ANALYSIS OF THE ANTIMALARIAL DRUG RESISTANCE PROTEIN PfCRT
EXPRESSED IN YEAST

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ABSTRACT

Mutations in the novel membrane protein pfcrt were recently found to be essential for chloroquine resistance (CQR) in *P. falciparum*, the parasite responsible for most lethal human malaria (Fidock et al., (2000) Mol. Cell 6, 861-71). Pfcrt is localized to the digestive vacuolar (DV) membrane of the intraerythrocytic parasite and may function as a transporter. Study of this putative transport function would be greatly assisted by overexpression in yeast followed by characterization of membrane vesicles. Unfortunately, the very high AT content of malarial genes precludes efficient heterologous expression. Thus, we back-translated pfcrt to design idealized genes with preferred yeast codons, no long poly-A sequences, and minimal stem–loop structure. We synthesized a designed gene with a two-step PCR method, fused this to N and C terminal sequences to aid membrane insertion and purification, and now report efficient expression of wild type and mutant pfcrt proteins in the plasma membrane of *S. cerevisiae* and *P. pastoris* yeast. To our knowledge, this is the first successful expression of a full length malarial parasite integral membrane protein in yeast. Purified membranes and inside-out plasma membrane vesicle (ISOV) preparations were used to analyze wild type vs. CQR – conferring mutant pfcrt function, which may include effects on H+ transport (Dzekunov et al., (2000) Mol. & Biochem. Parasitol. 110, 107 – 124), and to perfect a rapid purification of biotinylated pfcrt. These data expand on the role of pfcrt in conferring CQR and define a productive route for analysis of important *P. falciparum* transport proteins and membrane associated vaccine candidates.
INTRODUCTION

Malaria causes approximately 2.5 million deaths annually, mostly children. Four malarial species infect humans, the most deadly being *Plasmodium falciparum*. For decades, malaria has been treated effectively with the 4-aminoquinoline chloroquine (CQ) \(^1\), however, CQ resistant (CQR) strains of *P. falciparum* have spread with considerable success and continue to evolve. Mutations in two genes (*pf mdr* and *cg2*) were previously suggested to mediate CQR, but more recently CQR was found to be caused by mutations in the *pfcrt* gene \(^1\). Pfmdr1 protein likely plays a modulatory role that influences the drug resistance profile \(^2\). Pfcrt protein is localized to the membrane of the parasite digestive vacuole (DV). The DV is the site of hemoglobin (Hb) digestion, which is a principle source of food for the parasite during rapid intraerythrocytic development. Mutant pfcrt may confer drug resistance by directly or indirectly lowering DV pH \(^3\), which quite effectively titrates soluble drug target (heme released from Hb) out of solution without compromising detoxification of heme to hemozoin \((4,5)\). There are actually multiple mutant pfcrt alleles that appear to cause drug resistance and that arose in a geographically distinct pattern \(^1\).

Further elucidating the molecular mechanism of CQR is essential. Pfcrt plays a critical role in CQR, probably via some yet to be determined transport function. The protein resides in a subcellular membrane within an intracellular parasite, so to experimentally study pfcrt transport function requires that transported substrates cross three membranes in a coordinated fashion. This is extremely difficult to manipulate experimentally. Since the technology required for fabricating membrane vesicles of various types (e.g. secretory, inside out plasma, right side out plasma, vacuolar) and for purifying and reconstituting polytopic integral membrane proteins is...
well developed for yeast, heterologous expression of pfcrt in yeast would obviously greatly assist further analysis of pfcrt transport function.

Unfortunately, a literature survey for successful heterologous expression of \textit{P. falciparum} genes is not encouraging. There is but one successful report of low level heterologous expression of a native \textit{P. falciparum} cDNA (dihydrofolate reductase – thymidylate synthase [DHFR – TS] protein [6]). A notorious feature of the \textit{P. falciparum} genome is its very high A,T content (7,8), thus, \textit{P. falciparum} genes reveal a markedly biased codon usage. Coding regions are approximately 70\% A+T, and are flanked by A+T-rich regions as high as 86\% (9 – 12). The high A+T content makes heterologous expression in both prokaryotic (bacterial) and eukaryotic (yeast, insect) systems extremely difficult if not impossible because: a) these species have their own preferred codon usage that is not dominated by A+T(13 – 16), b) A+T rich sequences tend to form putative polyadenylation (poly A) sites as well as efficiency and positioning elements in yeast that result in terminated, truncated, or poorly transcribed mRNAs (17 – 20).

Thus, expression of a synthetic DHFR – TS gene with optimized codon usage was found to increase the level of expressed protein (21). This result recently enticed others to construct genes with optimized yeast codon usage for \textit{Pfsub1} (a subtilisin-like protease), \textit{Pf msp-1} (a merozoite stage specific surface protein complex), and the antigen Pfs48/45 (22 - 24). These three studies have yielded some additional success, however, heterologous expression of \textit{P. falciparum} genes remains extremely difficult and no successful overexpression of a polytopic integral membrane protein has yet been reported. Polytopic integral membrane proteins are typically encoded by large genes that are more difficult to assemble synthetically. Also, aside from codon usage and poly A termination issues, the encoded proteins contain folding,
membrane translocation and membrane insertion sequences that can be species and membrane specific. These are not well defined for malarial membrane proteins.

*S. cerevisiae* yeast have been used as an important tool in the heterologous expression of proteins (25). Several membrane proteins have been heterologously expressed, among them, human mdr 1 (26, 27), mouse mdr 3 (28), CFTR (cystic fibrosis transmembrane conductance regulator) (29), monoamine transporter (30), Na⁺ channel (31), and dopamine receptor (32). Very few polytopic integral membrane proteins have been successfully expressed in *P. pastoris* yeast (33 – 35).

We have examined these issues and have constructed a synthetic *pfcrt* gene that harbors appropriate base pair content and other necessary features. We have modified the N and C – termini regions based on previous work wherein we were able to functionally express the human multidrug resistance protein (hu mdr 1, p-glycoprotein) in *S. cerevisiae* (27), have subcloned the resulting constructs into appropriate yeast expression vectors, and have achieved high level inducible expression of *pfcrt* and *pfcrt* – biotin acceptor domain fusion (*pfcrt* - bad) proteins in the plasma membrane of *P. pastoris*. More modest constitutive expression in *S. cerevisiae* was also achieved. Using the successfully expressed wild type gene as template, we have also created a CQR associated mutant *pfcrt* allele and have overexpressed the mutant *pfcrt* – bad protein. Analysis of membrane vesicles from these yeast supports the earlier suggestion (1, 3, 5) that *pfcrt* is involved in modulating H⁺ transport.
MATERIALS AND METHODS

Materials

Cloned Pfu polymerase was from Stratagene (La Jolla, CA). Rabbit anti-pfcrt IgG was a kind gift from Dr. T. Wellems (NIAID / NIH, Bethesda, MD). HRP-conjugated monkey anti-rabbit IgG, HRP-conjugated streptavidin and ECL detection reagents were from Amersham (Piscataway, NJ). Prestained SDS-PAGE molecular markers were from BIO-RAD (Hercules, CA). Immobilized monomeric avidin resin was from Pierce (Rockford, IL). Yeast and bacteria growth media reagents were from Difco (Sparks, MD). Oligonucleotides were custom made by MWG-Biotech (High Point, NC). Dodecyl maltoside (DM) was from Calbiochem (San Diego, CA). Plasmids and sequencing primers relevant for subcloning and gene expression in P. pastoris yeast were purchased from Invitrogen ([Carlsbad,CA] version L Picchia expression kit). All other reagents were reagent grade or better and purchased from Sigma (St. Louis, MO).

