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Archives of Biochemistry and Biophysics 452 (2006) 119–128
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Functional reconstitution of purified chloroquine resistance membrane transporter expressed in yeast

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Received 16 May 2006, and in revised form 10 June 2006
Available online 5 July 2006

Abstract

Malaria is one of the major parasitic diseases. Current treatment of malaria is seriously hampered by the emergence of drug resistant cases. A once-effective drug chloroquine (CQ) has been rendered almost useless. The mechanism of CQ resistance is complicated and largely unknown. Recently, a novel transmembrane protein, *Plasmodium falciparum* chloroquine resistance transporter (PfCRT), has fulfilled all the requirements of being the CQ resistance gene. In order to elucidate the mechanism how PfCRT mediates CQ resistance, we have cloned the cDNA from a CQ sensitive parasite (3D7) and tried to express it in *Pichia pastoris* (*P. pastoris*) but with unsuccessful results due to AT-rich sequences in the malaria genome. We have therefore, based on the codon usage in *P. pastoris*, chemically synthesized a codon-modified *pfcrt* with an overall 55% AT content. This codon-modified *pfcrt* has now been successfully expressed in *P. pastoris*. The expressed PfCRT has been purified with immuno metal affinity chromatography (IMAC) and then reconstituted into proteoliposome. It was found that proteoliposomes have a saturable, concentration and time-dependent CQ transport activity. In addition, we found that proteoliposomes with resistant $PfCRT^r$ (K76T or K76I) showed an increased CQ transport activity compared to liposomes with lipid alone, or proteoliposomes reconstituted with sensitive $PfCRT^s$ (K76) protein. This activity could be inhibited by nigericin and decreased with the removal of Cl^-. This work suggests that PfCRT is mediating CQR in *P. falciparum* by virtue of its changes in CQ transport activity depending on pH gradient and chloride ion in the food vacuole.

Keywords: PfCRT; Yeast expression system; Proteoliposome; CQ transport activity

Malaria is a serious tropical disease caused by the parasite *Plasmodium*. More than one million people, mainly African children under the age of 5, die of malaria every year. Currently there is no vaccine available. Therefore, drug therapy is the best strategy to control malaria. However, the heavy use of drugs throughout the decades eventually led to drug resistant parasites. Resistance to drugs like dihydrofolate reductase (DHFR)[1] inhibitors (e.g., pyrimethamine) or heme metabolism inhibitors (e.g., chloroquine, CQ) has been observed clinically. The resistance to pyrimethamine is mainly due to the point mutations found in the target gene DHFR [1]. However, the mechanism of chloroquine resistance (CQR) is less well understood. Several CQR candidate genes have been proposed including *pfmdr1* (*Plasmodium falciparum* multi-drug resistance 1) [2,3], factors involved in blocking the access to the CQ target hematin [4], Na^+/H^+ exchanger (NHE) [5] and *cg2* [6]. However, they were found to be no association with CQR in a genetic cross between CQR and CQS strains [7,8]. This suggested that these genes might play a role in CQR, but they are not the only determinant for resistance [9–11]. Therefore, there may be some other more important players in CQR.

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[1] *Abbreviations used:* CQ, chloroquine; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; IMAC, immuno metal affinity chromatography; DHFR, dihydrofolate reductase; CQR, chloroquine resistance; NHE, Na^+/H^+ exchanger.

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.06.017

Recently, a new gene *pfcrt* (or *cg10*), which was a highly disrupted open reading frame, was found to be linked to CQ resistance phenotype in the genetic cross as well as in the field isolates [12,13]. This gene is on chromosome 7 with 10 kb away from *cg2*. Unlike *cg2*, the resistant allele of *pfcrt*, when transferred to the sensitive parasites, can mediate a full level of CQ resistance [12,13]. It was found that amino acid position 76 of PfCRT was important in CQ resistance. This is demonstrated by the CQ-sensitive strain (Sudan 106/1) which contained all but one amino acid observed in CQR parasites FCB. 106/1 contained K76 whereas FCB contained K76T. It was proposed that this K76 was the critical residue that caused strain 106/1 to be CQS. In addition, resistant parasites isolated by *in vitro* CQ selection resulted in the emergence of CQR mutants with K76I mutation in PfCRT, further confirming the importance of this residue in mediating CQR [12]. Several field surveys also indicated that the *pfcrt* was associated with CQR [14–16]. All these point to the notion that *pfcrt* is the long sought-after CQR gene. The question now is how it mediates CQR.

The primary sequence of PfCRT is 424 amino acids in length and it is predicted to have 10 transmembrane domains. It was found to be present in a logical localization, digestive vacuole, where CQ is expected to work [12]. In a recent paper, a detailed bioinformatics analysis revealed that PfCRT was a member of the drug/metabolite transporter superfamily [17,18]. PfCRT protein was queried against the Entrez Conserved Domain Database and was found to have significant hits to DMT superfamily conserved domains. The K76T mutation in PfCRT protein has been identified as a crucial determinant of PfCRT-mediated CQ resistance [13]. This mutation lies towards the C-terminal end of TMD1, where it is predicted to be involved in substrate recognition [18]. The possible function of PfCRT is to transport CQ. The K76, located at the vacuole face of the membrane, is predicted to be involved in repelling the protonated (cationic) form of chloroquine (CQ^{2+}) and preventing it from interacting with the transporter. The CQR conferring K76T or K76I removes the positive charge, allowing CQ^{2+} to interact with and be transported by the PfCRT. However, this remains to be investigated experimentally.

In addition, it has been found that the food vacuole pH in the CQ resistant parasites was lower than that of the sensitive counterparts. A pH difference of 0.43 in CQR parasites shift the balance towards insoluble hematin which cannot bind to CQ, resulting in lowered overall CQ accumulation [19]. This observation was controversial as previous findings were suggesting the opposite [20]. It has therefore been suggested that PfCRT might be involved in the regulation of food vacuole pH.

In order to fully understand the mechanism of CQ resistance, we need an *in vitro* system to study PfCRT's function. *P. falciparum* is notorious to be transfected efficiently. Other heterologous systems including *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris* have been successfully used to overexpress plasmodial genes [21–23]. The *Pichia* system is of particular interest due to its high expression level and the ease of producing soluble proteins. Despite this, AT rich sequences have been reported to cause premature transcription termination in *Pichia* [24,25]. The *Plasmodium* sequence is particularly AT-rich, sometimes up to 85% and is a major concern if PfCRT is to be overexpressed efficiently. Zhang et al. has recently reported to use *Pichia* system to overexpress PfCRT and has performed preliminary characterization to demonstrate that PfCRT may be cooperating with endogenous ATPase in alternating the lumen pH [26]. Here, we report the use of similar overexpression system to successfully overexpress PfCRT in *Pichia*. The expressed PfCRT was purified and then reconstituted into proteoliposomes, which was used to investigate the function of PfCRT.

