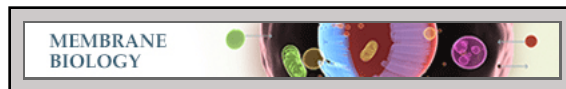


Membrane Biology:
**Purified E255L Mutant SERCA1a and
Purified PfATP6 Are Sensitive to
SERCA-type Inhibitors but Insensitive to
Artemisinin**



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Purified E255L Mutant SERCA1a and Purified PfATP6 Are Sensitive to SERCA-type Inhibitors but Insensitive to Artemisinins^{*[5]}

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The antimalarial drugs artemisinins have been described as inhibiting Ca²⁺-ATPase activity of PfATP6 (*Plasmodium falciparum* ATP6) after expression in *Xenopus* oocytes. Mutation of an amino acid residue in mammalian SERCA1 (Glu²⁵⁵) to the equivalent one predicted in PfATP6 (Leu) was reported to induce sensitivity to artemisinin in the oocyte system. However, in the present experiments, we found that artemisinin did not inhibit mammalian SERCA1a E255L either when expressed in COS cells or after purification of the mutant expressed in *Saccharomyces cerevisiae*. Moreover, we found that PfATP6 after expression and purification from *S. cerevisiae* was insensitive to artemisinin and significantly less sensitive to thapsigargin and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone than rabbit SERCA1 but retained higher sensitivity to cyclopiazonic acid, another type of SERCA1 inhibitor. Although mammalian SERCA and purified PfATP6 appear to have different pharmacological profiles, their insensitivity to artemisinins suggests that the mechanism of action of this class of drugs on the calcium metabolism in the intact cell is complex and cannot be ascribed to direct inhibition of PfATP6. Furthermore, the successful purification of PfATP6 affords the opportunity to develop new antimalarials by screening for inhibitors against PfATP6.

Malaria is a major infectious disease with about 500 million cases and one million deaths every year. Human malaria is caused by five species of protozoan parasites of the genus *Plasmodium* transmitted to humans by female *Anopheles* mosquitoes. Infec-

tions with *Plasmodium falciparum* are responsible for the most severe disease and deaths. Without a vaccine, drug treatment is a critical part of malaria control strategies. However, drug-resistant parasites are severely compromising the effectiveness of many therapies (1, 2). The artemisinins have maintained their efficacy despite the emergence of drug resistance to other classes of antimalarial drugs, although even this class now exhibits clinically relevant resistance in some areas (3). Artemisinins kill parasites faster (4) and with less toxicity (5) than other drugs, but their mechanism of action is not yet clearly known. Artemisinin is extracted from *Artemisia annua*, an herb long used to treat fevers in traditional Chinese medicine, and derivatives have been developed to improve their potency, solubility, stability, and pharmacokinetic properties (6–9). Artemisinins are sesquiterpene trioxane lactones that contain an endoperoxide bridge essential for antimalarial activity (10). They are effective against the blood stages of *Plasmodium* and especially the early ring and sexual stages (gametocytes) of the parasite life cycle (4, 11, 12). Artemisinins and derivatives accumulate preferentially in infected erythrocytes (13, 14) and are mainly located in parasite membranes and their neutral lipids, where the accumulation patterns are endoperoxide-dependent (15, 16). The generation of free radicals by artemisinins may be critical for killing parasites, an observation that is consistent with the importance of the endoperoxide bridge for drug efficacy (17). The nature of these radicals and how they are generated is debated, with hypotheses including roles for iron- or heme-catalyzing Fenton-like reactions (18, 19).

Artemisinin inhibits the endocytosis of red blood cell cytoplasmic macromolecules by the parasite (20), possibly via an increase in the cytosolic level of Ca²⁺. Efficacy against *Toxoplasma gondii* has also been related to changes in calcium homeostasis in this member of the apicomplexan family of parasites that includes *Plasmodium* spp. (21, 22). Several P-type ATPases (PfATP6, a sarco(endo)plasmic reticulum calcium ATPase (SERCA)⁴-type protein; PfATP4, a PTM (plasma membrane ATPase-related)-like

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

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⁴ The abbreviations used are: SERCA, sarcoplasmic reticulum Ca²⁺-ATPase; SR, sarcoplasmic reticulum; BAD, biotin acceptor domain; DDM, dodecyl-maltoside; C₁₂E₈, dodecyl octaethylene glycol monoether; DOPC, dioleoyl-phosphatidylcholine; Tg, thapsigargin; BHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; CPA, cyclopiazonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; LM, light membrane.

protein; and three putative ATPases that seem to belong to the Golgi-endoplasmic reticulum-type family) and a single $\text{Ca}^{2+}/\text{H}^{+}$ exchanger were identified to be involved in the maintenance of calcium homeostasis in *P. falciparum* (23).

Recently, Krishna and co-workers (24–26) observed that the ATPase activity of the single SERCA of *P. falciparum*, PfATP6, expressed in *Xenopus laevis* oocyte was inhibited by artemisinin ($K_i \sim 150$ nM). Isobologram analysis and competition studies with fluorophore derivatives localizing to parasites were consistent with a common target for artemisinin and thapsigargin (Tg), a specific inhibitor of SERCA-type proteins, because an antagonism was observed in the action of these drugs. PfATP6 and SERCA1 share an overall 40% identity with a well conserved transmembrane region, whereas the cytosolic sequence of the parasite Ca^{2+} -ATPase contains about 200 additional residues. Mutation studies on PfATP6 expressed in oocytes suggested that, in particular, Leu²⁶³ modulates the sensitivity of this enzyme to artemisinin (26). This experiment was in part based on the finding that rabbit SERCA1, whose Tg binding site is near Phe²⁵⁶ (27), is insensitive to artemisinin, and its amino acid sequence contains at the homologous position of Leu²⁶³ a glutamate (Glu²⁵⁵). When the Leu²⁶³ of PfATP6 was mutated to glutamate, sensitivity to artemisinin was decreased (26), and conversely when the glutamate residue of SERCA1 was mutated to a leucine, SERCA1 became sensitive to artemisinin (26). These results suggest that PfATP6 is a target for artemisinins, with further support derived from correlation between certain point mutations in PfATP6 in field isolates showing reduced *in vitro* sensitivity to artemether (28) and dihydroartemisinin (29), although all of the cases of artemisinin resistance are not related to these mutations but revealed some polymorphism (3, 19, 30, 31).

Up to now, only two of these transporters (PfATP6 and PfATP4) and mutated SERCA1a E255L have been studied in the *X. laevis* oocyte system (24, 26, 32). In order to further examine the interaction of artemisinins with PfATP6 and SERCA1a E255L mutant, it is important to characterize in more detail (functionally and structurally) those Ca^{2+} -ATPases. For that purpose, expression in alternative systems (we investigated COS-1 and yeast cells) and purification of the proteins is required. Our group recently developed a method to purify rabbit SERCA1a by affinity chromatography after its expression in yeast (33), and this method was successfully used for studying and crystallizing wild type (34) and mutated SERCA1a (35). In the present study, yeast expression was applied to purify and functionally characterize SERCA1a E255L and PfATP6 and to study the effects of artemisinin and other drugs when combined with detergent or lipids.

