Uptake of proteins and degradation of human serum albumin by \textit{Plasmodium falciparum} – infected human erythrocytes

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Abstract

**Background:** Intraerythrocytic malaria parasites actively import obligate nutrients from serum and export proteins and lipids to erythrocyte cytoplasm and membrane. The import of macromolecules in the malaria parasite has been the subject of many debates. To understand the import of macromolecules by the parasite, we studied the uptake of proteins by \textit{Plasmodium falciparum} infected human erythrocyte.

**Methods:** Proteins were biotin labelled individually, purified on a gel filtration column and added to uninfected and infected asynchronized culture. The uptake of these proteins by malaria parasites was determined by western blot analysis of parasite pellet and their different fractions using streptavidin-horseradish conjugate. To further confirm this import, we studied the uptake of \textsuperscript{125}I-labelled proteins by western blot analysis as well as used direct immunofluorescence method.

**Results:** Here we show that biotin labelled and radio-iodinated polypeptides of molecular sizes in the range of 45 to 206 kDa, when added in the culture medium, get direct access to the parasite membrane through a membrane network by by-passing the erythrocyte cytosol. The import of these polypeptides is ATP-dependent as sodium azide treatment blocks this uptake. We also show that malaria parasites have the ability to take up and degrade biotin labelled human serum albumin, which has been shown to be essential for the parasite growth.

**Conclusions:** These results can be used, as a basis to explore the role of human serum albumin in the intraerythrocytic development of parasites, and this in turn can be an important adjunct to the development of novel antimalarial drugs.

**Background**

During the asexual erythrocytic stage of their life cycle, the malaria parasite \textit{Plasmodium falciparum} grows and propagates within the red blood cells (RBCs) of their host. Within RBC, a single intraerythrocytic parasite reproduces asexually to produce 16 to 32 progeny within 48 h. The high rate of multiplication necessitates efficient trafficking of solute and macromolecules between the external medium and the parasites. Trafficking pathways in malaria-infected erythrocytes are complex and the solute passing between the parasite and plasma must traverse a series of three membranes, those of RBC, the parasitophorous vacuole and the parasite [1]. It has largely been recognized that malaria parasites usually import low molecular weight nutrients such as polyols, amino acids, lipids, nucleosides, inorganic anions and cations from the plasma.
[2–4]. During the last decade, macromolecule uptake by malaria-infected erythrocytes has been the subject of contention among different groups. Using fluorescent macromolecules, Pouvelle and co-workers [5] have shown that intraerythrocytic P. falciparum can endocytose dextran, protein A and an IgG₂ antibody. It was shown that these molecules do not cross the erythrocyte or parasitophorous vacuole membranes, but rather gain direct access from the external medium to the parasite through a duct. Based on their findings, they proposed a parasitophorous duct pathway for the direct access of macromolecules through the formation of aqueous channels. These findings were further supported by Loyevsky et al. [6] who showed that desferrioxamine and phloridzin drugs that inhibit parasite growth in culture find direct access to the parasite from external medium. In another report, Goodyer et al. [7] proposed two distinct pathways for the macromolecular transport. Recently, Bonday et al. [8] showed how the different recombinant fragments of RBC δ – aminolevunlate dehydratase (ALAD) were imported into the parasite cytoplasm were used for further analysis. [8].

**Parasite Culture**

P. falciparum culture (3D7 strain) was grown essentially by the method of Trager and Jensen [15] in RPMI 1640 medium, supplemented with 10% heat inactivated pooled human serum and O +ve washed human RBC. The culture flasks were incubated at 37°C in a CO₂ (5%) incubator. Parasite cultures were synchronised by treating the culture with 5% sorbitol [16]. Parasitemia was measured from methanol fixed and Giemsa-stained smears.

**Isolation of parasite and parasite membranes from infected red blood cells**

The parasitized RBCs were harvested by low speed centrifugation (800 × g, 5 min) and washed five times with incomplete RPMI medium. The pellet solutions were suspended in an equal volume of 0.15% saponin in PBS (weight/volume), the final concentration of saponin was 0.075% and incubated at 37°C in a shaking water bath for 20 min to allow complete lysis of the RBC. The lysate was centrifuged at 1000 × g for 10 min at 4°C. The supernatant was collected and the parasite pellet was washed five times with cold PBS at 4°C [8,17]. To isolate the parasite membrane fraction, the parasite was lysed for 1 h at 4°C in 20 mM Tris-HCl buffer, pH 7.5 containing 0.2% (weight/volume) Triton X-100, and was sonicated briefly. The lysate was spun at 12000 × g for 30 min and the membrane pellet (Triton X-100 insoluble fraction) and the parasite cytoplasm were used for further analysis. [8].

**Expression and purification of recombinant P. falciparum histidine rich protein-2 (PfHRP-2)**

Plasmid containing the gene encoding for PfHRP-2 in a pET-3d vector was transformed into E. coli BL21 (DE3) cells and the transformed E.coli were grown in LB media containing 100 µg ampicillin/ml. Expression of the proteins was induced by the addition of 0.4 mM IPTG. For the large-scale purification of proteins, one litre of the cultures was grown and induced by IPTG [18]. The protein was purified by metal-chelate chromatography on Ni⁺²-nitroacetate column using imidazole for elution [19].

