A Malaria Parasite-encoded Vacuolar H\textsuperscript{+}-ATPase Is Targeted to the Host Erythrocyte*

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The asexual development of malaria parasites inside the erythrocyte is accompanied by changes in the composition, structure, and function of the host cell membrane and cytoplasm. The parasite exports a membrane network into the host cytoplasm and several proteins that are inserted into the erythrocyte membrane, although none of these proteins has been shown to have enzymatic activity. We report here that a functional malaria parasite-encoded vacuolar (V)-H\textsuperscript{+}-ATPase is exported to the erythrocyte and localized in membranous structures and in the plasma membrane of the infected erythrocyte. This localization was determined by separation of parasite and erythrocyte membranes and determination of enzyme marker activities and by immunofluorescence microscopy assays using antibodies against the B subunit of the malarial V-H\textsuperscript{+}-ATPase and erythrocyte (spectrins) and parasite (merozoite surface protein 1) markers. Our results suggest that this pump has a role in the maintenance of the intracellular pH (pH\textsubscript{i}) of the infected erythrocyte. Our results also indicate that although the pH\textsubscript{i}, maintained by the V-H\textsuperscript{+}-ATPase is important for maximum uptake of small metabolites at equilibrium, it does not appear to affect transport across the erythrocyte membrane and is, therefore, not involved in the previously described phenomenon of increased permeability of infected erythrocytes that is sensitive to chloride channel inhibitors (new permeation pathway). This constitutes the first report of the presence of a functional enzyme of parasite origin in the plasma membrane of its host.

Plasmodium falciparum, one of the agents responsible for malaria, is an obligate intracellular parasite belonging to the phylum Apicomplexa. The asexual development of malaria parasites inside the erythrocyte is accompanied by changes in the composition, structure, and function of the host cell membrane and cytoplasm (1, 2). Upon invasion of the erythrocyte, the malaria parasite establishes a parasitophorous vacuole inside which it develops. In addition, the parasite interacts with its host cell and its environment by exporting a membrane network into the cytoplasm of its host cell and by inserting a number of proteins in the erythrocyte plasma membrane (3). As a result of these changes P. falciparum-infected erythrocytes show abnormally high permeability toward amino acids (1), sugars and polyols (2), purines (4), cations (5, 6), and anions (7, 8). The mechanism responsible for this increased permeability has a strong preference for anions over cations and is blocked by drugs known to inhibit anion-selective channels (9). The increased permeability, therefore, appears to be the result of the parasite activating or inserting an anion-selective channel in the membrane of the erythrocytes (9). Electrophysiological evidence for the presence of an anion-selective channel in the plasma membrane of P. falciparum-infected erythrocytes (10) supports this proposal (9). This channel has a high permeability to chloride ions, but it is not known whether it is encoded by the parasite genome (10). Interestingly, vacuolar H\textsuperscript{+}-ATPases (V-H\textsuperscript{+}-ATPases)\textsuperscript{3} are associated with chloride channels in the plasma membrane of numerous cells (11–15), and some stages of malaria parasites selectively invade V-H\textsuperscript{+}-ATPase-containing cells (16). Normal erythrocytes, however, do not possess a V-H\textsuperscript{+}-ATPase. Several parasite proteins are inserted into the erythrocyte membrane, but none of these proteins has been shown to have enzymatic activity (3).