EXPERIMENTAL PROCEDURES

Chemicals—All chemical products were purchased from Sigma unless specified otherwise. PfuTurbo[®] DNA polymerase was from Stratagene, and Electroelution G-capsules were from G-Biosciences (AGRO-BIO, La Ferté Saint Aubin, France). Phusion[®] high fidelity polymerase was from Finnzymes (Ozyme, Saint Quentin en Yvelines, France). Restriction and modification enzymes were from New England Biolabs (Ozyme, Saint Quentin en Yvelines, France). High activity bovine thrombin was from Calbiochem, and the streptavidin-Sepharose[™] High Performance was purchased from GE

Healthcare. All products for yeast and bacteria cultures were purchased from Difco (BD Biosciences). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, and egg yolk phosphatidylcholine were from Avanti Polar Lipids (Alabaster, AL); L- α -phosphatidylinositol from soybean (catalog no. P0639) was from Sigma. N-Dodecyl- β -D-maltoside (DDM) was from Anatrace (Maumee, OH), and octaethylene glycol mono-*n*-dodecyl ether (C_{12}E_8) was purchased from Nikkol Chemical (Tokyo, Japan). Precision protein standards were from Bio-Rad. Immobilon-P membranes were from Millipore (Bedford, MA). Avidin-peroxidase conjugate was from Sigma. Phosphoenolpyruvate (catalog no. P3637), L-lactic dehydrogenase solution from bovine heart (catalog no. L1006), and pyruvate kinase preparation type VII from rabbit muscle (catalog no. P7768) were from Sigma.

COS-1 Cell Experiments—Site-directed mutagenesis of cDNA encoding SERCA1a inserted into the pMT2 vector (36) was carried out using the QuikChange site-directed mutagenesis kit (Stratagene), and the mutant cDNA was sequenced throughout. To express wild type or E255L mutant cDNA, COS-1 cells were transfected using the calcium phosphate precipitation method (37). Microsomal vesicles containing the expressed proteins were isolated by differential centrifugation (38). The concentration of expressed Ca^{2+} -ATPase was determined by an enzyme-linked immunosorbent assay (39) and by determination of the maximum capacity for phosphorylation with ATP (“active site concentration”; see Ref. 40). ATPase activity was determined by following the liberation of P_i (41) in the presence of 4 μM calcium ionophore A23187 to prevent inhibition caused by rebinding of Ca^{2+} to the lumenally facing Ca^{2+} sites (40). Inhibition assays were performed at 25 °C or 37 °C by first preincubating microsomal vesicles together with the drug over an 8-min period and then measuring the ATPase activity for 10 or 30 min, respectively.

Yeast Transformation and Selection of Individual Clones—The *Saccharomyces cerevisiae* yeast strain W303.1b/Gal4 (*a*, *leu2*, *his3*, *trp1::TRP1-GAL10-GAL4*, *ura3*, *ade2-1*, *can^r*, *cir⁺*) was the same as previously described (42). Transformation was performed according to the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (43). Growth conditions and criteria for expression of the Ca^{2+} -ATPase were carried out as described for the test of individual clones and for the expression on minimal medium (42, 44). A colony streaked onto a minimum medium storage plate was toothpicked into minimum medium (0.1% bactocasamino acids, 0.7% yeast nitrogen base, 2% glucose (w/v), 20 $\mu\text{g}/\text{ml}$ adenine) and grown at 28 °C for 24 h with shaking (200 rpm). For each assay, 500 μl of the minimum medium precultures were centrifuged for 5 min at 4 °C and $1000 \times g_{\text{av}}$ (rotor AM2.19, Jouan MR22i) and resuspended in 5 ml of minimum medium with 2% galactose instead of glucose to induce expression. These cultures were incubated at 28 °C for 18 h with shaking. For each culture, 4 $A_{600\text{ nm}}$ were centrifuged for 5 min, at 4 °C and $8000 \times g_{\text{av}}$. After washing with water, the pellets were resuspended in cooled 2% trichloroacetic acid. Glass beads were added, and the suspensions were mixed with a vortex at maximal speed for 8 min at room temperature to break the cells. The tubes were then

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placed on ice, and glass beads were sedimented. The supernatant was kept on ice. After three washes with 2% trichloroacetic acid, all of the collected supernatants were gathered. The resulting solution was kept for 15 min on ice for protein precipitation. Then the samples were centrifuged for 15 min, at 4 °C and $30,000 \times g_{av}$. The pellets were resuspended in 100 μ l of 50 mM Tris-Cl, pH 7.5. These samples were analyzed by Western blotting after SDS-PAGE in order to choose which clones were best expressed.

Expression of SERCA1a E255L in Fernbach Flasks—Growth conditions of yeast and induction of the expression of the mutant were the same as previously published for native SERCA1a expressed in yeast (33).

Growth of Yeast Cells and Large Scale Expression of PfATP6 Using a Fermentor (Techfors-S Apparatus, INFORS HT, Massy, France)—This method is based on the one developed for SERCA1a in Fernbach flasks with the following modifications: 20 liters of YPGE2X were inoculated with 1.2 liters of a culture at exponential phase in minimum medium ($\sim 6 \times 10^6$ cells/ml). Culture was performed at 28 °C under high aeration (1 volume of air/volume/min; stirring rate 300 rpm) at the beginning of the culture and then regulated to maintain a dioxygen saturation of 20% until the cell density reached 3×10^8 cells/ml. The culture was then cooled to 18 °C, the regulation of dioxygen saturation was stopped, and stirring rate was maintained at 300 rpm, but aeration was lowered to 0.15 volume of air/volume/min. Thirty minutes later, a solution of sterile galactose (500 g/liter) was added to a final concentration of 20 g/liter, and the culture was continued for 13 h (45).

Preparation of Light Membrane Fractions—The light membrane (LM) fraction was obtained after breaking yeast cells with glass beads and differential centrifugation of the crude extract as described previously (42). They were finally resuspended in Hepes-sucrose buffer (20 mM Hepes-Tris (pH 7.5), 0.3 M sucrose, 0.1 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride) at a final volume corresponding to 0.5 ml/g of the initial yeast pellet. The membranes can be stored at -80 °C until use. The amount of the protein of interest is estimated by Western blot analysis using the appropriate antibody.

Solubilization and Batch Purification of PfATP6 by Streptavidin-Sepharose Chromatography—These procedures are described in the [supplemental material](#).

Protein Estimation and Ca²⁺-ATPase Quantification—Protein concentrations were measured by the bicinchoninic acid procedure (46) in the presence of 2% SDS (w/v) with bovine serum albumin as a standard. SERCA1a from rabbit muscle (SR), used as a standard for protein estimation, was prepared as previously described (47). Ca²⁺-ATPase quantification was performed either by a Coomassie Blue staining gel after SDS-PAGE or Western blot using known amounts of SR as standards.

SDS-PAGE and Western Blotting—For SDS-PAGE, samples were mixed with an equal volume of denaturing buffer, heated at 90 °C for 2 min, and loaded onto Laemmli-type 8% (w/v) polyacrylamide gels (48). The amounts of proteins or volumes of initial samples loaded in each well are indicated in the figure legends. After separation by SDS-PAGE, gels were stained with Coomassie Blue, or proteins were electroblotted onto polyvi-

nylidene difluoride Immobilon P membrane (49). For each gel, molecular mass markers (Precision Protein standards, Bio-Rad) were loaded.

The Western blotting was followed by detection with avidin-peroxidase for the recognition of biotinylated proteins or by immunodetection with the polyclonal antibody anti-SERCA1a 79B (a gift from A.-M. Lompré, INSERM, France) as described previously (33).

Immunodetection with Anti-PfATP6—For immunodetection with anti-PfATP6 antibody, the polyclonal antibody anti-PfATP6 generated in goat from the peptide CQSSNKKDK-SPRGINK (the sequence from Q to K corresponds to the 574–588 region of PfATP6) was used. Anti-PfATP6 antibodies were purchased from Bethyl Laboratories. After electroblotting, membrane was blocked for 10 min in PBST (90 mM K₂HPO₄, 10 mM KH₂PO₄ (pH 7.7), 100 mM NaCl, 0.2% (v/v) Tween 20) containing 5% powdered skim milk. The primary antibody (1:10,000) was then added to the solution and incubated for 1 h at room temperature. The membrane was washed once for 10 min in PBST and then incubated with horseradish peroxidase-conjugated secondary rabbit anti-goat antibody (1:10,000) in PBST containing 5% powdered skim milk. After three washes with PBST for 10 min each, detection of proteins was performed with ECL (GE Healthcare). The chemiluminescence signal was acquired with a GBox HR 16 apparatus coupled with GeneSnap acquisition software and analyzed with GeneTools analysis software (Syngene, Ozyme, France).

