BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

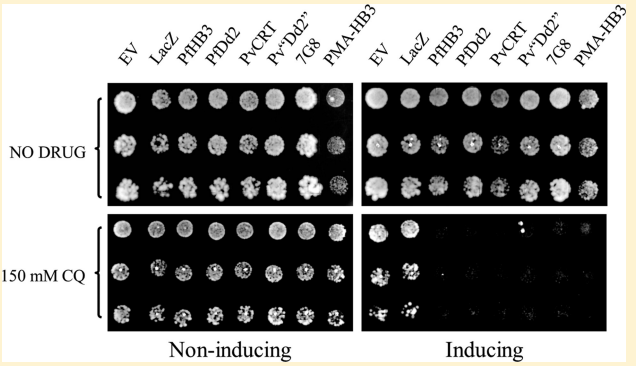
pubs.acs.org/biochemistry

Analysis of Chloroquine Resistance Transporter (CRT) Isoforms and Orthologues in *S. cerevisiae* Yeast

Nicholas K. Baro, Chaya Pooput, and Paul D. Roepe*

Department of Chemistry, Department of Biochemistry and Molecular Biology, and Center for Infectious Diseases, Georgetown University, 37th and O Streets, NW Washington, D.C. 20057, United States

ABSTRACT: Previous work from our laboratory optimized MeOH-inducible expression of the *P. falciparum* malarial parasite transporter PfCRT in *P. pastoris* yeast. These strains are useful for many experiments but do not allow for inducible protein expression under ambient growth conditions. We have therefore optimized galactose-inducible expression of PfCRT in *S. cerevisiae* yeast. We find that expression of PfCRT confers CQ hypersensitivity to growing yeast and that this is due to plasma membrane localization of the transporter. We use quantitative analyses of growth rates to compare hypersensitivity for yeast expressing various PfCRT isoforms. We also report successful high level inducible expression of the *P. vivax* orthologue, PvCRT, and compare CQ hypersensitivity for PvCRT vs PfCRT expressing yeast. We test the hypothesis that hypersensitivity is due to increased transport of CQ into yeast expressing the transporters via direct ^{3}H-CQ transport experiments and analyze the effect that membrane potential has on transport. The data suggest important new tools for rapid functional screening of PfCRT and PvCRT isoforms and provide further evidence for a model wherein membrane potential promotes charged CQ transport by PfCRT. Data also support our previous conclusion that wild type PfCRT is capable of CQ transport and provide a basis for understanding the lack of correspondence between PvCRT mutations and resistance to CQ in the important malarial parasite *P. vivax*.



Since the isolation of the *pfcrt* gene 10 years ago,[1] much has been learned regarding the encoded PfCRT protein and its role in conferring chloroquine resistance (CQR) in *P. falciparum* malarial parasites.[2,3] Geographically distinct patterns of amino acid substitutions in PfCRT are associated with a variety of different CQR phenotypes in *P. falciparum* isolates from around the globe. The different phenotypes are further characterized by cross resistance patterns to other drugs and variable contributions from additional allele inheritances, gene mutations, or gene overexpression events that work in concert with PfCRT mutations to cause antimalarial multidrug resistance.[4−6] One additional gene that appears to be involved in multidrug resistance is *pfmdr1*, which encodes a human P-glycoprotein orthologue that is mutated and/or alternately expressed in many CQR isolates.[7−9]

Further elucidation of the PfCRT molecular mechanism is essential for controlling current and newly evolving drug resistance phenomena and for developing second tier drug therapies. As one example of the former, CQR has been found in the related malarial parasite *P. vivax*, similar to *P. falciparum*,[10,11] albeit in a different geographically disposed pattern. However, unlike the case for PfCRT and *P. falciparum*, amino acid substitutions in the PvCRT orthologue are apparently not required for *P. vivax* CQR.[10] It would be helpful to translate concepts elucidated for *P. falciparum* CQR toward progress in understanding *P. vivax* CQR. Unfortunately, many approaches for studying CQR in *P. vivax* are inaccessible since there are no convenient laboratory-based methods available for culturing *P. vivax*. Heterologous expression of key *P. vivax* proteins, compared side-by-side with their *P. falciparum* orthologues, might provide one avenue for elucidating some aspects of *P. vivax* biology. For example, a model for why PvCRT mutations are apparently not related to *P. vivax* CQR could conceivably be generated from such experiments.

Previously, we have shown that both wild type ("HB3" isoform) and mutant CQR associated ("Dd2" [SE. Asian] and "7G8" [S. American]) isoforms bind CQ.[12,13] Surprisingly, affinity constants for equilibrium binding of ^{3}H-CQ were found to be similar for HB3 and Dd2 PfCRT.[12] Subsequent use of a CQ photoaffinity analogue and mass spectrometry allowed us to map a single specific quinoline binding site for HB3 PfCRT.[13] The predicted site lies proximal to PfCRT residues that have been found to be mutated in CQR conferring isoforms.

At low external doses of CQ, infected red blood cells harboring CQR parasites accumulate less CQ relative to CQS.[14−16] Also, CQ is known to concentrate within the parasite digestive vacuole (DV),[16,17] and subcellular PfCRT

Received: June 15, 2011
Revised: July 8, 2011
Published: July 11, 2011

protein localization includes high levels within the DV membrane.[1,18] It has thus been proposed that PfCRT mediates CQ transport from the DV in some fashion in order to lower CQ accumulation within the DV. As summarized elsewhere,[2] there is considerable support for this overall model, yet a number of issues remain to be resolved. Two recent studies have provided direct evidence for PfCRT-mediated CQ transport. One uses oocytes injected with *pfcrt* cRNA to measure slow, but heightened, accumulation of ^{3}H-CQ into the oocytes vs time,[19] and the other uses purified PfCRT protein reconstituted into proteoliposomes (PLs) and a novel fluorescent CQ reporter to measure pH gradient (ΔpH) and electrical potential (ΔΨ) stimulated efflux from the PLs.[20] Some differences in the two studies are found; in particular, estimates of turnover (drug transported per PfCRT per second) are much slower for the oocyte experiments relative to PL, and the PL study reports transport by both HB3 and Dd2 PfCRT isoforms, whereas the oocyte study suggests that only Dd2 PfCRT is capable of drug transport (see ref 2 for a more detailed discussion).

For these reasons and others, we have endeavored to devise additional convenient model systems for the study of PfCRT isoforms as well as PvCRT and other malarial transport proteins. Metabolic induction of transporters in *S. cerevisiae* growing under ambient conditions is in theory another such system, but high AT bias and unusual gene structure for some *P. falciparum* genes, as well as cellular targeting issues, often complicate application of these techniques. In this study, we have perfected pYES2-based galactose-inducible expression of PfCRT and PvCRT. We use this tool to further analyze questions related to PfCRT-mediated CQ transport and to also test for putative CQ transport by the important PfCRT orthologue PvCRT.

MATERIALS AND METHODS

Materials. Yeast DOB media and DOB with galactose and raffinose were obtained in powder form from MP Biomedicals (Solon, OH). Cell culture plastics were from BD Falcon. Glass beads for yeast cell lysis were from B. Braun Biotech (Allentown, PA). Anti-HexaHis-HRP and anti-V5-HRP antibodies were from Qiagen (Valencia, CA) and Invitrogen (Carlsbad CA), respectively. ^{3}H-CQ was from American Radiolabeled Chemicals Inc. (St. Louis, MO). All other chemicals were reagent grade or better, purchased from Sigma (St. Louis, MO), and used without additional purification.

Yeast Strains and Methods. CH1305 (MATa *ade2 ade3 ura3-52 leu2 lys2-801*) was supplied by J. F. Cannon.[21] Solid and liquid media were prepared as described in Sherman *et al.*[22] and included synthetic complete (SC) media lacking one or more specified amino acids as well as rich medium (YPAD or YPD). Induction of CRT protein expression was achieved by adding 2% galactose and 1% raffinose to SC media (then called SGR media) lacking uracil. Standard procedures were used for yeast manipulations.[22] Parental host strains were maintained in YPAD or YPD media and grown at 30 °C. Yeast transformations were done by the lithium acetate procedure[23] with 1 μg of pYES2 backbone containing specified construct (into the same host strain for each set of plasmids described.) Transformants were plated onto SD media lacking uracil. All subsequent cultures originated from frozen stock glycerol suspensions of individual clones.

