amino acids) and another (cDNA 2) with an additional 65 bp insert from intron 2. These were considered as authentic transcripts, produced by alternate splicing from a common precursor transcript. cDNA 1 was found to successfully rescue a FC-null mutant of *E. coli*. Studies in this laboratory led to the identification of another transcript when RT-PCR was carried out on RNA isolated directly from the parasite [12]. This transcript contained a 45 bp insert from intron 4 in addition to the 65 bp insert. However, this transcript could not be expressed in *E. coli*, although the reading frame was intact. Therefore, the C-terminal fragment (750 bp), with the sequence identical to that from cDNA 1, was expressed in *E. coli* to give a 32.5 kDa protein. MALDI analysis of this fragment gave an authentic peptide from the C-terminal PfFC sequence. Antibodies to this 32.5 kDa protein were used for the determination of the localization of PfFC in the parasite by immunoelectron microscopy. Localization in the apicoplast was concluded from the multi-membrane structure of the organelle.

In the present study, RT-PCR was carried out on total RNA from the parasite using the primers 5′-GCCCGGATCCATGGACGTACAGGATTTTCTGAATTGTAAC-3′ (forward primer) and 5′-GCCGGAATTC TTACACCCATCCTATTATATTTTGTTC TATAAG-3′ (reverse primer) to obtain full length PfFC cDNA based on putative PfFC gene sequence (MAL13P1.326, PlasmoDB database). The restriction enzyme sites are underlined. The PCR product was cloned into pRSETA using the BamHI–EcoRI sites. The inserts released with the restriction enzymes from the DNA isolated from three different clones (clones I–III) of the seven analyzed, ranged from 0.9 kb to 1.1 kb in size (Fig. S1B). DNA sequence analysis was carried out for all these clones and the partial derived amino acid sequence in the reading frame permitting maximum read out is presented in Fig. S1C. Clone I carried only a 10 amino acid sequence identity from the start methionine codon with that of clone III and was followed by stop codons. The rest of the sequence was entirely different from the annotated versions of PfFC sequence. There was yet another clone similar to clone I with additional variations beyond this 10 amino acid stretch (data not presented). Clone II carried a 79 amino acid stretch identical to that of clone III. This was followed by a four amino acid sequence, EPKY, that is included as the third exon as per one of the two versions in PlasmoDB database 5.4. Beyond this point, clone II had the intron 3 sequence (as per version (i) in Fig. S1A) and carried a stop codon after a stretch of another five amino acids from EPKY exon. This clone, which appears to be similar to the cDNA 2 sequence of Sato and Wilson [13], had only intron 3 but not introns 1, 2 and 4, thereby suggesting that the transcript of clone II could be an authentic splice variant. Clone III sequence was identical to the cDNA 1 sequence reported by Sato and Wilson [13] and did not carry the 'EPKY' exon or any of the introns.

Of the seven clones analyzed, two belonged to clone I, three to clone II and two to clone III. These results suggest that the diverse transcripts of PfFC could be present in similar proportions. Indeed, the content of the clone II transcript, as revealed by semi-quantitative RT-PCR analysis was more than the completely processed clone III transcript (Fig. S2A). Also, RT-PCR analysis carried out with PfFC forward primer and a reverse primer flanking the sequences of exon 3 and intron 3 (as per version (i) in Fig. S1A) gave rise to a product of 0.27 kb. However, no product was obtained with reverse primer flanking the sequences of exon 4 and intron 4 whereas, two products of 0.36 kb and 0.42 kb were obtained with reverse primer flanking the sequences of exons 4 and 5, further confirming the abundance of clone II transcript containing intron 3 sequence (Fig. S2B).