Preparation of Lipids—Phospholipids dissolved in chloroform were dried in a stream of nitrogen. Dried phospholipids were then dissolved at 5 mg/ml in C₁₂E₈ (20 mg/ml).

Detergent Removal and Relipidation—After purification and before glycerol concentration adjustment, PfATP6 was concentrated with the aid of a 100 kDa cut-off concentrator unit (Centricon YM100, Millipore). Egg yolk phosphatidylcholine was then added to concentrated PfATP6 at a final concentration of 1 mg/ml and a lipid/protein ratio of 3:1 (w/w). To remove detergent, Bio-beads SM2, prepared as described (50), were added to the solution at a Bio-beads/detergent ratio of 200:1 (w/w), and the whole solution was gently stirred at 18 °C for 3 h. Bio-beads were then removed, and the solution was kept at 4 °C.

ATPase Activity Measurement—ATPase activity was assayed using a spectrophotometric method as described (51, 52). In general, from 1 to 10 μ g of proteins was used in 2 ml of reaction buffer (50 mM Tes/Tris, pH 7.5, 0.1 M KCl, 6 mM MgCl₂, 0.3 mM NADH, 1 mM phosphoenolpyruvate, 0.1 mg/ml lactate dehydrogenase, 0.1 mg/ml pyruvate kinase containing 0.1 mM Ca²⁺ and 0.2:0.05 mg/ml C₁₂E₈/DOPC). Changes in reaction conditions, detergents, amount of proteins, and variations in reaction temperature are indicated in the figure legends. The reaction was started by the addition of 5 mM ATP to the medium and stopped by the addition of a final concentration of 750 μ M EGTA. The difference between the slopes obtained before and after the addition of EGTA is considered to be due to the Ca²⁺-ATPase activity. To obtain the specific activity, the concentration of Ca²⁺-ATPase (SERCA1a E255L or PfATP6) was determined from Coomassie Blue-stained gels after SDS-PAGE.

Inhibition Assays—The inhibition assays were performed by enzymatic spectrophotometry as described above except for

vanadate because this inhibitor oxidizes NADH in a coupled enzyme system (see [supplemental material](#)).

The drugs used (stock solutions at 15 mM Tg, 20 mM 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (BHQ), 3 mM cyclopiazonic acid (CPA), 10 mM artemisinin, 10 mM artemisone, and 10 mM dihydroartemisinin) were dissolved in DMSO. The effect of DMSO alone was taken into account, and we corrected for it when calculating the specific effect of the inhibitors.

All of the inhibition assays were performed by the addition of 2 μ l of each drug solution. The effect of inhibitors was investigated by adding them during the ATPase turnover. In some experiments, the protein was preincubated with these drugs for a few min (but longer incubations were not more efficient) before the ATP addition, which triggers the start of the reaction (as explained in the figure legends).

Because Fe^{2+} ions were sometimes used in the assays, a 10 mM FeSO_4 solution was freshly prepared and kept on ice (53). In some cases, we also used Fe^{2+} in the presence of metal chelators as suggested (54).

RESULTS AND DISCUSSION

Enzymatic Properties of the SERCA1a E255L Mutant Expressed in COS-1 Cells—We found that the SERCA1a E255L mutant, previously reported as being sensitive to artemisinin (26), could be expressed in COS-1 cells to a level similar to that of the wild type protein. To estimate the catalytic turnover of the expressed proteins, we measured the maximum rate of Ca^{2+} -activated ATP hydrolysis in the presence of the calcium ionophore A23187 to avoid the “back inhibition” imposed by Ca^{2+} accumulated in the microsomal vesicles. The catalytic turnover rate of SERCA1a E255L mutant calculated by relating the ATPase activity to the maximal phosphorylation capacity was very similar to that of wild type, in agreement with earlier experiments showing that mutation of Glu²⁵⁵ by Ala or His does not affect maximal turnover (55). The inhibition by thapsigargin, a specific inhibitor of SERCA proteins (56), was nearly complete for both wild type and E255L mutant (Fig. 1, Tg). This result indicates, on one hand, that the turnovers measured are the result of the expressed Ca^{2+} -ATPases and, on the other hand, that Glu²⁵⁵ does not play a decisive role for thapsigargin sensitivity despite the fact that this residue is located at the binding site (27). Then we measured the effect of artemisinin alone or together with Fe^{2+} because it was suggested that artemisinin could require the presence of iron to be an efficient inhibitor (24). Both in the presence and absence of Fe^{2+} , there was no inhibitory effect of artemisinin, even at high drug concentration (Fig. 1, ART and ART+ Fe^{2+}).

Study of the Mutant SERCA1a E255L and Purification and Enzymatic Properties after Yeast Expression—The yeast light membranes containing the SERCA1a E255L mutant (endoplasmic reticulum and secretion vesicles) were prepared by membrane fractionation, as described previously (33). From 1 liter of culture containing about 35 g of yeast, 325 mg of membrane proteins with the SERCA1a E255L mutant was obtained in the light membrane fraction. The subsequent solubilization with DDM and tag-mediated affinity purification by streptavidin chromatography were performed as described (35). After thrombin cleavage, only SERCA1a E255L devoid of tag was

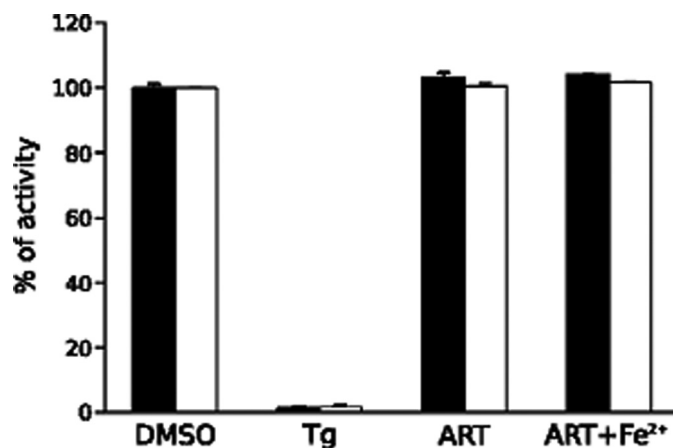


FIGURE 1. Effect of drugs on COS-1 cell-expressed SERCA1a wild type and E255L mutant. The rate of Ca^{2+} -activated ATP hydrolysis was determined at 25 °C in the presence of 4 μ M Ca^{2+} ionophore A23187 in a medium containing 50 mM TES/Tris (pH 7.0), 100 mM KCl, 7 mM MgCl_2 , 1 mM EGTA, 0.9 mM CaCl_2 (giving a free Ca^{2+} concentration of 3 μ M), and 5 mM ATP. The effect of 1.5 μ M Tg, 10 μ M artemisinin (ART), and 10 μ M artemisinin together with 10 μ M FeSO_4 (ART+ Fe^{2+}) were tested on wild type SERCA1a (black bars) and E255L mutant (open bars). The inhibitor was added dissolved in DMSO, giving a final DMSO concentration of 5% (v/v), and turnover rates are shown as a percentage of the turnover rate measured when the same amount of DMSO was added without inhibitor. S.E. values are indicated by the error bars on the columns.

eluted from the resin, leading to the recovery of 150 μ g of mutated protein at a concentration of about 30 μ g/ml as determined by Coomassie Blue gel staining and Western blotting (see Fig. 2, A and B). The protein was well purified, as shown by the Coomassie Blue-stained gel (to about 70%, most of the impurity being due to phenylmethylsulfonyl fluoride-inhibited thrombin; Fig. 2A).