Recent work has identified a protein export element in the N terminus of some exported proteins required for transport across the parasitophorous vacuole membrane (17, 18). It is not clear what additional mechanisms are required for trafficking of these proteins across the erythrocyte cytosol and for their insertion in the erythrocyte membrane. Membrane-bound compartments, such as Maurer clefts, a Golgi-like apparatus linked to and addressing parasite proteins to the host cell surface, and a tubulovesicular network have been suggested to be involved in this process (19). Some of these structures can be stained by dyes used to identify acidic compartments such as acridine orange and lysosensor green (20, 21). Acidification of the intracellular compartments in eukaryotic cells is usually through the activity of a vacuolar-type H\textsuperscript{+}-ATPase, but it is not known whether a similar enzyme is present in the Maurer clefts or in the tubulovesicular compartments of infected erythrocytes. Recently, a vacuolar-type H\textsuperscript{+}-translocating pyrophosphatase (V-H\textsuperscript{+}-PPase) previously described in malaria parasites (22, 23) that is widely utilized for the acidification of intracellular compartments in bacteria (24), plant vacuoles (25), and acidocalcisomes (26) was identified by proteomic analysis in the Maurer clefts (19), but its presence in these structures was not confirmed by co-localization studies.

Previous reports have established the presence of a functional V-H\textsuperscript{+}-ATPase in the plasma membrane of Apicomplexa parasites with an important role in the regulation of their intracellular pH (pH\textsubscript{i}) (27–29) and plasma membrane potential (30). Bafilomycin A\textsubscript{1}, a specific inhibitor of V-H\textsuperscript{+}-ATPases when used at low concentrations (31), produced a marked decrease in the steady state pH\textsubscript{i} of Toxoplasma gondii tachyzoites (27) or P. falciparum trophozoites (28, 29) and decreased the recovery of pH\textsubscript{i} after acidification as well as markedly inhibited the

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\textsuperscript{3} The abbreviations used are: V-H\textsuperscript{+}-ATPase, vacuolar H\textsuperscript{+}-ATPase; BCECF, 2’,7’-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein; Pipes, 1,4-piperazinediethanesulfonic acid; MSP-1, merozoite surface protein 1.
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proton efflux rate from either isolated parasite (27, 29) and decreased the plasma membrane potential of *P. falciparum* trophozoites (30). Immunocytochemical studies demonstrated the presence of this pump in the plasma membrane of these parasites (27, 29). We report here that functional malaria parasite-encoded V-H\(^{+}\)-ATPase is localized to membranous structures in the cytosol and in the plasma membrane of infected erythrocyte and could have a role in the regulation of intracellular pH of the infected cells.

**EXPERIMENTAL PROCEDURES**

Parasites—*Plasmodium berghei* berghei (strain NK65) and *P. falciparum* (clones HB3 or Dd2) trophozoites and infected erythrocytes enriched to parasitemias of >60% were obtained as described before (22, 32). To prepare *P. falciparum* or *P. berghei* plasma membrane vesicles, we adapted a method developed for other parasitic protozoa (33). This method resulted in a 3-fold purification of merozoite surface protein 1 (MSP-1) s compared with total homogenates and analyzed by immunoblotting with specific antibodies (34, 35) (data not shown). Trophozoites (5 x 10\(^{6}\) cells for each preparation) were centrifuged and washed 3 times with buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO\(_4\), and 10 mM Hepes-Tris, pH 7.2) and once with hypotonic medium (400 mM mannitol, 10 mM KCl, 2 mM EDTA, 20 mM Hepes, pH 7.20) (mannitol buffer) containing a protease inhibitor mixture (1 mM phenylmethanesulfonyl fluoride, 0.15 mg/ml soybean trypsin inhibitor, 10 \(\mu\)M leupeptin, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 10 \(\mu\)M trans-epoxysuccinyl-l-leucylamino(-guanidino) butane). The cell pellet was mixed with acid-washed glass beads (75–150 \(\mu\)m in diameter) at a ratio of 1:4 (wt/wt of beads). The cells were disrupted by abrasion in a chilled mortar until 90% disruption was achieved, as determined by optical microscopy. The glass beads, unbroken cells, and large debris were removed by centrifugation at 3000 \(\times\) g for 15 min at 4°C. The supernatant was subjected to differential centrifugation, first at 17,000 \(\times\) g for 30 min at 4°C and then at 105,000 \(\times\) g for 1 h at the same temperature. The resulting pellet was resuspended in about 0.5 ml of medium containing 150 mM KCl, 2 mM MgCl\(_2\), 75 mM Hepes, pH 7.2 (resuspension buffer) and used for immunoblotting assays or transport studies. To isolate erythrocyte ghosts, packed infected or non-infected erythrocytes (~500 \(\mu\)l) were washed once with lysis buffer containing 125 mM sucrose, 65 mM KCl, 2 mM MgCl\(_2\), 2 mM EDTA, 20 mM Hepes, pH 7.2, and the protease inhibitors mixture described above, resuspended in 2.5 ml of the same buffer containing 0.5% saponin, and incubated for 5 min at 37°C. The lysate was diluted 10-fold in the same buffer and centrifuged first at 1,000 \(\times\) g for 10 min, then at 2,000 \(\times\) g for 10 min, and finally at 14,000 \(\times\) g for 5 min. The dark parasites were pelleted at the bottom of the tube, and the fragments of erythrocyte membranes formed a visible band on top of the parasite pellet. The supernatant and layer containing the erythrocyte membranes were diluted in 100 ml of lysis buffer and centrifuged at 105,000 \(\times\) g for 60 min at 4°C. The band corresponding to the erythrocyte membranes was removed very carefully and resuspended again in 100 ml of mannitol buffer. After centrifugation at 105,000 \(\times\) g for 60 min at 4°C, the white erythrocyte membranes were resuspended in resuspension buffer. Giemsa staining confirmed the purity of the erythrocyte membrane preparation.