Experimental procedures

General reagent

Restriction enzymes and all other routine molecular reagents were purchased from either New England Biolabs or Gibco/BRL Life Technologies. The *P. pastoris* expression kit containing *P. pastoris* KM71 (*his4 aox1*::ARG4), intracellular expression vector (pPICZA) was purchased from Invitrogen. *n*-Dodecyl-D-maltoside (DDM), octyl-D-glucopyranoside and CHAPS were obtained from Amresco. Imidazole was purchased from Sigma (catalog number I-0250). Ni–NTA agarose resin was obtained from Qiagen. Rabbit anti-His polyclonal IgG and goat anti-rabbit IgG-HRP were from Santa Cruz Biotechnology. Super-Signal Substrate Western Blotting kit (Pierce) was used for detection in the Western hybridization.

Cloning of pfcrt cDNA and construction of the pfcrt expression vectors pPICZA-CRT

pfcrt gene was PCR amplified from mixed blood stages of *P. falciparum* (strain 3D7) cDNA library (unpublished). The primers used were designed to anneal the 5′ and 3′-end of the coding region with the following sequence: (A) 5′-ATTCGAAAATGGAATTCGCAAG-3′ (underlined *Sfu*I/*Eco*RI); (B) 5′-TTGGGTACCAATTGAATCGACG TTGGT-3′ (underlined *Kpn*I). PCR was carried out using 0.75 unit of Advantage® Polymerase (Clontech) in a 25 μL reaction volume with an initial denaturation step at 94 °C for 1 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, elongation at 60 °C for 1.5 min and then a final elongation step at 68 °C for 10 min. The 1.3 kb PCR fragment was inserted into the *Sfu*I/*Kpn*I sites of the vector of pPICZA and sequenced, yielding a construct named pPICZA-CRT.

Design of synthetic pfcrt

The sequence of the codon-modified *pfcrt* gene was designed according to the *P. pastoris* codon usage table

[26,27]. This was achieved by using the CODOP program (a generous gift of Liz Carpenter; Imperial Biol. Sci.; liz.carpenter@ic.ac.uk) with minor modifications as described before [26,28]. A total of 66 sets of 40-mers from both strands with 20-bp overlap were made. The oligonucleotides (both forward and reverse direction) coding the amino acid position 76 of PfCRT was designed with degeneracy to correspond to the K76T position of PfCRT that is known to be important for CQR. In addition, the Kozak sequence CACCATGG was incorporated in the extreme 5′-oligonucleotide for optimal initiation of translation and six additional histidine codons were introduced just prior to the stop codon in the extreme 3′-oligonucleotide. Finally, the 5′ and 3′ sequences were added, in order to enable versatile cloning of the gene in expression vectors.

Gene assembly and amplification

The synthetic *pfcrt* gene was constructed following the procedure described in Ref. [26] with some modifications. 2.5 pmol of each oligonucleotide was mixed in 20 μL of PCR assembly mixture (2 μL of 10 × Adanvatage® buffer, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/mL BSA, 0.2 mM each of dNTPs, 1.25 U of Advantage® DNA polymerase). The assembly PCR program was performed with 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s. Subsequently, 2 μL of the assembly mixture was diluted 10-fold in 20 μL PCR mixture (2 μL of 10× Advantage buffer, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/mL BSA, 0.2 mM each dNTPs, 3 U of Advantage® DNA polymerase and the two outermost primers at 1 μM each) and the second PCR program was started. It consisted of a denaturation step of 94 °C for 30 s, followed by 20 cycles of 94 °C for 30 s, 68 °C for 45 s and 72 °C for 90 s and a final incubation cycle at 72 °C for 10 min. The PCR product was analyzed on 0.8% agarose gel, purified with PCR-Purification Kit (Qiagen), cloned into pGEM-T vector (Promega) and transformed into *E. coli* strain XL-1 Blue (Stratagene). DNA sequencing was determined to confirm no mutations in it. The gene was digested with *Eco*RI and *Not*I and cloned into pPICZA vector, transformed into XL-1 Blue cells under the selection of 25 μg/mL Zeocin.

Growth of P. pastoris in shake flasks

The synthetic genes were cloned into the *P. pastoris* pPICZA vector using the *Eco*RI and *Not*I sites of the polylinker region, yielding two constructs named pPICZA-PfCRT^r, pPICZA-PfCRT^s, respectively. The constructs were transformed into the *P. pastoris* KM71 strain according to guidelines given by Invitrogen. Transformants were plated on YPD agar plates containing 1 mg/mL of Zeocin. Each construct was selected for expression trials in 100 mL MGYH medium (1.34% w/v yeast nitrogen base without amino acid, 1% v/v glycerol, 0.4 mg/L biotin and 40 mg/L histidine) and incubated at 30 °C with shaking to OD_{600} over 2.0, then the cells were centrifuged in sterile bottles (2500*g* for 10 min) and resuspended in 20 mL MMH medium (1.34% w/v yeast nitrogen base without amino acid, 0.5% v/v methanol, 0.4 mg/L biotin and 40 mg/L histidine). Incubation was continued for 72 h with further additions of methanol (0.5% v/v) at 24 and 48 h. Cells were finally collected by centrifugation at 2500*g* for 5 min at 4 °C, and store at −80 °C for further use.

Growth of P. pastoris cells in a fermentor

Fermentation was performed according to guidelines given by Invitrogen using a bench top fermentor with slight modification according to Lerner-Marmarosh et al. [29]. The incubation was started with 5 L of Fermentation basal salts medium (0.93 g/L $CaSO_4$, 18.2 g/L K_2SO_4, 14.9 g/L $MgSO_4{\cdot}7H_2O$, 4.13 g/L KOH, 26.7 mL/L 85% H_3PO_4, 4% v/v glycerol, 9 g/L $(NH_4)_2SO_4$, 4 g/L histidine, and 4.35 mL/L PTM1 trace salts) and approximately 1000 mL (20%) of start-up culture generated in baffled shake flasks in MGYH. Fermentation proceeded in glycerol batch phase, glycerol fed-batch phase and methanol fed-batch phase. After three phases, cells were centrifuged at 2500*g* for 10 min at 4 °C, washed twice with distilled water, and frozen at −80 °C.