To determine the effect of artemisinin on the purified SERCA1a E255L mutant, the specific ATPase activity of the protein was measured spectrophotometrically by a coupled enzyme system. We found that the maximal rate of ATP hydrolysis of the SERCA1a E255L mutant was slightly smaller than the specific activity of the wild type SERCA1a protein, overexpressed in yeast and measured under the same conditions. The wild type SERCA1a has the same specific activity as the wild type enzyme isolated from rabbit sarcoplasmic reticulum, indicating that yeast expression and purification is a valid method to study SERCA proteins (34), as also later confirmed for mutants of that protein (35). The Ca^{2+} -dependent ATPase activity of the mutant, like that of the wild type, could be stopped both by thapsigargin (a specific inhibitor for SERCA-type ATPases) and by EGTA (chelating agent of calcium ions) (Fig. 2C, experiment A), supporting the suggestion that the main calcium pumping function of this protein is retained in the mutant. To perform the assay under optimal conditions, we have adopted the use of lipid/detergent mixtures. The presence of DOPC in the assay media, forming mixed micelles with C_{12}E_8 , increased both the stability and enzymatic activity of solubilized Ca^{2+} -ATPase. We found that optimal conditions were obtained in the presence of 0.2 mg/ml C_{12}E_8 and 0.05 mg/ml DOPC (Fig. 2C, experiment B), and this resulted in a large increase of the specific activity (Fig. 2C, compare experiment B and experiment A). The phospholipid-dependent increase in activity of purified P-type ATPases had already been

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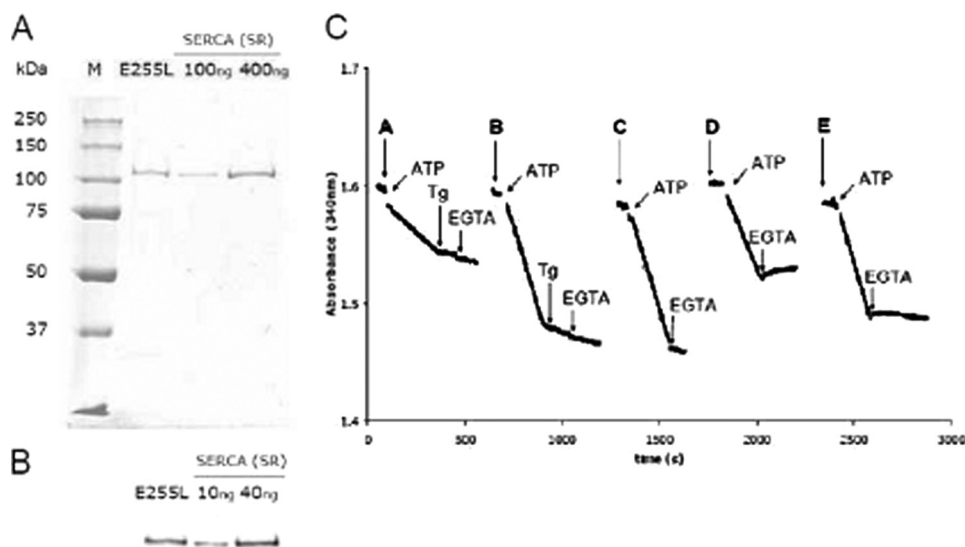


FIGURE 2. Characteristics of purified SERCA1a E255L mutant; purification and ATPase activity. *A*, SDS-PAGE followed by Coomassie Blue staining. 7.5 μ l of the eluted fraction was loaded (E255L), and to quantify the amount of SERCA-E255L in this sample, 100 and 400 ng of the wild type SERCA (SR) preparation were loaded. *M*, molecular mass markers. *B*, Western blot analysis with anti-SERCA1a antibodies. 0.75 μ l of the eluted fraction (E255L) and 10 and 40 ng of the SERCA (SR) preparation were loaded. *C*, ATPase activities at 25 °C of SERCA1a E255L in $C_{12}E_8$ (1 mg/ml) and $C_{12}E_8$ /DOPC (0.2/0.05 mg/ml) and inhibition assays with thapsigargin and artemisinin. Purified SERCA1a E255L was diluted to 0.75 μ g/ml in the ATPase assay medium. The medium contained also 125 μ M Ca^{2+} and either $C_{12}E_8$ (1 mg/ml) (*experiment A*) or $C_{12}E_8$ /DOPC (0.2/0.05 mg/ml) (*experiments B–E*), and hydrolytic activity was monitored continuously with a coupled enzyme system by recording NADH oxidation at 340 nm. The reactions were triggered by the addition of 5 mM ATP and stopped by the addition of 750 μ M EGTA. The inhibition effect of 1.5 μ M Tg was first checked (*experiments A and B*), and then the effect of artemisinin was tested by preincubation of the protein with 10 μ M artemisinin (*experiment C*) and 10 μ M Fe^{2+} + 10 μ M artemisinin (*experiment E*). The effect of Fe^{2+} + DMSO was controlled by the addition of 10 μ M Fe^{2+} + 2 μ l of DMSO (*experiment D*). In this and subsequent figures, the initial decrease in NADH absorbance due to a small amount of ADP in our ATP solution was removed.

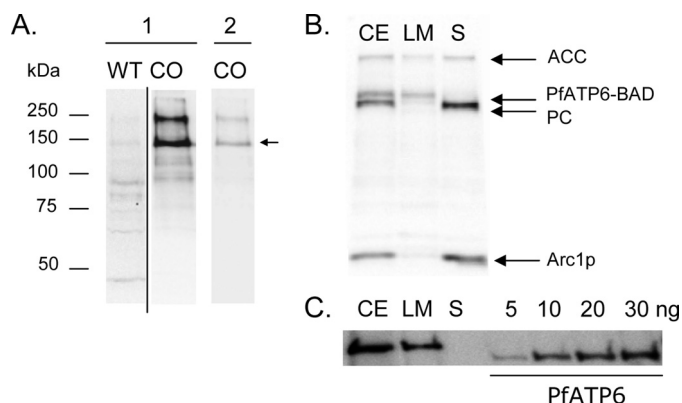


FIGURE 3. Expression of PfATP6. *A*, on minimal medium. Shown is Western blot analysis with anti-PfATP6 antibodies of the level of expression of PfATP6-BAD expressed from the wild type cDNA of Pfatp6 (*WT*) and the codon-optimized one (*CO*). 10 μ l of each protein solution after the expression assay were loaded, corresponding to 8 and 10 μ g of proteins for the *WT* and *CO* samples, respectively. 1 and 2 correspond to a signal acquisition of 14 and 2 min, respectively. The arrow indicates the band corresponding to monomeric PfATP6-BAD. *B*, on rich medium. Shown are analyses of fractions recovered during the membrane preparation by Western blot analysis with avidin peroxidase. Fractions of 2 μ g of total proteins of crude extract (*CE*) and 1 μ g of total proteins of LM and soluble fraction (*S*) were loaded. The arrows indicate the biotinylated proteins endogenous of the yeast *S. cerevisiae* (acetyl-CoA carboxylase (*ACC*), 250 kDa; pyruvate carboxylase (*PC*), 120 kDa; Arc1p protein (*Arc1p*), 45 kDa). *C*, analyses of these fractions by Western blot with anti-PfATP6 antibodies. To quantify the amount of PfATP6-BAD in the LM fraction, 5, 10, 20, and 30 ng of purified PfATP6 supplemented with 1 μ g of total proteins of light membrane fraction of yeast expressing SERCA1a were loaded.

observed (see Ref. 58, with the Na^+/K^+ ATPase as a recent example). The addition of artemisinin to the SERCA1a E255L mutant solubilized in phospholipid/ $C_{12}E_8$ medium did not

inhibit Ca^{2+} -ATPase activity (Fig. 2C, *experiment C*). The effect of artemisinin in the presence of iron (Fe^{2+}) was also tested (Fig. 2C, *experiment E*) and compared with the effect of iron alone (Fig. 2C, *experiment D*). There was no inhibitory effect of artemisinin and iron on the SERCA1a E255L mutant ATPase activity, whereas the combination gave rise to a slight increase in activity.

