Copper pathways in *Plasmodium falciparum* infected erythrocytes indicate an efflux role for the copper P-ATPase

Dominique RASOLOSON*, Lirong SHI*, Curtis R. CHONG†, Bjorn F. KAFSACK* and David J. SULLIVAN, Jr*[1]

*The Malaria Research Institute, W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205, U.S.A., and †Medical Scientist Training Program, Department of Pharmacology, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, U.S.A.

Copper, like iron, is a transition metal that can generate oxygen radicals by the Fenton reaction. The *Plasmodium* parasite invades an erythrocyte host cell containing 20 μM copper, of which 70 % is contained in the Cu/Zn SOD (cuprozinc superoxide dismutase). In the present study, we follow the copper pathways in the *Plasmodium*-infected erythrocyte. Metal-determination analysis shows that the total copper content of Percoll-purified trophozoite-stage-infected erythrocytes is 66 % that of uninfected erythrocytes. This decrease parallels the decrease seen in Cu/Zn SOD levels in parasite-infected erythrocytes. Neocuproine, an intracellular copper chelator, arrests parasites at the ring-to-trophozoite stage transition and also specifically decreases intraparasitic levels of Cu/Zn SOD and catalase. Up to 150 μM BCS (2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid), an extracellular copper chelator, has no effect on parasite growth. We characterized a single copy PfCuP-ATPase (*Plasmodium falciparum* copper P-ATPase) transporter, which, like the *Cryptosporidium parvum* copper P-ATPase, has a single copper-binding domain: 'Met-Xaa-Cys-Xaa-Xaa-Cys'. Recombinant expression of the N-terminal metal-binding domain reveals that the protein specifically binds reduced copper. Transcription of the PfCuP-ATPase gene is the highest at late ring stage/early trophozoite, and is down-regulated in the presence of neocuproine. Immunofluorescence and electron microscopy indicate the transporter to be both in the parasite and on the erythrocyte membrane. Both the decrease in total copper and the location of the PfCuP-ATPase gene indicate a copper-efflux pathway from the infected erythrocyte.

Key words: chaperone, chelator, copper, neocuproine, P-ATPase transporter, *Plasmodium falciparum*.

INTRODUCTION

Malaria infects hundreds of millions of people, and causes over one million deaths each year as one of the four most important infectious diseases in the world [1]. Efforts to prevent and control the disease have been hindered by the development of resistance to therapeutic agents, increasing the need for the development of new antimalarial drugs [2]. Both the widely used quinoline and the artemisinin class of drugs are thought to interact with metals to kill *Plasmodium* parasites via free-radical intermediates [3,4]. As reactive metals, both iron and copper can participate in the free-radical-producing Fenton reaction [5]. The iron chelator DFO (deferoxamine) has been demonstrated to kill *Plasmodium falciparum in vitro* and enhance the clearance of *P. falciparum* in human infections, possibly by a toxic radical mechanism [6,7]. Likewise, many copper chelators can inhibit *P. falciparum* parasites *in vitro* [8,9]. One approach to antimalarial drug discovery is to investigate the copper metabolic pathways in *P. falciparum* in order to determine if copper chelators can be enhanced as antimalarials.

Copper is an essential trace element for all organisms as a required cofactor in many enzymes. As a reduction–oxidation-reactive metal, toxic free ionic copper is carefully sequestered by specific cellular mechanisms. Indeed, in *Saccharomyces cerevisiae*, less than one copper molecule per organism is in the free ionic state, despite a total concentration of 70 μM [10]. Erythrocytes contain about 20 μM copper, of which 70 % is contained in Cu/Zn SOD (cuprozinc superoxide dismutase) [11]. During the ingestion of the host cell cytoplasm, Cu/Zn SOD, along with haemoglobin, is taken up via the cytostome and is transported to the parasite's food vacuole, where the digestion occurs [12]. The fate of copper from the ingested Cu/Zn SOD is not known. In the present study, we address additional pathways concerning copper metabolism in *Plasmodium*. First, does the copper content in parasite-infected erythrocytes increase or decrease? Secondly, what is the effect of extracellular as well as intracellular copper chelators on the growth of malaria parasites and on the Cu/Zn SOD content? Thirdly, how does the PfCuP-ATPase (*P. falciparum* copper P-ATPase) transporter respond to copper chelation? Fourthly, what is the metal-binding specificity of the PfCuP-ATPase? Fifthly, where is the PfCuP-ATPase transporter located in the parasite-infected erythrocyte? In the present paper, we demonstrate that copper content decreases in infected erythrocytes, and that extracellular copper chelation does not inhibit parasite growth. The intracellular copper chelator, neocuproine, inhibits parasites and also reduces Cu/Zn SOD both in *Plasmodium*-parasite-infected erythrocytes, as well as in saponin-purified parasites. The PfCuP-ATPase, the largest of all copper transporter homologues, binds reduced copper and is located not only on parasite membranes, but also on the surface of the infected erythrocyte. Overall, the data suggest that *P. falciparum* parasites export copper to minimize its toxicity.

Abbreviations used: AAS, atomic absorption spectrophotometry; BCA, bicinchoninic acid; BCS, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; Cu/Zn SOD, cuprozinc superoxide dismutase; HRPII, histidine-rich protein II; Hsp70, heat-shock protein 70; MBD, metal-binding domain; MBP, maltose-binding protein; ORF, open reading frame; PfCuP-ATPase, *Plasmodium falciparum* copper P-ATPase; RT, reverse transcriptase; TC-PCR, tandem competitive PCR.

[1] To whom correspondence should be addressed (e-mail dsulliva@jhsph.edu).

EXPERIMENTAL

Cell culture

P. falciparum (strain 3D7) was maintained in culture plates at 37 °C in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated human serum at a haematocrit of 2 % under a O_2/CO_2/N_2 (1:1:18) atmosphere [13]. For studies on stage-specific expression, cultures were synchronized by sorbitol lysis [14].

Trace metal determinations

Synchronized trophozoite-stage-infected erythrocytes were isolated using the Percoll–sucrose gradient method, washed and resuspended in PBS treated with Chelex 100 (Sigma) [15]. Similarly, uninfected erythrocytes were used as a control. The measurements of total copper in whole cells were determined by AAS (atomic absorption spectrophotometry) using a PerkinElmer model 4000 graphite furnace at a wavelength of 324.8 nm and 15 mA lamp current. The following times and temperatures were used: drying at 140 °C for 30 s with 30 s ramp, charring at 1200 °C for 30 s with 30 s ramp and atomization at 2000 °C for 5 s. The S.D. is from triplicate biological preparations with quadruplicate AAS determinations.