Preparation of microsomes from P. pastoris

Pichia pastoris microsomes were prepared as follows: yeast was harvested by low speed centrifugation. After centrifugation at 2500*g* for 10 min, cell pellet was resuspended in ice-cold yeast homogenization buffer (0.33 M sucrose, 300 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM DTT) at a concentration of 0.5 g wet wt cells per mL. Protease inhibitors were added as follows: 2 μg/mL pepstatin A and leupeptin, 0.5 μg/mL chymostatin, 1 mM PMSF. After adding 1.5 g glass beads (0.45–0.5 mm diameter) per gram of yeast, the cells were mechanically disrupted for six times, 1 min each, under liquid CO_2 cooling in a Braun homogenizer (type 853033). The homogenate was centrifuged at 3500*g* for 15 min at 4 °C, and the pellet was discarded, and the supernatant was centrifuged at 14,000*g* for 30 min at 4 °C. These steps removed unbroken cells, nuclei, and mitochondrial fractions, which contained little PfCRT as shown by Western blotting. The supernatant was ultracentrifuged at 200,000*g* for 90 min at 4 °C and the pellet containing the microsomes was resuspended in buffer A (50 mM Tris–HCl, pH 7.4, 10% v/v glycerol containing protease inhibitors as above) to the same volume as before the 200,000*g* centrifugation step. The microsome fraction at this stage may be stored at −80 °C until use.

Solubilization of microsomes and purification by Ni–NTA agarose

The microsome pellet was resuspended in buffer A containing 1.2% DDM plus protease inhibitors as above on ice. The suspension was mixed by inversion and incubated at

4 °C for 30 min on a rotator. It was then centrifuged at 10,000*g* for 30 min. The supernatant was added to Ni–NTA agarose resin (Qiagen) which was pre-equilibrated in buffer A at a ratio of 1 mL of the packed resin per 100 mg of solubilized microsomal protein. The slurry was incubated at 4 °C for 3 h on a rotator. The resin with bound protein was transferred into a column and washed with 30 bed-volumes of buffer A containing 0.1% DDM and then with 5 volumes of buffer A containing 0.1% DDM plus 20 mM imidazole. PfCRT was eluted eight times; 0.5 mL each of buffer A containing 0.1% DDM plus 250 mM imidazole.

Western blotting

Protein was electroblotted onto PVDF Immobilon P membrane (MilliPore) after separation by SDS–PAGE gels according to the procedure described by Towbin et al. [30]. After electroblotting, the blocking step was started by incubating for 1 h at room temperature in TTBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% powdered skim milk. The membrane was incubated with the primary antibody (1:3000) for 1 h in TTBS at room temperature, washed with TTBS for three times, 5 min each and then incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (1:2500) in TTBS for 1 h at room temperature, followed by washing and detection with the SuperSignal Substrate Western Blotting kit (Pierce). The chemiluminescence signal was analyzed by a LumiImager (Roche). Primary antibody against PfCRT was a generous gift of Dr. Thomas Wellems of the NIH.

Reconstitution of PfCRT into proteoliposomes

Liposomes were prepared according to a modified method of Putman et al. [31]. The *E. coli* total lipid extracts and egg yolk phosphatidylcholine (Avanti Polar Lipids) were dissolved in chloroform at a concentration of 10 mg/mL, respectively, and stored at −80 °C. For the preparation of liposomes, the *E. coli* total lipid extract and egg yolk phosphatidylcholine in a 3:1 ratio (w/w) were evaporated to dryness using N_2 gas to produce a film, which was subsequently lyophilized for 2 h to remove the last traces of solvent. The lipids film was resuspended in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 mg/mL and then vortexed vigorously to make it dissolve completely. The lipid suspension was then subject to more than five times of freeze (in liquid nitrogen) and thaw (in a 40 °C water bath). At this stage, the liposomes were stored at −80 °C until further use.

PfCRT protein was reconstituted into liposomes according to the procedure described by Putman et al. [31]. Typically, the liposomes were slowly thawed and extruded (11 times) through a 400 nm polycarbonate filter to obtain unilamellar liposomes with a relatively homogeneous size. Subsequently, the liposomes were diluted to 4 mg of lipid/mL with 50 mM potassium phosphate (pH 7.0) and saturated with detergent, which was followed by measuring the $OD_{540\,nm}$, as described by Paternostre et al. [32]. The purified PfCRT protein was mixed with the detergent-saturated liposomes (1 μmol of DDM/mg of lipid) at an appropriate ratio of protein to lipid and incubated for 30 min at room temperature under gentle agitation. The detergent was removed by adsorption to polystyrene beads (Bio-Beads SM2, Bio-Rad) as described previously [33]. Usually, Bio-Beads SM2 was added at a weight of 80 mg/mL and the samples were incubated for 1 h at room temperature. Fresh Bio-Beads were added twice and the samples were incubated at 4 °C for overnight and at room temperature for 1 h, respectively. Finally, the proteoliposomes were collected by centrifugation (200,000*g* for 1 h at 4 °C), resuspended in 0.25 M sucrose and 0.01 M Tris–HCl (pH 7.4) for CQ transport activity. Liposomes for control experiments were prepared in the same way as proteoliposomes except for the addition of protein. The proteoliposomes were stored at −80 °C until use.

Efficiency of PfCRT reconstitution

The PfCRT concentration in proteoliposomes preparations was determined by Western blot. The samples with serial dilutions of reconstituted PfCRT and the purified PfCRT samples of known concentration were separated on the SDS–PAGE side by side and then transferred to PVDF membrane. Comparison of densitometry of western blot of reconstituted PfCRT and purified PfCRT, the amount of the PfCRT protein originally added to the mixture recovered in the proteoliposomes was calculated.

Orientation of PfCRT on proteoliposomes

The orientation of PfCRT on the proteoliposomes was determined using antibodies raised against the C-terminal 6-histidine tagged of PfCRT. Proteoliposomes (containing about 1 μg purified PfCRT) were treated with 1% DDM or untreated for 1 h at 4 °C. Subsequently, the proteoliposomes were incubated for 2 h at 4 °C with anti-his polyclonal antibody. Twenty microliters of protein G-Agarose suspension was added to it and the mixture was incubated at 4 °C on a rocker platform for overnight. Proteoliposomes were then collected by centrifugation at 10,000*g* for 30 s at 4 °C. After washing four times with PBS containing 0.5% BSA, proteoliposomes were separated by SDS–PAGE. The proteins were blotted onto PVDF membranes and amount of bound antibody was determined by Western blotting using anti-PfCRT polyclonal antibody and densitometry.