**Chemicals and Solutions**—Bafilomycin A\(_1\) was from Kamiya Biomedicals (Thousand Oaks, CA). 2',7'-Bis-(carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF) acetoxymethyl ester, free BCECF, and the EnzChek phosphate assay kit were from Molecular Probes. A recombinant protein comprising 219 amino acids of *P. falciparum* V-H\(^{+}\)-ATPase B subunit was produced in *Escherichia coli* and used to obtain polyclonal antibodies in rabbits as described before (36). Secondary antiserum, molecular weight markers, and Coomassie Blue protein assay reagent were from Bio-Rad. Concanamycin A (folimycin) and monoclonal antibodies against human spectrin (α and β) were from Sigma. Polyclonal antibodies and monoclonal antibody 89.1, which react with a conserved epitope within the N-terminal 83-kDa fragment of MSP-1, were kindly provided by Dr. A. Holder (National Institute for Medical Research, London, UK). The enhanced chemiluminescence detection kit was bought from Amersham Biosciences. All other reagents were analytical grade. Loading buffer (same as buffer A plus 15 mM sucrose), standard buffer (135 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), and 10 mM Hepes-Tris), and high potassium buffer (140 mM KCl, 5 mM glucose, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), Hepes-Tris) were adjusted to pH 7.4 unless otherwise indicated.

**Intracellular pH (pH\(_i\)) Measurements**—pH\(_i\) was measured fluorometrically using BCECF. Cells were washed twice in buffer A and suspended to a final density of 1 x 10\(^{7}\) cells/ml in loading buffer and 9 \(\mu\)M BCECF acetoxyethyl ester. The suspensions were incubated for 20 min in a 30°C water bath with mild agitation. Subsequently the cells were washed twice with standard buffer to remove the extracellular dye and resuspended to a final density of 1 x 10\(^{8}\) cells/ml and kept at 4°C. For pH\(_i\) measurements, a 50-\(\mu\)l aliquot of the cell suspension was diluted into 2.5 ml of the appropriate buffer (final density 5 x 10\(^{7}\) cells/ml) in a cuvette placed in a thermostated (30°C) Hitachi F-2000 spectrofluorometer. All measurements were completed within 2 h of loading. The fluorescence ratio, with wavelengths for excitation set at 505/440 nm and for emission at 530 nm, was recorded and translated into pH values on the basis of the ratios obtained at various pH values using high potassium buffer and nigericin (37). Acid loading was accomplished by preincubating 5 x 10\(^{7}\) cells in 50 \(\mu\)l of buffer containing 40 mM NH\(_4\)Cl at 30°C for 15 min (NH\(_4\)Cl prepulse technique) (37). Cells were then centrifuged and resuspended in various buffers with or without inhibitors.