Western blot analysis

Synchronized parasites at ring stage were grown in the presence or absence of copper chelator for 14 h. Both intact and saponin-purified cells were isolated. An equal number of cells was solubilized in sample buffer and separated by SDS/PAGE, transferred on to a nitrocellulose membrane (Protean Nitrocellulose, BA 85; Schleicher & Schuell) and probed with antibodies detected by ECL® (enhanced chemiluminescence; Amersham Biosciences). Antibodies against Cu/Zn SOD, catalase, spectrin and Hsp70 (heat-shock protein 70) were obtained from Sigma and 2G12 [anti-HRPII (histidine-rich protein II)] was kindly provided by Dr Diane Taylor (Department of Biology, Georgetown University, Washington, DC, U.S.A.) [16].

Drug inhibition

Synchronized parasite-infected erythrocytes were incubated with or without drug treatment overnight at 37 °C. Copper chelator neocuproine (2,9-dimethyl-1,10-phenanthroline) and BCS (2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid disodium salt) (Sigma) were used either freshly prepared or diluted from a stock solution stored at − 20 °C. The parasite growth was monitored in blood smears stained with DiffQuick stain (Dade-Behring Deerfield, IL, U.S.A.). The inhibitory effect of BCS and neocuproine on the parasites was measured by incorporation of radioactive hypoxanthine from triplicate 200 μl aliquots of synchronized parasites [17]. Percentage inhibition was calculated from the geometric mean parasitaemias of triplicate test and control wells as 100 × (control − test)/control.

TC-PCR (tandem competitive PCR)

Total RNA was isolated from synchronized *P. falciparum* cells 3D7 with or without drug treatment at 8 h intervals using TRIzol® reagent (Invitrogen). Total RNA was treated with RNase-free DNase I (Roche) in 100 mM sodium acetate, pH 5.0, re-purified and mixed with Superscript II RT (reverse transcriptase) and poly(T) to synthesize cDNA. To quantify the amount of cDNA for PfCuP-ATPase, a constant amount of cDNA was mixed with a series of dilutions of competitor plasmid containing a 540 bp product corresponding to PfCuP-ATPase nucleotide positions 997–1537, containing a introduced novel *Eco*R1 restriction site [18]. The competitor plasmid also contained the mutated β-tubulin insert as a constant expression control. After PCR, the individual products were digested with *Eco*RI and quantified using Kodak ID 2.0.2 image analysis Software (Ultra-Violet Products). After correction, the PfCuP-ATPase competitor plasmid/cDNA ratio was calculated for individual reactions and analysed [18]. Intron analysis utilized *PfHRPII* primers [19] and PfCuP-ATPase primers: forward 5′-ACATAATTTTATGTGTATTG-AAGG-3′ and reverse 3′-ACAAGCATCAGCACTGTCCATA-5′.

Expression of recombinant PfCuP-ATPase MBD (metal-binding domain)

To determine the ability of PfCuP-ATPase to bind heavy metals, MBP (maltose-binding protein) (New England Biolabs) fused to the putative PfCuP-ATPase MBD with the 'Met-Xaa-Cys-Xaa-Xaa-Cys' motif was constructed (MBP–MBD). The 1696 bp segment of the PfCuP-ATPase MBD was amplified using primers MBD-Forward (5′-TCTAGAGCCATGGCTAAATTGTCATTA-AC-3′) and MBD-Reverse (3′-AAGCTTTATGTTCATTATTTT-GGCTAC-5′). Protein concentrations were measured by Coomassie-plus protein assay (Pierce) using BSA as a standard.

Copper binding

Isolated MBP–MBD (5 μmol) was mixed with 100 μM $CuCl_2$, with or without 1 mM ascorbate. Unbound copper was removed by overnight dialysis in sodium phosphate buffer (Slide-A-Lyzer Cassette®; Pierce) at 4 °C and by adding cation-chelating resin, Chelex 100 (Sigma), to the dialysis buffer. The copper binding to MBP and the oxidation state of bound copper were assessed by a BCA (bicinchoninic acid) protocol according to [20], in which protein–copper complexes are disrupted by acid denaturation, and the copper is detected upon release. BCA is specific for reduced copper, Cu^+, forming a complex that is detectable at 360 nm. Ascorbate (1 mM) must be added to the sample following acid denaturation to yield a Cu^+–BCA complex. Solutions of $CuCl_2$ in sodium phosphate buffer were used as standards, and affinity-purified MBP served as a negative control.

The involvement of cysteine residues in co-ordination of copper was demonstrated by the ability of the metal to protect cysteine residues in the MBD against labelling with the cysteine-direct fluorescent reagent CPM [7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin] (Molecular Probes) [21].

Antibody production, purification and PfCuP-ATPase localization

Purified MBP–MBD (total 1 mg/ml) was used to immunize rabbits. Antibody responses were assessed by Western blot analysis, and the rabbits were bled 2 weeks after the fourth boost. Antiserum was affinity-purified by elution from MBP–MBD bound to Affigel-15 column (Bio-Rad). The localization of the copper transporter within parasites was determined using immunofluorescence and electron microscopy. For immunofluorescence, parasite blood films were air-dried, then slides were fixed with ice-cold methanol/acetone (1:1, v/v), blocked with 2 % BSA in PBS for 1 h at room temperature (23 °C), and incubated with affinity-purified antibody, pre-immune serum or antibody with the immunizing protein (5 μg/ml) for 1 h at room temperature [22]. After rinsing three times with PBS, slides were allowed to react with goat anti-rabbit IgG conjugated to fluorescein (Texas Red; Jackson Immunoresearch Laboratories) for 1 h at room temperature in the dark. Fluorescence imaging was performed on a Nikon Eclipse E800 microscope at a magnification

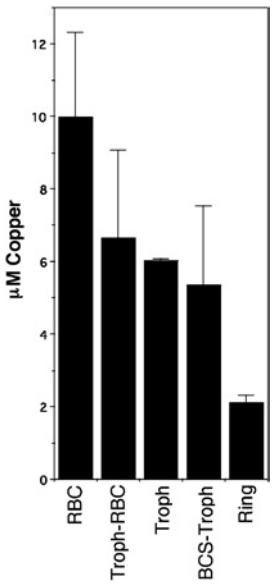

Figure 1 Copper content decreases in infected erythrocytes and isolated parasites