Plasmids. A codon-optimized version[24] of the wild type *P. falciparum* chloroquine resistance transporter (*pfcrt*) cDNA sequence, designed with a 5′- Kozak sequence and both V5 and polyhistidine C-terminal epitope tags, was synthesized by Genescript (Piscataway, NJ) and cloned into vector pUC57 to create pUC57/PfHB3vh. The PfHB3vh fragment was restricted from pUC57/PfHB3vh with *Kpn*1 and *Eco*RI and subcloned behind the GAL1 promoter of *Kpn*1/*Eco*RI-digested plasmid pYES2 (kindly provided by Dr. William Fonzi, Georgetown University) to create pYES2/PfHB3vh. To generate PfHB3$_{PMA}$, encoding HB3 PfCRT fused in frame to the N-terminal domain of the yeast plasma membrane ATPase (PMA), the 333-bp fragment encoding the first 111 amino acids of PMA was synthesized by Genescript and inserted into pUC57/PfHB3vh to create pUC57/PfHB3$_{PMA}$vh. In this construct, the 50 N-terminal amino acids of wild type (isoform HB3) PfCRT were replaced by the N-terminal 111 amino acids of yeast plasma membrane ATPase (PMA1). The PfHB3$_{PMA}$vh fragment was restricted from pUC57/PfHB3$_{PMA}$vh with *Kpn*1 and *Eco*RI and subcloned behind the GAL1 promoter of *Kpn*1/*Eco*RI-digested plasmid pYES2. pYES2/PfDd2vh and pYES2/Pf7G8vh, encoding epitope tagged CQR-associated Dd2 and 7G8 isoforms of PfCRT, respectively, were created by ligating fragments of pPIC3.5/PfDd2h and pPIC3.5/Pf7G8h (see refs 12 and 13), digested with *Sex*AI and *Aar*I, into *Sex*AI/*Aar*I-digested pYES2/PfHB3vh (see Table 1).

Table 1. Plasmids Used in the Present Study

plasmid	feature	source
pYES2	GAL1 PROM_MCS_V5_HIS, URA3 2μ	commercial vector
pPIC3.5/HB3hb	PfHB3hisbad, HIS	ref 12
pYES2.1/LacZ	LacZ, URA3, 2μ	commercial vector
pUC57/PfHB3vh	pBR322, PfHB3vh	present study
pUC57/PfHB3$_{PMA}$vh	pBR322, PfHB3$_{PMA}$vh	present study
pYES2/PfHB3vh	PfHB3vh, URA3, 2μ	present study
pYES2/PfHB3$_{PMA}$vh	PfHB3$_{PMA}$vh, URA3, 2μ	present study
pYES2/PfDd2vh	PfDd2vh, URA3, 2μ	present study
pYES2/Pf7G8vh	Pf7G8vh, URA3, 2μ	present study
pYES2/PvCRTvh	PvCRTvh, URA3, 2μ	present study
pYES2/PvCRT$_{Dd2}$vh	PvCRT$_{"Dd2"}$vh, URA3, 2μ	present study

To express *P. vivax* chloroquine resistance transporter (PvCRT), pYES2/PfHB3vh was linearized with *Bst*EII and pPICZ-A/PvCRTmh containing the native *pvcrt* cDNA was linearized with *Sac*II. After filling in, fragments were further restricted with *Kpn*I and ligated to plasmid pYES2 to create pYES2/PvCRTvh. Site-directed mutagenesis via the Stratagene QUICKChange method was used to set V5 and hexaHis epitope tags back in frame. pYES2/PvCRTvh was used to create a "Dd2-like" isoform of PvCRT. Primers Pv^{Q}271^{E}, Pv^{N}326^{S}, Pv^{I}356^{T}, Pv^{M}371^{I}, and Pv^{MIK}74−76^{IET} (see Table 2) were used to create pYES2/PvCRT$_{"Dd2"}$vh. All final plasmid constructs were confirmed by direct DNA sequencing.

Isolation of Yeast Membrane Fractions. Overnight cultures of yeast cells grown in inducing SGR-ura medium to midlog phase were harvested at 2000*g* for 5 min at 25 °C. Cells were washed in harvest buffer (100 mM glucose/50 mM imidazole/5 mM DTT, pH 7.5) and lysed by rapid mixing in

Table 2. Oligonucleotides Used in the Present Study

name	sequence
$PfDd2^{R}$ 371^{I}	5′-CTTAGCAGGTGATGTCGTAATAGAACCACGTTTGTT-3′
PvCRTinsrG(5′)	5′-CACCCCGGGCCGGGTCACCCATTCG-3′
PvCRTinsG(3′)	5′-CGAATGGGTGACCCGGCCCGGGGTG-3′
$Pv^{Q}271^{E}$	5′-CACCCTCCCATTTTTGAAGGAGATCAATTTGCCCTTCTCGG-3′
$Pv^{N}326^{S}$	5′GAAAACCTTCATAGCCTATTCCTTTTTCAGCATTTGCGACAATTTAATC-3′
$Pv^{I}356^{T}$	5′-GCATACAGGGACCAGCCACGGCCATCGCCTACTA-3′
$Pv^{M}371^{I}$	5′-GGAGACGCCGTCATACAACCCCGCATGTT-3′
$Pv^{MIK}74-76^{IET}$	5′-TCTGCTGATTATCGTTTACCTGTGCGTATGTGTAATAGAGACGCTGCTGGCAAAGAGGACTC-3′

the presence of glass beads (1.00−1.05 mm diameter). Suspensions (10 g wet weight of cells, 20 mL of breaking buffer [0.25 M sucrose/0.1 M glucose/50 mM imidazole/1.0 mM $MgCl_2$, pH 7.50], equal volume of glass beads) were homogenized using a BioSpec BeadBeater (Bartlesville, OK) at 3000 rpm for 20 min at 4 °C (for large-scale preparations) or, for 30 s followed by 30 s on ice, 8 times, for small-scale preparations. Suspensions were decanted away from the glass beads and centrifuged twice at 1000*g* and once at 3000*g* for 5 min each, to remove unlysed cells and cellular debris. The supernatant was collected and spun again at 100000*g* to collect the crude membrane (CM) fragment. To prepare pure plasma membrane (PM) fractions, these fragments were acid-precipitated via the procedure of Goffeau and Dufour.[25] CM or purified PM fraction was stored in suspension medium (10 mM imidazole/1 mM $MgCl_2$) and analyzed by Western blot and amido black assays.

Western Blotting. Strains expressing HB3 *Pf*CRT, Dd2 *Pf*CRT, 7G8 *Pf*CRT, *Pv*CRT, LacZ, or empty vector were grown at 30 °C to OD^{600} ∼ 1.0 in either inducing SGR or noninducing SD media lacking uracil. Cellular membrane fractions were prepared as described above, protein content was determined via amido black, and aliquots stored at −80 °C. For Western blot analysis, gel loading dye was added, extracts denatured at 65 °C for 5 min, and proteins first resolved by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (SDS-PAGE) 12% at 110 V for ∼2 h. Proteins were then transferred to poly(vinylidene difluoride) membranes in transfer buffer (20% methanol/150 mM glycine/20 mM Tris base/0.1% SDS) at 40 mA for 16 h. Membranes were then washed with phosphate buffered saline-Tween 20 (PBS-T) solution twice (10 min each), blocked with 5% milk in PBS-T for 1 h, incubated with either anti-HexaHis-HRP or anti-V5-HRP for 1 h, washed three times with PBS-T (5 min each), and detected using Amersham Hyperfilm ECL (Piscataway, NJ).

Colony Formation Assays. To test the effects of CQ on growing yeast cells expressing CRTs, strain CH1305 harboring CRT plasmids (see above), pYES2/LacZ, or empty pYES2 vector was initially assessed by colony spot assays (see Results). Cell suspensions used to inoculate agar plates were grown at 30 °C to mid-exponential phase in noninducing SD media lacking uracil. Suspensions were measured, equilibrated to an OD = 0.1, and transferred to wells of a 96-well plate. Three 10-fold dilutions were made for each cell suspension with a multichannel pipet, and 5 μL from each well was then replica plated side-by-side on either noninducing SD or inducing SGR media lacking uracil and vs specified concentrations of CQ (see Results).