On the basis of these results and the results obtained with microsomes of COS-1 cells expressing the same mutant, we were unable to confirm that the mutation of Glu²⁵⁵ to Leu in SERCA1a determines the sensitivity to artemisinin, as described previously after expression of E255L SERCA1a mutant in oocyte (26). There is thus no evidence for an artemisinin binding site with a putative localization in the binding region for thapsigargin on SERCA1a.

Study of PfATP6 and Expression and Purification of PfATP6 in Yeast— Because in our hands the expression of PfATP6 in COS-1 cells was not

successful, as previously reported (24), we then proceeded to investigate the expression of that plasmodial protein PfATP6 in yeast. Production of PfATP6 using an assay of expression on minimal medium as described under “Experimental Procedures” was attempted in parallel from the wild type and a codon-optimized gene (the gene of PfATP6 was the same as the one used in Refs. 24 and 26). Although the codon adaptation index (which is a measure of the similarity of the codon usage of a gene to that of the proposed host organism (59)) is high (0.843) for the wild type PfATP6 gene and *S. cerevisiae*, we designed a sequence that took into account optimal codon usage for yeast and removed most of the poly(A) or T tracts in the native sequence. Gene optimization increased the codon adaptation index to a very high value (0.959) while leaving the GC content almost unchanged (27.9% for the wild type sequence and 28.6% after modification). The use of this modified gene enormously increased the expression of PfATP6, as can be seen by immunodetection with anti-PfATP6 antibodies (Fig. 3A). An aggregated form of PfATP6-BAD is also present near 250 kDa. This is likely to be due to the trichloroacetic acid precipitation used for the recovery of the total protein content; this represents a drastic denaturing treatment of proteins that can lead to their aggregation. When expressing PfATP6-BAD from its wild type cDNA, several bands of proteins were revealed in low amounts by anti-PfATP6 antibodies, generally of lower size than the expected molecular mass of the monomeric protein. The presence of these lighter proteins can be explained by the AT-rich composition of the PfATP6 gene as

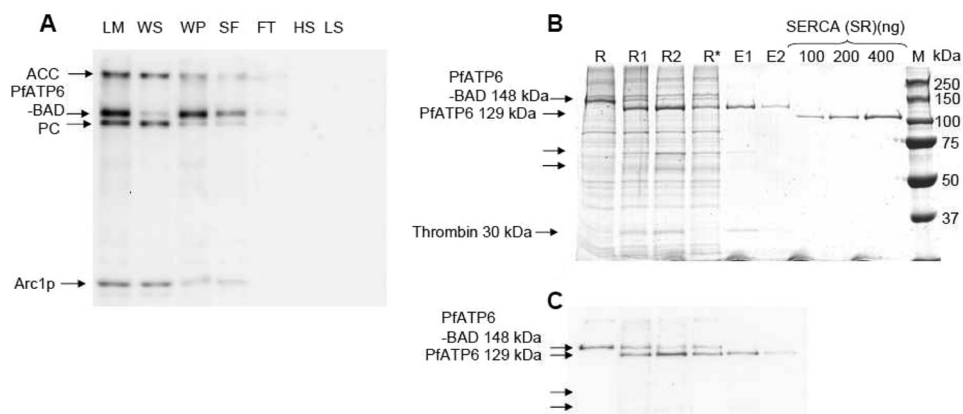


FIGURE 4. Purification of PfATP6 on streptavidin-Sepharose. *A*, Western blot analysis with avidin peroxidase of the washing, the solubilization, and the purification steps before thrombin cleavage of PfATP6. 0.5 μ l of each fraction was loaded (corresponding to 5 μ g of total proteins in the LM lane, for example). In order to remove soluble biotinylated proteins (acetyl-CoA carboxylase (ACC), 250 kDa; pyruvate carboxylase (PC), 120 kDa; Arc1p protein (Arc1p), 45 kDa), LM were diluted in washing buffer at 10 mg/ml and centrifuged. The resulting supernatant (WS) was discarded, and the pellet was resuspended in solubilization buffer (WP) with the same volume as the LM fraction and centrifuged. DDM was added, and solubilization was performed as described under "Experimental Procedures." The supernatant corresponding to the solubilized fraction (SF) was added to the resin, and the flow-through fraction (FT) was removed. The resin was then washed with high salt buffer (HS) and low salt buffer (LS). *B*, analyses of the resin and the eluted fractions by SDS-PAGE followed by Coomassie Blue staining. Except for the molecular mass marker (M) and the SERCA (SR) fractions, 7.5 μ l of each fraction were loaded. To check the action of thrombin in the cleavage of PfATP6-BAD (% of cleavage) and the efficiency of elution, the resin before (R) and after the first incubation with thrombin (about 60% of cleavage, R1) and after the second one (about 90% of cleavage, R2) and also the resin after elution (R*) were analyzed. To quantify the amount of PfATP6 recovered after the first (E1) and the second (E2) elution, 100, 200, and 400 ng of the wild type SERCA (SR) preparation were loaded. The arrows indicate potential nonspecific cleavages of PfATP6. *C*, analyses of the same fractions as in *B* by Western blot with anti-PfATP6 antibodies. Only 0.75 μ l were loaded in each lane. Cleavage products of PfATP6 are hardly detectable (indicated by the arrows).

described for other plasmodial proteins expressed in *S. cerevisiae* (60) and in *Pichia pastoris* (60–62). Indeed, AT-rich regions in a gene may form hairpins and mimic a termination signal of transcription and therefore result in the synthesis of truncated proteins. Consequently, we decided to use the optimized construct for a large scale expression of PfATP6. Like the mutant SERCA1a E255L, we were able to express PfATP6 at a high level by the use of a fermentor for the yeast culture. With this equipment, 50 g of wet cells were recovered per liter of culture.

After preparation of the light yeast membrane fraction, Western blot analyses with avidin peroxidase and anti-PfATP6 antibodies confirmed that PfATP6-BAD had been expressed and had undergone *in vivo* biotinylation (see Fig. 3B). Because PfATP6 is a SERCA-type protein with a location in the endoplasmic reticulum, we have focused on the light membrane fraction (endoplasmic reticulum) in the next steps. The amount of PfATP6-BAD contained in the LMs represents about 2% of the total protein content as determined by Western blot revealed with anti-PfATP6 antibodies (see Fig. 3C) and 8 mg/liter of culture. We also evaluated that about 30% of it was biotinylated (2.4 mg; data not shown) and therefore subject to purification by affinity chromatography. It can be noted that naturally biotinylated yeast proteins (acetyl-CoA carboxylase (ACC), 250 kDa; pyruvate carboxylase (PC), 120 kDa; Arc1p protein (Arc1p), 45 kDa) are mainly eliminated with the soluble fraction (see Fig. 3B). Nevertheless, because some of the soluble proteins remain bound to the LM fraction, we included one or two additional washing steps of the membranes with a high KCl buffer that helped to remove the major part of the remaining

contaminant proteins before the solubilization (see Fig. 4A, compare lanes WS and WP). Then the membranes were solubilized with DDM, a mild detergent used successfully with SERCA1a-BAD. The same detergent/protein ratio (3:1, w/w) was used except that the protein and detergent were 5 times more concentrated than as described previously with SERCA1a-BAD (33). Under these conditions, the solubilization was about 25% (see Fig. 4A, compare lanes WP and SF).