Accumulation of ^{3}H-CQ in proteoliposomes

^{3}H-CQ uptake in proteoliposomes was measured by a modification of the method of Frances J. Sharom's [34]. Incubations were performed at room temperature in 100 μL final volumes in 1.5 mL eppendorf vials. Proteoliposome preparations were diluted in transport buffer (0.25 mol/L sucrose, 10 mmol/L Tris–HCl, 5 mmol/L $MgCl_2$, 3 mmol/L ATP), and

pre-incubated with unlabeled chloroquine (200-fold of ^{3}H-CQ concentration) for 15 min in 20 μL volumes. To initiate CQ accumulation, 80 μL of transport buffer containing appropriate concentration of ^{3}H-CQ (5 Ci/mmol, American Radiolabeled Chemicals, Inc) was added. At specific time, the accumulation experiments were terminated by addition of 1 mL of ice-cold transport buffer. Proteoliposomes were harvested using a Hoeffer filtration manifold on glass fiber filters (ADVANTEC GC50, Toyo Roshi Kaisha, Ltd.), which had been pre-wetted in PBS. After the filters were washed twice with 5 mL of ice-cold PBS, the filters were dissolved in 5 mL of scintillation fluid (Beckman) after drying overnight. The radioactivity was measured using a liquid scintillation counter (LS 6500 Scintillation System, Beckman). Specific CQ accumulation was calculated by subtracting the counts obtained with 200-fold excess unlabeled CQ from those obtained with ^{3}H-CQ alone. All experimental points were carried out at least in triplicate. Standard error of means was calculated where possible, and they were indicated as error bars in the figures.

Results

Our goal is to use *P. pastoris* to overexpress PfCRT for *in vitro* functional studies. We have used reverse transcriptase polymerase chain reaction (RT-PCR) to amplify the *pfcrt* gene using cDNA from mixed blood-stages of a CQ-sensitive strain of *P. falciparum* (3D7). This *pfcrt* gene was cloned into the *P. pastoris* vector pPICZA to give pPICZA-CRT. However, when transformed into *P. pastoris*, it failed to produce any detectable PfCRT as measured by Western blotting using anti-polyhistidine (lane 1 in Fig. 1) or anti-PfCRT antibody (data not shown). The reasons are that the AT-rich sequence of *pfcrt* (71% A-T in the coding region) might cause premature transcriptional termination [25,35] and the codon usage of *Plasmodium* is different from that of *P. pastoris* [15,27,36].

Codon modification of pfcrt

The unsuccessful expression result prompted us to consider changing the codon usage of *pfcrt* according to that of the *P. pastoris*. There were precedents where modification of codon usage could help in overcoming the expression problems of plasmodial proteins in *P. pastoris* [28,35]. Care was taken to lower the A-T percentage. Based on the codon usage of *P. pastoris* and the codon frequency of a highly expressed gene, *AOX1*, and using a software written to design oligonucleotides for complete gene synthesis (CODOP, a generous gift of Liz Carpenter), we have redesigned the DNA sequence of *pfcrt* while keeping the amino acid sequence unchanged. A degeneracy in oligonucleotides allowed both K76 and K76T to be made. K76I mutant was generated from K76T using standard site directed mutagenesis. Both K76T and K76I mutations have been demonstrated to be important in CQR [12]. Six histidines were added at the C-terminus for purification and detection purposes. The newly designed *pfcrt* has a lowered overall A-T content of 54.6%, compared to the 71% in the native *pfcrt*.

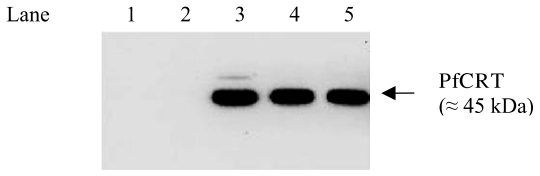

Fig. 1. Expression of PfCRT in *P. pastoris*. Total extracts of *P. pastoris* microsomes were separated by SDS–PAGE, transferred onto PVDF membrane and analyzed by Western blotting using anti-polyhistidine antibody. Similar results were obtained using anti-PfCRT antibody (data not shown). Expression was performed in shake flasks. No detectable PfCRT can be found in *P. pastoris* expressing native PfCRT (lane 1) or control vector (lane 2). In contrast, a band of about 45 kDa can be observed in *P. pastoris* expressing codon-modified PfCRT (lanes 3, 4 and 5). Lane 1, pPICZA-CRT (native PfCRT); lane 2, pPICZA alone; lane 3, pPICZA-PfCRT^{r} (K76); lane 4, pPICZA-PfCRT^{s} (K76T); lane 5, pPICZA-PfCRT^{r} (K76I).

Expression of PfCRT in membrane fractions of P. pastoris

Clones containing pPICZA-PfCRT^{s} (K76), pPICZA-PfCRT^{r} (K76T or K76I) were picked to test for the presence of expressed PfCRT by Western blot analysis using either anti-polyhistidine or anti-PfCRT antibody, which gave similar results: a band of about 45 kDa in size was detected (expected: 49.5 kDa). Typical results using anti-polyhistidine antibody is shown in Fig. 1 (lanes 3–5). There were no significant differences in expression levels among the three samples. No signal was present in the control containing native PfCRT (pPICZA-CRT) or vector alone (pPICZA) (lanes 1 and 2 in Fig. 1). The redesign of codon usage of *pfcrt* was therefore successful in allowing full length PfCRT to be expressed in *P. pastoris*.

Purification of PfCRT

Six histidines were introduced at the C-terminus of the PfCRT during gene assembly. Immobilized metal affinity chromatography (IMAC) was used to purify PfCRT from the microsome fraction. Different detergents have been tried including 1% Triton X-100, 1% CHAPS, 1% *n*-octyl-D-glucopyranoside and 1% DDM to solubilize PfCRT with the 1% DDM being the most effective in preventing aggregation of PfCRT. After IMAC purification, it can be seen, based on Coomassie Blue-stained gel, that PfCRT can be purified to almost homogeneity (Fig. 2). The yield was about 200 μg purified PfCRT per 3 g of wet cells. The yields were almost the same for sensitive PfCRT^{s} (K76) and resistant PfCRT^{r} (K76T or K76I).