Quantitative Growth Rate Analysis. Susceptibilities of yeast strains to CQ were quantitatively assessed by automated growth curve analysis in liquid media. Cell suspensions were grown at 30 °C to mid-exponential phase in noninducing SD media lacking uracil. Cells density was adjusted to OD = 0.1 and cells transferred to 96-well plates containing 200 μL of SD or SGR media lacking uracil plus various concentrations of drug. Plates were wrapped in parafilm and placed in a Tecan GENious plate reader (Durham, NC) and growth curve data collected using the following parameters: measurement wavelength, 595 nm; number of flashes, 3; number of kinetic cycles, 125; kinetic interval, 30 min; valid temparature range, 28−32 °C; orbital shake duration, 30 s. For ΔpH and Δψ dependent growth curve analysis yeast harboring isoforms of CRT were assayed in synthetic complete media containing additional 100 mM KCl and buffered with 100 mM MES−Tris (see Results). Growth under each condition was measured in triplicate via back-dilution of the strain grown under normal noninducing conditions (SD media lacking uracil). These experimental conditions are designed to indirectly probe sensitivity of yeast strains in response to decreases in ΔpH. Under normal conditions, medium external pH (pH_{ex}) ∼ 5.0 and the yeast PM maintains a high ΔpH but low Δψ.[26,27] Alkalinization of the external medium lowers ΔpH, and cells then use PM K^+ channels to increase Δψ such that substantial Δψ compensates for loss of ΔpH.[26,27]

[^{3}H]CQ Accumulation Assay. Transformed CH1305 derived strains were grown to OD^{600} 0.5−1.0 in 300 mL of SGR medium. Cells were counted with a hemacytometer, centrifuged 1000*g* for 5 min, washed twice in transport buffer (2.0% glucose/10 mM MES, pH 6.1), and resuspended in approximately 1/100th of the original culture volume in assay buffer (2×10^9 cells mL^{-1}). For uptake experiments, 50 μL of cell suspension was added to 150 μL of transport buffer containing ^{3}H-CQ at a final concentration of 1 μM (0.01 mCi/mM). At specific times, reactions were stopped by addition of 3 mL of ice-cold quench buffer and cells were rapidly filtered through Whatman GF/C filters (Piscataway, NJ). Filters were immediately washed twice with 3 mL of ice-cold quenching buffer (50 mM CQ/20 mM $NaC_6H_7O_7$/5 mM $MgSO_4$/1 mM EDTA/1 mM K_2HPO_4/1 mM $CaCl_2$/1 mM NaCl) transferred to scintillation vials containing 14 mL of Ecoscint A scintillation cocktail (National Diagnostics, Atlanta, GA) and radioactivity measured using a Beckman LS6500 scintillation counter (Brea, CA). Uptake at 0 time was determined by simultaneously mixing cells in ice-cold transport buffer media and ice-cold wash buffer. For kinetic analyses, uptake rates were obtained in triplicate for each strain as described in Results.

RESULTS

Previous results from our group showed that codon optimization of the 72% A-T *pfcrt* gene was required for

dx.doi.org/10.1021/bi200922g | *Biochemistry* 2011, 50, 6701−6710

efficient heterologous expression of PfCRT protein in either *P. pastoris* or *S. cerevisiae* yeast.[24] The former expression system provides tightly controlled, AOX1 promoter mediated induction by MeOH and acceptable yields of protein for purification and proteoliposome reconstitution.[13,20] However, the system does not allow for analysis of PfCRT effects on growing yeast. The latter used a constitutively active, noninducible Ste6 promoter. Using the pYES system and codon optimized *pfcrt*, we are now able to strongly induce PfCRT expression in tightly controlled fashion for growing yeast using galactose (Figure 1A). Expression is similar for multiple isoforms of PfCRT (Figure 1A,B). Also, using slightly modified *pvcrt* cDNA (see Methods), we are able to use this system for similar inducible heterologous expression of the PfCRT orthologue PvCRT (Figure 1B, lanes 4, 5 vs lanes 1−3). Figure 1C shows that 50−60% of the PfCRT protein expressed in these yeast is PM localized (see caption) and that the protein can be exclusively localized to the PM via attachment of a N-terminal leader sequence from the yeast H^+ ATPase (see ref 28).

Unlike previous heterologous expression methods, these galactose-inducible strains allow us to carefully quantify CRT-dependent phenotypes for actively growing yeast via careful side-by-side comparison of transformed strains grown in glucose (SD medium) vs galactose (SGR medium). Since PfCRT amino acid substitutions confer reduced susceptibility to CQ growth inhibitory effects in *P. falciparum*, we hypothesized that expression of PfCRT might also alter the sensitivity of growing yeast to the drug. Indeed, Figure 2 shows that inducing PfCRT or PvCRT using SGR medium (+galactose/raffinose, −glucose; right-hand side of Figure 2) significantly reduces growth in a CQ concentration - dependent fashion. That is, yeast can tolerate CQ in the growth medium up to about 150 mM without appreciable effects on growth (Figure 2, left-hand side), and expression of PfCRT or PvCRT isoforms in and of themselves similarly does not affect growth (compare top panels "no drug" for the left-hand [noninducing] and right-hand [inducing conditions] sides, Figure 2). However, when PfCRT or PvCRT proteins are expressed, increasing the concentration of external CQ progressively inhibits colony formation in a CRT specific fashion (Figure 2, right-hand side, compare two left most rows for "EV" [empty vector] and "LacZ" [gal inducible β galactosidase expression], vs other rows for CRT expressors).

Taking the results shown in Figures 2 and 1C together, one obvious hypothesis is that, similar to plasma membrane expression of PfCRT in oocytes,[19] PfCRT at the yeast plasma membrane facilitates influx of toxic CQ, promoting increased sensitivity to the drug, in a [CQ]-dependent fashion. The similar CQ-dependent growth inhibited phenotype of yeast expressing the PMA−PfCRT fusion protein (row 8, right-hand side of Figure 2) supports this idea, since expression of the fusion protein is PM-specific (Figure 1C). We expect PM topology of the expressed CRT protein to conserve cytosolic disposition of cytosolic domains, and indeed, such a topology is strongly supported by earlier work with inside-out vesicles made from yeast constitutively expressing PfCRT.[24] Since PfCRT protein expressed in the DV is believed to facilitate transport of CQ from inside the DV to the cytosol of the malarial parasite,[2] then this yeast PM topology would be predicted to facilitate influx of CQ from growth medium to the yeast cytosol.