Then ~600 μ g of *in vivo* biotinylated and solubilized PfATP6 was added to 2 ml of streptavidin-Sepharose resin. Among the non-retained proteins, a part corresponded to PfATP6-BAD (see Fig. 4A, lane FT). This could be due to either exceeding the binding capacity of the resin or an inappropriate folding of the biotin acceptor domain. The thrombin cleavage between the sequence of PfATP6 and the biotinylated acceptor domain was followed by a Coomassie Blue staining gel (Fig. 4B) and

by immunodetection with anti-PfATP6 antibodies (Fig. 4C). Then only PfATP6 devoid of BAD tag was eluted from the resin, and the corresponding fractions were well purified (Fig. 4B, lanes E1 and E2). An approximate concentration of 30 μ g/ml was determined for the first elution and of 10 μ g/ml for the second elution fraction. About 20% of PfATP6 was still retained on the resin (lane R*). The purity of the protein reached about 70% (the remaining 30% being mainly due to thrombin) as evaluated from the color density of the Coomassie Blue-stained gel. In conclusion, the purification procedure gave a total amount of at least 160 μ g of purified PfATP6 starting from 1 liter of yeast culture and therefore a yield of 26% compared with the amount of biotinylated and solubilized PfATP6-BAD added to the resin.

Other plasmodial membrane proteins produced in *P. pastoris* and purified over Ni²⁺-nitrilotriacetic acid were generally obtained in better yield (60–62). Despite our lower yield, we can expect to recover PfATP6 with good functional properties by our procedure because the yeast *in vivo* biotinylation that our protocol implies tends to select properly folded proteins (34).

One possible point of concern is the possibility that yeast expression of PfATP6 could induce molecular modifications of the enzyme. Therefore, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry under the conditions that we formerly designed for large membrane proteins or their fragments (63). Briefly, after PfATP6 streptavidin purification, we performed gel filtration high pressure liquid chromatography to reduce the DDM content, and then the protein was concentrated up to 2 mg/ml. Two controls were performed; SERCA1a was purified from rabbit SR, and SERCA1a was yeast-expressed and purified. In all three cases,

SERCA Pumps and Artemisinin

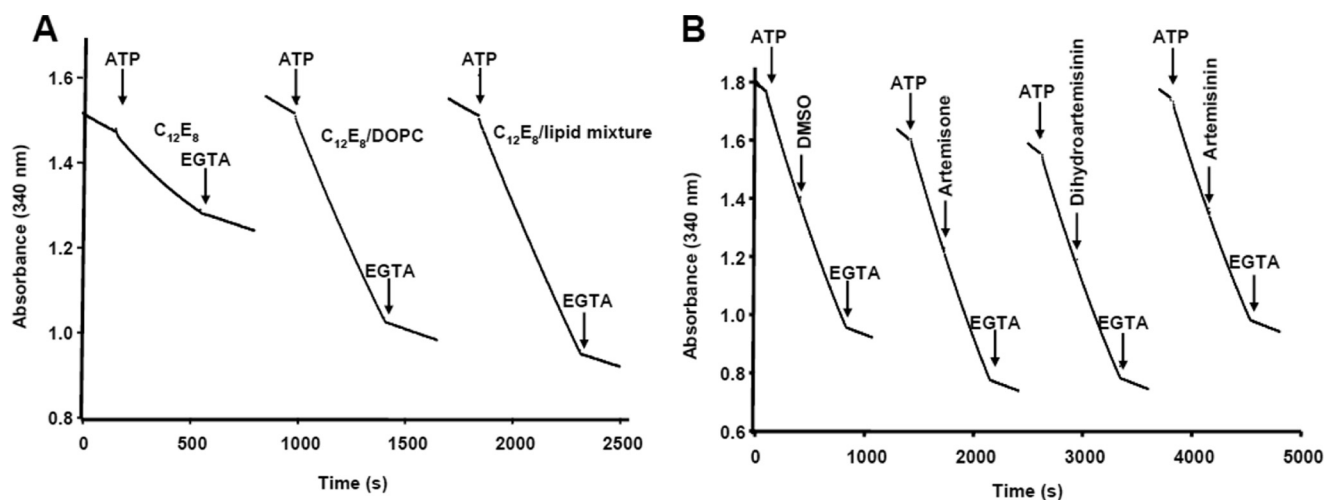


FIGURE 5. ATPase activities of purified PfATP6 protein and effect of artemisinin drugs. *A*, activity was measured at 25 °C and pH 7.5 with 10 μ g of proteins in 2 ml of assay buffer (see legend to Fig. 1). The reaction was triggered by the addition of 5 mM ATP into the medium and stopped by the addition of a final concentration of 750 μ M EGTA. The reactions were carried out in the presence of C₁₂E₈ (0.2 mg/ml), C₁₂E₈/DOPC (0.2/0.05 mg/ml), and C₁₂E₈/lipid mixture (0.2/0.05 mg/ml). *B*, the reactions were performed with 5 μ g/ml purified PfATP6 at 25 °C in C₁₂E₈/DOPC (0.2/0.05 mg/ml). Artemisinins were added with 2 μ l of a solution at 10 mM prepared in DMSO. The effect of various artemisinins was tested by the addition of 10 μ M (final concentrations) of the drugs during the ATP hydrolysis of the enzyme.

the determined molecular mass was very close to the expected molecular mass. In the case of PfATP6, we found a mass of 140,053 Da, whereas the expected mass is 139,994 Da. Under similar conditions, rabbit SR SERCA1a gave 109,497 Da for an expected mass of 109,489 Da, and yeast expressed SERCA1a (which is slightly larger due to DNA construction (34)) gave 110,090 Da for an expected mass of 110,069 Da.⁵ Under these conditions, the error associated with this type of measurement is 0.05–0.1%, so that this excludes large modification, such as partial proteolysis, glycosylations, etc.

Study of the Enzymatic Properties of the Soluble Purified PfATP6—We first measured the specific ATPase activity of the purified PfATP6 with the aid of the coupled enzyme assay as described for SERCA1a E255L and in the presence of 1 mg/ml C₁₂E₈ (data not shown) or 0.2 mg/ml C₁₂E₈ (Fig. 5*A*, left). As can be seen, the major part of ATP hydrolysis was stopped by the addition of EGTA, indicative of calcium-dependent ATPase activity. However, even at higher C₁₂E₈ concentrations (data not shown), we observed a decrease of the hydrolytic rate with time, suggesting that under these conditions with pure detergent, the protein is inactivated during turnover. By the addition of lipids (0.05 mg/ml of DOPC) together with 0.2 mg/ml C₁₂E₈, we were able to maintain a stable hydrolysis rate of ATP that was still inhibited by EGTA (Fig. 5*A*, middle). A similar stabilization was obtained in the presence of other lipids (1,2-dioleoyl-*sn*-glycero-3-phosphoserine, egg yolk phosphatidylcholine, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (data not shown)) or a lipid mixture consisting of 48.4% DOPC, 43% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, and 8.6% L- α -phosphatidylinositol (Fig. 5*A*, right). The phospholipid composition of this mixture was based on the membrane lipidic composition of *P. falciparum* as described (64). At a C₁₂E₈/DOPC ratio of 0.2:0.05 mg/ml, the specific activity at 25 °C of the purified PfATP6

was 1.7 μ mol of hydrolyzed ATP \cdot min⁻¹·(mg of PfATP6)⁻¹, which is about 30% of the activity of rabbit SERCA1a at this temperature.

We then measured the rate of hydrolysis of ATP, carried out by PfATP6, as a function of different calcium concentrations (data not shown). PfATP6 is activated by a low concentration of free Ca²⁺ (*p*Ca \sim 7), and optimal activities are obtained in the *p*Ca interval 6–4 with a maximal stimulation around *p*Ca 4. Above this concentration, the activity is gradually inhibited by the increasing amount of free Ca²⁺. This *p*Ca dependence profile is also in agreement with the one described for rabbit SERCA1a solubilized in C₁₂E₈ (51).