**Biotinylation and radio-iodination of proteins**

Biotinylation of proteins was carried out using an ECL protein biotinylation kit according to the manufacturer’s
Infected erythrocytes (4.8x10^8 trophozoites) were harvested from parasite cultures containing 10% human serum and incubated with BFA at 5µg/ml or with monensin at 5µg/ml. After the treatment, infected RBCs were detected at 214 nm.

Radio-iodination of protein was done using Iodo-Beads according to the manufacturer’s instructions. Iodinated proteins were purified using Sep-Pak Plus C18 cartridge.

**Uptake of proteins and their analysis**

To study the uptake of proteins, biotin labelled or radio-iodinated proteins were diluted to the appropriate concentration using 1X RPMI 1640 to a final pH 7.4. P. falciparum cultures (5% parasitaemia; 10 ml) were incubated for different times with labelled polypeptides. After the incubation, parasite pellets and their different fractions were purified as described above and the labelled proteins in these fractions were analysed by SDS-PAGE and western blot analysis.

For SDS-PAGE analysis, purified parasite pellets or membrane fractions were solubilized in SDS sample buffer with (5%) β-mercaptoethanol, boiled and electrophoresed on 7.5% or 10% SDS-polyacrylamide gels. In case of radio-iodinated proteins, the gels were dried and exposed directly to X-ray film. To detect biotin labelled proteins, proteins were electrotransferred on nitrocellulose membrane. The membranes were subsequently blocked with 5% milk or casein, incubated with streptavidin-fluorescein isothiocyanate (FITC) for 2 h at room temperature. The slides were washed with PBS several times before visualization by fluorescence microscopy.

**Direct immunofluorescence**

Samples from parasite cultures incubated with biotinylated proteins were taken and processed for immunofluorescence. Smears of infected red blood cells were made on glass slides and allowed to dry for 10 min then fixed with acetone: methanol (9:1 vol/vol) mixture at -20°C and dried again. Slides were washed with PBS several times, blocked with 1% bovine serum albumin in 0.5% Tween 20 and subsequently, incubated in 1:100 diluted streptavidin-fluorescein isothiocyanate (FITC) for 2 h at room temperature. The slides were washed with PBS several times before visualization by fluorescence microscopy.

**Results**

**Uptake of proteins by P. falciparum-infected human erythrocytes**

The uptake of macromolecules by infected human RBC has been mostly studied using fluorescent labelled molecules and microscopic examination of the treated parasites [5,7,10]. However, the existence of macromolecular import has been the subject of debate because of the experimental designs [9–11]. To shed more light on macromolecular import into the malaria infected human RBCs; we used biotin labelled as well as radio-iodinated proteins of different molecular weights. Five proteins, egg albumin (45 kDa), recombinant PfHRP-2 (66 kDa), HSA (68 kDa), β galactosidase (116 kDa) and β amyrase (206 kDa) were biotin labelled individually, purified on a gel filtration column to remove free biotin and added to uninfected and infected asynchronized culture. The uptake of these proteins by malaria parasites was determined by western blot analysis of the parasites lysate after saponin lysis of infected RBCs. Surprisingly, all the biotin labelled proteins were taken up only by infected RBCs (Fig. 1A, lanes 5,6,7 and 8) and not by uninfected cultures incubated for 6 h with biotin labelled protein (Fig. 1A lane 2). Infected cultures incubated with unlabelled proteins did not show any band on the western blot (Fig. 1A lane 3). Analysis of
the infected RBC cytoplasm after saponin lysis also did not show the presence of biotin labelled – polypeptides (Fig. 1A, lane 4). To further confirm this import, we studied the uptake of $^{125}$I labelled recombinant PfHRP-II and egg albumin. Both these proteins were also taken up by the infected RBC as shown in Fig. 1B (lanes 1 and 4). Uptake of proteins by malaria parasite was also confirmed by direct immunofluorescence (Fig. 1C).

We next studied the location of biotin labelled proteins in different compartments of parasite. Analysis of parasite cytoplasm and parasite membrane fractions using streptavidin-horseradish conjugate showed that the labelled...
proteins were localized only in Triton X-100 insoluble fraction of the parasite (Fig. 2 lane 3). Localization of biotin labelled polypeptide in Triton X-100 insoluble fractions indicated that import of labelled protein may takes place through membranes.