In view of these complexities observed, study was undertaken to examine the localization of a functionally active FC of *P. falciparum*. Since clone III DNA sequence is identical to that of cDNA 1 that was earlier shown to successfully rescue a FC-null mutant of *E. coli* [13], it was used to express the recombinant protein in *E. coli* Rosetta2(DE3)pLysS strain, providing tRNAs for rare codons. Induction was carried out with 1-thio-β-galactopyranoside (IPTG) at 30 °C for 4 h. The cells were suspended in lysis buffer containing 50 mM Tris–HCl buffer, pH 7.5, 0.5 M NaCl, 10% glycerol and 0.1% Triton X-100 and the soluble enzyme obtained after sonication and centrifugation at $50{,}000 \times g$ for 1 h was loaded onto Ni^{2+}-nitrilotriacetic acid (NTA) column. The column was sequentially washed with lysis buffer containing 30, 75 and 100 mM imidazole. The recombinant protein was finally eluted with lysis buffer containing 250 mM imidazole (Fig. 1A). Following dialysis of the eluate against the lysis buffer, enzyme assays were carried out immediately. The same preparation was also used to raise antibodies in mice. The purified recombinant PfFC (rPfFC) of 43 kDa reacted positively with both anti-histidine tag (Fig. 1B) and anti-PfFC antibodies (Fig. 1C, Lane 1) in Western blots. Also, Western blot analysis of the parasite cytosol and solubilized total organellar fraction with anti-PfFC antibodies revealed the presence of an approximately 41 kDa native PfFC in the solubilized total organellar fraction, but not in the cytosol (Fig. 1C, Lanes 2 and 3). Enterokinase cleavage was also carried out with rPfFC to check whether the presence of his-tag alters its properties and the general protocols used have been described earlier [10,11].

The purified rPfFC did not show absorbance peak at 330 nm, typical of Fe–S proteins (Fig. 1D). While mammalian FCs contain a labile [2Fe–2S] centre, the enzyme from prokaryotes, yeast and plants does not contain the same [14]. Gel filtration analysis with Superdex-G200 analytical fast-performance liquid chromatography (FPLC) column (bed volume = 25 ml), indicated that the functional recombinant enzyme is a dimer with native molecular weight of 78 kDa (Fig. 1E).

There are issues related to the assay of FC enzyme as discussed in detail by Hunter et al. [15]. The enzyme catalyzes iron incorporation into protoporphyrin IX to give protoheme. Protoporphyrin IX is highly hydrophobic and aggregates or stacks in solution. To overcome this problem, more water soluble substrates such as mesoporphyrin and deuteroporphyrin IX are generally used. Again, ferrous iron is highly unstable in aqueous solution particularly at neutral to basic pH and zinc is often used to assay the enzyme. Hunter et al. [15] have pointed out that the routine use of non-natural substrates is not a good indicator of the results that would be obtained with natural substrates and have optimized conditions for the assay of FC with Fe^{2+} and protoporphyrin IX in short assay time conditions, avoiding the use of metal chelating agents. These conditions, including the protocols for the preparation of the substrates were followed in the present study.

Briefly the reaction mixtures contained FC reaction buffer (0.1 M MOPS, 0.4 M NaCl and 0.2% (v/v) tween-80, pH 7.0), 3 μM protoporphyrin IX, 0.025–300 μM divalent transition metal ion and 0.12 μM of purified rPfFC in a total volume of 2 ml. The reaction was carried out at 30 °C and initiated by the addition of metal ion. The absorbance was continuously monitored as a function of time at 407 nm in the case of iron and cobalt or at 417 nm for zinc, nickel and copper. Control reactions in the absence of enzyme were also carried out.

The results presented in Fig. 1F indicate that iron is the preferred substrate for rPfFC, followed by the other metal ions nickel, copper and cobalt. Table 1 summarizes the kinetic properties of rPfFC. In comparison with mouse and yeast FCs [15], the unique features of rPfFC are that zinc is a poor substrate, the Km and Kcat/Km values being 0.19 ± 0.04 μM and $0.53 \pm 0.06 \times 10^5\ M^{-1}\ S^{-1}$ respectively and that copper, which shows a sigmoidal response with mouse FC gives a hyperbolic curve with PfFC. Hunter et al. [15] have shown with the mouse and yeast FCs that the metal ions copper,