Copper content was determined by AAS in uninfected erythrocytes (RBC), 2.1 ± 0.50 ng/mg; trophozoite-infected erythrocytes (Troph-RBC), 4.2 ± 1.50 ng/mg; saponin-purified trophozoites (Troph), 15.2 ± 0.08 ng/mg; 100 μM BCS-treated saponin-purified trophozoites (BCS-Troph), 13.5 ± 5.48 ng/mg; and saponin-purified rings (Ring), 10.75 ± 1.10 ng/mg. These numbers were multiplied by 300 mg/ml, 100 mg/ml, 25 mg/ml and 12.5 mg/ml for RBC, Troph-RBC, Troph and Rings respectively to obtain a ng/ml value that was converted into molar concentration [24]. The S.D. is from triplicate biological preparations with at least quadruplicate AAS determinations.

of 100×. For electron microscopy, cells were fixed in 2.5 % (v/v) glutaraldehyde for 24 h at 4 °C, post-fixed in 1 % (w/v) osmium tetroxide in PBS, and dehydrated through ascending grades of ethanol: 70, 90, and 100 %. Cell preparations were then treated with propylene oxide for 30 min before embedding in EPON™ resin (SPI-Chem, West Chester, PA, U.S.A.) at 60 °C overnight. Ultra-thin sections cut with a diamond knife were stained with Toluidine Blue and visualized by light microscopy, or stained with uranyl acetate and lead citrate and examined using a JEOL 200FX transmission electron microscope [23].

RESULTS

Determination of copper content

Copper concentration in human serum is in the range 11–24 μM, largely due to caeruloplasmin. The normal erythrocyte has copper concentrations in the range 14–24 μM with 20 μM most commonly cited [11]. We measured copper levels by AAS, along with protein content and cell numbers in preparations of uninfected erythrocytes, Percoll-purified trophozoite-infected erythrocytes and saponin-isolated purified parasites (Figure 1). While the ng/mg values are slightly higher for parasite isolations, the concentration decreases because an intact infected erythrocyte has one-third of the protein content of an uninfected erythrocyte, and an isolated parasite has one-tenth of the protein content of an uninfected erythrocyte [24]. The determined 9.98 ± 2.3 μM concentration for copper in uninfected erythrocytes is at the low range of expected values. However, the 6.6 ± 2.4 μM concentration for the Percoll-purified infected trophozoites and 6.0 ± 0.03 μM for saponin-purified trophozoites is lower than our preparation of normal erythrocytes. BCS treatment does not affect trophozoite

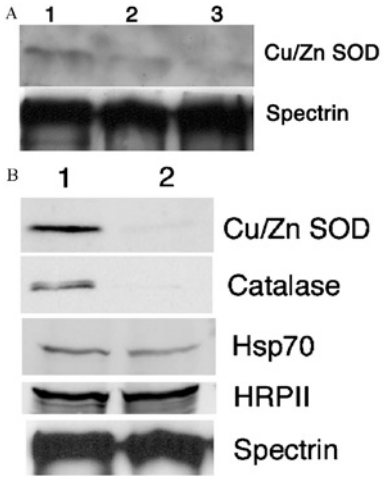

Figure 2 Erythrocyte and parasite Cu/Zn SOD content decreases

(**A**) Uninfected erythrocytes (lane 1) and intact synchronized parasite-infected erythrocytes harvested by Percoll purification at trophozoite (lane 2) and schizont (lane 3) stages were separated by SDS/PAGE, and probed with antibodies against human Cu/Zn SOD and spectrin, an abundant erythrocyte membrane-bound protein. A decrease in Cu/Zn SOD is apparent in the late stages of erythrocyte infection. (**B**) Equal amounts of protein from saponin-purified parasites without (lane 1) or with (lane 2) 14 h of neocuproine treatment were separated by SDS/15 % PAGE, blotted and probed with anti-Cu/Zn SOD and catalase. Antibodies against Hsp70, HRPII, spectrin and Band III were used as controls. Cu/Zn SOD content in neocuproine-treated isolated parasites is decreased compared with that of the control.

copper content. Saponin-purified rings have a 2.1 ± 0.2 μM concentration. The copper concentration of our complete medium with 10 % serum is 2.4 μM. Overall, this indicates that the *P. falciparum*-infected erythrocyte has less total copper than the uninfected erythrocyte.

Cu/Zn SOD content

Cu/Zn SOD is the most abundant copper-containing enzyme in erythrocytes, and may be the main source of bioavailable copper for the *Plasmodium* parasite. We followed the fate of Cu/Zn SOD protein in infected erythrocytes, rather than activity. Synchronized *P. falciparum*-infected erythrocytes, purified to more than 90 %, infected erythrocytes at trophozoite stage and schizont stage, had a decrease in Cu/Zn SOD content (Figure 2A). The level of Cu/Zn SOD in saponin-purified parasites treated with neocuproine decreased compared with untreated trophozoites harvested at the same time (Figure 2B). The Cu/Zn SOD level in neocuproine-treated parasites was also lower than that of isolated ring-stage parasites harvested at the time of drug application (results not shown). Similar content of human spectrin, parasite Hsp70 and *PfHRPII* is observed in both fractions, while the human erythrocyte-derived catalase declined like Cu/Zn SOD. Purified *P. falciparum* digestive vacuoles contained non-degraded Cu/Zn SOD, as shown by Western blot analysis (results not shown). This is consistent with the findings of Fairfield et al. [25,26] demonstrating lysosomal localization of host SOD in murine *Plasmodium*.

Effect of copper chelators (neocuproine and BCS) on growth of *P. falciparum*

To distinguish parasite sources of copper, synchronized cultures of early rings or schizonts were exposed to both intracellular and extracellular copper chelators. The IC_{50} for the intracellular copper chelator, neocuproine, was 300 ± 8.6 nM at 48 h and

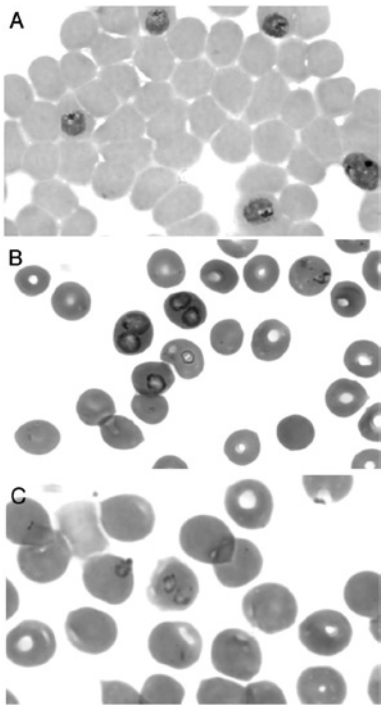

Figure 3 Effect of copper chelators on growth of *P. falciparum* synchronized parasites

Synchronized parasites at ring stage were incubated overnight with or without copper chelators. (**A**) Slide of the control parasites at trophozoite stage stained with DiffQuick. (**B**) Extracellular copper chelator BCS at 150 μM does not inhibit the development to trophozoite stage. (**C**) The intracellular copper chelator neocuproine (10 μM) arrests the growth of the parasites at ring stage.