Strains and Growth Conditions

The E. coli strain NM522 (hsd Δ 5 Δ (lac-pro)F - lacI lacZ ΔM15 pro supE) was used for all bacterial work. S. cerevisiae strain 9.3 (Mat a leu2 ura3 trp1 ade2 trk1Δ trk2Δ ena1::His3::ena4) was kindly provided by Dr. Alonso Rodriguez-Navarro, Universidad Politechnica, Madrid, Spain. P. pastoris strains KM71 and GS115 (arg4 his4 aox1::ARG4 and his4) were from Invitrogen. 9.3 yeast harboring pYHZHB3crt, pYHZHB3crt – bad, pYHZDd2crt - bad or pYKM77 were selected for growth in synthetic complete media lacking uracil (SC-ura) supplemented with 100 mM KCl. P. pastoris strains KM71 and GS115
harboring pPIC35HB3crt, pPIC35HB3crt – bad, pPIC35Dd2crt - bad or pPIC35 were selected for growth in minimal glycerol media (MGM) lacking histidine.

**Designing the synthetic (yeast optimized) wild type Pfcr gene**

The Pfcr-hb3 gene sequence (wild type Pfcr) was obtained from Genebank (http://www.ncbi.nlm.nih.gov) and we used the CODOP program (generously provided by Dr. Elisabeth P Carpenter, Division of Protein Structure, National Institute for Medical Research, London, UK [22]) to back translate the encoded protein sequence. We allowed CODOP to back translate pfcr into a theoretically optimized pfcr gene using a *S. cerevisiae* yeast codon usage table (http://www.kazusa.or.jp/codon). The process was repeated many times with different random seeding values. All the polyA and premature codon patterns in a selected sequence were then screened and destroyed by a second layer of codon engineering. We suspect disrupting poly A is more important than 100 % optimization of codon usage (see Results, Table I caption). A Kozak consensus (GCCGCCACCAUGG) was included and adjusted at -3 (to A) and +4 (to G). Finally, this theoretically optimized pfcr gene was divided up into a collection of 40 base fragments encoding both DNA strands and the melting temperature (Tm) for the 20 bp overlap regions in each primer set (see Results) were calculated. All primer set Tm were then adjusted to reside in the range 56-64 C by yet a third round of codon adjustment. Vector NTI software was also used to check all primers for repeats, palindromes, hairpins and dimers. In the final theoretical optimized pfcr gene AT% is reduced from 72% to 55% (see Results).
Synthesis of the optimized wild type pfcrt (HB3crt) gene

A total of 66 40-mers were made that encoded both strands of the theoretically optimized pfcrt gene. We then followed procedures suggested in (22, 36) with some modifications. Equal volumes of all 66 40-mers (1.5 µM each) were combined and the resultant mixture was diluted 10-fold in 100 µl of a PCR mixture (20 mM Tris HCl (pH8.8) / 2 mM MgSO4 / 10 mM KCl / 10 mM [NH4]2SO4 / 0.1% Triton X-100 / 0.1 mg/mL nuclease-free BSA / 0.2 mM each dNTP / 2.5 U Pfu polymerase). The initial PCR program was one denaturation step at 94 C for 1 min, followed by 25 cycles of 94 C (30 sec), 52 C (30 sec) and 72 C (2 min) and then a final incubation at 72 C for 10 min. 10 µl of this “assembly solution” was then diluted 10-fold in 100 µl of similar PCR mixture, but with 1 µM each of the 5’ and 3’ flanking primers (#1, #34 in the sequence of 66). The amplifying PCR program used was one denaturation step at 94 C for 1 min, followed by 25 cycles at 94 C (45 sec), 68 C (45 sec) and 72 C (5 min) and then a final incubation cycle at 72 C for 10 min.

Synthesis of wild type Pfert – TCBD (HB3crt – bad) fusion gene

A fragment from the P. shermanii transcarboxylase biotin acceptor domain (tcbd) is a convenient biotin tag for monitoring protein expression and facilitating purification (37). Isolated plasmid YEp352/BIO6 containing this fragment (kindly provided by Dr. A. Tzagoloff, Columbia University) was PCR amplified with two designed primers that created convenient overlap and restriction sites (tcbd 5’ primer: (5’) GACAGTAT CATCACTCAAGCGGCCGCGAG GCTTCGAG CTCGGTAC CCGGGGAT CCGGT (3’); tcbd 3’ primer: (5’) GGCCAGTG CCAAGCTTGC ATGCTTGC AGGT (3’). A Not I restriction site was engineered, a Pst I site was destroyed and a Hind III site was retained (underlined). Purified
synthetic \textit{pfert} and purified \textit{tcbd} fragment were combined and PCR amplified to form one larger PCR product containing an in-frame fusion of wild type \textit{pfert} and \textit{tcbd} (\textit{HB3crt – bad}).

\textbf{Synthesis of mutant Pfert – bad (\textit{Dd2crt – bad}) fusion gene}

Several different mutant \textit{crt} alleles have been associated with chloroquine resistance (CQR) (1), including the \textquotedblleft Dd2\textquotedblright allele. This allele differs from the CQS associated allele (what we will call the \textquotedblleft HB3\textquotedblright allele) at 8 codons (74, 75, 76, 220, 271, 326, 356, 371). Thus, we designed a collection of 12 oligonucleotides (sequences available from the authors as supplemental information) that overlapped in 6 locations to create 8 codon mutations (encoding M74I, N75E, K76T, A220S, Q271E, N326S, I356T, and R371I amino acid substitutions, see [1]). These oligos also directed silent codon point mutations that created restriction sites flanking each codon mutation (or group of mutations). The created restriction sites (which facilitated subcloning and which will facilitate future work studying other resistance-associated \textit{pfert} alleles) were Nru I (flanking codon 74 - 76), EcoR V (flanking codon 220), Afl II (flanking codon 271), Cla I (flanking codon 326), Apa I (flanking codon 356), and Sac II (flanking codon 371).