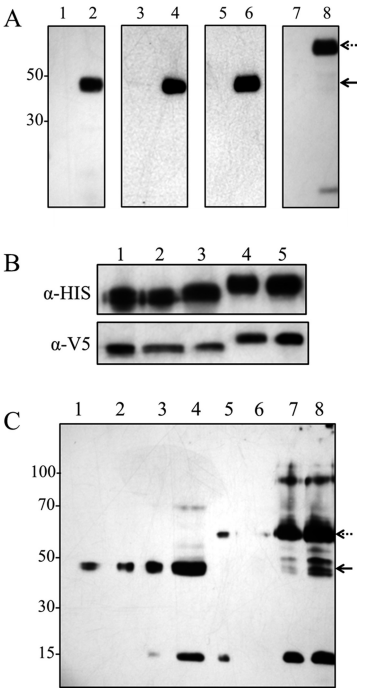


Figure 1. Western blot analysis of gal-inducible CRT protein expression. (A) Crude membrane preparations of *S. cerevisiae* transformed with different isoforms of PfCRT grown under non-inducing glucose conditions (lanes 1, 3, 5, 7) vs inducing galactose/raffinose (lanes 2, 4, 6, 8). Each lane contains 10 μg of protein. Each blot uses crude yeast membranes (total membrane fraction) prepared from CH1305 strains transformed with pYES2/PfHB3vh (lanes 1 and 2), pYES2/PfDd2vh (lanes 3 and 4), pYES2/Pf7G8vh (lanes 5 and 6), or pYES2/PfHB3$_{PMA}$vh (lanes 7 and 8). The V5-tagged proteins were detected with anti-V5-HRP. Top arrow (dashed) points to PfHB3$_{PMA}$vh protein; bottom (solid) arrow points to normal length PfCRT proteins. (B) Crude membranes were prepared from CH1305 strains transformed with pYES2/PfHB3vh (lane 1), pYES2/PfDd2vh (lane 2), pYES2/Pf7G8vh (lane 3), pYES2/PvCRTvh (lane 4), or pYES2/PvCRT$_{Dd2}$vh (lane 5) and equivalent membrane protein (quantified by amido black assay) run side-by-side. Top panel shows anti-pentaHis staining; bottom shows anti-V5 staining, both of which indicate that approximately equivalent levels of the isoforms and orthologues are expressed in the different strains. (C) Comparison of relative amounts of PfHB3vh and pYES2/PfHB3$_{PMA}$vh in plasma membrane fractions. Lanes 1 and 5 are 5 μg of crude membranes prepared from CH1305 strains transformed with pYES2/PfHB3vh and pYES2/PfHB3$_{PMA}$vh. Lanes 2 and 6 are 5 μg of pellet harvested at 75000*g* (intermediate step in pure plasma membrane preparation, harboring non-plasma membrane cellular membranes) from CH1305 strains transformed with pYES2/PfHB3vh and pYES2/PfHB3$_{PMA}$vh. Lanes 3, 4 and 7, 8 contain 5 and 10 μg, respectively, of pure plasma membranes harvested from CH1305 strains transformed with pYES2/PfHB3vh and pYES2/PfHB3$_{PMA}$vh. Top arrow indicates PMA1-fusion PfHB3 isoform, and bottom arrow indicates PfHB3 isoform. High mass bands near 100 kDa in lanes 7, 8 are likely PMA1−HB3CRT aggregates, and the band near 15 kDa is an apparent protease degradation product. Densitometry of bands indicated approximately 50−60% of CRT is within the plasma membrane fraction without N-terminal modification, but for pYES2/PfHB3$_{PMA}$vh, >10 times is found in the plasma membrane relative to all other cell membranes.

Interestingly then, in these colony formation assays (Figure 2) the most pronounced CQ-dependent growth inhibition is seen for PvCRT expressing yeast (row 5, right-hand side of Figure 2), suggesting that even though PvCRT mutations have

dx.doi.org/10.1021/bi200922g | *Biochemistry* 2011, 50, 6701−6710

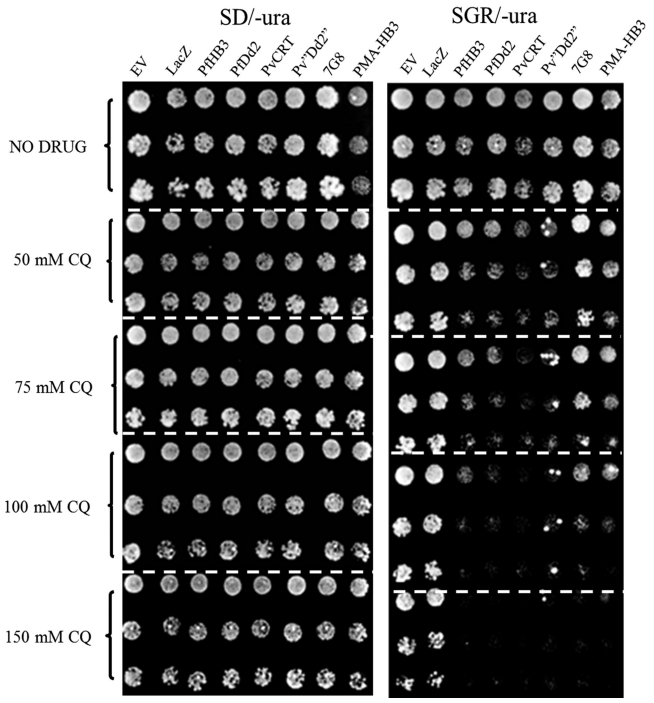


Figure 2. Plate assays for CQ and CRT-dependent growth inhibition. Susceptibilities of CH1305 yeast harboring pYES2 ("empty vector" or "EV"), pYES2/LacZ, pYES2/PfHB3vh, pYES2/PfDd2vh, pYES2/Pf7G8vh, pYES2/PvCRTvh, pYES2/PvDd2vh, or pYES2/PfHB3$_{PMA}$vh. Suspensions for inoculating plates were grown at 30 °C to mid-exponential phase in SD media − uracil. Suspensions equilibrated to OD = 0.1 were transferred to 96-well plates, 10-fold serial dilutions were made, and 5 μL of each (10^4, 10^3, 10^2 cells; top, middle, and bottom row, respectively, each "box" defined by white dashed lines) was then replica plated on SD or SGR media − uracil, ±concentrations CQ. Plates were incubated side-by-side for ~3 days at 30 °C.

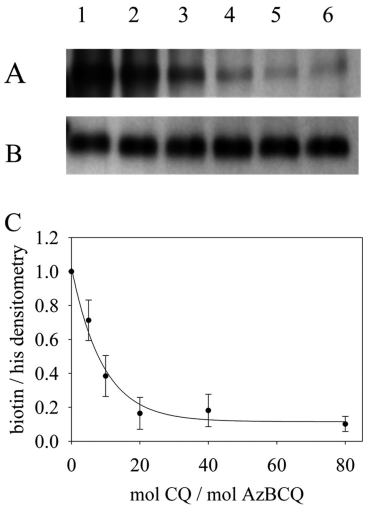


Figure 3. AzBCQ photolabeling of partially purified PvCRT. (A) Representative streptavidin-HRP detection of AzBCQ photolabeled PvCRT vs competition with underivitized CQ (see refs 9 and 13 for detailed description of methods). Lane 1: no CQ competitor; lanes 2–6: 5-, 10-, 20-, 40-, and 80-fold molar excess of CQ relative to AzBCQ, respectively. (B) PentaHis-HRP detection of PvCRT in the same samples as in (A), showing equal amounts of protein were used in each photolabeling reaction (see ref 13). (C) Plot of densitometry of bands as in (A) vs [CQ] competitor. As described,[13] results from three different experiments (three photolabeling reactions, three separate sets of gels) were averaged; data represent mean ± SEM. Fitting this curve shows that 50% maximal inhibition of PvCRT CQ analogue photolabeling occurs at (8.5 ± 1.3)-fold molar excess of underivatized CQ.

not yet been associated with *P. vivax* CQR, this orthologue also interacts with CQ. As shown in Figure 2 (5th, 6th rows, right-hand side), mutating PvCRT to a "Dd2-like" PvCRT (see Materials and Methods) did not significantly change the hypersensitivity phenotype. To test interaction between CQ and PvCRT further, we partially purified wild type PvCRT protein from these yeast, reconstituted the protein into proteoliposomes, and examined AzBCQ photolabeling as previously described in depth for PfCRT protein.[13] As shown in a representative blot (Figure 3), 50% competition of photolabeling with the high affinity CQ analogue is achieved at ~8-fold molar excess of underivitized CQ (Figure 1C). Previously, we found that 50% competition of photolabeling occurred at 22-, 25-, and 6-fold excess underivitized CQ for HB3, Dd2, and 7G8 PfCRT isoforms, respectively.[13] Thus, under de-energized conditions CQ probe apparent affinity for PvCRT is the second highest we have yet measured for any CRT protein.

Colony formation assays are a convenient phenotypic screen, but to more rigorously quantify effects of PfCRT and PvCRT expression on yeast CQ hypersensitivity, we performed quantitative growth analyses (Figure 4). In these experiments, seeding yeast density was normalized and strains aliquoted in triplicate into wells containing inducing (SGR, "B" right-hand side Figure 4) or noninducing (SD, "A" left-hand side, Figure 4) media. In each case, solid lines in each panel in Figure 4 are growth in the absence of CQ, whereas dotted lines are growth in the same medium containing 100 mM CQ. Again, quantitative growth analysis shows that at this concentration CQ alone has no effect on growth of any of the strains, assuming CRT protein is not being induced (left-hand side, Figure 4, compare solid vs dotted lines). Similarly, when strains harboring either empty vector (top panel, right-hand side Figure 4) or vector expressing β galactosidase (data not shown) are grown in inducing medium, no significant effect is seen ± CQ (e.g., compare solid vs dotted line, top right panel Figure 4). However, when CRT is induced (panels 2–5 from top, right-hand side Figure 4) the yeast clearly grow much slower in the presence of 100 mM CQ, consistent with the colony formation data shown in Figure 2. Notably, yeast expressing wild type PfCRT ("HB3" isoform, second panel from top, Figure 4) grow as slowly as do yeast expressing the mutant PfCRT isoforms found in CQR *P. falciparum* ("Dd2" and "7G8" isoforms, third and fourth panels from top, left-hand side Figure 4). Similar to earlier work with purified protein reconstituted into proteoliposomes,[20] this suggests that wild type PfCRT is also capable of transporting CQ under some conditions. Interestingly, yeast expressing wild type PvCRT grows the slowest (bottom panel, right-hand side).