Reconstitution of PfCRT into proteoliposomes

Most of the reconstituted proteoliposome were unilamellar and regularly shaped observed under a typical light micrograph (Fig. 3). CQ transport in proteoliposomes containing PfCRT formed at varying lipid–protein ratios were determined. The specific transport activity was highest at

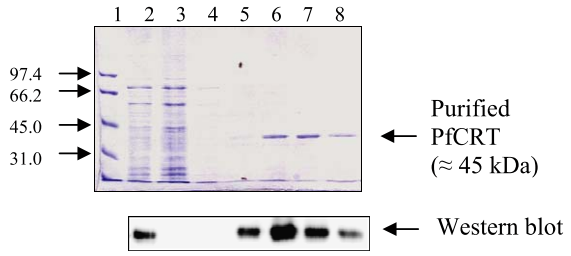

Fig. 2. Purification of PfCRT using Ni–NTA agarose. Microsome fraction pPICZA-PfCRT^s (from fermentor) was solubilized in detergent and subject to Ni–NTA agarose purification. All samples were separated by SDS–PAGE and stained with Coomassie Blue. Western analysis using anti-polyhistidine antibody was performed on these fractions and the result was shown below the gel. (1) size marker; (2) microsome fraction pPICZA-PfCRT^r (from fermentor) solubilized in 1.2% DDM; (3) flow through; (4) fraction washed with 20 mM imidazole buffer A (0.1% DDM); (5–8) fractions eluted with 0.5 mL each of elution buffer containing 250 mM imidazole.

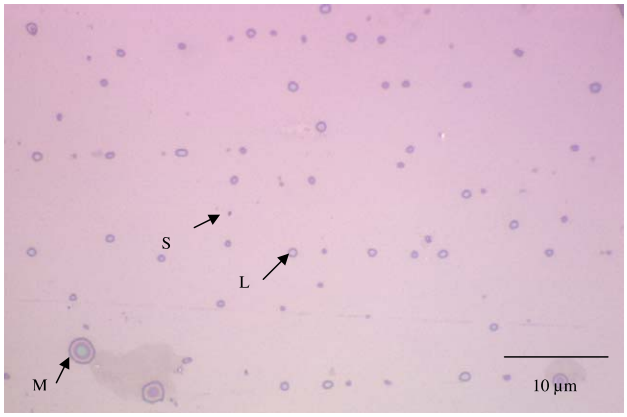

Fig. 3. Light micrograph of proteoliposomes (1000×). The reconstituted proteoliposome was reflected in the micrograph. M, multilamellar vesicle; L, large unilamellar vesicle; S, small unilamellar vesicle. Most of them were large unilamellar vesicles.

lipid-to-protein ratios of 150 (w/w) (data not shown). The both of sensitive and resistant proteoliposomes exhibited the same efficiency of reconstitution (about 30%), and the orientation of PfCRT protein incorporated into proteoliposomes was almost the same for sensitive PfCRT^s (K76) and resistant PfCRT^r (K76T or K76I) (about 50%).

PfCRT^r-containing proteoliposomes transport CQ in a saturable manner

We hypothesize that PfCRT is involved in the transport of CQ in the food vacuole of *P. falciparum*. The relationship between PfCRT mutations and CQ resistance has been well established by several studies, as reviewed by Wellems and Plowe [37]. The mechanism by which PfCRT mediates CQ resistance, however, is not clear. It has been suggested that PfCRT might transport CQ but this hypothesis has never been formally tested. Here, we used the proteoliposomes containing PfCRT^r (K76T) to test if PfCRT can transport ^{3}H-CQ. Fig. 4A shows that resistant proteoliposomes (K76T) can accumulate ^{3}H-CQ in a concentration-dependent manner. The ^{3}H-CQ transport activity was specific because it can be competed by excess unlabeled CQ. The specific ^{3}H-CQ transport into proteoliposomes (K76T) approached saturation at 487 pmol/mg PfCRT/min, with half-maximal accumulation at a concentration of about 280 nM ^{3}H-CQ.

To determine if ^{3}H-CQ was indeed transported into the lumen of the PfCRT-containing proteoliposomes rather than binding onto the proteoliposomes surface, we have determined the radioactivity that remained associated with proteoliposome membranes after addition of detergent (DDM). It was found that small amount of DDM was enough to reduce the PfCRT^r (K76T) proteoliposome-associated ^{3}H-CQ radioactivity (Fig. 4B). This was due to the rupture of proteoliposome membrane and releasing the ^{3}H-CQ from the lumen. In contrast, the addition of equivalent amount of DDM cannot lower the control liposome-associated ^{3}H-CQ radioactivity (Fig. 4B). This result suggests that radioactivity in resistant proteoliposome (K76T) is derived from ^{3}H-CQ inside the proteoliposome lumen and ^{3}H-CQ is indeed transported into the proteoliposome whereas the radioactivity in liposome control is derived from the binding of ^{3}H-CQ onto the membrane surface.

Another way to demonstrate that ^{3}H-CQ is indeed transported into the lumen of the proteoliposome is to measure the effect of the osmolarity on ^{3}H-CQ count. Increasing the osmolarity will reduce the proteoliposome volume and will lower the amount of ^{3}H-CQ that can be transported into the lumen. If ^{3}H-CQ is only binding on the proteoliposome surface, changes in osmolarity will have no effect on the count. Fig. 4C shows that ^{3}H-CQ count is lowered when the sucrose concentration is increased. This highly suggests that the ^{3}H-CQ count is due to the transport of ^{3}H-CQ into the proteoliposome lumen.

It was found that the ^{3}H-CQ uptake activity was dependent on the amount of PfCRT used (Fig. 4D). The uptake activity reached almost saturation when 0.6 μg of PfCRT is used. The initial uptake within the first minute is linearly proportional to incubation time (Fig. 4E) and it reached almost equilibration after 1 min and the level remained the same for a period of 7 min (see inset of Fig. 4E).

