The human and mouse malaria parasites, *Plasmodium berghei* and *Plasmodium falciparum*, respectively, synthesize heme *de novo* following the standard pathway observed in animals despite the availability of large amounts of heme, derived from red cell hemoglobin, which is stored as hemozoin pigment. The enzymes, δ-aminolevulinate dehydrase (ALAD), coproporphyrinogen oxidase, and ferrochelatase are present at strikingly high levels in the *P. berghei* infected mouse red cell in *vivo*. The isolated parasite has low levels of ALAD and the data clearly indicate it to be of red cell origin. The purified enzyme preparations from the uninfected red cell and the parasite are identical in kinetic properties, subunit molecular weight, cross-reaction with antibodies to the human enzyme, and N-terminal amino acid sequence. Immunogold electron microscopy of the infected culture indicates that the enzyme is present inside the parasite and, therefore, is not a contaminant. The parasite derives functional ALAD from the host and the enzyme binds specifically to isolated parasite membrane in *vitro*, suggestive of the involvement of a receptor in its translocation into the parasite. While, ALAD, coproporphyrinogen oxidase, and ferrochelatase from the parasite and the uninfected red cell supernatant have identical subunit molecular weights on SDS-polyacrylamide gel electrophoresis and show immunological cross-reaction with antibodies to the human enzymes, as revealed by Western analysis, the first enzyme of the pathway, namely, δ-aminolevulinate synthase (ALAS) in the parasite, unlike that of the red cell host, does not cross-react with antibodies to the human enzyme. However, ALAS enzyme activity in the parasite is higher than that of the infected red cell supernatant. We therefore conclude that the parasite, while making its own ALAS, imports ALAD and perhaps most of the other enzymes of the pathway from the host to synthesize heme *de novo*, and this would enable it to segregate this heme from the heme derived from red cell hemoglobin degradation. ALAS of the parasite and the receptor(s) involved in the translocation of the host enzymes into the parasite would be unique drug targets.

Chloroquine resistance in malarial parasite is assuming serious proportions, and the necessity to discover new drugs to treat malaria is a major challenge. This calls for the identification of newer drug targets.

Studies in this laboratory have revealed that the human malaria parasite, *Plasmodium falciparum*, synthesizes heme *de novo*. The parasite is able to incorporate [2-14C]glycine and 4-14C-labeled δ-aminolevulinate (ALA), but not 1-14Cglutamate into heme, indicating that the parasite manifests the glycine pathway observed in liver and erythrocytes. ALA synthase and ALA dehydrase activities were also detected in the parasite (1). The synthesis of heme *de novo* by the parasite in the intraerythrocytic stage is rather surprising, since the parasite obtains a surplus of heme from the degradation of the red cell hemoglobin. However, this heme is stored as the inert hemozoin pigment (2, 3). Further, most hemoflagellates, protozoic protozoa in both plants and animals, need preformed heme for growth (4).

*L. tarentolae* was found to contain free porphyrins but required intact iron-protoporphyrin for growth in culture, suggesting that it may lack ferrochelatase, the enzyme required to incorporate iron into protoporphyrin (5). *Trypanosoma cruzi* was shown to require hemin/hemoglobin for growth. It was reported to manifest ALA dehydrase activity, but-polorphobilinogen deaminase was absent. However, functional ferrochelatase could be detected, leading to the conclusion that the parasite has lost part of its heme pathway due to mutations in some of the enzyme sequences, necessitating preformed hemin requirement (6).