**Effect of inhibitors on protein import to the infected human red blood cells**

To further understand the mechanism of import of proteins into infected RBCs we investigated the effect of sodium azide on the uptake of biotin labelled HSA and recombinant PfHRP-2. Sodium azide treatment of malaria parasite has been shown to considerably deplete ATP levels in the parasite [20]. As shown in Fig. 3A there was a significant decrease in the uptake of these proteins by the parasites after sodium azide treatment (lanes 2 and 3), compared to the uptake shown by untreated parasites (lanes 1 and 4). We also investigated the effect of BFA and monensin on the import of biotin labelled recombinant PfHRP-2. Both BFA and monensin have been previously used effectively in eukaryotic cells as well as in *P. falciparum* to study the export of different proteins [20,21]. Both these inhibitors did not affect the uptake of recombinant PfHRP-2 (Fig. 3B, lanes 2 and 3). These studies thus suggested that the import of proteins into the parasites is insensitive to BFA and monensin but dependent on ATP.

**Uptake of human serum albumin and its degradation by *P. falciparum***

To investigate the fate of proteins imported into the parasite, we carried out a time course study. The uptake of three different biotin labelled proteins, namely egg albu-
by the parasite from the culture medium. We wondered if this uptake is specific for PfHRP-2 and extended this study to other polypeptides of different molecular weights. Surprisingly, we found that all biotin labelled/radio-iodinated proteins, when added in the culture medium were, found to be localized in the parasite pellets and in particular in Triton X-100 insoluble fraction regardless of their size. We did not observe any of the labelled protein in host cell cytoplasm. Their results indicated that polypeptides, when added to the culture medium, gain direct access to the parasite regardless of their molecular weights and primary sequences. This situation is however, different to the process of translocation of malaria proteins in different compartments of the parasite which seem specific to signal sequences [26]. In comparison to earlier approaches where uptake of macromolecules was studied using confocal/electron microscopy, we studied the uptake by SDS-PAGE and western blot analysis. While we were studying this import of polypeptides, Bonday et al. [8] demonstrated the import of different polypeptide fragments of ALAD into the parasite. Nyalwidhe et al. [27] reported that nonpermeant biotin derivatives gain access to the parasitophorous vacuole in P. falciparum-infected erythrocytes permeabilized with streptolysin O, where the derivatives gain access to the vacuole lumen but not to the parasite cytosol. These results thus supported the previous observation of Pouvelle et al. [5], which suggested that macromolecules from the external medium get access into the intracellular parasite by-passing the host cell cytoplasm. Since we performed the experiments under normal culture conditions, carefully checked for the purity of macromolecules, ruled out the binding of biotin labelled molecules uninfected RBC membranes and localized this labelled molecules by SDS-PAGE in infected RBCs, we believe that these results and their interpretation are valid and cannot be attributed to contamination of the tracers by low molecular weight impurities or due to the degradation of labelled macromolecules into smaller molecules. Recently, we demonstrated the inhibitory effect of falcipain’s dsRNAs to the parasites suggesting the permeability of these macromolecules to the infected human erythrocytes [28].

The export of several parasite proteins (PfEMP2 and PfHRP2) has been shown to be mostly dependent on a BFA dependent pathway [24]. Low levels of BFA insensitive export have also been shown [25]. To understand the mechanism of import or uptake of protein by the parasite, in the present study, we treated the parasite cultures with BFA, monensin or sodium azide. BFA and monensin,
which inhibit intracellular transport of protein through the endoplasmic reticulum/Golgi complex, did not affect the uptake of different proteins, while sodium azide, which is known to deplete ATP in the parasite [20], reduced the uptake of proteins. These results were in agreement with the earlier observation by Pouvelle et al. [5] where uptake of rhodamine-dextran was shown to be inhibited by depletion of ATP.

It has been shown that *in vitro* serum albumin and its associated fatty acids are essential for intraerythrocytic development and cell cycle progression of *P. falciparum* [23]. We extended our studies to understand the fate of HSA taken up by *P. falciparum*. Surprisingly, our experiments provided evidence that HSA is the only protein of those investigated here that undergoes proteolysis inside the parasite in a time-dependent manner. To further explore the proteolysis of HSA by the parasite, HSA was incubated with parasite extract overnight and analysed on a C18 column. A similar proteolytic study has been earlier carried out on haemoglobin by Kolakovich et al. [29]. As shown in Fig. 5B, most of the HSA was found to be cleaved at neutral pH by the parasite extract. Taken together, our data suggest the uptake and breakdown of HSA by the malaria parasite. Serum albumin is known as a lipid carrier protein in blood, and it has been shown earlier that both lipids as well as serum albumin are essential for optimum parasite growth *in vitro* [23,30]. It may be that the sole role of HSA is to provide lipids to the parasite for its growth, but it is tempting to speculate, based on the results of the present study, that degradation of HSA inside the parasite may serve as an additional source, along with haemoglobin, for the amino acid pool required by the parasite for its growth. Further investigations to elucidate the role of HSA will extend our understanding of lipid and protein uptake by malaria parasites and may help identify additional chemotherapeutic targets to combat the persistent and deadly malaria parasite *P. falciparum*.

**Authors’ contributions**
AE carried the practical work, PM supervise and participated in the biotinylation and immunoflourescence works, VSC the group leader participated in its design and co-ordination. All authors read and approved the manuscript.
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