After performing these growth curve analyses multiple times, doubling times for each well (δ) were then determined by converting growth vs time to semilog plots and calculating slope. Figure 5 summarizes these averaged data for strains growing in the presence of 100 mM CQ under either noninducing conditions (black bars) or inducing conditions (gray bars) and at either low external pH (= 5.0, panel A) or

dx.doi.org/10.1021/bi200922g | *Biochemistry* 2011, 50, 6701–6710

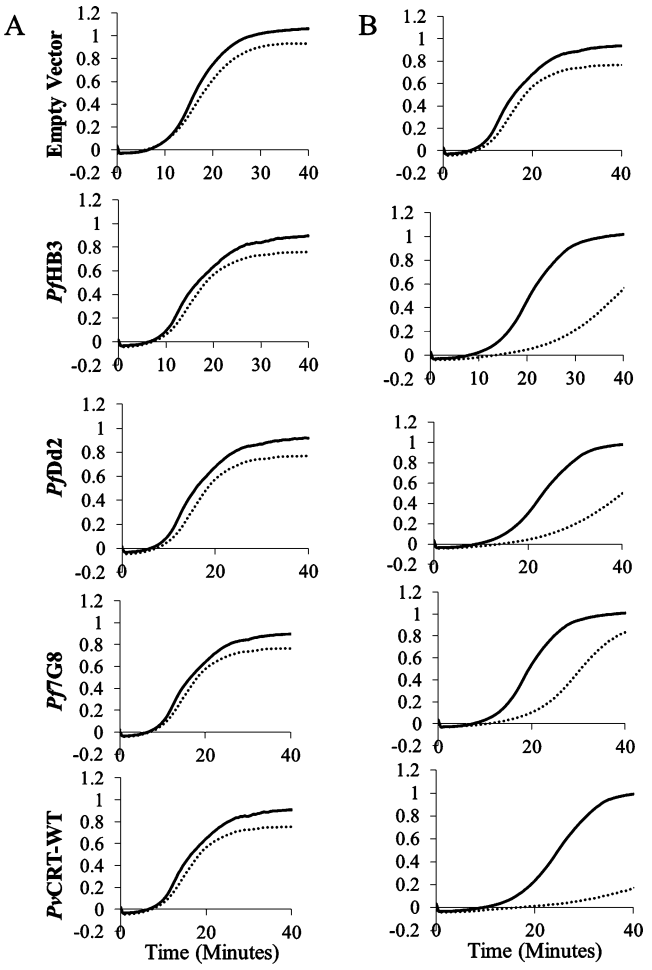


Figure 4. Quantitative growth curve analysis. Susceptibilities of yeast strain CH1305 harboring pYES2 (empty vector, top), pYES2/PfHB3vh, pYES2/PfDd2vh, pYES2/Pf7G8vh, or pYES2/PvCRTvh were assessed by quantitative growth curve analysis in liquid medium, pH = 5.0. Cultures were inoculated with 10^5 cells/mL synthetic complete medium with (A, left side panels) 3% glucose (SD-ura) alone (solid lines) or with 100 mM CQ (dashed) or (B, right side panels) synthetic complete medium with 2% galactose and 1% raffinose (SGR-ura) alone (solid) or SGR-ura with 100 mM CQ (dashed). Average growth curves from three replicates are shown; see Figure 5 for curve fit analysis.

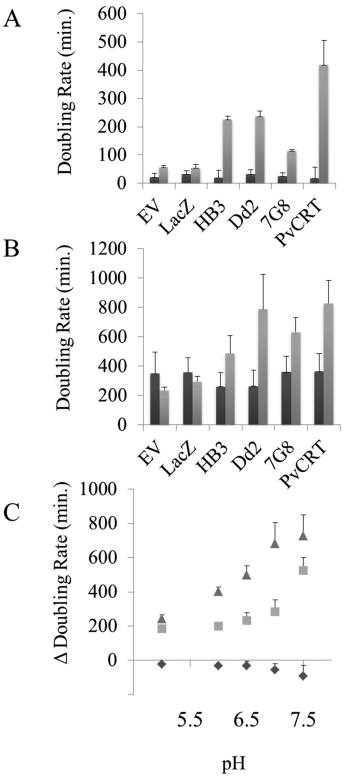


Figure 5. Summary of quantitative growth analysis. Doubling rates for data obtained as in Figure 4 were computed as described. (A) Inducing SGR medium, pH = 5.0 with (gray bars) or without (black bars) 100 mM CQ. (B) Inducing SGR medium, buffered to pH = 7, with (gray bars) or without (black bars) 75 mM CQ. (C) Induction and pH/ΔΨ-dependent effects on growth rate. Growth rates obtained for noninducing medium +75 mM CQ were subtracted from rates obtained for inducing medium +75 mM CQ. The growth rate differences for yeast expressing Dd2 PfCRT (top, triangles) were significantly higher relative to yeast expressing HB3 PfCRT (middle, squares) for all pH/ΔΨ ($P \leq 0.05$, unpaired t tests in each comparison). As expected, growth under the two conditions did not differ significantly for yeast harboring empty vector (bottom, diamonds). The data represent the means (+SEM) of four independent experiments.

high external pH (pH = 7.5, panel B, see Materials and Methods). We wished to inspect growth effects at a range of pH because as external pH is raised for growing yeast, plasma membrane ΔpH is reduced, but ΔΨ is increased to preserve electrochemical driving force for essential membrane transport (see Materials and Methods and refs 26 and 27).

At low external pH (high ΔpH, ΔΨ near zero), the growth inhibition due presumably to CRT mediated influx of CQ is again most pronounced for yeast expressing PvCRT (Figure 5A, far right-hand side). Also, growth inhibition for strains expressing HB3 wild type (CQS associated) PfCRT is similar to that for strains expressing mutant (CQR associated) Dd2 PfCRT, and at this pH/ΔΨ, CQR associated mutant 7G8 PfCRT confers less dramatic growth inhibition relative to HB3 wild type.

However, as external pH is raised to 7.5, such that ΔpH is near zero (internal pH for yeast is ~8.0) but ΔΨ is near maximum,[26,27] the growth inhibition effect promoted by 7G8 PfCRT and Dd2 PfCRT is noticeably more severe than that promoted by HB3 PfCRT (Figure 5, panel B). That is, elevating ΔΨ has a more significant effect on growth inhibition caused by mutant (CQR associated) PfCRT isoforms (from 100−200 to 300−500 min for 7G8 and Dd2 PfCRTs) than by wild type (CQS associated) PfCRT (from ~200 to ~250 min) or than the PvCRT orthologue (~400 min in both cases; compare Figure 5A vs 5B). To examine this more closely, Figure 5C shows a plot of the difference in strain doubling times (growth in the presence of CQ under CRT inducing conditions minus growth plus CQ under noninducing conditions; [y-axis]) vs pH (x-axis) for strains expressing no PfCRT (◆) or HB3 (■) vs Dd2 (▲) PfCRT. Control doubling rate (◆) is mildly dependent on pH but is not slowed by CQ; instead, a very mild stimulation by glucose is seen at higher pH (yielding points just below the x-axis). Strains expressing PfCRT are slowed in their growth upon addition of CQ in a pH/ΔΨ-dependent manner. The effect is exacerbated quite significantly as pH/ΔΨ are increased, with the change for Dd2 PfCRT expressing yeast greater than for

dx.doi.org/10.1021/bi200922g | Biochemistry 2011, 50, 6701–6710

yeast expressing wild type PfCRT. A similarly heightened effect is seen for 7G8 PfCRT expressing yeast (not shown).