The malarial parasite contains cytochromes (7, 8), and studies in this laboratory have demonstrated a requirement for heme to sustain optimal protein synthesis (9). *De novo* synthesis of heme is vital to the malarial parasite, since treatment of the culture with succinylacetone, a specific inhibitor of ALA dehydrase, leads to death of the parasite. It has, therefore, been concluded that the parasite utilizes the heme synthesized *de novo* for metabolic purposes, whereas it stores the surplus heme, which is toxic, derived from hemoglobin degradation as inert hemozoin pigment (1, 9). In view of these results and the possibility that the heme biosynthetic pathway of the malarial parasite could be a new drug target, studies have been carried out on the possible origin of the enzymes of the pathway. The results indicate the interesting feature that the parasite genome may not code for all the enzymes of the pathway. While

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The abbreviations used are: ALA, δ-aminolevulinate; ALAS, δ-ami-nolevulinate synthase; ALAD, δ-aminolevulinate dehydrase; CPO, coproporphyrinogen oxidase; FC, ferrochelatase; IRS, infected red cell super-natant; URS, uninfected red cell supernatant; SA, succinylacetone; RBC, red blood cell; GST, glutathione-S-transferase; GST-XR, gluta-thione S-transferase-retinoid X receptor fusion proteins; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBG, porphobilinogen.
**Heme Biosynthesis in the Malarial Parasite**

The parasitic enzyme ALAS is essential in the biosynthesis of heme. The parasites acquire the necessary enzymes from host red blood cells to complete their heme biosynthesis. The study of ALAS and other enzymes involved in heme biosynthesis in parasites can provide insights into drug resistance mechanisms.

**EXPERIMENTAL PROCEDURES**

**Materials**

Antibodies raised against purified human ALAS and FC preparations from the human red blood cells in rats (10) were used. Antibodies to human red cell ALAS and full-length mouse erythroid ALAD clone were gifts from Dr. Gloria C. Ferreira, University of South Florida and Dr. Terry Bishop, Johns Hopkins University, respectively. [2-14C]Glycine and [4-14C]-labeled succinylacetone were obtained from BRT (Bombay) and NEN Life Science, respectively.

**Methods**

**Parasite Maintenance and Isolation—**Swiss mice were injected with *P. berghei*, and blood was removed at different stages of parasitemia ranging from 30 to 80%. The parasitized red cells as well as the red cells from uninfected mice (control) were spun down and put into overnight culture as described for *P. falciparum* (11). In some experiments, *P. falciparum* maintained on O human red blood cells in culture (about 8% parasitemia) was described earlier (11). In other experiments, the parasites were spun down and put into overnight culture (8 h), during which time the cells were isolated and lysed with 0.15% (w/v) saponin (12). The parasite pellet as well as the infected red cell supernatant (IRS) were obtained by centrifugation. The red cells from uninfected mice were processed similarly to isolate the uninfected red cell supernatant (URS) and the membrane pellet.

**Heme Synthesis by *P. berghei* in Culture—**Mice were injected with *P. berghei* and blood was removed at different stages of parasitemia ranging from 30 to 80%. The parasitized as well as control red blood cells were spun down and put into overnight culture (8 h), during which time the parasites were incubated with [4-14C]ALA (1 μCi) or [2-14C]Glycine (12.5 μCi). The red cells were pelleted, and the parasite pellet, IRS, and URS were isolated after lysis. The fractions were extracted with ethyl acetate/acetate acid (3:1). The extract was washed with water and then 1.5 N HCl to remove the precursor used and all the porphyrins. The final ethyl acetate layer was washed twice with water to remove acid and used to measure radioactivity incorporated into heme (13). Separate analysis of the ethyl acetate layer by chromatography has revealed that the radioactivity is entirely accounted for by heme (14).

**Radioactivity incorporation into heme was measured in the parasite and mouse red blood cells by saponin lysis as described earlier.** The parasites were then lysed in 20 mM Tris-HCl (pH 7.5) containing 0.2% Triton X-100 by repeated homogenization, and the membrane fraction was pelleted at 8000 × g (15 min). The membrane pellet was washed with PBS. The membrane pellet was suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.2% Triton X-100 for 1 h at 4 °C. The lysate was spun down at 12,000 × g for 30 min, and the pellet was washed with PBS. The membrane pellet was suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% Triton X-100 and an aliquot (200 μg of protein) was incubated with partially purified ALAD from mouse RBCs at room temperature for 1 h with gentle shaking. A similar incubation was carried out with mouse red blood membrane and tryptophan-treated, washed parasite membrane. Another control employed was the incubation of bacterially expressed glutathione S-transferase and glutathione S-transferase-retinoic acid X receptor fusion proteins with the parasite membrane under similar conditions. After the incubations, the membrane fractions were reisolated by centrifugation and washed repeatedly with PBS, and the pellet was then subjected to SDS-PAGE (10% gels) followed by Western analysis with anti-ALAD and anti-erythroid protein antibodies. In addition, the membrane fraction was also examined through immunoelectron microscopy as described earlier.