**Proton Efflux Measurements**—To measure extracellular pH (pH\(_e\)) reflecting proton efflux by cells, 1 x 10\(^{6}\) cells were washed and resuspended in a weakly buffered (0.1 mM Hepes-Tris, pH 7.4) standard solution containing 0.38 \(\mu\)M BCECF (free acid) (37).

**Enzyme Assays**—ATPase was assayed by measuring released phosphate using the EnzChek phosphate assay kit as described before with the microtitre plate modification (38) and using ATP (1 mM) instead of PP\(_i\), and also by measuring the decrease in A\(_{340}\) in a coupled enzyme assay at 30°C (33). Each sample (5 \(\mu\)l) was mixed with 100 \(\mu\)M of reaction mixture containing 30 mM Hepes, 200 mM KCl, 4 mM MgCl\(_2\), 50 \(\mu\)M EGTA, 1 mM phosphoenolpyruvate, 5 mM ATP, pyruvate kinase at 14 units/ml, lactate dehydrogenase at 20 units/ml, and 0.3 ml of NADH, pH 7.6. Acetylcholine esterase activity was determined as described previously (39). All assays were performed using a PowerWave 340i plate reader (BioTek Instruments) at 30°C. Proton uptake measurements with acridine orange were done as described previously (22).

**Radioisotope Influx Measurements**—The unidirectional influx into *P. falciparum* (clone HB3)-infected and uninfected human erythrocytes of methionine, thymidine, and choline was estimated from the uptake of [\(^{35}\)S]methionine, [\(^{3}H\)]thymidine, and [\(^{3}H\)]choline, respectively, into cells washed 4 times by centrifugation (5 min and 1000 \(\times\) g) and then resuspended in Hepes-buffered saline (125 mM NaCl, 5 mM KCl, 25 mM Hepes, 5 mM glucose, pH 7.4) as described before (9). Normal or infected erythrocytes enriched (22) at 60–90% parasitemia (5 x 10\(^{8}\) cells) were incubated with furosemide or bafilomycin A\(_1\) at the concentrations indicated under “Results.” Measurement commenced with the addition of radiolabeled substrate. The sample was mixed thoroughly, and at regular intervals aliquots of the suspension
were transferred to microcentrifuge tubes containing 0.8 ml of ice-cold isotonic saline layered over 0.25 ml of dibutyl phthalate, and these were centrifuged immediately (10,000 \times g, 30 s). After sedimentation of the cells below the oil, the aqueous supernatant solution was removed by aspiration, and the radioactivity remaining on the walls of the tubes was removed by rinsing the tubes four times with water. The dibutyl phthalate was aspirated, and the cell pellet was lysed with 0.1% (v/v) Triton X-100 (0.5 ml) and deproteinized by the addition of 5% w/v trichloroacetic acid (0.5 ml) followed by centrifugation (10,000 \times g, 10 min). Radioactivity was measured using a \( \beta \)-scintillation counter.