As suggested above, one obvious hypothesis for these effects on growth is that PfCRT at the PM of the yeast is facilitating heightened transport of CQ into the yeast. To test this more directly, we performed ^{3}H-CQ uptake experiments. Figure 6

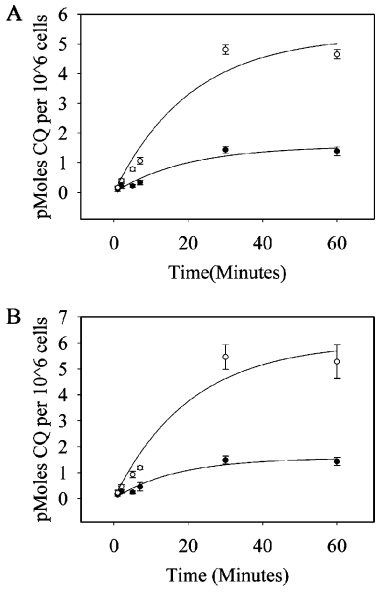


Figure 6. ^{3}H-CQ accumulation in yeast +/− CRT expression. CH1305 harboring pYES2 (closed circles) or pYES2/PfDd2vh (open circles) were grown to exponential phase in SGR − ura, harvested, and assayed for [^{3}H]-CQ uptake at pH 5.5 (A) or 7.5 (B). The data represent the means (±SD) for 3−5 separate experiments.

shows representative ^{3}H-CQ accumulation vs time for induced Dd2 PfCRT yeast (open circles) and induced yeast harboring empty vector (closed circles) at pH 5.5 (panel A) and 7.5 (panel B). Clearly, expression of PfCRT in the induced yeast leads to increased accumulation of ^{3}H-CQ. Figure 7 summarizes 30 min accumulation data for various strains assayed at low (black bars) vs high (gray bars) pH. Consistent with the PfCRT isoform-dependent growth effects discussed above, yeast strains expressing mutant (CQR associated) Dd2 and 7G8 PfCRT accumulate more drug at high pH (low ΔpH, high ΔΨ) vs low pH (high ΔpH, low ΔΨ; see caption), whereas yeast expressing HB3 wild type PfCRT or PvCRT accumulate similar levels at both pH/ΔΨ. Figure 8 and Table 3 summarize initial rate analysis of these uptake data. Importantly, neglecting slower background CQ accumulation (yeast grown under inducing conditions but harboring empty vector), the rank order of initial rate of drug transport is PvCRT > Dd2 PfCRT > 7G8 PfCRT > HB3 PfCRT at high ΔpH/low ΔΨ. Consistent with plateau data (Figure 7), rate does not increase for HB3 PfCRT or PvCRT expressing yeast at lower ΔpH/higher ΔΨ but does increase for 7G8 and Dd2 PfCRT expressing yeast, such that at high ΔΨ the rank order of initial rate of drug transport is PvCRT ∼ Dd2 PfCRT ∼ 7G8 PfCRT ≫ HB3 PfCRT (Table 3).

DISCUSSION

Mutated PfCRT is believed to cause resistance to the antiparasitic effects of CQ via mediating increased flux of CQ

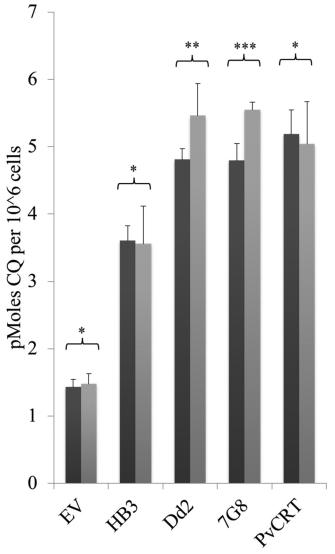


Figure 7. Summary of ^{3}H-CQ accumulation data for different yeast strains CH1305 harboring pYES2 ("EV"), pYES2/PfHB3vh, pYES2/PfDd2vh, pYES2/Pf7G8vh, or pYES2/PvCRT were grown to exponential phase in SGR − ura, harvested, and assayed for [^{3}H]-CQ uptake at 30 min (near plateau, see Figure 6) at either pH 5.5 (black bars) or pH 7.5 (gray bars). Similar results are obtained at 60 min time (not shown, see Figure 6). Each bar represents the average of three separate experiments. *P > 0.2, **P = 0.08, ***P < 0.01. Differences in uptake at pH 7.5 vs 5.5 are significant for 7G8 ($P \leq$ 0.05, unpaired t tests) and suggestive for Dd2 (P = 0.08, unpaired t tests), but not significant for any of the other strains.

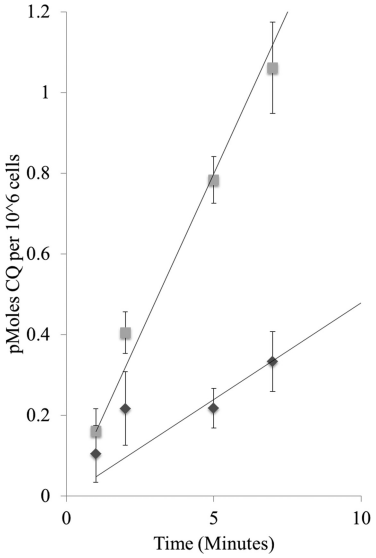


Figure 8. Representative initial rate analysis of ^{3}H-CQ accumulation (see also Table 3 for a summary). Gray squares are CH1305 expressing pYES2/PfDd2vh, and black diamonds are CH1305 expressing pYES2. Data were obtained at pH 5.5 and are the results of three experiments, ±SD. See Table 3 for a summary of initial rate data.

from the malarial parasite DV.[2,19,20] Data in this paper help to further elucidate key features of that transport. Previous yeast heterologous expression of PfCRT via MeOH induction or via constitutively active Ste6 promoter[24] did not allow for convenient analysis of PfCRT function in actively growing

dx.doi.org/10.1021/bi200922g | Biochemistry 2011, 50, 6701−6710

Table 3. Initial Rates of ^{3}H-CQ Accumulation (fmol CQ/min/10^6 Cells) for Yeast Expressing Various CRT Isoforms/Orthologues (See Figure 8 for Representative Raw Data)[a]

	pH	
	5.5	7.5
EV (none)	47.9	50.5
HB3 Pf	120.2	120.5
Dd2 Pf	160.0	181.7
7G8 Pf	150.5	176.9
PvCRT	188.6	183.0

[a]Data shown are average of three determinations.

yeast under ambient growth conditions. However, the high fidelity of the glu/gal induction system now provides new convenient growth assays (Figures 2 and 4) that can be used to characterize the function of various PfCRT and PvCRT isoforms. The simple growth assays that are now possible should be useful for screening putative PfCRT and PvCRT inhibitors and/or drug resistance reversal agents that act via interaction with PfCRT. Although often overlooked, PfCRT has no strong homologues other than additional *Plasmodium* CRT proteins and appears to be an essential protein.[29] Although not yet proven for *P. vivax*, it is likely that PvCRT is also essential. These proteins therefore represent important, unique targets for development of new antimalarial drugs. Rapid screening of candidate CRT inhibitors vs CQ hypersensitivity might provide one inexpensive way to prioritize lead compounds.

We find a reasonably good correspondence between the degree of CQ-induced growth inhibition vs heightened rate of CQ accumulation promoted by expression of CRT protein (Figures 4 and 5 vs Table 3). Since yeast intracellular compartmentalization of the diprotic weak base CQ is complex, involving the yeast vacuole and other compartments, we suggest altered initial rates of CQ transport are a less ambiguous indicator of CRT function than net accumulation at 30 min. That is, net accumulation at ≥30 min is due to a variety of overlapping complex effects, many of which do not involve PfCRT or PvCRT. We also note that the protein−polysaccharide outer membrane of yeast changes cellular permeability of CQ relative to what would be seen for intact parasites (14−16). Nonetheless, relative differences in accumulation across the yeast strains qualitatively parallel the effects seen for initial rates and are again consistent with trends in altered growth phenotypes due to expression of CRTs. Our model to explain these data is simple: expression of the known CQ transporter PfCRT at the PM of yeast promotes heightened accumulation of toxic CQ, leading to significant growth inhibition of these yeast.