A sequence of PCR reactions was required to synthesize the full length optimized \textit{Dd2crt – bad} gene using the optimized \textit{HB3crt – bad} gene (wild type Pfert – TCBD fusion gene described above) as initial template. First, six PCR fragments (A,B,C,D,E,F) were amplified from \textit{HB3crt – bad} using the 6 complimentary sets of 12 oligos that created the codon and restriction site mutations described above. These were purified, combined in pairs of two, and PCR amplified to yield three larger fragments I, II, III; (e.g. Frag I encoding codons 1-225 was created using Frag A,B as template), etc. By using this sequential strategy, the entire \textit{Dd2crt –}
bad gene was constructed via tiered PCR, subcloned into pYKM77 as described below, and colony amplified and purified.

**Construction of recombinant plasmids**

Plasmid pYKM77 (kindly provided by Drs. K. Kuchler and J. Thorner) was isolated and restricted with Pst I and Hind III. The synthetic HB3crt gene, the synthetic HB3crt- bad fusion gene, and the synthetic Dd2crt – bad gene were each trimmed with Pst I and Hind III and ligated to the 5.4 kbp vector. Recombinant plasmids were isolated from bacterial transformants and analyzed by restriction. Candidate pHZHB3crtbad (encoding the wild type fusion protein under control of a modified Ste 6 promoter), pHZDd2crtbad (encoding mutant crt fusion protein) and pHZHB3crt (encoding wild type pfcrt) plasmids were sequenced in both directions using oligos from the gene assembly steps, the BigDye Terminator cycle sequencing program and ABI prism 373 software. None of the dozen or so sequences that were fully sequenced in each case properly encoded the proteins of interest. Typically, 2 – 3 unwanted point mutations were found in the fully assembled recombinant clones. However, by combining restriction fragments from these constructs (using sites fortuitously placed during the initial gene design) our final synthetic genes encoding full length proteins of the correct sequence were ligated together.

These genes were then also subcloned into the vector pPIC3.5 to create pPIC35hb3crt, pPIC35hb3crtbad and pPIC35dd2crtbad for expression in *P. pastoris*. The pPIC vector harbors an inducible promoter activated by MeOH, as described in Results.
**Yeast Transformation**

Yeast were transformed by the lithium acetate method with 2 µg of target plasmid and 10 µg of carrier plasmid to enhance transformation efficiency. Transformants were plated on selective SC-ura (*S. cerevisiae*) or MGM (*P. pastoris*) agar plates.

**Isolation of Yeast Crude and Plasma Membranes**

Yeast cells were grown to midlog phase and crude cellular membranes were isolated via a glass bead lysis protocol (27) and stored at –80°C. Plasma membranes were purified via the acid precipitation method of Goffeau and Dufour (38), and clear plasma membrane pellets were resuspended in glycerol containing solution and stored at –80°C.

**Partial Purification of Biotinylated wild type pfcrt - bad Protein**

Crude membranes were resuspended to a protein concentration of 1 mg/mL in solubilization buffer (0.75% DM / 500 mM NaCl / 50 mM Tris – Cl (pH 7.50) / 250 mM sucrose / 20% (v/v) glycerol / 1 mM MgATP / 1 mM MgCl₂ / 6.5 mM DTT) and mixed gently for 1 hr at 4°C. The unsolubilized material was removed by centrifugation (100,000 x g / 1 hr / 4°C). DM extracts were loaded on immobilized monomeric avidin resin which had been pre-equilibrated in column wash buffer (0.1% DM / 250 mM NaCl / 50 mM Tris Cl (pH 7.5) / 250 mM sucrose / 20% (v/v) glycerol / 1 mM MgCl₂ / 6.5 mM DTT). The column was washed with 6 bed volumes column wash buffer. Biotinylated wild type pfcrt - bad protein was eluted with 2 bed volumes of elution buffer (2 mM D-biotin in column wash buffer).
**Preparation of ISO Plasma Membrane Vesicles**

Inside-out plasma membrane vesicles (ISOV) were isolated following the procedure described by Menendez et al. (39) with some modifications (27). In particular, ISOV were fabricated with 100 mM KCl inside the vesicles so that H⁺ pumping could be analyzed with symmetrical high Cl⁻ on either side of the plasma membrane. When elevating [salt], [sucrose] was adjusted to conserve osmolality.

**Yeast vacuole isolation**

Our method is adapted from Ohsumi and Anraku (40) with some minor modifications.

**Parasite Digestive Vacuole Isolation**

The *P. falciparum* DV was isolated following (41), with some modifications. 5 ml culture suspensions of twice - synchronized mid trophozoites of the Sudan 106 / 1 strain (at ~12% parasitemia) were washed three times in PBS, pH 7.4. Each 5 ml sample was resuspended in PBS containing 0.15% saponin, incubated for 5 min, and centrifuged to collect trophozoites. The isolated trophozoites were washed repeatedly in ice-cold PBS until the supernatant was clear, resuspended in 10 vol of ice-cold trituration buffer (0.25 M sucrose / 10 mM NaPi [pH 7.10] / 0.5% streptomycin sulfate) and triturated three times on ice using a 27-gauge needle. The suspension was centrifuged (20,000 g / 2 min / 4 C), supernatant was discarded, and the pellet resuspended in 5 vol of 2 mM MgSO₄ / 100 mM KCl / 10 mM NaCl / 25 mM Hepes / 25 mM NaHCO₃ / 5 mM NaPi [pH 7.10]). To 1 ml of the suspension 20 µl of 5 mg/ml DNAseI was added and the suspension was incubated at 25 C for 5 min, followed by centrifugation as before. The supernatant was discarded and the pellet was resuspended in 5 vol of ice-cold trituration
buffer and triturated once again. The suspension was layered on 7 ml of ice-cold 42% Percoll solution containing 0.25 M sucrose / 1.5 mM MgCl$_2$ / 10 mM NaPi (pH 7.10), and centrifuged (16,000 g 30 min, at 4°C). The bottom layer containing the purified food vacuoles was washed three times in the same ice-cold buffer, resuspended in 200 µl washing buffer, and stored at – 80 C.

**Western blot and biotin detection**

14% SDS-PAGE gels were run at 110 V for about 2 hrs, proteins transferred onto PVDF membranes at 40 mAmp overnight, and western or biotin detection blots were performed the next day. For westerns, membranes were washed with PBS-Tween solution for 30 min, blocked with 5% milk in PBS-T for 1 hr, incubated with primary Ab for 1 hr, washed 3 X with PBS-T (15 min each), incubated with secondary Ab (whole anti-rabbit IgG - HRP) for 1 hr and washed again. Biotin detection was essentially the same, with streptavidin -HRP in PBS-T (1:5000 dilution) as the primary (and only) Ab. Membranes were stripped with 62.5 mM Tris HCl (pH 6.80) / 2% SDS / 100 mM B-mercaptopethanol at 50 C for 30 min. with gentle shaking.