**SDS Electrophoresis and Preparation of Western Blots**—Aliquots of normal and infected erythrocyte ghosts and tophozoite plasma membrane vesicles (10 \( \mu l \), about 30 \( \mu g \) of protein) were mixed with 10 \( \mu l \) of 125 mM Tris/HCl (pH 7.0), 10% (w/v) \( \beta \)-mercaptoethanol, 20% (w/v) glycerol, and 4.0% (w/v) bromophenol blue as tracking dye and boiled for 5 min before application to SDS-10% polyacrylamide gels (10 \( \mu g \)/lane). Electrophoresed proteins were transferred to nitrocellulose with a Bio-Rad transblot apparatus. After transfer, the nitrocellulose was blocked in 5% nonfat dry milk in 0.1% Tween 20, phosphate-buffered saline and kept overnight at 4 °C. A dilution of the antibodies in blocking buffer was applied at room temperature for 60 min as indicated in the figure legend. The nitrocellulose was washed 3 times for 20 min each with 0.1% Tween 20, phosphate-buffered saline and incubated with secondary anti-mouse or anti-rabbit antibody (1:20,000) in blocking buffer at room temperature for 30 min. Immunoblots were visualized on radiographic film (Eastman Kodak Co.) using the ECL chemiluminescence detection kit according to the instructions of the manufacturer (Amersham Biosciences).

**Immunofluorescence Microscopy**—P. falciparum (clones HB3 and Dd2)-infected human erythrocytes were fixed with 4% freshly prepared formaldehyde plus 0.1% grade I glutaraldehyde in PHEM buffer (60 mM Pipes, 25 mM Heps, 10 mM EGTA, and 2 mM MgCl\(_2\), pH 7.2) and stored at 4 °C. The samples were deposited on coverslips and permeabilized with methanol at -20 °C for 20 min, blocked with 1% bovine serum albumin in 0.1% Triton X-100/PHEM, and incubated with the primary antibodies for 1 h at room temperature.

**FIGURE 1.** Effects of bafilomycin A\(_1\) on \( \text{pH}_i \) in P. falciparum- and P. berghei-infected erythrocytes and on acridine orange uptake by plasma membrane vesicles from P. berghei trophozoites. A, BCECF-loaded infected (clone Dd2) erythrocytes were centrifuged and resuspended in standard buffer, and the \( \text{pH}_i \) was allowed to stabilize. Bafilomycin A\(_1\) was then added at different concentrations, and the cells were incubated for the times indicated. B and C, BCECF-loaded erythrocytes infected with P. falciparum (B, clone HB3) or P. berghei (C) were acidified by the \( \text{NH}_4 \) prepulse method and resuspended in standard buffer with (BAF) or without (Control) the concentrations of bafilomycin A\(_1\) indicated. D and E, P. falciparum (D, clone Dd2) or P. berghei (E) infected erythrocytes were resuspended in a weakly buffered (0.1 mM Heps-Tris pH 7.4) standard solution containing 0.38 mM BCECF (free acid) in the absence (Control) or presence (BAF) of 10 nM bafilomycin A\(_1\). F and G, human normal erythrocytes treated as in B and A, respectively. H, P. berghei plasma membrane vesicles (50 \( \mu g \) protein/ml) were added to a buffer containing 130 mM KCl, 2 mM MgSO\(_4\), 50 \( \mu M \) EGTA, and 10 mM Heps (pH 7.2) in the absence (trace b) or presence (trace a) of 5 nm bafilomycin A\(_1\). Acridine orange (AO; 3 \( \mu M \)), 1 mM ATP, 2 \( \mu M \) nigericin (NIG), 10 mM NH\(_4\)Cl, or 5 nm bafilomycin A\(_1\) were added where indicated by the arrows. Traces shown in A–H are representative of three independent experiments conducted with separate cell preparations.
V-H⁺ -ATPase in P. falciparum