326 ± 24.6 nM at 96 h by the radioactive hypoxanthine-incorporation assay, whereas no inhibition was seen up to 100 μM BCS at either time-point. Addition of 150 μM of the extracellular copper chelator BCS throughout the erythrocytic cycle of *P. falciparum* had no visible effect on the growth of the parasites (Figure 3B). When the neocuproine (10 μM) was added to synchronized cultures of early rings, parasite growth ceased (Figure 3C). However, the addition of 10 μM neocuproine to synchronized cultures of schizonts had no effect on schizont development to rings, but again inhibited the ring-to-trophozoite transition. Pre-incubation of uninfected erythrocytes overnight with 10 μM neocuproine, followed by a washout of drug and infection of these treated erythrocytes, had no effect on parasite development. The data indicate that neocuproine acts specifically at a ring-to-trophozoite transition stage, and that extracellular copper is not required by *P. falciparum* parasites.

PfCuP-ATPase protein sequence analysis

The *P. falciparum* genomic source PlasmoDB (http://plasmoDB.org) indicates that PfCuP-ATPase (PFI0240c) is located on chromosome 9 on the negative strand as a two-exon gene with a large 7342 bp first exon and a small 350 bp second exon, translating to a protein with 2564 amino acids with a predicted molecular mass of 298 622 Da. This exon structure is confirmed by PCR of cDNA and genomic DNA (Figure 4A). The full-length cDNA ORF (open reading frame) is more than 1000 amino acids larger than orthologues of the copper P-ATPase family. Additionally, the PfCuP-ATPase is 588 amino acids larger than the *Plasmodium yoelii* (PY00066) orthologue with one 230-amino-acid insert at amino acid number 530 of *P. yoelii* (600 for *P. falciparum*). The additional 350 extra amino acids are distributed in 40–60-amino-acid inserts every 100–200 amino acids between the two *Plasmodium* genes. We find partial sequences of the copper transporter in the unfinished *Plasmodium* genomes of *Plasmodium vivax*, *Plasmodium knowlesi*, *Plasmodium chabaudi* and *Plasmodium reichnowi*. A multiple sequence alignment with yeast, bacterial and mammalian orthologues is available at (http://plasmodb.org/cgi-bin/plasmodb/showOrthologGroup.pl?expID=4813&group=781547&view=align). The Kyte–Doolittle hydropathy plot of PfCuP-ATPase, alignment with orthologues and the TMAP, THMM, TMpred and TOPRED membrane prediction programs all suggest that PfCuP-ATPase contains ten transmembrane domains (Figure 4B). The first six and last two correspond to transmembrane domains of orthologues, while both *P. yoelii* and *P. falciparum* are predicted to have two more transmembrane domains within the extramembrane loop containing the ATP-binding motifs. Like all P-ATPases, the PfCuP-ATPase gene sequence possesses the invariant phosphorylation Asp^{1941}-Lys-Thr-Gly-Thr^{1945} motif containing the aspartate residue phosphorylated by ATP in the reaction cycle [27] and the Gly^{2454}-Asp-Gly-Ile-Asn-Asp-Cys-Phe^{2461} motif believed to be involved in ATP binding [28]. Specifically, there is a 250-amino-acid insert containing the extra two predicted transmembrane domains in both the *P. falciparum* and *P. yoelii* sequences after the Asp-Lys-Thr-Gly-Thr phosphorylation motif, but before the Gly^{2454}-Asp-Gly-Ile-Asn-Asp-Cys-Phe^{2461} ATP-binding motif shown (Figure 4B). PfCuP-ATPase also contains distinctive features of copper P-ATPases, including one metal-binding motif Met^{403}-Xaa-Cys-Xaa-Xaa-Cys^{408} at the N-terminal region preceding the first predicted transmembrane domain, and the intramembranous cation-transduction motif Cys^{1897}-Pro-Xaa^{1899} believed to function in heavy-metal transduction [29].

Search for metallochaperones and other metal-binding proteins in *P. falciparum*

Eukaryotic cells have a specialized family of proteins that serve to deliver copper for the enzymes that require the metal. These molecules are known globally as copper chaperones [30].We took a two-pronged *in silico* approach in our attempt to identify novel metallochaperones in the *P. falciparum* genome. In the first approach, we 'blasted' (BLASTP) the protein sequence of 12 previously characterized metallochaperones of both prokaryotic and eukaryotic origins in Table 1 against the PlasmoDB Version 4.0 ORFs >50 amino acids database. We identified a putative orthologue of the widely conserved mitochondrial cytochrome *c* oxidase assembly protein COX17 [31], but were unable to identify any further copper chaperones, most notably the copper chaperone to copper transporters that contain a Met-Xaa-Cys-Xaa-Xaa-Cys domain [30].

Our second approach made use of PlasmoDB Version 4.0 Motif Search Tool. We identified three molecular patterns characteristic of metal-binding proteins in PROSITE (metallothionein, PS00203; ferritin, PS00540; heavy-metal-associated domain, PS01047) and used these plus the copper-binding motif Met-Xaa-Cys-Xaa-Xaa-Cys to develop candidate metallochaperones from the ORFs >50 amino acids in the genomic database. Ideally, metallochaperones are of low molecular mass and lack transmembrane domains to facilitate rapid diffusion; we therefore limited our candidates to ORFs of 50–300 amino acids in size without transmembrane domains. We excluded further candidates with known similarity to metal-binding domains unrelated to metallochaperones, such as zinc-finger domains or enzymes with known metal cofactors. Notably, the *Plasmodium* genome has