**Determination of the Number of Binding Sites for ALAD on the Parasite Membrane—**Full-length mouse erythroid ALAD-dDNA was cloned and expressed with histidine tag in the T7 polymerase promoter based vector, pRSETB. The transformed *E. coli* cells were grown in 5 ml S-Sepharose and phenyl-Sepharose chromatography. The last two column steps were repeated with the preparation obtained to get the pure protein. The preparation from human red blood cells was used to raise antibodies in rabbits.

**Western Blot Analysis—**The purified ALAD preparations (75 ng) from the parasite and mouse red blood cells were analyzed on SDS-PAGE (10% gels) and transferred to nitrocellulose for immunoblot analysis. Similarly, membrane fractions (8000 × g pellet, 100 μg of protein) from the parasite, IRS, and URS were used for Western analysis of ALAS, CPO, and FC. The crude soluble fraction (75 μg of protein) was also used for analysis of ALAD. The blots were screened with antibodies to the appropriate enzyme preparations, diluted in the range 1:200 to 1:1000. The blots were finally analyzed with goat anti-rabbit antibodies conjugated to alkaline phosphatase.

**N-terminal Sequence Analysis—**Purified ALAD from the parasite (*P. berghei*) was subjected to N-terminal sequence analysis using a Shimadzu gas phase protein sequencer (PSQ1).

**ImmunoGold Electron Microscopy—**Immunogold electron microscopy was carried out (20) with the isolated parasite (*P. berghei*), as well as with the infected and uninfected red cells. These were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and embedded in LR Gold resin. Sections (60–70 nm) were cut and picked on copper grids. In experiments where purified ALAD was incubated with parasite membrane to demonstrate binding, the membrane preparation was first briefly washed to get a homogenous preparation. After incubation with ALAD and fixation, the membranes were fixed in 2% osmium tetroxide and examined under the electron microscope.

**Influence of Succinylacetone Treatment of the Host Red Cell on ALAD Activity of the Parasite (*P. falciparum*)—**Succinylacetone is known to be an irreversible inhibitor of ALAD activity (21). Fresh human RBCs in complete medium were incubated with succinylacetone (500 μM in final concentration) overnight at 4 °C. These as well as RBCs not treated with succinylacetone (control) were washed thoroughly with culture medium and then incubated with PBS containing 3% bovine serum albumin and incubated for 2 h with ALAD antibodies (1:1000 dilution), washed again, and then incubated with goat anti-rabbit IgG conjugated to gold particles (10 nm in diameter) in PBS containing 1% bovine serum albumin. The grids were stained with 2% osmium tetroxide and examined under the electron microscope.

**Purification of ALAD from the Parasite (*P. berghei*) and RBCs—**Purification of ALAD from the parasite and RBCs was carried out (20) with the isolated parasite (*P. berghei*), as well as with the infected and uninfected red cells. These were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and embedded in LR Gold resin. Sections (60–70 nm) were cut and picked on copper grids. In experiments where purified ALAD was incubated with parasite membrane to demonstrate binding, the membrane preparation was first briefly washed to get a homogenous preparation. After incubation with ALAD and fixation, the membranes were fixed in 2% osmium tetroxide and examined under the electron microscope.