**ATPase Activity Assays**

We followed the method of Chifflet and colleagues (42) with some modifications. Briefly, ISOVs were mixed with ATP in an assay buffer containing 50 mM Tris – Mes, pH 7.5, 90 mM NH$_4$Cl, 5 mM MgCl$_2$ and 0.01% NaN$_3$. The reaction was initiated at 37 C for one minute and quenched by the addition of SDS and ammonium molybdate. Absorbance of the generated inorganic phosphate – molybdate complex was measured at 700 nm.
Proton pumping Assay

Proton pumping assays were performed with ISOV essentially as described (27, 43) with some modifications. Namely, 2 μM acridine orange (AO) was used in the assay instead of 20 μM, excitation was at 490 nm instead of 465 nm, and the transport buffer composition in most experiments was 330 mM sucrose / 100 mM KCl / 10 mM Mes – Tris / 4 mM MgCl₂ / 1 mM DTT. Some experiments substituted K glutamate for KCl and MgSO₄ for MgCl₂ when Cl⁻ free conditions were desired.
RESULTS

Fig. 1 is a summary of synthetic (yeast optimized) wild type pfcrt and wild type pfcrt – tcbd gene assembly, and Table 1 shows the final codon composition of the constructed wild type pfcrt gene. Once an optimized wild type crt gene was designed using CODOP and other computer based algorithms (see Methods), the two strands of the gene were divided into 66 40 – mer oligonucleotides with 20 bp overlap regions. Sequences of the overlap regions were then adjusted to yield similar Tm, which we find to be very important for efficient assembly of a gene this size in the first PCR step (cf. Fig. 1). After 25 cycles, trace levels of assembled full length gene (not visible on a conventional agarose DNA gel) were then amplified in the second PCR step (Fig. 1). After DNA sequencing of recombinant clones (which confirms the error rate of the polymerase) a full length optimized gene was assembled from several restriction fragments that contained no errors. As summarized in Table 1, importantly, the overlap Tm, stem loop structure, and poly A region modifications we made to the initially designed gene (see Methods) led to the final gene having a non negligible number of yeast codons that are not “highly preferred”. Therefore, since neither the native wild type or CQR associated pfcrt sequences, nor a wild type sequence optimized at the N terminal 50 codons is expressed in yeast, but the final synthetic genes are (see below), efficient expression of malarial genes in yeast requires conversion of most, but not necessarily all, “unpreferred” codons.

The optimized wild type pfcrt gene was subcloned into a yeast expression vector based on pYKM77 (26) that was previously modified for high level expression of the human mdr 1 protein in yeast (27, 43), to create pHZHB3crt. Also, the optimized gene was fused in frame to a 270 nucleotide region of the P. shermanii transcarboxylase gene (Fig 1, step 3) that encodes the
minimal biotin acceptor domain (37) and similarly subcloned to create pHZHB3crtbad. The wild type \textit{crt–bad} gene was also used as template to construct pHZDd2crtbad encoding CQR associated mutant \textit{crt} (see Methods). \textit{S. cerevisae} were transfected with each construct, as well as similar constructs harboring the native \textit{P. falciparum} HB3 (CQS) and Dd2 (CQR) \textit{crt} alleles. As shown in Fig. 2, the optimized HB3 and Dd2 gene sequences were constitutively and efficiently expressed whereas the native gene sequences were not.

In addition, \textit{pfcrt}, \textit{HB3crt-bad} and \textit{Dd2crt–bad} genes were appropriately restricted and subcloned into the vector pPIC3.5 (Invitrogen). This nonfusion vector integrates at the \textit{AOXI} (alcohol oxidase) locus when linearized prior to transformation, followed by histidine selection. Thus, expression of the cloned gene is induced by a convenient metabolic switch (glycerol to MeOH as the sole carbon source). As shown in Fig. 3, optimized HB3crt (lanes A3, B3) and optimized HB3crt–bad (lanes A4, B4) were even more efficiently expressed in \textit{P. pastoris}, and inducible expression plateaued in approximately 12 hrs (Fig. 3C). Dd2crt–bad is expressed to similar levels in \textit{P. pastoris}, but the time course for efficient plasma membrane insertion is slower relative to HB3crt–bad (Fig. 4). Whether this result implies a physiologically significant alteration in processing of the CQR associated mutant within the native \textit{P. falciparum} environment requires additional detailed study.

Since the levels of expression were higher in \textit{P. pastoris} and at plateau were equal for HB3crt–bad vs. Dd2crt–bad, subsequent functional analysis was performed with \textit{P. pastoris} membranes harvested 16 hrs post induction. In our analysis presented below, our negative controls were isolated after identical MeOH induction treatments performed side–by–side.

Previously (27) we developed a convenient assay for monitoring pH gradient formation in yeast ISOV preparations. Since mutant \textit{pfcrt} protein appears to be involved in modulating the
pH gradient across the DV membrane (3) we analyzed pH gradient formation in ISOV from control KM71 / pPIC3.5 vs. KM71 / pPIC35HB3crtbad vs. KM71 / pPIC35Dd2crtbad strains (Fig. 5). Importantly, the experiments shown in Fig. 5 were with ISOVs fabricated with high (100 mM) [Cl–] inside so that we better approximated the conditions of the native *P. falciparum* DV (likely high internal [Cl–]). Interestingly, Fig. 5 shows that in the presence of symmetrical 100 mM [Cl–] the magnitude of ΔpH formed in ISOV harboring Dd2crt – bad (dashed lines) is conspicuously higher than in control (light solid line), but ΔpH for ISOV harboring HB3crt – bad (heavy solid line) is similar. In Figs. 5A,C we show two comparisons between six different independent ISOV preparations to demonstrate reproducibility (see also Fig. 6 left hand side).