In contrast to expression of PfCRT in yeast, PvCRT expression did not require extensive codon optimization.[24] The *P. vivax* genome is ∼58% A + T content whereas *P. falciparum* is ∼81% A + T.[30] Correspondingly, *pvcrt* cDNA is 54% A + T whereas the *pfcrt* is 72%. Interestingly, we find that the PvCRT orthologue promotes more significant growth inhibition and appears to transport CQ *better* than do the mutant (CQR associated) Dd2 and 7G8 PfCRT isoforms. This provides a surprising, but simple, hypothesis for why PvCRT mutations have not been associated with CQR in *P. vivax*, even though CQR clearly exists for this species and is on the rise.[11] Namely, since the wild type PvCRT transporter is already as capable of CQ transport as are the mutant PfCRT isoforms, there is perhaps little to "gain" (in terms of further increased drug transport ability) by acquisition of PvCRT mutations. These data and arguments then suggest that CQ IC_{50} should be intrinsically higher for *P. vivax* relative to *P. falciparum*. This is predicted because CQ IC_{50} is related to the relative intra-DV accumulation of CQ.[15,16] IC_{50} assays for *P. vivax* are difficult since currently the parasite cannot be conveniently cultured *in vitro*. However, available data that compare *P. falciparum* and *P. vivax* patient isolates side-by-side with the same assay[31] show that CQ IC_{50} is indeed intrinsically higher for *P. vivax* relative to *P. falciparum*. That is, at least with respect to growth inhibition by CQ, *P. vivax* is intrinsically more resistant to CQ because of a higher endogenous (wild type PvCRT mediated) ability to allow diffusion of accumulated CQ^{2+} back out of the DV. We note that this does not necessarily mean that *P. vivax* are more resistant to cytocidal effects of CQ relative to *P. falciparum*, since DV accumulation of CQ and CQ LD_{50} do not appear to be related.[16]

Growth inhibition and direct ^{3}H-CQ transport data in this paper strongly support the notion that, similar to wild type PvCRT, wild type CQS-associated isoform HB3 PfCRT is able to transport CQ in some fashion. Currently, there are differing conclusions on this point.[2] Martin *et al.*[19] do not measure appreciable CQ uptake for oocytes expressing HB3 PfCRT but do measure uptake for oocytes expressing Dd2 PfCRT. In constrast, Paguio *et al.*[20] reported that both HB3 and Dd2 isoforms of PfCRT were capable of mediating electrochemically downhill, $\Delta\Psi$-dependent, transport of a fluorescent CQ analogue. Some possible explanations for the discrepancy have been discussed previously[2] and include the important point that membrane potentials in the two experimental systems (live oocytes vs reconstituted proteoliposomes) are very different.

Also, data in this paper further support the notion that CQ transport by PfCRT is stimulated by $\Delta\Psi$ (see also ref 20). This is not unexpected since the physiologic environment for PfCRT and PvCRT is the DV membrane, across which a high electrochemical potential (positive inside) is expected. PfCRT topology in the parasite DV membrane places cytosolic domains at the negative side with respect to $\Delta\Psi$ (and at alkaline pH) and intra-DV domains of PfCRT facing positive (and acid pH). For growing yeast, external pH is acid and $\Delta\Psi$ across the plasma membrane is positive outside and negative inside; thus, PfCRT topology in yeast plasma membrane preserves the physiologic orientation with respect to electrochemical driving force. Because yeast are capable of generating high $\Delta\Psi$ when external pH is artificially raised,[26,27] membrane potential stimulatory effects for CQ transport by PfCRT can be observed, similar to how generation of high artificial $\Delta\Psi$ for proteoliposomes promotes increased transport of a fluorescent CQ analogue.[20]

Interestingly, an increase in CQ transport upon elevating $\Delta\Psi$ is only found for mutant (CQR associated) Dd2 and 7G8 PfCRT isoforms. Neither wild type HB3 PfCRT nor wild type PvCRT appeared to be stimulated by higher $\Delta\Psi$ with respect to growth inhibitory phenotypes or with respect to whole cell ^{3}H-CQ accumulation. Consistent with previous work with purified PfCRT and a fluorescent CQ probe,[20] we suggest that electrochemical potential differences across the parasite DV membrane are important for heightened electrochemically passive CQ^{2+} facilitated diffusion by mutant PfCRT in CQR

parasites. In support of this concept, some studies have published data that show mutant PfCRT promotes lower intra-DV pH (0.2−0.4 pH unit higher DV membrane ΔpH[32−34]); however, other studies have not measured these small DV ΔpH changes.[35−37] If cytosolic pH remains constant for CQR parasites (or is perhaps even elevated for some particularly drug resistant strains, see ref 38), the increased driving force presumably provided by this increased ΔpH may promote reduced DV $[CQ^{2+}]$ for CQR parasites. Increases in $\Delta\Psi$ across the DV membrane for CQR parasites may indeed exist, but this awaits additional experimental measurement. Currently, reliable quantification of $\Delta\Psi$ for an organellar membrane of a living intracellular parasite is technically challenging, but further work may be able to test these predictions. In any case, clear differences in DV volume for CQS vs CQR malarial parasites strongly suggest altered osmolyte traffic exists for the CQR DV.[39] If these osmolytes include ions or charged solutes for which the DV membrane normally has high permeability, perturbation of $\Delta\Psi$ is expected.

AUTHOR INFORMATION

Corresponding Author

*Tel: (202) 687-7300. Fax: (202) 687-6209. E-mail: roepep@georgetown.edu.

Funding

This work was supported by NIH grants AI056312 and AI090832.

ACKNOWLEDGMENTS

We thank Drs. Tom Wellems and Juliana Sa (NIAID/NIH) for *pvcrt* cDNA, Dr. William Fonzi (Georgetown) for pYES2 and assistance with growth curve analyses, Dr. John F. Cannon (BYU) for strain CH1305, and Dr. Choukri Ben Mamoun (Yale University) for pYES2/LacZ, PMA N-terminal cDNA, and very helpful discussions. We also thank our laboratory colleagues for experimental help.

REFERENCES

(1) Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M., Sidhu, A. B., Naudé, B., Deitsch, K. W., Su, X. Z., Wootton, J. C., Roepe, P. D., and Wellems, T. E. (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol. Cell 6*, 861−871.

(2) Roepe, P. D. (2011) PfCRT − Mediated Drug Transport in Malarial Parasites. *Biochemistry 50*, 163−171.

(3) Valderramos, S. G. (2006) Fidock DA.Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol. Sci. 11*, 594−601.

(4) Ferdig, M. T., Cooper, R. A., Mu, J., Deng, B., Joy, D. A., Su, X. Z., and Wellems, T. E. (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol. Microbiol. 52*, 985−997.

(5) Patel, J. J., Thacker, D., Tan, J. C., Pleeter, P., Checkley, L., Gonzales, J. M., Deng, B., Roepe, P. D., Cooper, R. A., and Ferdig, M. T. (2010) Chloroquine susceptibility and reversibility in a *Plasmodium falciparum* genetic cross. *Mol. Microbiol. 78*, 770−787.

(6) Sá, J. M., Twu, O., Hayton, K., Reyes, S., Fay, M. P., Ringwald, P., and Wellems, T. E. (2009) Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proc. Natl. Acad. Sci. U. S. A. 106*, 18883−18889.

(7) Wilson, C. M., Serrano, A. E., Wasley, A., Bogenschutz, M. P., Shankar, A. H., and Wirth, D. F. (1989) Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*. *Science 244*, 1184−1186.

(8) Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M., Forsyth, K., Kemp, D. J., and Cowman, A. F. (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *P. falciparum*. *Nature 345*, 255−258.

(9) Pleeter, P., Lekostaj, J. K., and Roepe., P. D. (2010) Purified *Plasmodium falciparum* multi-drug resistance protein (PfMDR 1) binds a high affinity chloroquine analogue. *Mol. Biochem. Parasitol. 173*, 158−161.