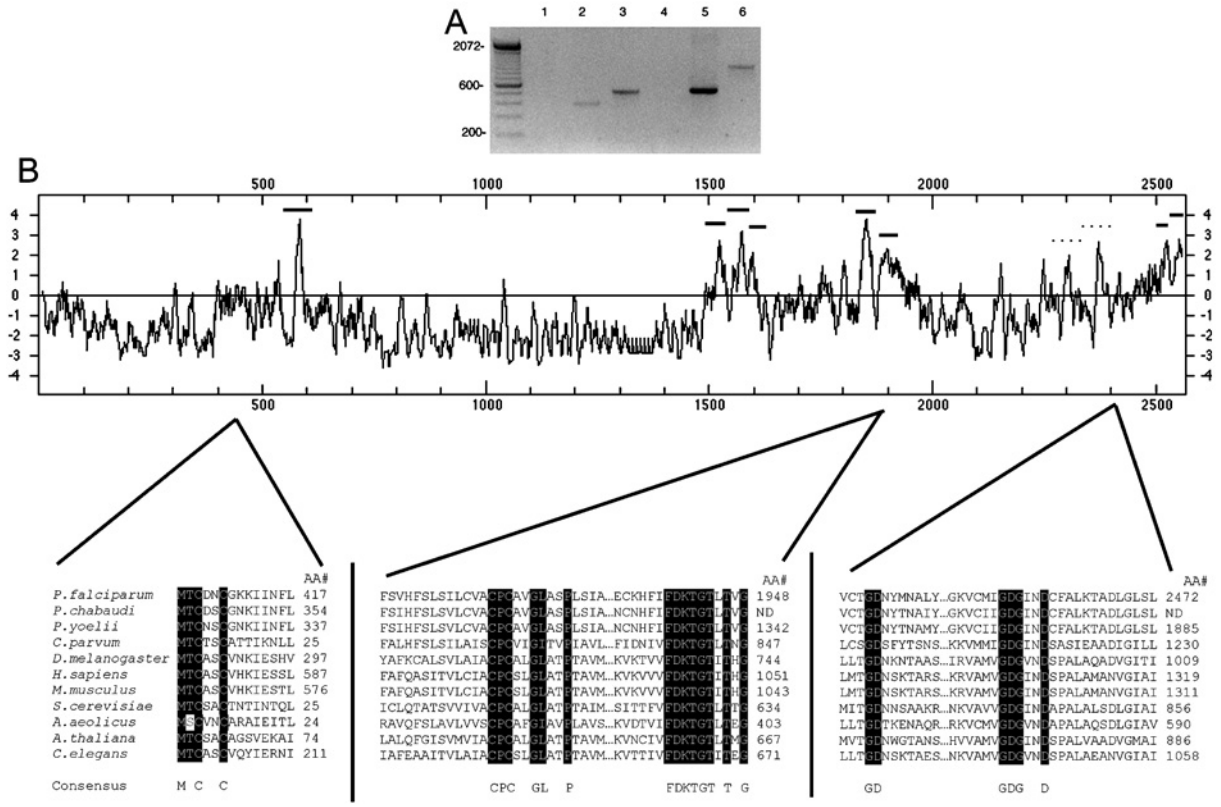

Figure 4 Genomic structure of PfCuP-ATPase reveals multiple large amino acid inserts

(**A**) PCR verification of small 3′ exon. Total RNA was reverse-transcribed and amplified with primers for PfCuP-ATPase (lanes 1–3) and *PfHRPII* (lanes 4–6). No template control (lane 1 and 4), RT-cDNA (lanes 2 and 5) and genomic DNA (lanes 3 and 6). (**B**) Hydropathy plot of PfCuP-ATPase using the Kyte–Doolittle algorithm shows eight similar transmembrane domains (lines) and the presence of a region approx. 800 amino acids long between the first and second transmembrane domains. The extra two potential transmembrane domains within the ATP-binding motif are marked with a dotted line. The alignment of the deduced protein sequence of PfCuP-ATPase from the 3D7 isolate with its orthologues reveals that cysteine residues that co-ordinate heavy-metal binding and the ATP phosphorylation domain Asp-Lys-Thr-Gly-Thr are completely conserved among all 11 transporters. Identical amino acids are shaded in black. *P. chabaudi* is partial sequence. *A. aeolicus*, *Aquifex aeolicus*.

Table 1 Metal chaperone orthologues

Metallochaperone	Source	GenBank® accession number	Functional family	Orthologue found
Prokaryote				
ureE	*Klebsiella aerogenes*	P18317	Ni siderophore	No
AAM94017	*Chlamydomonas reinhardtii*	AAM94017	Putative Cu chaperone	No
Ferredoxin I	*Pseudomonas aeruginosa*	NP_252311	Ferredoxin	No
FepC	*Pseudomonas aeruginosa*	NP_252847	Fe siderophore	No
Fungus				
Copper chelatin	*S. cerevisiae*	P07215	Metallothionein	No
COX17	*S. cerevisiae*	S62056	Cu chaperone	Yes
ATX1	*S. cerevisiae*	S47930	Cu chaperone	No
LYS7	*S. cerevisiae*	NP_013752	Cu chaperone	No
Sit1p	*S. cerevisiae*	NP_010849	Ferroxamine transport	No
Plant				
CCH	*Arabidopsis thaliana*	AAC33510	Cu chaperone	No
T52130	*Arabidopsis thaliana*	T52130	Putative Cu and Zn chaperone	No
Animal				
Ferroportin I	*Mus musculus*	AAF82036	Ferroportin	No

no orthologous sequence to metallothioneins, ferritins or heavy metal domains. The orthologue to the copper chaperone in yeast that loads the copper P-ATPase with mirror Met-Thr-Cys-Asp-Asn-Cys motif is absent in *P. falciparum*. Of three low-molecular-mass proteins, less than 1400 amino acids containing Met-Xaa-Cys-Xaa-Xaa-Cys, PFI1555w (185 amino acids) has four transmembrane domains with a Met-Ser-Cys-Gln-Lys-Cys motif, and an orthologue only in *P. yoelii*. PF14_0617 (278 amino acids), with a Met-Arg-Cys-Lys-Glu-Cys motif, has one transmembrane domain and matches only a gene in *P. yoelii*. However,

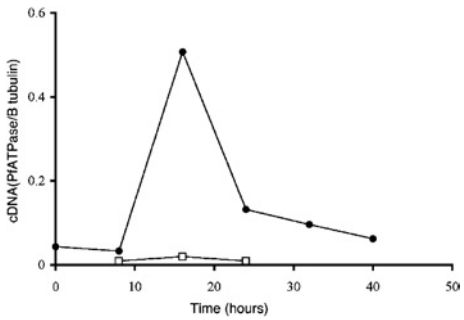

Figure 5 PfCuP-ATPase transcripts peak at ring to trophozoite stages

The amount of PfCuP-ATPase and β-tubulin cDNA from each time-point obtained from synchronized parasite cultures in the absence (●) or presence (□) of neocuproine was calculated from luminometric data by linear regression, and plotted as the ratio of PfCuP-ATPase/β-tubulin (vertical axis). Very little PfCuP-ATPase mRNA relative to β-tubulin is detectable in very early stages of erythrocyte development. Our results show that PfCuP-ATPase mRNA has the most expression at late ring or early trophozoite stage of the life cycle. The absolute β-tubulin values varied little in the presence or absence of neocuproine. Results are means of two separate RNA isolation experiments.