**Parasite Membrane—**The parasite membranes were isolated from infected mouse red blood cells by saponin lysis as described earlier. The parasites were then lysed in 20 mM Tris-HCl buffer (pH 7.5) containing 0.2% Triton X-100 for 1 h at 4 °C. The lysate was spun down at 12,000 × g for 30 min, and the pellet was washed with PBS. The membrane pellet was suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% Triton X-100 and an aliquot (200 μg of protein) was incubated with partially purified ALAD from mouse RBCs at room temperature for 1 h with gentle shaking. A similar incubation was carried out with mouse red blood membrane and tryptophan-treated, washed parasite membrane. Another control employed was the incubation of bacterially expressed glutathione S-transferase and glutathione S-transferase-retinoic acid X receptor fusion proteins with the parasite membrane under similar conditions. After the incubations, the membrane fractions were reisolated by centrifugation and washed repeatedly with PBS, and the pellet was then subjected to SDS-PAGE (10% gels) followed by Western analysis with anti-ALAD and anti-erythroid protein antibodies. In addition, the membrane fraction was also examined through immunoelectron microscopy as described earlier.

**Specific Binding of Host Red Cell ALAD to the Parasite (*P. berghei*) Membrane—**The parasites were isolated from infected mouse red blood cells by saponin lysis as described earlier. The parasites were then lysed in 20 mM Tris-HCl buffer (pH 7.5) containing 0.2% Triton X-100 for 1 h at 4 °C. The lysate was spun down at 12,000 × g for 30 min, and the pellet was washed with PBS. The membrane pellet was suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% Triton X-100 and an aliquot (200 μg of protein) was incubated with partially purified ALAD from mouse RBCs at room temperature for 1 h with gentle shaking. A similar incubation was carried out with mouse red blood membrane and tryptophan-treated, washed parasite membrane. Another control employed was the incubation of bacterially expressed glutathione S-transferase and glutathione S-transferase-retinoic acid X receptor fusion proteins with the parasite membrane under similar conditions. After the incubations, the membrane fractions were reisolated by centrifugation and washed repeatedly with PBS, and the pellet was then subjected to SDS-PAGE (10% gels) followed by Western analysis with anti-ALAD and anti-erythroid protein antibodies. In addition, the membrane fraction was also examined through immunoelectron microscopy as described earlier.
of LB medium (inoculated from an overnight culture) and after 2 h of growth (OD 0.6), 1 mM isopropyl-1-thio-β-D-galactopyranoside and 200 μCi of [35S]methionine were added. After another 2½ h of incubation, labeled ALAD was isolated from E. coli cells and purified on a nickel-nitrotriacetic acid (Qiagen) column, using a single-step affinity procedure. The parasite membrane (2.4 mg of protein) was solubilized using 2.5% Triton X-100, 0.5% sodium cholate, and 0.5 mM NaCl for suspension followed by sonication. The insoluble debris was removed by centrifugation, and the solubilized membrane was loaded onto nitrocellulose membrane filters. These filters were then blocked with 5% skimmed milk powder in TTBS (0.1% Triton X-100, 50 mM Tris-buff ered saline) containing 1% bovine serum albumin. The nitrocellulose membrane was then incubated with increasing amounts of labeled ALAD for 1 h in the presence and absence of a 100-fold excess of nonradioactive ALAD. The nitrocellulose membrane filters were washed thrice with TTBS and dried, and radioactivity was measured. The data were used to generate the saturation curve and the Scatchard plot to calculate the dissociation constant and the number of binding sites.

RESULTS

De Novo Heme Synthesis in P. berghei and P. falciparum—In the case of P. berghei, blood from infected and uninfected mice was spun down, and the cells were incubated with [2-14C]glycine or [4-14C]ALA for 8 h in culture. In the case of P. falciparum, the parasite maintained in culture was incubated with the radiolabeled precursors overnight. The cells were then pelleted down, washed, and lysed with saponin, and the parasite pellet, IRS, and URS were used for measurement of radioactivity in the heme fraction. The results presented in Fig. 1 (A and B) indicate that similar results are obtained with P. berghei and P. falciparum. The parasite-infected red cell synthesizes 5–8-fold more heme than the uninfected red cell. Certain interesting features of the incorporation pattern of the precursors into heme are also evident. With [4-14C]ALA as precursor, 80% of the radioactive heme is recovered in IRS, whereas with [2-14C]glycine as precursor, the bulk of the radioactive heme is recovered in the parasite pellet.