(10) Nomura, T., Carlton, J. M., Baird, J. K., del Portillo, H. A., Fryauff, D. J., Rathore, D., Fidock, D. A., Su, X., Collins, W. E., McCutchan, T. F., Wootton, J. C., and Wellems, T. E. (2001) Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. *J. Infect. Dis. 183*, 1653−1661.

(11) Baird, K. J. (2009) Resistance to therapies for infection by *Plasmodium vivax*. *Clin. Microbiol. Rev. 22*, 508−534.

(12) Zhang, H., Paguio, M., and Roepe, P. D. (2004) The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. *Biochemistry 43*, 8290−8296.

(13) Lekostaj, J. K., Natarajan, J. K., Paguio, M. F., Wolf, C., and Roepe., P. D. (2008) Photoaffinity labeling of the *Plasmodium falciparum* chloroquine resistance transporter with a novel perfluorophenylazido chloroquine. *Biochemistry 47*, 10394−10406.

(14) Geary, T. G., Jensen, J. B., and Ginsburg, H. (1986) Uptake of [3H]chloroquine by drug-sensitive and -resistant strains of the human malaria parasite *Plasmodium falciparum*. *Biochem. Pharmacol. 35*, 3805−3812.

(15) Hawley, S. R., Bray, P. G., Mungthin, M., Atkinson, J. D., O'Neill, P. M., and Ward, S. A. (1998) Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum in vitro*. *Antimicrob. Agents Chemother. 42*, 682−686.

(16) Cabrera, M., Paguio, M. F., Xie, C., and Roepe, P. D. (2009) Reduced digestive vacuolar accumulation of chloroquine is not linked to resistance to chloroquine toxicity. *Biochemistry 48*, 11152−11154.

(17) Sullivan, D. J. Jr., Gluzman, I. Y., Russell, D. G., and Goldberg, D. E. (1996) On the molecular mechanism of chloroquine's antimalarial action. *Proc. Natl. Acad. Sci. U. S. A. 93*, 11865−11870.

(18) Cooper, R. A., Ferdig, M. T., Su, X. Z., Ursos, L. M., Mu, J., Nomura, T., Fujioka, H., Fidock, D. A., Roepe, P. D., and Wellems, T. E. (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Mol. Pharmacol. 61*, 35−42.

(19) Martin, R. E., Marchetti, R. V., Cowan, A. I., Howitt, S. M., Bröer, S., and Kirk, K. (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science 325*, 1680−1682.

(20) Paguio, M. F., Cabrera, M., and Roepe., P. D. (2009) Chloroquine transport in *Plasmodium falciparum*. 2. Analysis of PfCRT-mediated drug transport using proteoliposomes and a fluorescent chloroquine probe. *Biochemistry 48*, 9482−9491.

(21) Nigavekar, S. S., and Cannon, J. F. (2002) Characterization of genes that are synthetically lethal with *ade*3 or *leu*2 in *Saccharomyces cerevisiae*. *Yeast 19*, 115−122.

(22) Sherman, F., Baim, S. B., Hampsey, D. M., Gooodhue, C. T., Friedman, L. R., and Stiles, J. I. (1986) in *Translational Control, Current Communications in Molecular Biology* (Matthews, M. B., Ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

(23) Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *Bacteriology 153*, 163−168.

(24) Zhang, H., Howard, E. M., and Roepe, P. D. (2002) Analysis of the antimalarial drug resistance protein Pfcrt expressed in yeast. *J. Biol. Chem. 277*, 49767−49775.

(25) Goffeau, A., and Dufour, J. P. (1988) Plasma membrane ATPase from the yeast *Saccharomyces cerevisiae*. *Methods Enzymol. 157*, 528−533.

(26) Fritz, F., Howard, E. M., Hoffman, M. M., and Roepe, P. D. (1999) Evidence for Altered Ion Transport in *S. cerevisiae* Overexpressing Human MDR 1 Protein. *Biochemistry 38*, 4214−4226.

(27) Orij, R., Postmus, J., Ter Beek, A., Brul, S., and Smits, G. J. (2009) *In vivo* measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth. *Microbiology 155*, 268−278.

(28) Downie, M. J., El Bissati, K., Bobenchik, A. M., Nic Lochlainn, L., Amerik, A., Zufferey, R., Kirk, K., and Ben Mamoun, C. (2010) PfNT2, a permease of the equilibrative nucleoside transporter family in the endoplasmic reticulum of *Plasmodium falciparum*. *J. Biol. Chem. 285*, 20827−20833.

(29) Waller, K. L., Muhle, R. A., Ursos, L. M. B., Verdier-Pinard, D., Fujioka, H., Roepe, P. D., and Fidock, D. A. (2003) Chloroquine resistance modulated *in vitro* by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT). *J. Biol. Chem. 278*, 33593−33601.

(30) Carlton, J. M., Adams, J. H., Silva, J. C., Bidwell, S. L., Lorenzi, H., Caler, E., Crabtree, J., Angiuoli, S. V., Merino, E. F., Amedeo, P., Cheng, Q., Coulson, R. M., Crabb, B. S., Del Portillo, H. A., Essien, K., Feldblyum, T. V., Fernandez-Becerra, C., Gilson, P. R., Gueye, A. H., Guo, X., Kang'a, S., Kooij, T. W., Korsinczky, M., Meyer, E. V., Nene, V., Paulsen, I., White, O., Ralph, S. A., Ren, Q., Sargeant, T. J., Salzberg, S. L., Stoeckert, C. J., Sullivan, S. A., Yamamoto, M. M., Hoffman, S. L., Wortman, J. R., Gardner, M. J., Galinski, M. R., Barnwell, J. W., and Fraser-Liggett, C. M. (2008) Comparative Genomics of the Neglected Human Malarial Parasite *Plasmodium vivax*. *Nature 455*, 757−763.

(31) Russell, B., Chalfein, F., Prasetyorini, B., Kenangalem, E., Piera, K., Suwanarusk, R., Brockman, A., Prayoga, P., Sugiarto, P., Cheng, Q., Tjitra, E., Anstey, N. M., and Price, R. N. (2008) Determinants of in vitro drug susceptibility testing of *Plasmodium vivax*. *Antimicrob. Agents Chemother. 52*, 1040−1045.

(32) Bennett, T. N., Kosar, A. D., Ursos, L. M. B., Dzekunov, S., Sidhu, A. B. S., Fidock, D. A., and Roepe, P. D. (2004) Drug resistance-associated pfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. *Mol. & Biochem. Parasitol. 133*, 99−114.

(33) Reeves, D. C., Liebelt, D. A., Lakshmanan, V., Roepe, P. D., Fidock, D. A., and Akabas, M. H. (2006) Chloroquine-resistant isoforms of the *Plasmodium falciparum* chloroquine resistance transporter acidify lysosomal pH in HEK293 cells more than chloroquine-sensitive isoforms. *Mol. Biochem. Parasitol. 150*, 288−299.

(34) Naude, B., Brzostowski, J. A., Kimmel, A. R., and Wellems, T. E. (2005) *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. *J. Biol. Chem. 280*, 25596−25603.

(35) Hayward, R., Saliba, K. J., and Kirk, K. (2006) The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. *J. Cell Sci. 119*, 1016−1025.

(36) Klonis, N., Tan, O., Jackson, K., Goldberg, D., Klemba, M., and Tilley, L. (2007) Evaluation of pH during cytostomal endocytosis and vacuolar catabolism of haemoglobin in *Plasmodium falciparum*. *Biochem. J. 407*, 343−354.

(37) Kuhn, Y., Rohrbach, P., and Lanzer, M. (2007) Quantitative pH measurements in *Plasmodium falciparum*-infected erythrocytes using pHluorin. *Cell. Microbiol. 9*, 1004−1013.

(38) Bennett, T. N., Patel, J., Ferdig, M. T., and Roepe, P. D. (2007) Altered *Plasmodium falciparum* Na^+/H^+ Exchange Activity is Correlated with Quinine Resistance Mol. *Biochem. Parasitol. 153*, 48−58.

(39) Gligorijevic, B., McAllister, R., Urbach, J., and Roepe, P. D. (2006) Spinning Disk Confocal Microscopy of Intraerythrocytic Malarial Parasites II: Quantification of Digestive Vacuolar Volume. *Biochemistry 45*, 12411−12423.