MAL6P1.300 (482 amino acids), with the motif Met-Leu-Cys-Glu-Lys-Cys, has multiple orthologues all containing Met-Xaa-Cys-Xaa-Xaa-Cys motifs in other *Plasmodium* species as well as *S. cerevisiae*, *Mus musculus*, *Homo sapiens*, *Escherichia coli*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana* and *Anopheles gambiae*, but whose function is unknown. Only TMAP predicts a transmembrane domain. This is the best candidate for a putative PfCuP-ATPase copper chaperone by *in silico* searching based on the yeast motifs.

Transcription of PfCuP-ATPase with and without drug treatment

We characterized the pattern of PfCuP-ATPase mRNA expression using synchronized cultures of asexual stages. The technique of TC-PCR was used to quantify precisely the amount of PfCuP-ATPase mRNA relative to that of a housekeeping gene, β-tubulin (Figure 5). We confirmed the precision and reproducibility of this ratiometric method by assessing the gene copy number of PfCuP-ATPase relative to that of β-tubulin, using genomic DNA as a template in two independent experiments. We showed that expression of the copper transporter is under developmental control during the life cycle of the parasite, with an early peak of mRNA expression at 16 h. Very little PfCuP-ATPase mRNA, relative to that of β-tubulin, is detectable in the early stage of erythrocyte development. We also monitored the gene expression when the synchronized parasite cultures were treated with neocuproine. Our result reveals that PfCuP-ATPase transcription at 16 h is down-regulated in the presence of neocuproine. Absolute levels of mRNA for β-tubulin are approximately the same with or without drug treatment at the 16 h time-point.

PfCuP-ATPase copper binding

We showed that the MBP–MBD protein in the presence of ascorbate binds copper, whereas no copper binding occurs for the control MBP alone (Figure 6A). The metal binding is approximately equivalent to one copper ion bound per MBD, consistent with Menkes P-ATPase cation binding [32]. If ascorbate is not added during the initial incubation with $CuCl_2$, then no copper is bound. This suggests that the PfCuP-ATPase MBD does not bind Cu(II). To examine the role that cysteine residues play in copper binding, the ability of the metal to protect cysteine residues in the metal-binding motifs against labelling with CPM was compared.

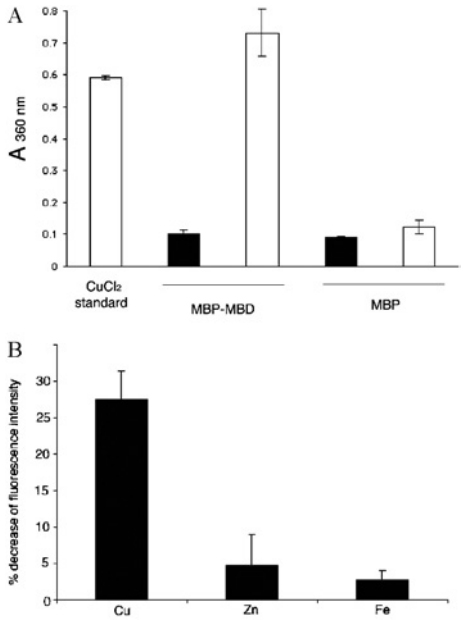

Figure 6 Cu^+ binds to MBD–MBP

(**A**) N-terminal domain fusion protein MBP–MBD or MBP was incubated with copper. Following dialysis overnight, in the presence (black bars) or absence (white bars) of reducing agent (1 mM ascorbate), each duplicate dialysis bag was collected and the BCA assay was used to determine the amount of bound copper. Absorbance (A_{360}) readings represent the amount of BCA–Cu^+ complex formed under each experimental condition. MBD–MBP proteins bound to Cu^+ in the presence of ascorbate, whereas no copper binding was observed for control proteins. The concentration of copper standard ($CuCl_2$) was equimolar to the amount of protein in the assays. Results are means ± S.E.M. of triplicate measurements from each duplicate dialysis bag. (**B**) MBD–MBP protein was isolated from *E. coli* cells grown in the presence of copper (Cu), zinc (Zn) or iron (Fe), and then labelled with CPM to monitor the metal binding of the Met-Xaa-Cys-Xaa-Xaa-Cys motif in PfCuP-ATPase. Fluorescence of labelled proteins separated by SDS/10 % PAGE was detected under a UV lamp. The densitometry of bands was quantified, and differences in fluorescence of CPM-labelled fusion proteins in the presence and absence of $CuCl_2$ were calculated from these data. A decrease in fluorescence intensity for MBD–MBP in presence of $CuCl_2$ indicates that copper binds to cysteine residues in the MBP–MBD, and protects these cysteine residues against labelling with fluorescent CPM. Fusion proteins from zinc- and iron-treated cells showed no decrease in fluorescence, indicating that the metals did not bind to cysteine residues. Results are means ± S.E.M. for four different experiments.

CPM labelling of MBP–MBD (+ascorbate) purified from *E. coli* cells grown in the presence or absence of copper was studied. We monitored the differences in the fluorescence under UV light and the band densities, and quantified them using image reader LAS-1000 software (Fujifilm). In the present study, we observed a decrease in fluorescence intensity for MBP–MBD in the presence of $CuCl_2$ when compared with an identical amount of protein without copper (Figure 6B). Binding of copper to the MBP-MBD domain significantly decreases the availability of the cysteine residues for modification with the cysteine-directed probe. This indicates that cysteine residues are probably directly involved in the co-ordination of copper. MBP–MBD isolated from *E. coli* transformants cultured with 0.5 mM $ZnCl_2$ and $FeCl_2$ were similarly tested for the ability to interact with CPM. We saw insignificant binding of zinc or iron to the cysteine residues for the fusion protein from zinc- and iron-treated cells.