The ATPase activity of PfATP6 was also measured at different pH (see supplemental Fig. S1). The pH optimum was, at pH 7–7.5, 1.7 μ mol of hydrolyzed ATP \cdot min⁻¹·(mg of PfATP6)⁻¹, whereas the activities at pH 6.5 and 8 were low. This is in agreement with what was measured previously for SERCA1a (65).

Effect of Artemisinins—We then proceeded to test the effect of artemisinin and some derivatives on PfATP6. By the addition of 10 μ M artemisinin, we were not able to detect any inhibition (whatever the temperature in the range of 20–37 °C) because 90% or more of the activity was always measured after the addition of this drug to PfATP6 (Figs. 5*B* and 7 and supplemental Fig. S2*A*), whereas under these conditions, PfATP6 was previously reported to be completely inhibited (24, 26). We also tested the effects of 10 μ M artemisone or 10 μ M dihydroartemisinin (Fig. 5*B*) and lower and higher concentrations of the artemisinins (1–100 μ M for artemisinin and 1–500 μ M for artemisone) but were never able to demonstrate any effect on PfATP6-dependent Ca²⁺-ATPase activity (data not shown). Moreover, with a preincubation of 10 min with artemisinin, we obtained the same result.

As was done with SERCA1a E255L, we also checked the effect of artemisinin in the presence of iron (10 μ M Fe²⁺) by comparing the effect with that obtained in the presence of iron alone (see supplemental Fig. S2, *B* and *C*). This was carried out by preincubating PfATP6 with iron together with artemisinin before trigger-

⁵ D. Cardi, C. Marchand, and M. le Maire, unpublished results.

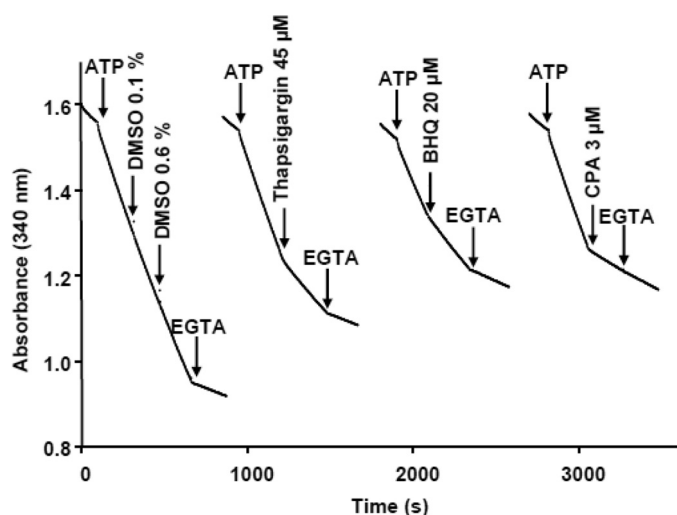


FIGURE 6. Effect of inhibitors of SERCA-type proteins on the ATPase activity of PfATP6. The reactions were performed with 5 $\mu\text{g/ml}$ purified PfATP6 at 25 $^{\circ}\text{C}$ in $\text{C}_{12}\text{E}_8/\text{DOPC}$ (0.2/0.05 mg/ml). They were triggered by the addition of 5 mM ATP and stopped by the addition of 750 μM EGTA. Inhibitors, diluted in DMSO, were added with 2 μl of the corresponding mother solution. First, the effect of DMSO on the activity of PfATP6 was monitored after the addition of 2 μl (DMSO 0.1%) and 4 μl (DMSO 0.6%) to PfATP6. Then the inhibitions of the enzyme by thapsigargin (45 μM), BHQ (20 μM), and CPA (3 μM) were tested.

ing the reaction. On the other hand, the Ca^{2+} -ATPase activity as well as the nonspecific activity was slightly decreased in the presence of Fe^{2+} . In addition, other artemisinin derivatives that were also tested, including artemisone, which was also used in the oocyte tests (26), and artesunate (see supplemental Fig. S2, D and E), did not inhibit the ATPase activity of PfATP6 either.

Effect of SERCA Inhibitors—We then tested the effect of specific inhibitors of mammalian SERCA proteins (see Fig. 6), including Tg, BHQ, and CPA, on PfATP6. These assays were also performed with the $\text{C}_{12}\text{E}_8/\text{DOPC}$ mixture at 25 $^{\circ}\text{C}$. A small degree of inhibition was observed with 1.5 μM Tg (data not shown), and Ca^{2+} -dependent ATP hydrolysis became clearly inhibited with 45 μM Tg (Figs. 6 and 7). This means that PfATP6 is significantly less sensitive than rabbit SERCA1a to Tg because the latter is completely inhibited by concentrations of Tg in the nanomolar range (66). These differences are well correlated with the observations of Varotti *et al.* (67), who, on parasites, found a similar difference in the maximum effect of Tg between *Plasmodium* parasites and mammalian cells. According to their results, for *Plasmodium*, 25 μM Tg were necessary to inhibit Ca^{2+} release in the cytoplasm, whereas 500 nM Tg was sufficient for producing the same effect in mammalian cells. Two other SERCA pump inhibitors were then assayed. We observed that 20 μM BHQ was able to inhibit about 50% of the activity of PfATP6 (see Figs. 6 and 7) and that the addition of 3 μM CPA resulted in an almost complete inhibition of this activity (see Figs. 6 and 7), similar to what is observed with rabbit SERCA1a and after expression of PfATP6 in oocytes. The effect of these inhibitors, which was checked in various conditions (detergent/lipid ratios, glycerol concentrations), clearly indicates that PfATP6 is a high affinity target for CPA in all of the conditions.⁶ Although less sensitive to Tg and

⁶ B. Arnou and J. V. Møller, unpublished results.

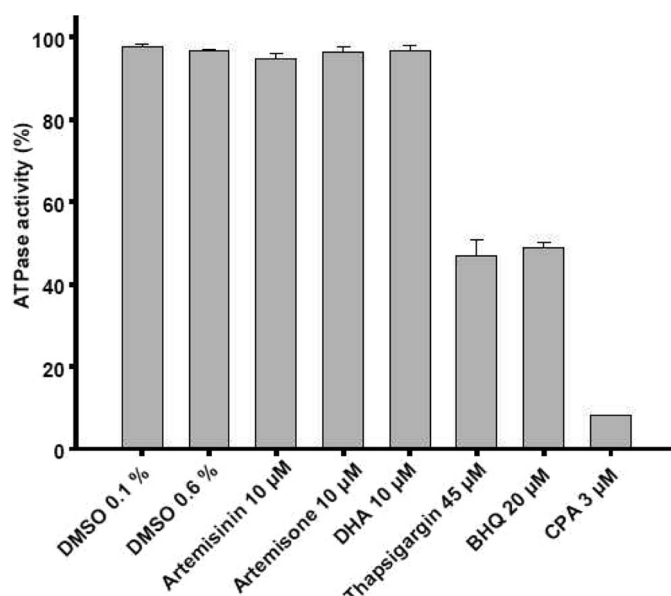


FIGURE 7. Summary of the effect of specific inhibitors of SERCA and artemisinin drugs on the ATPase activities of PfATP6 measured as described in the legends to Figs. 5 and 6. Each bar represents a minimum of three experiments \pm S.D. (error bars).

BHQ than rabbit SERCA1a, these results suggest that the purified PfATP6 qualitatively behaves the same way as a mammalian SERCA protein. Vanadate, another P-type ATPase inhibitor, was tested in a few experiments; close to 50% inhibition was obtained at low vanadate concentration (100 μM) and in the absence of EGTA (data not shown; the exact experimental conditions are described in the supplemental material).