TABLE ONE

| Sample                          | ATPase activity (% inhibition by 0.5 µM Baf. A₁) mmol/min × mg of protein | Acetylcholine esterase µmol/min × mg protein |
|--------------------------------|--------------------------------------------------------------------------|---------------------------------------------|
| **P. falciparum-infected erythrocytes** |                                                                           |                                             |
| Trophozoite membranes           | 28 ± 1.4 (81)⁺                                                          | 0.13 ± 0.04                                 |
| Erythrocyte membranes           | 68 ± 1.6 (76)⁺                                                          |                                             |
| **P. berghei-infected erythrocytes** |                                                                           |                                             |
| Trophozoite membranes           | 63 ± 7 (74)⁺                                                            | 0.09 ± 0.04                                 |
| Erythrocyte membranes           | 34 ± 6 (84)⁺                                                            | 0.94 ± 0.01                                 |
| **Normal erythrocyte membranes** |                                                                           |                                             |
| Human                           | 9 ± 3 (3)⁺                                                              | 2.1 ± 0.24                                  |
| Mice                            | 19 ± 4 (5)⁺                                                             |                                             |
|                                 | 22 ± 2 (5)⁺                                                             | 0.89 ± 0.05                                 |

⁺Coupled enzyme assay.
⁺⁺EnzChek assay.

albun, 2% normal goat serum, and 0.5% fish gelatin in PHEM buffer for 60 min, and then incubated with primary antibodies (V-H⁺ -ATPase, 1:100) in PHEM plus 1% fish gelatin for 3 h at room temperature followed by 3 washes and incubation with Alexa-488-conjugated goat-anti-mouse or Alexa-546-conjugated goat anti-rabbit antibodies (1:1000) in PHEM plus 1% fish gelatin for 45 min. After three more washes, the samples were mounted under a coverslip in phosphate-buffered saline, 50% glycerol and examined with an Olympus Fluoview FV 300 laser-scanning confocal microscope using optical sections of 0.1 µm or with an Olympus BX60 epifluorescence microscope. The distribution of staining was measured counting 100 infected erythrocytes (as indicated by 4,6-diamidino-2-phenylindole staining) in two different experiments.

RESULTS

Effect of Bafilomycin A₁ on Intracellular pHᵢ and Recovery from an Acidic Load in Infected Erythrocytes—We examined whether bafilomycin A₁ could affect the steady state pHᵢ and the recovery from acidification of whole, infected erythrocytes. Low concentrations of bafilomycin A₁ (10–100 nM) greatly decreased the steady state pHᵢ of infected erythrocytes (Fig. 1A) without affecting normal cells (Figs. 1G). Although recovery from acidification of uninfected erythrocytes, caused by a NH₄Cl prepulse, was not affected by bafilomycin A₁ (Fig. 1F), the inhibitor caused a marked inhibition of recovery or further acidification of either P. falciparum (Fig. 1B) or P. berghei (Fig. 1C)-infected erythrocytes. Bafilomycin A₁ also decreased the rate of proton efflux from P. falciparum (Fig. 1D) or P. berghei (Fig. 1E)-infected erythrocytes. These results suggest that either the parasites are the main source of protons and inhibition of proton efflux from the parasite to the erythrocyte cytosol limits their extrusion through the erythrocyte plasma membrane or that a bafilomycin A₁-sensitive proton pump is also located in the plasma membrane of the infected erythrocytes.

Presence of a V-H⁺-ATPase Activity in the Erythrocytes Plasma Membrane—To investigate whether a V-H⁺-ATPase was present in the plasma membrane of infected erythrocytes, we separated the parasites from infected erythrocyte plasma membranes and measured their bafilomycin A₁-sensitive ATPase activity (TABLE ONE). A considerable bafilomycin A₁-sensitive activity was retained in the infected erythrocyte plasma membranes that also contained high acetylcholine esterase activity (a marker for erythrocyte membranes (39)). Acetylcholine esterase activity was very low in the parasite membrane fraction. Normal erythrocytes had very low bafilomycin A₁-sensitive ATPase activity (TABLE ONE). Attempts to detect bafilomycin A₁-sensitive proton transport in ghosts from infected erythrocytes failed, probably because deficient resealing of membranes due to deformations induced by the parasite (3). However, we were able to detect bafilomycin A₁-sensitive proton transport by plasma membrane vesicles of isolated trophozoites (Fig. 1F).