Localization of PfCuP-ATPase

The affinity-purified antiserum to PfCuP-ATPase recognizes a 298 kDa band in *P. falciparum* trophozoites (Figure 7A). The band is not visible in uninfected erythrocytes, ring stages or with pre-immune serum, and is suppressed by pre-incubation

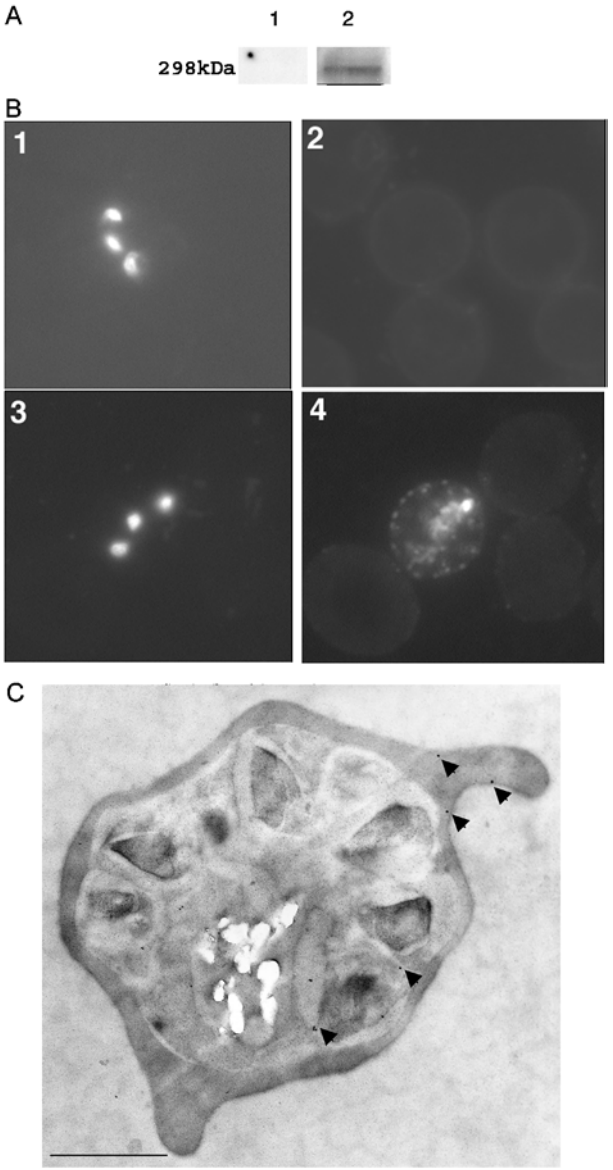

Figure 7 PfCuP-ATPase locates to both the plasma and erythrocyte membranes

(**A**) Western blot of PfCuP-ATPase in trophozoites using affinity-purified polyclonal antibody: pre-immune serum (lane 1), immunoaffinity-purified serum (lane 2). (**B**) Localization of native PfCuP-ATPase in trophozoite-infected erythrocytes using immunofluorescence microscopy: panels 1 and 3, DAPI (4,6-diamidino-2-phenylindole)-fluorescent image; panel 2, pre-immune serum (Texas-Red-labelled cells); panel 4, (Texas-Red-labelled cells) affinity-purified polyclonal antibody. The brightest signals were observed in late trophozoite and schizont stages. Signal was competed with MBP–MBD and not MBP alone. (**C**) Trophozoite/schizont-infected erythrocytes were stained with antibody against PfCuP-ATPase using a gold pre-embedding immunoelectron-microscopic method. The arrows indicate signals both on the parasite as well as on the erythrocyte membrane. The scale bar is 1 μm.

of the antiserum with the immunogen, and not by MBP alone. In immunofluorescence illumination of *P. falciparum* parasite blood smears, signal is seen on the surface of the infected erythrocyte in addition to the parasite (Figure 7B). No staining is visible in uninfected erythrocytes and ring-infected erythrocytes. Fluorescent signal disappears with competition of immunogen and not competition with MBP alone. Electron microscopy (Figure 7C) also shows a portion of signal both on the surface of erythrocytes and on membranes of a schizont-stage parasite in this case.

DISCUSSION

Copper is an essential trace element for all organisms, due to its action as a cofactor in many enzymes, but it is also potentially toxic because, like iron, copper can donate or receive electrons. Thus all organisms have devised cellular mechanisms for sequestration and detoxification that keep free ionic copper at low concentrations [33]. Many organisms are able to respond to increased extracellular copper with efflux mechanisms [34,35]. The finding of a decrease in copper content in the *Plasmodium*-infected erythrocyte also implies an efflux mechanism for its metabolism of copper. Extracellular copper chelation by BCS indicates that *Plasmodium* does not require import of copper from serum, but can rely on erythrocyte sources. Marva et al. [36] have also shown that 5 μM added copper sulphate inhibits more than 50 % of parasite growth, and the inhibition is reversed by 200 μM diethylenetriaminepenta-acetic acid (an impermeant metal chelator). Cu/Zn SOD is the most abundant copper-containing enzyme in the erythrocyte, and is believed to be one possible source of bioavailable copper for the *Plasmodium* parasite. The *Plasmodium* parasite synthesizes both an iron and manganese SOD and lacks a gene encoding a Cu/Zn SOD. During the ingestion of the host cell cytoplasm, erythrocyte Cu/Zn SOD and catalase, along with haemoglobin, are taken up via the cytostome and is deposited in the parasite's food vacuole, where host cytosol digestion occurs [12]. Fairfield et al. [37] proposed that Cu/Zn SOD may escape digestion and either remain within the food vacuole or emigrate into the parasite cytoplasm. However, the decrease in Cu/Zn SOD content shown here implies that degradation of Cu/Zn SOD occurs in the digestive vacuole, which would release both copper and zinc. Interestingly, transgenic mice created with overexpression of Cu/Zn SOD had similar levels of parasitaemias, despite levels of erythrocytic Cu/Zn SOD activity of 1.6 times the normal erythrocyte amount [38]. These transgenic mice also showed no difference in parasite-infected erythrocyte response to ascorbate or paraquat, indicating that host response, and not parasite development, was associated with increased mortality in these *Plasmodium berghei*-infected mice. This also indicates that these mice were able to tolerate an increase in erythrocyte copper content.