Next, it was of interest to examine the heme biosynthetic potential of the isolated fractions. These experiments were carried out with P. berghei, since high yields of the parasite could be obtained from infected mice. The IRS, URS, and the parasite pellet were individually incubated with [4-14C]ALA, and the incorporation of radioactivity into heme and intermediates of the pathway was measured. The results presented in Table I indicate certain interesting features. First, maximum heme synthesis is again seen in IRS that is ~4–5-fold more than URS or parasite pellet. The parasite pellet has a substantial amount of [4-14C]ALA remaining unutilized compared with URS or IRS. This correlates with the low levels of ALAD detected in P. berghei (this study) as well as in P. falciparum (1). Nevertheless, the parasite converts the porphyrin intermediates to heme efficiently and is more effective than the IRS. This suggests that FC, catalyzing iron incorporation into protoporphyrin, could be present at significant levels in the parasite.

Assay of Heme Biosynthetic Enzymes in P. berghei—The next step was to assay the activities of ALAS, ALAD, and FC in P. berghei and mouse IRS and URS fractions. ALAS and FC activities were detected in the 8000 × g pellet in each case, whereas ALAD activity was detected in the soluble fractions. The results presented in Table II indicate that ALAS activity in the parasite is 3–4-fold more than in IRS or URS. FC activity in the parasite is as high as that in IRS and is 9–10-fold more than in IRS. ALAD activity in the parasite is ~20-fold lower than IRS and 4–5-fold lower than in IRS. From the results presented, it is also clear that, whereas the activity of ALAS is the same in infected and uninfected RBC preparations, the activities of ALAD and FC are at least 5-fold greater in IRS than in URS.

Parasite and Red Cell ALAD Activities Are Due to the Same Protein Species—ALAD was purified to homogeneity from P. berghei (Fig. 2), mouse and human red blood cells. The human
**Table II**

**ALAS, ALAD, and FC activities in isolated fractions from infected (P. berghei) and uninfected red cells**

The parasite pellet, IRS, and URS fractions were isolated as described earlier. ALAS and FC were assayed in the membrane fraction (8000 × g pellet). ALAD was assayed in the soluble fraction after fractionation through DEAE-Sepharose to remove hemoglobin. The assay conditions are given under “Experimental Procedures.” The values represent mean ± S.D. obtained from three different experiments.

| Enzyme activity | ALAS* | ALADb | FCc |
|-----------------|-------|-------|-----|
| Parasite        | 0.130 ± 0.008 | 0.074 ± 0.006 | 1.17 ± (0.09 × 10⁶) |
| IRS             | 0.040 ± 0.012 | 3.879 ± 1.023 | 8.89 ± (0.81 × 10⁶) |
| URS             | 0.039 ± 0.008 | 0.442 ± 0.058 | 1.16 ± (0.06 × 10⁶) |

*Values for ALAS are in μm of PBG equivalent/mg of protein.

b Values for ALAD are in μm of PBG/mg of protein.

c Values for FC are cpm in heme/mg of protein.

**Fig. 2.** SDS-PAGE profile of purified ALAD from *P. berghei*. The enzyme was purified from *P. berghei* as described under “Experimental Procedures.” The purified preparation was analyzed on SDS-PAGE (10% polyacrylamide gel). Lane 1, molecular weight markers; lane 2, purified ALAD.

enzyme preparation was used to raise antibodies in rabbits. The parasite and mouse red cell (uninfected) enzymes appear identical on the basis of several criteria. Both of the purified preparations show a requirement for zinc. Succinylacetone at a concentration of 50 μM brings about 50% inhibition of the parasite enzyme. First, it was found that while the parasite fraction used to assay or purify ALAD from human RBC, 75 ng of purified protein was analyzed by SDS-PAGE (10% polyacrylamide gel). M, mouse RBC; P, parasite. B, sequence of 20 amino acids at the N terminus of purified ALAD from *P. berghei* and mouse RBC.

