GENERAL SUMMARY

Human malaria is caused by infection with the protozoan parasite of the genus *Plasmodium* which is transmitted by *Anopheles* mosquitoes Of the four species of *Plasmodium* known to infect humans, *Plasmodium falciparum* causes the most severe form of the disease Despite global efforts to control and eradicate the disease, it remains a major and growing threat in most parts of the world but mainly in sub-Saharan Africa and South East Asia Annually about 300-500 million people are affected by malaria and about 3 million affected people, mainly children, die The spread of chloroquine resistance in the malaria parasite, *P falciparum* in particular, has led to an urgent need for the development of newer antimalarial agents, for which the identification of new drug targets is essential

Previous work done in this laboratory has shown that *P falciparum* is capable of heme biosynthesis *de novo*, although it acquires plenty of heme from the host red blood cell hemoglobin The parasite aggregates the heme derived from hemoglobin to be stored as hemozoin pigment and depends on the biosynthetic heme to fulfill its requirement for protein synthesis and to provide the prosthetic group for parasite cytochromes The heme biosynthetic pathway of the malarial parasite is a potential drug target, as inhibition of δ-aminolevulinate dehydratase (ALAD), the second enzyme of the pathway, with a specific inhibitor, succinylacetone, leads to the death of *P falciparum* in culture Earlier, detailed studies of the heme biosynthetic pathway in the *in vivo* model using the rodent malarial parasite *P berghei* have added a new 'twist', in that the parasite imports ALAD, and perhaps the subsequent enzymes of the pathway, from the host red blood cell to sustain heme synthesis Mouse red cell ALAD was shown to bind to solubilized *P berghei* membrane with specificity and high affinity in a pH dependent manner This differential binding of ALAD to the parasite membrane was used to identify a 65kDa parasite membrane protein which might be a receptor for ALAD import ALAD-ΔNC, a N- and

C- terminal truncated recombinant protein of mouse red blood cell ALAD was shown to bind and compete with full length ALAD for binding to solubilized *P berghei* membrane Subsequently it was shown that *P falciparum* also imports ALAD from infected human red blood cell Addition of ALAD-ΔNC to *P falciparum* in culture at 2-5μM concentration decreased the import of native human red blood cell ALAD by *P falciparum* causing death of parasite due to decreased heme biosynthesis

This thesis contains three main Chapters In **Chapter – I**, a brief review of the literature pertaining to various aspects of parasite biology, which are relevant to the work done in this study, has been documented The results obtained in this study are presented in detail in **Chapter – II** and **Chapter – III** and are summarized below

Chapter – II ***Studies on the import of host ALAD and other proteins by the malaria parasite with special reference to parasite heme synthesis***

In this study, further evidence for the import of host red cell ALAD by the malaria parasite has been provided using Western blotting, immunofluorescence and immuno electronmicroscopic techniques It was shown that red cell ALAD is imported by the parasite through the cytostome pathway and is present in parasite cytoplasm and food vacuole Having observed the effects of ALAD-ΔNC protein on *P falciparum* growth in culture, it was of interest to see whether the protein has to enter the infected erythrocyte in order to manifest its effect For this purpose [S^{35}]-methionine labeled ALAD-ΔNC was used along with [S^{35}]-methionine labeled GAL4 (an unrelated protein of yeast origin) as control Unexpectedly, both the proteins were recovered intact from the parasite cell The proteins were not detected in the cytosol or membrane of both infected and uninfected erythrocyte Immunolocalization studies were used to verify the import of ALAD-ΔNC and GAL4 and these studies also revealed that import of ALAD-ΔNC but not that of GAL4 led to a decrease in host ALAD levels in the parasite As was the case for the imported red cell ALAD, both ALAD-ΔNC and GAL4 were present intact in the parasite food vacuole