Based on these results, we next tested another prediction of one hypothesis that has been offered for pfcrt function, namely, that it might perform some type of anion transport (perhaps Cl–) in order to assist maintenance of the high chemical gradient in H+ that likely exists across the DV membrane (44). Presumably, similar to other endo membranes that maintain high ΔpH, dissipation of the electrical potential difference caused by H+ influx, via counter balanced diffusion of Cl–, is required to maintain high DV membrane ΔpH. Fig. 5B,D shows that upon substituting Cl– in the pH gradient assay buffer with equimolar glutamate there is nearly complete normalization of ΔpH formation for the three ISOV preparations. Again, two comparisons using six independent preparations are shown to demonstrate reproducibility. Therefore, increased ΔpH formation provided by mutant crt – bad is Cl– dependent. As shown in Fig. 6, the increased ΔpH for Dd2crt – bad ISOV is also more sensitive to verapamil (VPL) relative to control or HB3crt – bad ISOV. This is consistent with partial reversal of resistance due to Dd2crt by similar [VPL] (44 and references within).
Since the amplitude of ATP–dependent ∆pH formation was increased via expression of Dd2crt–bad, we next analyzed liberation of inorganic phosphate from these ISOV preparations (Fig. 7). ISOV from KM71 / Dd2crt - bad yeast fabricated with high [Cl⁻] inside (dotted bars) showed elevated release of inorganic phosphate relative to control (closed bars) or KM71 / HB3crt - bad (hatched bars) ISOV. This effect was again reversed by VPL (Fig. 7, center) and partially reversed by FCCP (Fig. 7, right hand side). Release of Pi was inhibited by vanadate with similar Ki for the two preparations (not shown), consistent with either a stimulation or upregulation of the endogenous (presumably PMA1–like [45]) H+ ATPase found in the P. pastoris plasma membrane. To test the latter, we analyzed H+ ATPase levels in the plasma membranes of control vs. Dd2crt – bad vs. HB3crt - bad yeast via western blot. As shown in Fig. 8 (for two independent sets of preparations, the same preparations as those used for experiments in Fig. 5), to the best of our ability to ascertain, the plasma membrane ATPase levels are nearly identical for the three strains even though the apparent H⁺ translocation ability and ATPase activity is higher for Dd2crt – bad.

Finally, obviously, additional detailed studies that expand upon these initial observations would be greatly assisted by purification of the biotinylated enzyme. Fig. 9 shows that a combination of membrane purification, membrane wash, solubilization with DM, and monomeric avidin chromatography results in a rapid (at least 2000 fold, see caption) purification of HB3crt - bad protein.
DISCUSSION

Our results may be summarized as follows:

1) The *P. falciparum* gene critical for evolution of CQR, *pfcrt*, was optimized for expression in the yeast *S. cerevisiae*. Expression was detected, and higher expression of the same constructs were found in the yeast *P. pastoris*. To our knowledge, this is the first successful demonstration of overexpression of a polytopic integral membrane protein from *Plasmodia* in yeast.

2) Wild type (HB3) and CQR associated mutant (Dd2) *crt – bad* are found in the plasma membrane of both strains of yeast. Levels are not affected by adding the bad domain, but rate of folding or of membrane incorporation in *P. pastoris* appears slower for Dd2*crt – bad* vs. HB3*crt – bad*.

3) Initial studies indicate that when ISOV are created with high internal [Cl\textsuperscript{-}] and diluted into equimolar Cl\textsuperscript{-}, Dd2*crt – bad* stimulates release of inorganic phosphate and H\textsuperscript{+}-pumping ability via the PMA1 H\textsuperscript{+}-ATPase, in a Cl\textsuperscript{-} dependent fashion. This effect is also inhibited by VPL.

4) Rapid purification of *pfcrt – bad* is accomplished via DM solubilization and avidin – biotin affinity chromatography.

These results have a variety of important implications. There is tremendous interest in the transport biology of *P. falciparum* because alterations in that transport are linked to drug resistance which causes millions of deaths annually. Recently, mutations in the *pfcrt* gene were linked to CQR in *P. falciparum* (1), but the function of the polytopic integral membrane protein encoded by this gene is unknown. Since the protein product is localized to the DV membrane,
since a high $\Delta \text{pH}$ is physiologically required across this membrane, since lowered accumulation of weak base antimalarials is found in resistant parasite DV, and since mutations in pfcrt have been linked to possible changes in DV pH (3 – 5), it is likely that pfcrt performs some type of molecular membrane transport function. Elucidation of this function within the native DV membrane (a subcellular membrane within a cell within another cell) would be exceedingly difficult, if not impossible. Thus, heterologous expression in yeast followed by biochemical characterization of well defined yeast membrane vesicle preparations offers one conspicuously attractive route for further analysis. Unfortunately, as described earlier, the base composition of malarial genes makes this impossible unless the gene is optimized to reflect preferred yeast codon bias and to eliminate deleterious mRNA structures. Only a few examples of gene optimization have been reported, with varying levels of success, and yeast optimization of no genes the size of pfcrt (to our knowledge) has yet been attempted. Moreover, since polytopic integral membrane proteins are notorious for exhibiting processing and membrane insertion difficulties when expressed heterologously, it is justifiably thought by most investigators that attempting heterologous expression of malarial genes encoding polytopic membrane proteins is a very risky proposition. Our results show that, assuming the gene is designed as described, it is less risky than initially anticipated.

There are two reasons why this simple result is very important. The first is, again, tremendous interest is focused on membrane transport pathways of \textit{P. falciparum}. For example, nucleotide transport (46), sugar transport (47), and H$^+$ transport by unusually intriguing pyrophosphate hydrolyzing pumps (48) are all catalyzed by specific transporters and all offer unique insight into \textit{Plasmodia} biology and (hopefully) therapeutic intervention. With the
continued growth of malaria as a shockingly severe global health threat, developing methods for rapidly analyzing these transporters is vital.

Second, a number of integral membrane proteins expressed by *Plasmodia* at various stages of development could (in theory) serve as antigens in vaccine development. They are externally disposed and hence accessible to immune surveillance. Better methods for efficiently expressing and purifying these proteins would presumably be welcome. We believe this detailed, methodical, yeast–based approach is one such method, and our initial data with pfcrt support this notion.

Beyond these technical issues, our results are also interesting because they suggest ways in which mutant pfcrt proteins (e.g. Dd2crt) cause drug resistance in *P. falciparum*. Clearly we are just beginning a molecular–level analysis of wild type vs. various mutant pfcrt function and there is much yet to learn, but under conditions of high symmetrical [Cl\(^-\)] across the membrane, Dd2crt: 1) stimulates release of Pi from yeast plasma membranes 2) appears to increase the ΔpH that is formed in yeast ISOV. Moreover, the ΔpH effects are inhibited by either addition of VPL or the withdrawal of Cl\(^-\), and the ATPase enhancement in ISOVs is similarly inhibited by VPL and also by FCCP. Since CQR has previously been associated with alterations in DV pH (3, 5) it is not difficult to envision how these effects could contribute to CQR (5). One reason the ISOV system is of interest in this context is because in certain ways it mimics the endogenous physiology of the DV (e.g., bioenergetics dominated by a H\(^+\) ATPase with an extrafacially disposed ATP hydrolysis site).

However, first, it is important to point out that multiple interpretations of these data are formally possible. The first, which we do not favor, is that Dd2crt does not affect ΔpH under symmetrical [Cl\(^-\)], but promotes active translocation of the probe we use to measure ΔpH
(acridine orange) at a rate faster than passive diffusion. If this is the case, the transport is directly or indirectly Cl\(^-\) dependent. Active translocation of acridine orange (AO) at a rate faster than the (already extremely rapid) passive translocation under initial rate conditions would be quite surprising (5). Also, this model does not easily explain the elevated ATPase activity found in Dd2crt – bad yeast ISOV under these conditions (the assay is performed in the absence of AO, antimalarials, or any other small molecule putative substrate that might be envisioned via this model). On the other hand, AO does share some structural homology with the antimalarial quinicrine. The AO transport model requires energy, and since pfcr does not harbor recognizable ATP hydrolysis domains, the only energy available is the \(\Delta \mu H^+\) generated by the \(P. pastoris\) plasma membrane ATPase. Thus, if this is the case, Dd2crt might catalyze H\(^+\) / AO antiport. Clearly, testing this model requires an extensive and detailed array of additional kinetic and thermodynamic solute transport assays.