**Fig. 3.** Western blot analysis and N-terminal sequence of purified ALAD from *P. berghei* and uninfected mouse RBC. A, Western blot was carried out with antibodies to purified ALAD from human RBC. 75 ng of purified protein was analyzed by SDS-PAGE (10% polyacrylamide gel). M, mouse RBC; P, parasite. B, sequence of 20 amino acids at the N terminus of purified ALAD from *P. berghei* and mouse RBC.

**Table III**

[4-14C]ALA incorporation into heme in *P. falciparum* culture maintained on SA-treated RBCs

The experimental details are given under “Experimental Procedures.” The parasite was cultured in untreated (control) and SA-treated RBCs. The final culture (1.5 ml) was incubated with 1 μCi of [4-14C]ALA overnight, and radioactivity incorporated into the heme fraction isolated from the parasite and red cell supernatant was measured. The results presented are those obtained in a typical experiment.

| Fraction | Radioactivity in heme cpm/1.5 ml culture |
|----------|----------------------------------------|
| Parasite (control RBC) | 375 |
| Parasite (SA-treated RBC) | 45 |
| RBC supernatant (control) | 11,400 |
| RBC supernatant (SA-treated) | 1200 |

by measuring [4-14C]ALA incorporation into heme in the parasite and IRS fractions. The results presented in Table III indicate that incorporation of [4-14C]ALA into heme in the parasite and IRS fractions derived from succinylacetone-treated RBCs is around 10% of the corresponding fractions derived from control RBCs. ALAD activity assays from control, succinylacetone-treated, and mixed RBC lysates indicate that there is no free succinylacetone left to inhibit the enzyme activity or heme synthesis in the parasite (Table IV). It has also been noted that the parasitemia tends to fall when the parasite is cultured in succinylacetone-treated RBCs, and there is a striking inhibition of parasite growth after three or four subcultivations with such RBCs, indicating that the level of ALAD in the parasite is too low to sustain an optimal rate of de novo heme synthesis to support growth.

**Parasite (P. berghei) ALAD Is Not a Contaminant from the Host**—First, the parasite fraction used to assay or purify ALAD was thoroughly washed, till the washings did not carry a detectable enzyme activity. Second, the parasite membrane fraction as isolated after lysis of the intact parasite does not contain detectable ALAD enzyme activity or the protein species detectable by Western analysis under conditions where the activity can be demonstrated in the parasite cytosol fraction. Third, the recoveries of ALAS, ALAD, CPO, and FC proteins in the parasite do not follow the trend seen in the RBC supernatant (Fig. 6). Further, to rule out the presence of ALAD in the parasite fraction as an experimental contaminant, *in situ* immunogold labeling of the enzyme was carried out with the isolated parasite as well as with the infected red cell. In both
cases, immunoelectron microscopy indicates that the grains are seen inside the parasite and not on the periphery, supporting the possibility of import of the host enzyme into the parasite and ruling out experimental contamination (Fig. 4). It is also interesting to note that the infected red cell has many more grains than the uninfected red cell, confirming the enzyme activity model presented in Table II.

*Parasite (P. berghei) Membrane Specifically Binds Host Red Cell ALAD*—To examine whether a possible receptor for host red cell ALAD could exist on the parasite membrane, partially purified ALAD from the red cell was incubated with parasite membrane fraction for 1 h at 25°C, and the membrane fraction was reisolated, washed thoroughly, and then analyzed for ALAD protein by Western analysis after SDS-PAGE. The results presented in Fig. 5A indicate that the parasite membrane can specifically bind to host red cell ALAD. Under identical conditions, the red cell membrane has no sites for binding the protein. Incubation of the parasite membrane with bacterially expressed mammalian glutathione S-transferase and glutathione S-transferase-retinoic acid X receptor fusion protein does not lead to detectable binding of these proteins. Trypsinized and washed parasite membrane fails to bind ALAD (Fig. 5B, lanes 3–5). The ALAD protein can be recovered in the supernatant fraction, suggesting that the failure to detect membrane binding is not due to degradation of ALAD by leftover trypsin. Finally, the parasite membrane incubated with partially pure ALAD was reisolated, washed, and subjected to immunoelectron micrography. A specific association of the enzyme with the parasite membrane can be clearly demonstrated, while the red blood cell manifests negligible binding (Fig. 5C).