Western Blot Analysis of Parasite and Erythrocyte Membranes—Use of antibodies against a region of the P. falciparum V-H⁺-ATPase subunit B (36) recognized a polypeptide of 56 kDa present in both parasite and infected erythrocyte membrane preparations (Fig. 2, lanes 1 and 2). No background staining was observed with preimmune serum (data not shown) or in normal erythrocytes (Fig. 2, lane 3). No reaction was detected in normal (Fig. 2, lane 6) or infected erythrocyte (Fig. 2, lane 4) membranes using antibodies against MSP-1 (34), a plasma membrane marker for different stages of P. falciparum (40), and no reaction was detected in the parasite fraction using antibodies against spectrins α and β (Fig. 2, lane 8), markers for erythrocyte membranes (41). As expected, antibodies against spectrins (α and β) recognized two bands of high molecular mass (220–240 kDa) in normal erythrocytes (Fig. 2, lane 9) and the same bands plus additional low molecular weight bands in infected erythrocytes (Fig. 2, lane 7). These additional bands are probably proteolytic products generated by parasite proteases, as has been reported before (41). Antibodies against MSP-1 recognized a main band of 195 kDa and some lower molecular weight polypeptides in isolated trophozoites (Fig. 2, lane 5). Peptide mapping has shown that the smaller polypeptides are proteolytic fragments of the 195-kDa protein (34). No reactions were detected in all cases when normal serum was used (data not shown).

Immunofluorescence Localization of the V-H⁺-ATPase—The immunolocalization of the V-H⁺-ATPase in P. falciparum was also tested by immunofluorescence using the polyclonal antibody against the B subunit of the parasite V-H⁺-ATPase (36). Labeling of the plasma membrane of infected erythrocytes was more intense at early stages of parasite development (ring and early trophozoite stages, Fig. 3A). Some
infected erythrocytes showed predominant labeling of the parasites (Fig. 3C), whereas others showed labeling of both the parasite and the erythrocyte plasma membrane as well as a punctate staining in the cytosol of the erythrocyte (Fig. 3E). Fig. 3G shows no staining of uninfected erythrocytes. Fig. 4 shows the distribution of the different staining patterns in synchronized populations of two different strains of P. falciparum 6 h after infection. Erythrocyte surface labeling markedly predominates at this time.

Effect of Bafilomycin A₁ on the New Permeation Pathway—Because the presence of a V-H⁺-ATPase in the plasma membrane of infected erythrocytes could have a role in their higher permeability for small molecular weight metabolites, we measured the effect of bafilomycin A₁ on the P. falciparum-augmented uptake of different substrates into infected erythrocytes (Fig. 5). The initial rates of augmented transport of methionine and thymidine were similar with and without bafilomycin A₁ but were significantly decreased by furosemide (Figs. 5, 6A, and 6B), as has been reported previously (9). The malaria-augmented uptake of methionine, choline, and thymidine by infected erythrocytes was, however, significantly decreased at equilibrium (Fig. 5, A and B) in a dose-dependent manner (Fig. 5C), without affecting the transport of these substrates in normal erythrocytes (data not shown). Thus, the pH₇ maintained by the V-H⁺-ATPase is important in determining the maximum uptake of these substrates at equilibrium.