Neocuproine inhibits parasite growth specifically at ring-to-trophozoite stage transition. One mechanism is that neocuproine could enter the infected erythrocyte by diffusion and then chelate copper from copper-containing proteins. However, pre-treatment of uninfected erythrocytes followed by a washout of drug did not affect subsequent invasion and development of parasites. Our IC_{50} is approximately the same as the 100 nM seen when neocuproine was dosed pre-complexed with copper [8]. This indicates that neocuproine is still potent when delivered with copper already bound. Scheibel and Adler [39] proposed that the potency of the neocuproine is similar to that of other phenanthrolines with the formation of a lethal complex taken up by the cell. A radioactive uptake assay proved that neocuproine forms a complex with copper which rapidly enters into the parasitized cells in 10 min, compared with 6 h in normal erythrocytes [8]. Recent reports indicate that chelated copper is more redox active than free copper. Indeed, neocuproine induced hydrogen-peroxide-mediated DNA damage, whereas copper alone did not [40]. Additionally, a copper complex to buparvaquone was the most potent of the metal complexes in *P. falciparum* growth inhibition [41]. We suggest that,

under normal physiological conditions, copper does not participate in Fenton chemistry to produce free radicals, but that membrane-permeant copper chelators may increase redox activity of copper to participate in oxidative damage in *Plasmodium*. Neocuproine decreasing the Cu/Zn SOD may indicate that cytostomal ingestion is affected before inhibition of protein degradation of the food vacuole contents, which would preserve levels of some proteins from the parasite, but not preserve levels of ingested host proteins such as catalase and Cu/Zn SOD. Human erythrocyte catalase has been shown to be confined to the digestive vacuole and ingestion apparatus for erythrocyte cytosol [42].

Transport proton pumps are used by the cell to maintain homoeostatic levels of copper. We characterized the single copy of PfCuP-ATPase transporter in *P. falciparum*.

The predicted 298 kDa molecular mass of PfCuP-ATPase is double that of *C. parvum* and other eukaryotic Menkes and Wilson disease copper transporter orthologues because of inserts, even in important functional domains, common to many *Plasmodium* proteins [43,44]. The presence of the extra two transmembrane domains within the ATP-binding loops remains to be verified. The RT-PCR assay identified transcription highest at early trophozoite stage rather than late trophozoite/early schizont shown by Bozdech and DeRisi [45]. The discrepancy may be accounted for by their use of a reference control from pooled RNA samples representing all developmental stages, while the RT-PCR compared individual time-points with β-tubulin. Regardless, neocuproine decreased PfCuP-ATPase at this maximum transcription point. In yeast, copper deprivation increases copper ATPase transcription [46], while in bacterial systems, where the copper ATPase functions as an efflux molecule, copper deprivation decreases transcription [47]. At present, our data argue more for a chelator-metal inhibition of specific transcription, rather than deprivation of bioavailable copper by neocuproine decreasing the need for efflux. The copper chelator pyrrolidine dithiocarbamate has been shown to decrease Pax-8 mRNA level and its DNA-binding activity in rat thyroid follicular cells [48]. In endothelial cells, neocuproine also inhibited TNFα (tumour necrosis factor α)-induced protein expression of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 [49]. In a bacterial reporter system, a neocuproine–copper complex bound at transcription sites to inhibit RNA polymerase [50]. Microarray analysis will investigate this phenomenon in greater detail with regard to dose- and time-dependence of transcriptional changes.

The fusion protein MBP–MBD containing the metal-binding motif Met-Thr-Cys-Asp-Asn-Cys of PfCuP-ATPase *in vitro* selectively binds Cu^+ with a stoichiometry of 1:1, but does not bind zinc or iron. Like its orthologue in *C. parvum*, the cysteine residues in MBD in PfCuP-ATPase seem to be involved directly in the specificity of copper co-ordination [44]. In the *P. falciparum* genome database, the Met-Thr-Cys-Asp-Asn-Cys motif was found only in PfCuP-ATPase. In eukaryotic cells, copper chaperones can acquire the metal under conditions where metalloenzymes cannot, then function to deliver and transfer copper directly to specific cellular targets [33]. In essence, these molecules act to escort copper ions and protect them from copper-scavenging mechanisms. In yeast, extensive research on the pathways for copper transfer from copper transporter Ctr to metallochaperone or from chaperone ATX1 to copper transporter CCC2 indicate concordance with the chaperone metal-binding motif and transporter motif [33]. Despite annotation of the genome, many metal chaperones in *Plasmodium* remain to be described.

The immunolocalization studies suggests that the *Plasmodium* copper transporter is both on the plasma membrane of the parasite as well as on the surface of the erythrocyte. For transport out of erythrocytes, two separate targeting pathways would be required: one to the parasite plasma membrane and one to the erythrocyte surface. Many of the rifins, stevors and variant surface antigens, which contain single transmembrane domains, require delivery through the erythrocyte cytosol to the erythrocyte surface. The Mauer's clefts and specialized vesicles are two postulated potential pathway for delivery of parasite membrane proteins to the surface of the host cell [51]. The ATP-binding cassette PFGCN20 was demonstrated to localize to both the parasite and infected erythrocyte membranes [52].

In summary, we report that copper content decreases in the infected erythrocyte. We located the copper P-ATPase to both the parasite and erythrocyte surface, suggesting that *P. falciparum* avoids copper toxicity by efflux. The *P. falciparum* genome annotation indicates that many of the mammalian copper-containing proteins are absent, except for cytochrome *c* oxidase and the copper-binding protein *S*-adenosylhomocysteine [53]. Without a Cu/Zn SOD and many of the copper-requiring oxidases, the *Plasmodium* parasite may not require much bioavailable copper [54]. This agrees with the copper content analysis and observation of a decrease in Cu/Zn SOD content. While normal copper pathways avoid generating oxygen radicals, intracellular chelators of copper may exert toxicity by this mechanism.

We thank Valeria Culotta and Laran Jensen for assistance with AAS. We thank Leo Slater and Oluwatosin Gisanrin for critical reading of the manuscript. D. R. thanks Jan Tachezy. This work was supported by grants from Burroughs Wellcome Career Award in Biomedical Sciences, Pew Scholars Program in Biomedical Sciences and NIH (National Institutes of Health) RO1 AI45774-01. A NCCR (National Center for Cancer Research) grant GPDGCRC RR0052 supports the use of erythrocytes for culturing *P. falciparum*.

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Received 2 March 2004/19 April 2004; accepted 5 May 2004
Published as BJ Immediate Publication 5 May 2004, DOI 10.1042/BJ20040335