Attempts were then made to estimate the number of binding sites for ALAD on the parasite membrane. Initial attempts to label the native protein using radiolabeled iodine led to some degradation of the protein and irreproducible binding data. Therefore, the mouse erythroid ALAD was expressed in *E. coli* and biosynthetically labeled with [35S]methionine. Since the protein was expressed with a histidine tag, it could be purified on a nickel-nitrolotriacetic acid column. Once again, binding experiments with intact parasite membrane gave high background, perhaps due to difficulty in effective washing of the membrane by centrifugation. Therefore, binding of [35S]labeled ALAD to solubilized parasite membrane loaded on to nitrocellulose membrane filters was carried out. This approach gave reproducible results, and the binding of radioactive ALAD could be chased by the nonradioactive species to an extent of 85–90%. The binding of ALAD to NC filters in the absence of parasite membrane preparation was negligible. The saturation curve and the Scatchard plot are given in Fig. 6 (A and B). The nonspecific binding was deducted to generate the Scatchard plot. The data indicate a fairly high affinity of binding (dissociation constant, 6.3 ± 0.4 × 10⁻⁸) with the number of binding sites estimated at 1550 ± 26/parasite.

*Western Blot Analysis of ALAS, ALAD, CPO, and FC in Parasite, IRS, and URS*—Western analysis of ALAS, CPO, and FC in the membrane fractions of the parasite, IRS, and URS reveals interesting features (Fig. 7). First, antibodies to human erythroid ALAS do not cross-react with the parasite enzyme, although the mouse red cell membrane preparation shows a strong cross-reaction (~72 kDa). However, the membrane preparation used actually shows 3–4-fold more ALAS enzyme specific activity in the parasite than in the mouse red cell. CPO is hardly detectable in the parasite and URS, although strikingly large amounts are seen in IRS (~37 kDa). Separate experiments reveal that when more protein is loaded on gels, the parasite CPO can be shown to move with a mobility identical to that of the host CPO, both cross-reacting with antibodies to the

![Fig. 4. Immunogold electron microscopy of *P. berghei* trophozoites and infected and uninfected red cells.](image-url) The isolated parasite as well as the red blood cells were processed as described in the text. The preparations were incubated with ALAD antibodies and then treated with anti-rabbit IgG conjugated to gold particles. A, isolated parasite; B, parasite inside the red cell. Arrow indicates parasite boundary. C, a field of uninfected red cell. D, A field of infected red cell.
human enzyme (data not presented). FC protein levels in the parasite and IRS are about the same, with only traces detected in URS. Once again, the protein preparations from all three preparations have identical mobilities in SDS gels (42 kDa) and cross-react with antibodies to the human enzymes. The almost equal FC enzyme activity detected in the parasite and IRS (Table II) correlates with the Western blot analysis (Fig. 7). ALAD protein was analyzed in the soluble fraction. The bulk of the protein is seen in the IRS with lower levels in URS and parasite (Fig. 7). This result also correlates well with the enzyme activity pattern shown in Table II.

**DISCUSSION**

The present study on the localization of the enzymes of the heme-biosynthetic pathway in the malarial parasite indicates a dual origin for these proteins. The first enzyme of the pathway, namely ALAS, is detectable in the parasite membrane at a level significantly higher than that of the host red cell membrane in terms of activity. However, the parasite enzyme, unlike that of the host red cell enzyme, does not cross-react with antibodies against human erythroid ALAS. This suggests the parasite origin of the ALAS localized in the parasite membrane. This suggestion is supported by a recent report on the successful cloning of the ALAS gene from *P. falciparum* (24). Parasite ALAD, CPO, and FC fall into another category. These are induced in the host (mice) in response to parasite (*P. berghei*) infection, and an amount is then perhaps translocated into the parasite.