DISCUSSION

In this report we provide evidence that a functional malaria parasite-encoded V-H⁺-ATPase is exported into the host cytosol and plasma membrane. The inhibitory effects of bafilomycin A₁, a specific inhibitor of the V-H⁺-ATPase at the concentrations used (31), on the steady state pH₇, recovery from an acid load, and proton extrusion of both P. falciparum- and P. berghei-infected erythrocytes (Fig. 1) support an important role of this pump in the maintenance of the pH of the infected erythrocyte. These effects could be due to inhibition of the parasite plasma membrane-located V-H⁺-ATPase (28, 29). This is because BCECF has been shown to compartmentalize in the parasites when infected erythrocytes are loaded with its ester (29). However, separation of erythrocyte and parasite membranes of both P. falciparum and P. berghei revealed that a significant portion of the bafilomycin A₁-sensitive ATPase activity was retained in the erythrocyte plasma membrane fraction (Fig. 2). It has been pointed out that although infected erythrocyte ghosts are devoid of detectable contamination with proteins of the parasite cytoplasm and the parasite membrane, they do contain Maurer clefs linked to the erythrocyte membrane (19). We cannot rule out the possibility that this ATPase activity could be due to contamination of the erythrocyte membrane with these structures. However, immunofluorescence studies also indicated the localization of this pump in the erythrocyte plasma membrane (Fig. 3), predominantly at early stages of parasite development (Fig. 4), and recent proteomic studies of Maurer clefs failed to detect this enzyme (19).

The effect of bafilomycin A₁ on proton extrusion by infected erythrocytes (Figs. 1, D and E) together with the presence of a bafilomycin
A$_1$-sensitive activity in erythrocyte membranes (TABLE ONE) and with the localization studies (Figs. 2 and 3) support a functional role of the malaria-encoded V-H$^+$-ATPase in the plasma membrane of the erythrocyte. Our results suggest that this pump has a role in the maintenance of the intracellular pH of the host-parasite complex as indicated by the potent inhibitory effect of bafilomycin A$_1$ on the steady state pH, recovery from an acid load, and proton efflux from infected cells. Our results also indicate that although the pH maintained by the V-H$^+$-ATPase is important for maximum uptake of small metabolites at equilibrium, it does not appear to affect transport across the erythrocyte membrane and is, therefore, not involved in the previously described phenomenon of increased permeability of infected erythrocytes that is sensitive to chloride channel inhibitors (new permeation pathway).

V-H$^+$-ATPases located in the plasma membrane have been shown to energize a number of different processes related to homeostasis of ions and water in a variety of cells (13). The electrochemical proton gradient established by the V-H$^+$-ATPase provides the driving force for transport of different ions and molecules. For example, in the midgut of plant-feeding insect larvae, an apical V-H$^+$-ATPase energizes K$^+$ secretion via basolateral K$^+$ channels and apical K$^+$/H$^+$ antiport (13, 42). Insects counterbalance a salt and water load by secretion across their Malpighian tubule epithelia, and an apical V-H$^+$-ATPase plays a dominant role in this process (43). In the frog skin the proton pump provides the electrical driving force for Na$^+$ uptake from low Na$^+$ concentrations (44). The H$^+$ pump could support Cl$^-$ absorption by maintaining a high, outwardly directed HCO$_3^-$ gradient that drives Cl$^-$ uptake via the Cl$^-$/HCO$_3^-$ antiport or by hyperpolarizing the membrane potential, driving Cl$^-$ through channels across the membrane (44). This electrical potential difference might also be used to drive the movement of ions and small molecules and could in part explain the importance of the V-H$^+$-ATPase for maximum uptake of small metabolites at equilibrium.

The B subunit of the V-H$^+$-ATPase studied here lacks a signal sequence and the host cell targeting signal recently identified in the N terminus of many exported proteins (17, 18), implying a different mechanism for targeting this protein to the plasma membrane of the host cell. Other proteins that are known to traffic outside the parasites, such as the Maurer cleft protein PB1 and several chaperones (19), also lack this host targeting signal, and it has been proposed (19) that a distinctive class of double membrane vesicles exported from the parasite endoplasmic reticulum to the parasitophorous vacuole membrane may participate in the relocation of some parasite proteins to the erythrocyte.

Although several enzymes, such as phosphatases, serine-threonine kinases, and a putative ABC transporter have been shown to possess a
host–targeting signal (17, 18), it has not been demonstrated that these are exported as functional enzymes. This is the first report of the presence of a functional enzyme of parasite origin in the plasma membrane of malaria-infected erythrocytes.

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