The second possibility is that Dd2crt protein directly interacts with H\(^+\) ATPase, stimulating both hydrolysis of ATP and H\(^+\) pumping in the presence of high symmetrical [Cl\(^-\)]. This would be very interesting, but somewhat curious, since the H\(^+\)ATPase present in these membranes is presumably a P2 type H\(^+\) ATPase, whereas the H\(^+\)ATPase that is expected to be the partner of pfcr in DV membrane bioenergetics is presumably a V – type H\(^+\) ATPase (49). V and P2 type H\(^+\) ATPases do not share sequence homology other than in the ATP hydrolyzing domains. Clearly, co – expression of relevant ATPases and pfcr would be an avenue for future testing of this model, as would purification and co – reconstitution of pfcr and PMA1 vs. pfcr and vacuolar ATPase.

The third explanation, which we currently favor, is that pfcr protein directly or indirectly mediates passive movement of Cl\(^-\) that is perhaps gated by some antimalarial drugs. We suggest
that upon mutation of crt to confer CQR (e.g. Dd2crt) and under appropriate ionic conditions, the mutant crt catalyzes more efficient passive Cl⁻ transport under certain conditions such that even higher ΔpH are possible under these conditions. It is common (if not essential) for proteins that mediate the passive movement of Cl⁻ to exist in endomembranes across which a high ΔpH is maintained, and binding / inhibition of various ion transporters by amphipathic hydrophobic drugs is actually quite common. Such a function would assist formation of a higher ΔpH as found in this study, stimulate H⁺ ATPase activity of PMA1 in ISOV as found, be reversed by agents that also reverse resistance (e.g. VPL) as found, and obviously exhibit a simple dependency on Cl⁻ as found. Future studies with these preparations and others will help distinguish between the possibilities.
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FOOTNOTES:

1 Abbreviations: CQ, chloroquine; CQR, chloroquine resistant (resistance); DV, digestive vacuole; Hb, hemoglobin; DHFR – TS, dihydrofolate thymidylate synthase; bad, biotin acceptor domain; DM, dodecyl maltoside; SC, synthetic complete; MGM, minimal glycerol media; Tm, melting temperature; BSA, bovine serum albumin; PCR, polymerase chain reaction; tcbd, transcarboxylase biotin acceptor domain; DTT, dithiothreitol; ISOV, inside – out plasma membrane vesicles; PBS, phosphate buffered saline; NaPi, sodium phosphate buffer; HRP, horseradish peroxidase; AO, acridine orange; VPL, verapamil; PMA1, plasma membrane ATPase 1; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; Hepes, N-(2-Hydroxyethyl)piperazine-N¢-(2-ethanesulfonic acid).
Figure 1: Construction of the Synthetic pfcr Gene

A. 66 40-mer oligonucleotides were mixed and 24 cycles of PCR amplification were used to assemble a small amount of full length optimized pfcr gene (step one, top). A second round of PCR then amplified trace levels of full length synthetic pfcr (step two). The final gene contained a unique 5’ Pst I site and 3 ‘ Not I and Hind III sites. To fuse the tcbd biotin acceptor domain to the C terminus of pfcr, the tcbd fragment was amplified from YEp352-BIO6 using primers that created Not I and Hind III restriction sites and that removed a Pst I site, purified, and then combined with purified pfcr in an additional PCR reaction (step three).

Figure 2: Constitutive expression of optimized HB3crt – bad and Dd2crt – bad in plasma membrane fractions of S. cerevisiae.

A (top) is a western blot using the anti-pfcr antibody (kindly provided by Drs. T. Wellems and R. Cooper, NIH) B (bottom) is the same PVDF membrane stripped and re-probed with streptavidin-HRP to detect biotinylated proteins. Lane 1 is DV isolated from P. falciparum, and shows an immuno reactive band at approximately 48.6 kDa (see [1]), the predicted mass of wild type pfcr (which is not endogenously biotinylated). Lanes 2 and 5 are negative controls (9.3 S. cerevisiae transformed with empty vector), and lanes 3, 4, 6, and 7 are strain 9.3 S. cerevisiae expressing engineered HB3crt – bad, engineered Dd2crt – bad, endogenous P. falciparum HB3 and endogenous P. falciparum Dd2 alleles, respectively. Pfcr-bad membranes show an immunoreactive (A, top) and biotinylated (B, bottom) band that runs at a higher molecular weight (57.7 kDa) because of the added biotin acceptor domain, whereas yeast expressing the endogenous crt genes show no measureable immunoreactivity (Lanes A6, A7), even upon prolonged overexposure (not shown). Each lane contains 50 µg membrane protein.
**Figure 3:** Inducible expression of optimized HB3crt – bad in plasma membrane fractions of *P. pastoris*.

**A** Left is a representative western blot using anti-pfcrt antibody, and **B (right)** is the same PVDF membrane stripped and re-probed with streptavidin-HRP to detect biotinylated proteins (lanes B1 – B4). Lane (A1, B1) is DV isolated from the *P. falciparum* Sudan / 106 strain. Lane (A2, B2) KM71/ pPIC3.5 uninduced (no pfcrt, but harboring endogenously biotinylated yeast proteins); lane (A3, B3) KM71/ HB3crt after 24 hr MeOH induction (non biotinylated wild type pfcrt); lane (A4, B4) KM71/HB3crt - bad after 24 hr MeOH induction (biotinylated HB3crt). As in Fig. 2, KM71 pfcrt-bad membranes show an immunoreactive and biotinylated band that runs at a higher molecular weight because of the added biotin acceptor domain. Importantly, fusion of the *tcbd* encoded domain to pfcrt (Fig. 1 step 3) does not affect levels of pfcrt expressed (compare lanes A3, A4). Comparison between lanes B1 – B4 reveal expected endogenously biotinylated yeast proteins that are useful for verifying equivalent protein loading per lane and the consistency of membrane (ISOV) preparations. **C. (bottom)** shows the time course of HB3crt – bad MeOH induction in *P. pastoris*. Each lane contains 50 µg membrane protein, and crt – bad is detected with avidin - HRP. Odd numbered lanes are KM71/pPIC3.5 (control), even numbered lanes are KM71/HB3crt – bad, and the time points examined for each (proceeding left to right) are 0 (no induction) 1, 3, 6, 9, 12, 24, and 48 hrs. HB3crt - bad is fully induced in *P. pastoris* within 12 - 24 hrs. Cell fractionation (see Methods) followed by similar gel analysis reveals most expressed HB3crt and HB3crt – bad is found in the crude membrane fraction, with approximately 50 % of this localized to *P. pastoris* plasma membrane (not shown). More detailed sub cellular fractionation work will be reported elsewhere.
Figure 4: Time course of HB3crt – bad vs. Dd2crt – bad membrane incorporation. Lanes (1, 4, 7, 10, 13) are control KM71/pPIC3.5 samples at 0, 3, 6, 12, 24 hr post MeOH induction, respectively. Lanes (2, 5, 8, 11, 14) are KM71/HB3crt-bad samples, and lanes (3, 6, 9, 12, 15) are KM71/Dd2crt-bad samples at the same time points relative to control. Note that although conspicuous HB3crt – bad is present at 3 hrs (lane 5), Dd2crt – bad is not present until 6 hrs and even then is still at reduced levels relative to HB3crt (compare lane 9 to lane 8). However, at 12 and 24 hrs (compare lanes 12, 11 and 15, 14, respectively) Dd2crt – bad and HB3crt – bad levels normalize.