Study of PfATP6 Enzymatic Activity in Membranes—One of the differences between our ATPase assays and those done with oocytes is the presence of detergent. Consequently, because the detergent could interfere with artemisinin drugs, the protein was relipidated, and detergent was completely removed using Bio-beads (Fig. 8A). Relipidated PfATP6 was then submitted to the same type of inhibition assays at 37 $^{\circ}\text{C}$ but without any detergent in solution (Fig. 8B). Again, artemisone was not able to inhibit PfATP6 even in the presence of iron. These results then conclusively show that under our conditions, the purified PfATP6 enzyme is not inhibited by artemisinin and its derivatives.

Conclusions—Our work overcoming the obstacles of heterologous expression of a membrane protein from an apicomplexan organism in yeast has provided the first opportunity to study the functional properties of a purified SERCA of *P. falciparum*. This has revealed both similarities with (pH and $p\text{Ca}$ profile) and some differences from (drug sensitivity) that of the mammalian SERCA1a. This is not unexpected due to the sequence differences and the presence of a number of cytoplasmic insertions in PfATP6, in particular in the N-domain (68). Furthermore, the availability of a purified preparation has allowed us to test in a direct way the evidence for interaction of purified PfATP6 with artemisinin arising from previous studies after expression in the *Xenopus* oocyte membrane (24, 26). However, neither by the addition of artemisinin, nor by the further addition of Fe^{2+} to induce radical formation was it possible to observe an effect on the isolated system. We conclude that it is not possible to demonstrate an effect of arte-

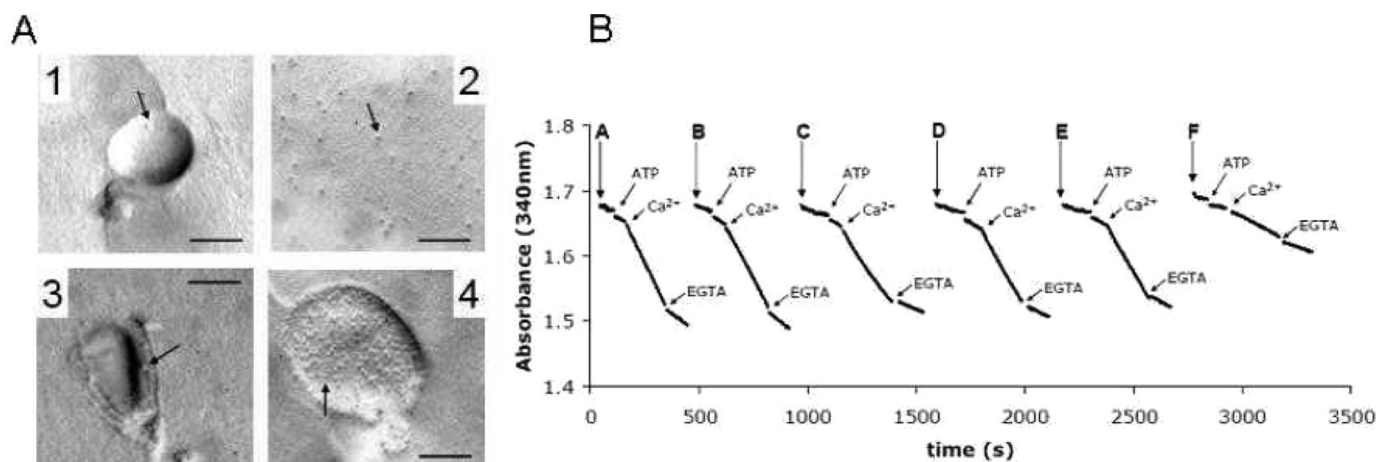


FIGURE 8. Effect of artemisinin and thapsigargin on the ATPase activity of relipidated PfATP6. A, freeze-fracture electron microscopy analysis of relipidated PfATP6 with egg yolk phosphatidylcholine. Bar, 100 nm. The sample is heterogeneous and contains different lipid structures: vesicles with one membrane protein (1), large membranous structures with dispersed proteins (2), multilamellar vesicles (3), and vesicles with membrane protein clusters (4). Each arrow shows one molecule of PfATP6. B, ATPase activities performed with 1.75 μg/ml reconstituted PfATP6 at 37 °C. They were triggered by successive additions of 5 mM ATP and 100 μM Ca²⁺ and stopped by the addition of 750 μM EGTA. Artemisinin was added with 2 μl of solutions at 10 or 100 mM prepared in DMSO. The effects of artemisinin were tested by preincubation of the protein in 150 μM EGTA with 10 μM artemisinin (experiment B), 10 μM Fe²⁺ + 2 μl of DMSO (experiment C), 10 μM Fe²⁺ + 10 μM artemisinin (experiment D), or 10 μM Fe²⁺ + 100 μM artemisinin (experiment E) before starting the reaction. The effect of 15 μM thapsigargin (experiment F) was tested in the same conditions. The effect of DMSO and Fe²⁺ + DMSO was controlled by the addition of 2 μl of DMSO (experiment A) and 10 μM Fe²⁺ + 2 μl DMSO (experiment C) to the protein before starting the reaction.

misinin on PfATP6 ATPase activity and that the explanation for the effect of artemisinin on the malarial parasite is probably more complex than originally thought. With respect to the oocyte data, it should be taken into account that the oocyte membranes represent a foreign and complex environment with hundreds of other proteins being present together with the expressed protein. The possibility cannot be excluded that these and other putative proteins or proteolipid components could interact with PfATP6 and with the tested drug and thereby affect ATPase activity. In oocyte also, using the same tests based on measurements of ATPase activity on membranes, Krishna and co-workers (26) reported a strong inhibition of artemisinin on the SERCA1 E255L homology mutant with a K_i of 315 nM. However, we demonstrate in the present paper that the Ca²⁺-dependent ATPase activity of this mutant in the endoplasmic reticulum of COS cells is not affected by artemisinin. The same is true for the purified mutant after yeast expression. Therefore, it would appear to be an oocyte-specific artemisinin inhibition, not extendable to other eukaryotic cells.

When trying to pinpoint the target of artemisinin or derivatives, many puzzling facts come to mind. Clearly, in a cellular context, artemisinin is affecting Ca²⁺ homeostasis as demonstrated, for example, with Ca²⁺-sensitive dyes, and it is now also used to kill/induce apoptosis of cancer cells with a likely effect on Ca²⁺ mobilization (e.g. see Refs. 69–71). However, in *P. falciparum*, the rise of cytosolic Ca²⁺ due to artesunate was also observed after prior thapsigargin addition, suggesting an intracellular target distinct from that of the endoplasmic reticulum (69). In other experiments, artemisinin was shown to induce swelling of mitochondria and to interfere with mitochondrial electron transport in a yeast model (see Ref. 72 and references therein), and it was suggested that an activated species of artemisinin could depolarize the mitochondrial membrane. However, this was not observed in

Toxoplasma exposed to artemisinin (21, 22). Because mitochondria are also a site of Ca²⁺ storage, this may still be related to the Ca²⁺ homeostasis effect, but it is by no means the only possible mechanism of action.

In other investigations of the target, it was shown that activated artemisinin formed covalent adducts with four major membrane-associated proteins, but only one of these could be classified, being a homolog of the translationally controlled tumor protein with a still unknown function in parasites (73, 74). In future experiments, it will be important to reconcile findings on the mechanisms of action of artemisinins obtained in apicomplexan parasites and in genetic studies, with findings from heterologous expression studies and with the present results to reassess the target of artemisinin. The present data do not support a direct action of artemisinins on PfATP6 (see also Ref. 57), but we cannot exclude the possibility that artemisinin may need some transformations before becoming active or that it could act indirectly on PfATP6 after binding to another protein. Alternatively, the drug may act on other proteins, such as Ca²⁺ channels involved in Ca²⁺ homeostasis. However, we note that with our procedure for purification of PfATP6, we have described a system with the potential for high throughput screening with novel classes of inhibitors acting against a key parasite transport protein.

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