Interestingly, there is no induction of ALAS in the IRS, once...
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again indicating a difference in its origin in the parasite. Preliminary studies reveal that in *P. falciparum* maintained in culture, there is no significant increase in ALAD in the IRS, unlike the situation seen in mice with *P. berghei* infection in *vivo*. This is to be expected, since the erythrocytes used to culture *P. falciparum* represent a terminally differentiated state and are incapable of RNA or protein synthesis. Therefore, the higher level of heme synthesis in parasite-infected cultures of *P. falciparum* should be due to the contribution of ALAS and perhaps FC from the parasite. The high levels of ALAD, CPO, and FC detected in *P. berghei*-infected mouse erythrocytes should be related to induction of these enzymes during the red cell maturation in *vivo*, perhaps due to stimuli provided by parasite-mediated red cell degeneration.

Detailed studies with ALAD reveal that the parasite (*P. berghei*) enzyme is identical to that of the host red cell but is not a contaminant. The immunogold labeling experiments and the demonstration of specific binding of host red cell ALAD to isolated parasite membrane suggest that this enzyme from the host may be translocated into the parasite through a receptor. These studies indicate that the host ALAD binds to the parasite membrane with high affinity, with the number of binding sites coming to about 1550/parasite. This can only be considered as a minimal estimate, since the binding studies have been carried out with solubilized membrane for reasons mentioned earlier.

A similar mechanism of translocation from the host may also operate, in the cases of CPO and FC, on the basis that these enzymes from the parasite have identical mobilities on SDS-PAGE with those of the host and that the parasite and host enzymes cross-react with antibodies to the human erythrocyte species, although further studies are needed to establish this point. However, unlike in the case of ALAD and CPO, the parasite has significantly high levels of FC. In mammalian mitochondria, both ALAS and FC, the first and last enzymes of the heme-biosynthetic pathway, are localized in the inner mitochondrial membrane (25). There is a possibility that FC like ALAS may also be coded for by the parasite genome, or it may be of dual origin.

While there is extensive literature on the transport of proteins made by the parasite into the host erythrocyte (20, 26, 27), internalization of host hemoglobin and its degradation in parasite food vacuoles lead to generation of amino acids for parasite protein synthesis and large amounts of heme, detoxified and stored as inert hemozoin pigment. Superoxide dismutase represents another example, where 20% of the activity in the parasite is accounted for by the parasite gene coding for the protein and the rest is accounted for by the host red cell superoxide dismutase internalized by the parasite. However, the adopted superoxide dismutase has not been proven to participate functionally in the parasite (28, 29). In the case of ALAD, it is clear that the entire functional enzyme of the parasite is derived from the host. Besides, it has not so far been possible to identify the ALAD gene in the parasite genome (studies in this laboratory), although this cannot be taken as evidence for its absence.

The results obtained with [4,14C]ALA incorporation into heme in cultures of *P. falciparum* and *P. berghei* indicate that 80% of the radioactivity is recovered in IRS. This correlates well with the higher levels of ALAD and the subsequent enzymes in IRS than in the parasite. In contrast to this situation, the results obtained with [2,14C]glycine incorporation into heme clearly indicate that 80% of the radioactivity is recovered in the parasite fraction. This suggests that this precursor is essentially handled by ALAS in the parasite membrane and that the level of this enzyme is also significantly higher than that of the red cell membrane enzyme. The results also indicate that there is no significant loss of the intermediates from the parasite into the IRS, or perhaps import of the intermediates from IRS into the parasite also does not take place. The parasite has evolved a mechanism to import the required enzymes rather than the biosynthetic intermediates or preformed heme. Heme should be essentially synthesized within the mitochondrion of the parasite, as is the case with mammalian mitochondria, and then distributed to the other parasite compartments. Thus, the parasite has evolved a strategy to segregate and divert the heme derived from red cell hemoglobin into the inert hemozoin pigment while making use of the heme synthesized *de novo* for metabolic purposes.

These studies have also identified new potential drug targets. These would be ALAS species unique to the parasite and the receptor(s) on the parasite membrane to import ALAD and perhaps the other enzymes of the pathway from the host. It would also be of interest to examine in this context whether the parasite employs additional mechanisms for making ALA.

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