Characterization of CQ transport activity from CQR and CQS PfCRT-containing proteoliposomes

Amino acid position 76 is critical to PfCRT's role in conferring CQR in *P. falciparum*. K76T and K76I are associated with CQR whereas K76 is associated with CQS. Here we studied the importance of residue 76 in affecting the CQ transport activity in *Pichia* microsomes. Fig. 5 shows that, by incubating with 150 nM of ^{3}H-CQ, the proteoliposomes containing PfCRT^r (K76T) or PfCRT^r (K76I) accumulated significantly higher amount of ^{3}H-CQ (166 ± 11 and 182 ± 6 pmol/mg PfCRT/min, respectively) than that of proteoliposomes containing PfCRT^s (K76) (118 ± 7 pmol/mg PfCRT/min). K76, K76T and K76I proteoliposomes all

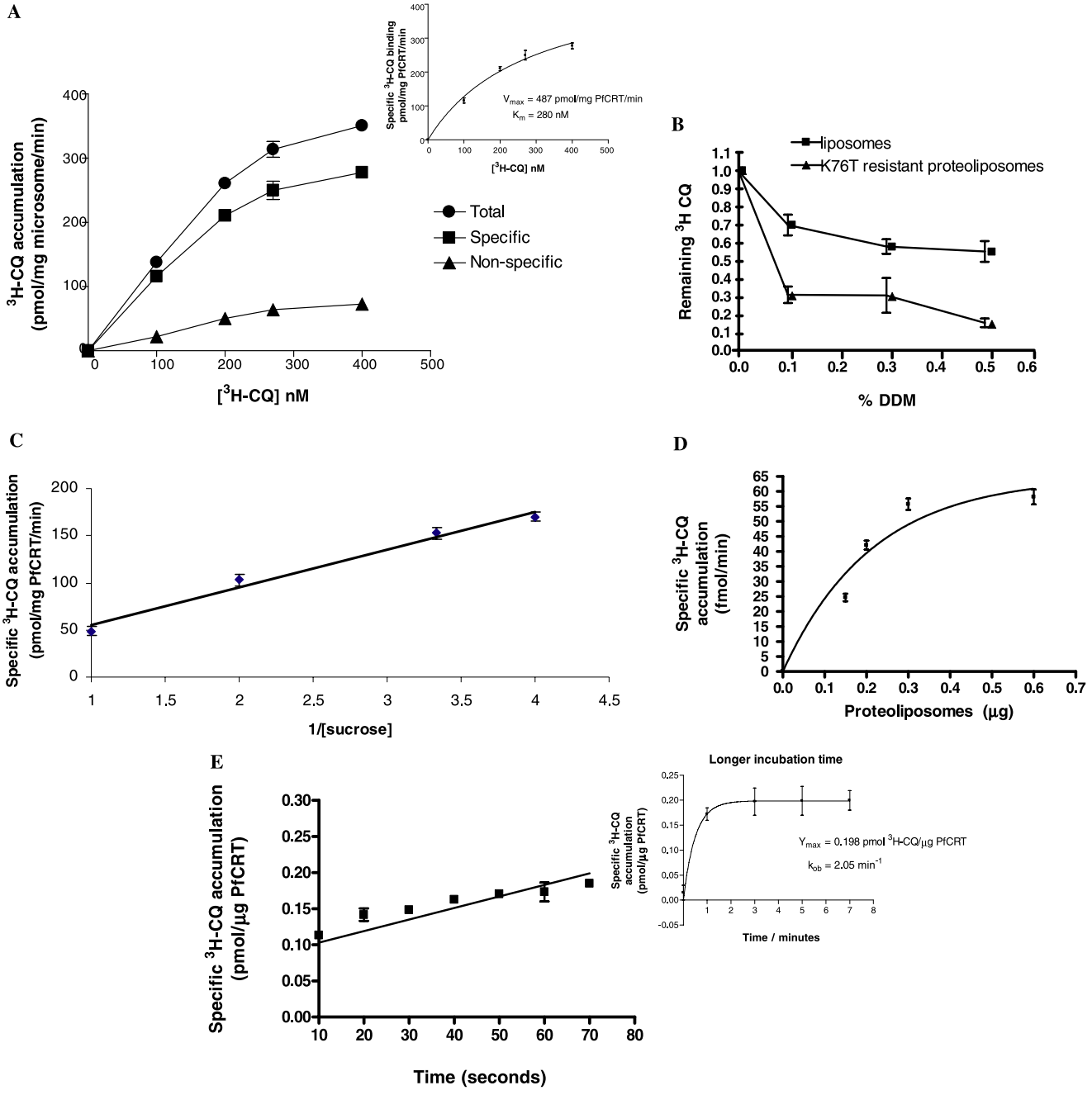

Fig. 4. Uptake of ^{3}H-CQ by proteoliposome containing resistance PfCRT^r (K76T). In general, proteoliposomes were incubated with the indicated amount of ^{3}H-CQ for 1 min. After incubation, the amount of radioactivity remaining in the proteoliposome was determined by rapid filtering, followed by washing and liquid scintillation counting. (A) Dependence of ^{3}H-CQ uptake on ^{3}H-CQ concentration. ^{3}H-CQ uptake into proteoliposomes containing 0.15 µg PfCRT^r (K76T) was measured using various ^{3}H-CQ concentrations. Incubation time was for 7 min. Total ^{3}H-CQ uptake (circle) and nonspecific ^{3}H-CQ uptake (triangle) in the presence of 200-fold unlabeled CQ were shown in the figure. Specific ^{3}H-CQ uptake (square) was calculated by subtracting nonspecific ^{3}H-CQ count from total ^{3}H-CQ count. Non-linear regression analysis using Michaelis–Menten model $Y = (V_{max} \times X)/(K_m + X)$ was shown in the inset. (B) Effect of DDM on CQ transport in proteoliposomes. Liposomes (proteoliposomes containing PfCRT^r, triangle; liposome, square) was first incubated with ^{3}H-CQ followed by treatment with various amounts of detergent (DDM). Data were expressed as percentage of CQ transport activity (means ± SE, $n = 3$) measured in the absence of DDM. Sample size and volume were identical to those used for measurement of CQ transport, so that the direct comparison could be made with transport experiments carried out in the presence of DDM. (C) Dependence of ^{3}H-CQ uptake on osmolarity. Proteoliposomes containing PfCRT^r (0.15 µg) were preincubated with transport buffer containing sucrose concentrations ranging from 0.25 M (isotonic) to 1.0 M steady-state ^{3}H-CQ uptake for 1 min. ^{3}H-CQ concentration was 150 nM. Each data point represents means ± SE ($n = 3$). (D) Dependence of ^{3}H-CQ uptake on PfCRT concentration. Samples containing 0.15–0.6 µg of PfCRT^r (K76T) were incubated for 1 min in a reaction buffer containing 150 nM ^{3}H-CQ. Each data point represents means ± SE, $n = 3$. (E) Time course of ^{3}H-CQ uptake into proteoliposomes containing 0.15 µg PfCRT^r. Proteoliposomes with PfCRT^r (K76T) were incubated with 150 nM ^{3}H-CQ for the indicated period of time. Each data point represents means ± SE, $n = 3$.