Further, we have shown that both albumin and transferrin are imported by *P falciparum* from serum present in the culture medium Detailed immuno electronmicroscopic studies have shown that albumin is present in intra erythrocytic vesicles budding from the red cell membrane, Maurer's cleft, PV/PVM, caveolae pinching into the parasite cytosol from parasite plasmamembrane, vesicles in parasite cytosol, and food vacuole The Maurer's cleft and the PV/PVM are known to be part of the TVN and it appears from our localization studies that proteins present in the extracellular medium can reach the PV through the TVN Studies on GFP import by the parasite also showed that TVN is involved in the import process The results presented also show that although the imported proteins are present in the food vacuole of the parasite, unlike hemoglobin, they are not degraded (only transferrin appeared to undergo some degradation) and remain intact It is possible the at least some of the host proteins, which are imported and present intact in the parasite food vacuole, can get translocated across the single membrane of the food vacuole and reach the parasite cytosol where they can be functionally useful for the parasite We have shown that the import phenomenon is not specific for *P falciparum* in culture by demonstrating the process in *P berghei* also

In order to study the mechanism by which proteins from the extracellular medium are imported by the intraerythrocytic parasite, a host of [S^{35}]-methionine labeled recombinant proteins were added to *P falciparum* culture All the proteins tested were imported by the parasite but to varying extent Import of high molecular weight proteins (e g, recombinant ALAD added to culture medium) was negligible Interestingly, none of the imported proteins were detected in the red cell cytosol of the infected cell showing that the proteins had direct access to the intraerythrocytic parasite During these studies, it was observed that import of heterologous proteins added to *P falciparum* culture was much better in the absence of serum from the culture medium It appears that serum proteins, owing to their presence in high concentration, are able to saturate the pathway through which proteins from extracellular medium are imported by the parasite Making use of these observations, further studies were done on the time course and temperature dependence of the import process using radiolabeled *E coli* lysates as the source of heterologous proteins in the absence of serum Uptake of proteins was saturating at 4hr

time and was completely abolished at low temperatures (4°C) Import of proteins up to 50-60 kDA in size was much more efficient than the other higher molecular weight proteins In addition Li^+ was also able to inhibit the import process Based on these observations it appears that *P falciparum* may import proteins from the red cell cytosol through the cytostome pathway while proteins from the extracellular medium may be imported into the infected red cell by fluid phase bulk endocytosis and reach the PV which are then internalized by the parasite At least some of the imported proteins could remain intact in the parasite and perform a functional role

Chapter – III *P falciparum ALAD – studies on its role in parasite heme biosynthesis*

While we have shown that host red cell ALAD is imported by the malaria parasite and is essential for parasite heme synthesis, data from parasite genome sequence showed that the parasite contains a gene that can code for ALAD By genetic complementation studies it had been shown that the parasite gene codes for a functional protein If the parasite expresses its own ALAD, why should it import host ALAD? If both host and parasite ALAD proteins are present in the parasite cell, is their subcellular localization different? What is the relative contribution of these proteins to *de novo* heme biosynthesis in the parasite? To answer these questions, recombinant ALAD was produced as both GST-tagged and His tagged proteins The recombinant proteins were enzymatically active and purified as octomers of molecular mass 345 kDa His-tagged *P falciparum* ALAD (PfALAD) was used for further biochemical characterization

PfALAD closely resembles plant and algal chloroplast ALAD in primary structure and was therefore predicted to have similar biochemical characteristics All ALAD proteins studied so far require metal ions for activity and are therefore classified as Zn^{2+}, Mg^{2+} or monovalent ion (K^+) dependent enzymes In plants, algae and some bacteria, allosteric stimulation of activity is seen in the presence of Mg^{2+} Studies on PfALAD revealed that although the pH optima of the enzyme was similar to that of plant ALAD (pH 8 5-9), it was active in the absence of metal ions Unlike other ALADs, PfALAD enzyme activity was not inhibited in the presence of EDTA However, stimulation of enzyme activity by