Figure 5: ATP-Dependent H⁺-Pumping by ISOVs in the presence (A,C) or absence (B,D) of symmetrical Cl⁻. An acridine orange (AO) – based assay (27) was used to assess formation of a ΔpH in ISOVs via the yeast plasma membrane H+ ATPase for KM71/pPIC3.5 (light solid line each panel) KM71/HB3crt – bad (heavy solid line) and KM71/Dd2crt – bad (dashed line). As the inside of the vesicle acidifies upon addition of 2 mM ATP (first arrow; a slight blip upon addition of ATP has been removed for clarity), AO is trapped via the weak base effect and forms aggregates, thus strongly quenching fluorescence (27, 40). After plateau, high [NH₄Cl], 5 μM nigericin, or 100 μM vanadate similarly dissipate the formed ΔpH (second arrow [27, 40]). 25 μg of ISOV (quantified by amido black assay [27]) are used in each experiment. Panels A and C as well as panels B and D compare two independent control (light solid lines) vs. two independent HB3crt – bad (heavy solid lines) vs. two independent Dd2crt - bad ISOV preparations (dashed lines) to demonstrate reproducibility. Panels A, C are in experiments performed in the presence of high symmetrical KCl (100 mM each side of the membrane).
whereas panels B,D, are the same ISOV but diluted into equimolar K glutamate (no symmetrical Cl\(^-\), but a high Cl\(^-\) gradient oriented outward).

**Figure 6: H\(^+\) - Pumping in the absence of external Cl\(^-\) and vs. verapamil.** Multiple experiments with multiple independent ISOV preparations (at least two preparations and at least two trials with each preparation in each case) were performed as described in Fig. 5, the results were averaged, and are expressed as the mean amplitude +/- standard error. Solid bars: control KM71 / pPIC3.5; hashed bars: KM71 / HB3crt – bad; dotted bars: KM71 / Dd2crt – bad ISOVs, respectively. As shown in Fig. 5, amplitudes are significantly higher for Dd2crt – bad ISOVs in the presence of symmetrical high Cl\(^-\) (left hand side). Amplitudes are reduced for all samples and also normalize (center) in the absence of Cl\(^-\) (similar [K glutamate] replacing KCl), and amplitudes nearly normalize in the presence of 5 \(\mu\)M verapamil (VPL; right hand side).

**Figure 7: ATPase activity for intact ISOVs.** ATPase activity was measured at pH 7.5 (see Methods) for KM71/pPIC3.5 (solid bars), KM71/ HB3crt - bad (hashed bars) and KM71/ Dd2crt - bad (dotted bars) in the presence of either symmetrical KCl with no drug (left) symmetrical KCl plus 5 \(\mu\)M VPL (center) or symmetrical KCl plus 10 \(\mu\)M FCCP (right). As in Fig. 6, we present the average (+/- S.E.) from at least two trials with at least two independent ISOV preparations of each type. The reactions were all initiated by addition of 1mM ATP and allowed to proceed for either 15 or 0 min in the presence and absence of 0.5 mM vanadate. In each case, the results are presented as percent of the vanadate – inhibitable activity (27) exhibited by the control ISOV at pH 7.5. At pH 7.5, in our hands, the vanadate-inhibitable specific activity (\(\mu\)mol P\(_i\) / mg protein / min) as determined by P\(_i\) standard curves is 0.1 – 0.2 for control ISOV.
This agrees reasonably well with activity reported for *S. cerevisiae* ISOV (39). To our knowledge, no ATPase activity has previously been quantified for *P. pastoris* ISOV.

**Figure 8: Quantification of PMA1 expressed in ISOV.** Western blot using anti – PMA1 (anti *S. cerevisiae* plasma membrane H⁺ - ATPase) antibody generously provided by Dr. Carolyn Slayman (Yale), was performed as described in methods. We show results from two independent sets of ISOV preparations; lanes 1 – 3 contain 30 µg highly purified plasma membrane protein (ISOV protein) for one set, and lanes 5 – 7 contain 20 µg ISOV protein for another set. These two independent sets of preparations are the same as those used for the experiments shown in Fig. 5. Lanes 1, 5 are KM71/pPIC3.5, lanes 2,6 KM71/HB3crt - bad, lanes 3,7 KM71/Dd2crt - bad, respectively. Elevated PMA1 expression is not the explanation for the increased H⁺ pumping found for KM71 / Dd2crt – bad ISOV (Fig. 5).