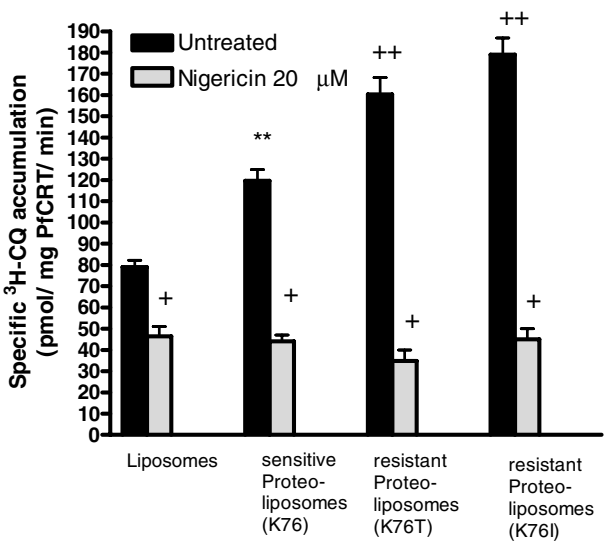

Fig. 5. Comparison of nigericin-inhibitable uptake of radiolabeled CQ by proteoliposomes containing PfCRT^r (K76T or K76I) or PfCRT^s (K76). The liposomes and proteoliposomes (0.15 μg) with the same amounts of phospholipids were pre-incubated with 20 μM nigericin for 15 min. ^{3}H-CQ uptake activity was then measured using 150 nM ^{3}H-CQ. Liposomes showed background levels of drug uptake for lipids alone. Data points represent means ± SE, $n = 3$. $^{**}P > 0.05$; $^{++}P < 0.05$ (compared to liposome control). $^{+}P < 0.05$ (in the absence and presence of nigericin in different liposomes).

have a significantly higher activity than that of liposome control (82 ± 10 pmol/mg PfCRT/min). This result highly suggests that the allelic polymorphism of the 76th amino acid of PfCRT observed in CQ resistance in *P. falciparum* (CQR: K76T, K76I; CQS: K76) is associated with the higher CQ transport activity (high CQ transport activity: K76T, K76I; low: K76).

Nigericin is an ionophore that permits K^+/H^+ exchange across membrane. It can, therefore, dissipate pH gradient. 20 μM nigericin reduced ^{3}H-CQ accumulation of all four liposomes to almost the same level (Fig. 5). This suggest that PfCRT mediated CQ transport is dependent on pH gradient.

Previous paper indicated that PfCRT^r-reduced CQ transport in microsomes was dependent on Cl^- [38]. However, it is not clear whether PfCRT itself mediates Cl^- movement by functioning as a chloride channel, or alternatively, Cl^- can activate other endogenous channels which can regulate CQ uptake. The reconstituted proteoliposomes could provide a clue for the association of PfCRT with Cl^- channel. The result showed that the CQ accumulation in proteoliposomes decreased with the removal of Cl^-. This suggests that PfCRT itself might mediate Cl^- movement. But a high concentration of Cl^- (140 mM), which resembles the conditions of the native *P. falciparum* DV, resulted in no enhanced CQ accumulation compared to low concentration of Cl^- (10 mM) (Fig. 6).

Discussion

Malaria is one of the world's leading killers among infectious diseases and the situation has worsened in the past decades due to the emergence of drug resistant parasites, especially CQR parasites. The mechanism of CQR remains unclear. PfCRT is currently one of the most promising candidates, but its role in mediating CQR is still not understood. The problem is mainly due to the lack of an *in vitro* system to study its function. Using total gene synthesis and modification of codon usage, we have successfully expressed PfCRT in *P. pastoris*. This method is both quick and effective in expressing malarial gene products by overcoming the problem of AT-rich malarial genome, which could potentially lead to the premature transcriptional termination. The expressed PfCRT protein was found to be located in the microsome fraction, which is the predicted location based on its primary amino acid sequence. No PfCRT protein was observed for non-modified PfCRT control microsomes, demonstrating that no PfCRT protein was expressed without codon modification.

Purification of membrane proteins requires detergent for solubilization. The choice of detergent is crucial, since some detergents can irreversibly inactivate the protein. DDM is a mild non-ionic detergent. Not only can it almost completely solubilize the microsomes, it also has the advantage of not

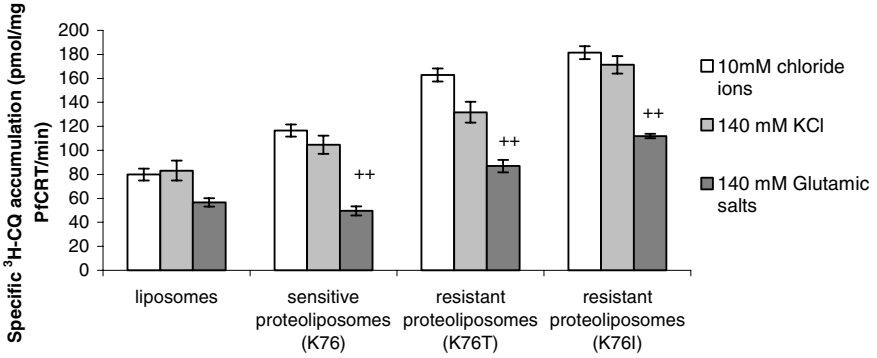

Fig. 6. Effect of chloride ion on CQ transport in proteoliposomes. Proteoliposomes (0.15 μg) were incubated with 150 nM ^{3}H-CQ in normal reaction buffer, 140 mM KCl buffer, or equimolar glutamic salts. ++, significant differences ($P < 0.01$) in the presence or absence of Cl^- in different proteoliposomes. Each data point represents means ± SE ($n = 3$).

inactivating of most proteins as observed by others [31]. We found that expression level of PfCRT in shake flasks was lower. Potential reasons for this include: a lack of pH control, inadequate aeration of cultures, or inability to control feeding of carbon sources at optimal rates. Therefore, the use of fermentor significantly increased the yield. The expression level was found to increase by roughly 10 times when compared to the shake flask method. This allows us to purify a large amount of protein by affinity chromatography (Ni–NTA). The large amount of purified PfCRT obtained could be used to reconstitute into proteoliposomes for characterization of the role of PfCRT in mediating CQR.