20-30% occurred in the presence of Mg^{2-} K^{+} was also capable of stimulating PfALAD activity at pH values between 6 5-8 These properties of PfALAD resembled that of *Pseudomonas aeruginosa* ALAD (PsALAD) However, unlike PfALAD, PsALAD was not active in the absence of metal ions In order to explain the observed properties of PfALAD, the structure of the protein was modeled using PsALAD crystal structure as the template From PfALAD model structure it was clear that – (i) due to certain deletions and insertions in the primary sequence of the protein, the relevant active site amino acids are prefixed in a catalysis ready orientation and therefore the protein is active in the absence of metal ions, and (ii) Mg^{2+} bound to the C-site metal binding pocket present at the subunit interface is responsible for the observed stimulatory effect by Mg^{2+} although it was earlier predicted by another group, based on amino acid sequence that the C-site may not exist in PfALAD Using antibodies specific for PfALAD it was shown by immuno-electronmicroscopy that the parasite ALAD is localized to the apicoplast Western blotting showed that PfALAD expression was very low Utilizing the fact that, the host ALAD is inhibited by EDTA while PfALAD is not it was shown that the parasite enzyme contributed only about 10% of the total ALAD activity in *P falciparum* in culture Thus both imported ALAD and parasite genome-encoded ALAD appear to be essential for parasite heme synthesis

Taken together, our studies indicate that the parasite may be synthesizing heme in two different compartments One site is the parasite cytosol utilizing imported host ALAD and other enzymes A second site is the apicoplast with parasite genome-encoded ALAD and other enzymes of the pathway Since, the parasite has a single apicoplast, unlike plants with numerous chloroplasts, apicoplast may not be able to provide all the biosynthetic heme required by the parasite The source of ALA in the apicoplast is still an open question Work from this laboratory has shown that parasite ALAS is localized to the mitochondria Due to close association of the mitochondria and the apicoplast in the parasite, there is a proposal that ALA could be transported into the apicoplast Also, it is now evident from the parasite genome sequence that all enzymes required for *de novo* heme biosynthesis, except uroporphyrinogen-III synthase, are encoded by the parasite Finding the subcellular localization of these enzymes will help us understand how this

pathway operates in the parasite and its unique features could offer us a novel antimalarial drug target

LIST OF PUBLICATIONS

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1 **Shanmugham Dhanasekaran**, Nagasuma R Chandra, B K Chandrasekhar Sagar, Pundı N Rangarajan and Govındarajan Padmanaban δ-Amınolevulınic Acıd Dehydrase from *Plasmodıum falcıparum* – Indıgenous *vs* Imported
In Press J Bıol Chem , 2004

2 P G Vathsala, A Pramanık, **S Dhanasekaran**, C Usha Devı, C R Pıllaı, S K Subbarao, S K Ghosh, S N Tewarı, T S Sathyanarayanan, P R Deshpande, G C Mıshra, M R Ranjıt, A P Dash, P N Rangarajan, G Padmanaban Chloroquıne-resıstant Pfcrt haplotype (SVMNT) ıs wıdespread ın *Plasmodıum falcıparum* - malarıa ın Indıa
In Press Amerıcan Journal of Tropıcal Medıcıne and Hygiene 2004

3 S Varadharajan, **S Dhanasekaran**, Z Q Bonday, P N Rangarajan and G Padmanaban (2002) Involvement of δ-aminolevulinate synthase encoded by the parasite gene in *de novo* haem synthesis by *Plasmodium falciparum*
Biochem J 367, 321-327

4 Z Q Bonday*, **S Dhanasekaran***, P N Rangarajan and G Padmanaban (2000) Import of host δ-aminolevulinate dehydratase into the malarial parasite Identification of a new drug target
Nat Med 6, 898-903
***ZQB** and **SD** made equal contributions to this study

5 Import of host proteins by the malaria parasite
Manuscript under preparation