**Figure 9: Partial Purification of Pfcr - bad.**

Avidin – HRP blot showing approximately 2000 fold purification of pfcr - bad protein from KM71 / pPIC3.5crtbad membranes. Lane 1, control membrane fraction (50 µg), lane 2, pfcr bad membranes (50 µg), lane 3, 1.0 µg DM solubilized pfcr bad membranes, lane 4, column wash buffer, lane 5, column fraction collected immediately after application of solubilized pfcr bad, lane 6, 0.02 µg of eluate after application of D – biotin elution buffer (see Methods). Comparing densitometry of these bands vs. protein assay quantification, we calculate approximately 2000 fold purification of pfcr - bad protein via a combination of membrane isolation, membrane wash, DM solubilization, and monomeric strep avidin chromatography.
| A.A. | Codon | P. falciparum usage | S. cerevisiae usage | # used in native Pfcrt | # used post optimization |
|------|-------|---------------------|----------------------|------------------------|-------------------------|
|      |       |                     |                      | After CODOP            | Final gene               |
| ARG  | CGA   | 2.4                 | 3.0                  | 2                      | 0                       | 0                       |
|      | CGC   | 0.5                 | 2.6                  | 0                      | 0                       | 0                       |
|      | CGG   | 0.2                 | 1.7                  | 0                      | 0                       | 0                       |
|      | CGU   | 3.4                 | 6.5                  | 3                      | 5                       | 5                       |
|      | AGA   | 16.7                | 21.3                 | 11                     | 8                       | 9                       |
|      | AGG   | 4.0                 | 9.3                  | 1                      | 4                       | 3                       |
| LEU  | CUA   | 5.3                 | 13.4                 | 1                      | 6                       | 6                       |
|      | CUC   | 1.7                 | 5.4                  | 1                      | 0                       | 0                       |
|      | CUG   | 1.4                 | 10.4                 | 0                      | 10                      | 13                      |
|      | CUU   | 8.6                 | 12.2                 | 10                     | 3                       | 1                       |
|      | UUA   | 49.3                | 26.3                 | 29                     | 12                      | 5                       |
|      | UUG   | 10.2                | 27.1                 | 5                      | 15                      | 21                      |
| SER  | UCA   | 18.0                | 18.8                 | 7                      | 5                       | 4                       |
|      | UCC   | 5.4                 | 14.2                 | 6                      | 6                       | 9                       |
|      | UCG   | 2.8                 | 8.6                  | 0                      | 0                       | 0                       |
|      | UCU   | 15.2                | 23.6                 | 6                      | 7                       | 5                       |
|      | AGC   | 3.8                 | 9.7                  | 3                      | 5                       | 8                       |
|      | AGU   | 21.7                | 14.2                 | 7                      | 6                       | 3                       |
| THR  | ACA   | 22.7                | 17.7                 | 9                      | 10                      | 6                       |
|      | ACC   | 5.6                 | 12.6                 | 5                      | 4                       | 10                      |
|      | ACG   | 3.8                 | 8.0                  | 0                      | 0                       | 0                       |
|      | ACU   | 12.8                | 20.2                 | 7                      | 7                       | 5                       |
| PRO  | CCA   | 13.3                | 18.2                 | 4                      | 4                       | 5                       |
|      | CCC   | 2.6                 | 6.8                  | 0                      | 2                       | 1                       |
|      | CCG   | 1.0                 | 5.3                  | 0                      | 0                       | 0                       |
|      | CCU   | 9.2                 | 13.6                 | 3                      | 1                       | 1                       |
| ALA  | GCA   | 12.8                | 16.2                 | 7                      | 7                       | 6                       |
|      | GCC   | 3.4                 | 12.6                 | 4                      | 3                       | 5                       |
|      | GCG   | 1.2                 | 6.1                  | 0                      | 0                       | 0                       |
|      | GCU   | 12.5                | 21.1                 | 7                      | 8                       | 7                       |
| GLY  | GGA   | 16.6                | 10.9                 | 10                     | 3                       | 3                       |
|      | GGC   | 1.7                 | 9.7                  | 2                      | 10                      | 6                       |
|      | GGG   | 2.9                 | 6.0                  | 0                      | 0                       | 0                       |
|      | GGU   | 16.9                | 23.9                 | 11                     | 10                      | 14                      |
| VAL  | GUA   | 17.9                | 11.8                 | 13                     | 6                       | 3                       |
|      | GUC   | 2.6                 | 11.6                 | 4                      | 3                       | 10                      |
|      | GUG   | 4.9                 | 10.7                 | 1                      | 4                       | 4                       |
|      | GUU   | 17.7                | 22                   | 7                      | 12                      | 8                       |
| LYS  | AAA   | 90.5                | 42.1                 | 23                     | 15                      | 0                       |
|      | AAG   | 19.4                | 30.8                 | 4                      | 12                      | 27                      |
| ASN  | AAC   | 18.8                | 24.9                 | 7                      | 16                      | 32                      |
|     | AAU  | 106.1 | 36  | 25  | 16  | 0  |
|-----|------|-------|-----|-----|-----|----|
| GLN | CAA  | 24.8  | 27.5| 10  | 8   | 6  |
|     | CAG  | 3.3   | 12.2| 0   | 2   | 4  |
| HIS | CAC  | 3.9   | 7.8 | 3   | 3   | 4  |
|     | CAU  | 19.6  | 13.7| 2   | 2   | 1  |
| GLU | GAA  | 63.7  | 45.9| 17  | 20  | 13 |
|     | GAG  | 10.0  | 19.1| 3   | 0   | 7  |
| ASP | GAC  | 8.6   | 20.4| 4   | 6   | 11 |
|     | GAU  | 55    | 37.8| 10  | 8   | 3  |
| TYR | UAC  | 5.7   | 14.7| 5   | 10  | 18 |
|     | UAU  | 46.2  | 18.8| 13  | 8   | 0  |
| CYS | UGC  | 2.5   | 4.7 | 2   | 6   | 9  |
|     | UGU  | 15.5  | 8.0 | 12  | 8   | 5  |
| PHE | UUC  | 7.3   | 18.2| 13  | 19  | 38 |
|     | UUU  | 34.5  | 26  | 25  | 19  | 0  |
| ILE | AUA  | 44.4  | 17.8| 13  | 9   | 0  |
|     | AUC  | 6.0   | 17.1| 4   | 14  | 41 |
|     | AUU  | 33.7  | 30.2| 29  | 23  | 5  |
| MET | AUG  | 21.1  | 20.9| 12  | 12  | 12 |
| TRP | UGG  | 5.3   | 10.3| 2   | 2   | 2  |
| TER | UAA  | 1.1   | 1.0 | 1   | 1   | 1  |
|     | UAG  | 0.2   | 0.5 | 0   | 0   | 0  |
|     | UGA  | 0.2   | 0.6 | 0   | 0   | 0  |
| Total| 425  | 425   | 425 |     |     |    |

**Caption for Table I. Comparison of Malarial vs. Yeast Codon Preferences and Codon Usage for Native vs. Yeast Optimized Pfcrt.** Use per 1000 codons is listed for *P. falciparum* and *S. cerevisiae* (http://www.kazusa.or.jp/codon) (column 3, 4). Codon usage in native *pfcrt* (5th column) was compared to preferred usage for *S. cerevisiae* (4th column). Subsequently, the optimized gene was analyzed for poly A and premature termination sequences and the sequence was further adjusted. Thus, for example, even though the yeast preferred codon for lysine is AAA, all lysine residues were coded with AAG to avoid poly A sequences (compare last two columns) in the final optimized gene.
STEP 1
(gene assembly)

STEP 2
(gene amplification)

STEP 3
(gene fusion)

Figure 1
Figure 2

A

1 2 3 4 5 6 7

57.7 kDa

48.6 kDa

B

1 2 3 4 5 6 7

57.7 kDa
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Analysis of the antimalarial drug resistance protein Pfcrt in yeast
Hanbang Zhang, Ellen M. Howard and Paul D. Roepe

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