In this study, *E. coli* total lipids and egg PC were employed to reconstitute purified PfCRT and proteoliposomes derived was used to study the functions of PfCRT. Here, most of reconstituted proteoliposomes were large unilamellar vesicles, which contained only a double layer membrane. This kind of liposomes was suitable for entrapment of aqueous materials and probably the best system for studying PfCRT function. In addition, this system could help to differentiate between direct effect mediated by PfCRT itself or through other molecular partners. The same reconstitution efficiency (30%) and orientation of PfCRT (50%) in the different proteoliposomes allowed us to compare transport activity of ^{3}H-CQ in different proteoliposomes and this activity was interpreted only by PfCRT action and not by its reconstitution efficiency or orientation.

The previous work proposed that PfCRT, being localized in digestive vacuole, might be involved in pH regulation. It has been reported that pH of digestive vacuole of Dd2 isolate (a CQR parasite with K76T allele of PfCRT) was lower than that of HB3 isolate (a CQS parasite with K76 allele of PfCRT) [19]. In addition, selection of 106/1 with increasing concentration of CQ resulted in the generation of CQR lines. PfCRT was found to have mutated from K76 to either K76I or K76N. The food vacuole of these CQR lines also has a lower pH than that of the parental line 106/1 (K76) [39]. It was proposed that the resistant allele of PfCRT causes a decrease in vacuolar pH, resulting in the diminished interaction between CQ and its target, hematin. However, the observation that the food vacuole of CQ-resistant parasites having a lower pH than its CQ-sensitive counterparts was opposite to what others observed [20]. The use of acridine orange in measuring vacuolar pH was challenged by Bray et al. as considerable amount of acridine orange fluorescence was found in cytoplasm and the fluorescence signal from the vacuole could be quenched [40]. Overall, it is difficult to accurately measure pH of food vacuole in the parasite.

The PfCRT-mediated CQ transport activity in the proteoliposomes can be explained in two ways: either PfCRT is directly responsible for CQ uptake, or PfCRT regulates pH across the proteoliposomal membrane and the subsequent pH gradient will drive the unprotonated CQ uptake into the lumen. In this study, we were not able to differentiate between these two possibilities. Zhang et al. recently reported the role of PfCRT in mediating a pH change, possibly by cooperating with an unknown ATPase in a similar *Pichia* expression system [26]. However, our study found that PfCRT-mediated CQ transport was inhibited by nigericin, suggesting that pH gradient might drive CQ transport. A function for PfCRT as a transporter depending on pH gradient would be consistent with most models proposed to explain CQR in *P. falciparum*. The higher ^{3}H-CQ transport activity in resistant PfCRT^{r} (K76T or K76I)-containing proteoliposomes suggest that PfCRT might mediate CQ transport of the food vacuole.

We believe that the PfCRT-mediated CQ transport activity observed *in vitro* (proteoliposomes) system is relevant to the *in vivo* CQR observed in parasites. Allelic polymorphism in amino acid 76 (CQR: T or I; CQS: K) of PfCRT has a major effect on its CQ transport activity. It has been found that PfCRT^{r} (K76T or K76I) exhibit a higher CQ transport activity compared to PfCRT^{s} (K76) in proteoliposomes, although background here in controls was somewhat high in proteoliposomes. High background is mainly due to binding of radiolabeled CQ to the lipids. Therefore, we hypothesize that PfCRT^{r} (K76T or K76I) mediates CQR in parasites by increasing the efflux of CQ out of the food vacuole, resulting in an overall lowered CQ accumulation in the CQR parasites. However, other research groups have measured lower CQ accumulation in resistant PfCRT^{r} (Dd2) yeast inside-out vesicles (ISOV) compared to the sensitive one (HB3) [38]. They suggested that the reduced CQ accumulation for Dd2 ISOV might be caused by resistant PfCRT^{r} either catalyzing downhill facilitated diffusion of CQ^{2+} molecules out of the ISOV or conducting H^{+}- (or perhaps HCO_3^{-}- or Cl^{-}-) coupled active transport of CQ^{2+} in the outward direction. Thus, they proposed that lower net CQ accumulation within the parasite food vacuole results in CQR parasites. Another group found no evidence for a direct or indirect function of PfCRT in Cl^{-} conductance [41]. But in our study, CQ accumulation in proteoliposomes was lower after the removal of Cl^{-}, suggesting that PfCRT-mediated CQ transport was Cl^{-} dependent.

There are eight amino acids of PfCRT that are different between the CQS (HB3 strain) and CQR (Dd2) alleles of PfCRT: M74I, N75E, K76T, A220S, Q271E, N326S, I356T, and R371I [12]. Both of transfection and epidemiological studies suggest that 76th amino acid of PfCRT is critical to CQR parasites. The 76th amino acid may contribute to the PfCRT's function as a CQR determinant in *P. falciparum* by virtue of its effect on the CQ transport activity across the food vacuole membrane. K76T has always been observed together with the other seven amino acids differences. The contribution of the other amino acids in CQR is unknown. In this study, we demonstrate that K76T or K76I, in the absence of any other amino acid changes, is sufficient to cause a significant increase in CQ transport activity compared to K76. If PfCRT is mediating CQR in *P. falciparum* solely by its CQ transport activity and the 76th amino acid

is the only important residue, one would expect to find CQR parasites in the field with only a single 76th amino acid mutation. The fact that single point mutant in CQR parasites cannot be found in the field suggests that other amino acid polymorphisms might also contribute to CQR by other yet-to-be determined mechanisms. The slight difference between resistant and sensitive PfCRT-containing proteoliposomes for CQ transport in this study might be because the 76th amino acid mutation is necessary but not sufficient to mediate resistance to CQ. There may be other amino acid mutations together to confer full-level of CQ resistance. The single 76th amino acid mutation was found to result in higher CQ accumulation, suggesting that the 76th amino acid may be involved in substrate recognition or binding. Whether other mutations besides 76th mutation in PfCRT play a role in CQ binding and/or recognition is now being investigated in our lab.

Acknowledgments

We are grateful to Dr. Iris Wong and Guoqing Liang for the helpful comments. This work was supported in part by the Hong Kong Research Grant Council CERG grant awarded to Dr. LMC Chow.

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