A plant-like vacuolar H^+-pyrophosphatase in *Plasmodium falciparum*

Shuhong Luo, Norma Marchesini, Silvia N.J. Moreno, Roberto Docampo*

Laboratory of Molecular Parasitology, Department of Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Avenue, Urbana, IL 61802, USA

Received 6 September 1999

Abstract Inorganic pyrophosphate promoted the acidification of a subcellular compartment in cell homogenates of *Plasmodium falciparum* trophozoites. The proton gradient driven by pyrophosphate was collapsed by addition of NH_4Cl or the K^+/H^+ exchanger nigericin and eliminated by the pyrophosphate analog aminomethylenediphosphonate. Pyrophosphatase activity was dependent upon K^+, and partially inhibited by Na^+. The presence of a plant-like vacuolar H^+-translocating pyrophosphatase (V-H^+-PPase) was confirmed using antibodies raised against conserved peptide sequences of the enzyme, which cross reacted with a protein band of 76.5 kDa. Immunofluorescence microscopy using these antibodies showed a general fluorescence over the whole parasites and intracellular bright spots suggesting a vesicular and plasma membrane localization. Together, these results indicate the presence in *P. falciparum* of a V-H^+-PPase of similar characteristics to those of the enzyme from plants.

Key words: Aminomethylenediphosphonate; Malaria; Vacuolar pyrophosphatase; *Plasmodium*

1. Introduction

Plasmodium falciparum is an obligate intracellular parasite belonging to the phylum Apicomplexa. It has been found recently that apicomplexan parasites possess several biochemical peculiarities in common with plants, such as the presence of a functional shikimate pathway [1], and a chloroplast-like organelle, termed the apicoplast [2–4]. These recent discoveries have been useful to explain the mechanism of action of some antiparasitic compounds [5] and to provide new opportunities for the development of novel antimalarial agents [4,6].

In previous work we demonstrated the presence of a plant-like vacuolar proton-translocating pyrophosphatase (V-H^+-PPase) in *Trypanosoma cruzi* [7], *Leishmania donovani* [8], and *T. brucei* [9], the causative agents of Chagas' disease, visceral leishmaniasis, and African sleeping sickness, respectively, and in the apicomplexan parasite *Toxoplasma gondii* (Rodrigues et al., unpublished results). Until recently, V-H^+-PPases had been described in detail only in plants (including the unicellular alga *Acetabularia* [10] along with a homologous H^+-pyrophosphate synthase in the photosynthetic bacterium *Rhodospirillum* [11]). An apparently vacuolar-type pyrophosphatase activity was detected in rat liver Golgi fractions [12] using very high concentrations of pyrophosphate (1–3 mM). However, it is not known whether it has similar properties to the plant enzyme.

In this report, we demonstrate that *P. falciparum* intraerythrocytic stages possess a V-H^+-PPase activity with features in common with the trypanosomatid and plant activities.

2. Materials and methods

P. falciparum clone HB3 was grown at 2% hematocrit and 10% parasitemia to the trophozoite stage by the method of Trager and Jensen [13]. To isolate the trophozoites, erythrocytes were washed twice in phosphate buffered saline (PBS) by centrifugation at 4°C at $1500\times g$ for 5 min. Infected erythrocytes were enriched using the Percoll method [14]. Infected erythrocytes within the 70% Percoll interphase were collected and contained predominantly trophozoites (85–90% of the red blood cells, the rest containing ring-stage and schizont forms) by examination of Giemsa-stained thin blood smears. To isolate the parasites, infected erythrocytes were lysed with 0.1 mg/ml saponin in PBS at room temperature for 5 min. After centrifugation at $1500\times g$ for 5 min at 4°C to remove red blood cell membranes, the parasites were washed five times in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM HEPES, pH 7.2). The parasites were resuspended at 1×10^8 cells/ml in the same buffer. Contamination of the preparation with red blood cells was negligible. Protein was measured using the Bio-Rad Coomassie blue method.

Nigericin, ammonium chloride and sodium pyrophosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acridine orange, 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), purine nucleoside phosphorylase (PNP), and the standard phosphate solution were from Molecular Probes, Inc. (Eugene, OR, USA). Aminomethylenediphosphonate (AMDP) [15] and polyclonal antisera, which had been raised against keyhole limpet hemocyanin (KHL)-conjugated synthetic peptides corresponding to the hydrophilic loops IV (antibody 324) and XII (antibody 326) of plant V-H^+-PPase [16], were kindly provided by Prof. Philip Rea, University of Pennsylvania. All other reagents were analytical grade.

Pyrophosphate-driven proton transport was assayed by measuring changes in the absorbance of acridine orange (A_{493}-A_{530}) in an SLM-Aminco DW 2000 dual wavelength spectrophotometer [7–9]. Cell homogenates were incubated at 30°C in 2.5 ml of a standard buffer containing 130 mM KCl, 2 mM $MgSO_4$, 10 mM HEPES (pH 7.2), 50 μM EGTA, 3 μM acridine orange, and 0.1 mM PPi (pH 7.2). Each experiment was repeated at least three times with different cell preparations, and Fig. 1 shows representative experiments. Pyrophosphatase activity, in terms of phosphate release, was determined as per Scott et al. [7] for assays using different buffers. Reaction mixtures contained 130 mM KCl, 10 mM K-HEPES, 2 mM $MgSO_4$, 50 μM EGTA, pH 7.2 (or other buffers, as described in Table 1), 0.1 mM MESG, 0.4 U/ml PNP, together with the cell membrane preparation (60 μg/ml) in a total volume of 0.1 ml. Activity was monitored by the increase in absorbance at 360 nm using a Power Wave 340 plate reader (Bio-Tek Instruments) at 30°C, and was calibrated for each buffer with a standard phosphate solution.

For SDS electrophoresis and preparation of Western blots *P. falciparum* trophozoites (1×10^9) were resuspended in 300 μl Dulbecco's PBS (Gibco BRL) containing proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride) and frozen at −70°C. Thawed cells were homogenized with a Teflon pestle at 4°C and aliquots (10 μl, about 10 μg of protein) were mixed with 10 μl electrophoresis buffer (125 mM Tris-HCl pH 7, 10% (w/v) β-mercaptoethanol, 20% (v/v) glycerol, 4.0% (w/v) SDS, 4.0% (w/v) bromophenol blue) and boiled for 5 min prior to application to 10% SDS-polyacrylamide gels. Electrophoresed proteins were trans-

*Corresponding author. Fax: (1) (217) 244-7421.
E-mail: rodoc@uiuc.edu

PII: S0014-5793(99)01353-8

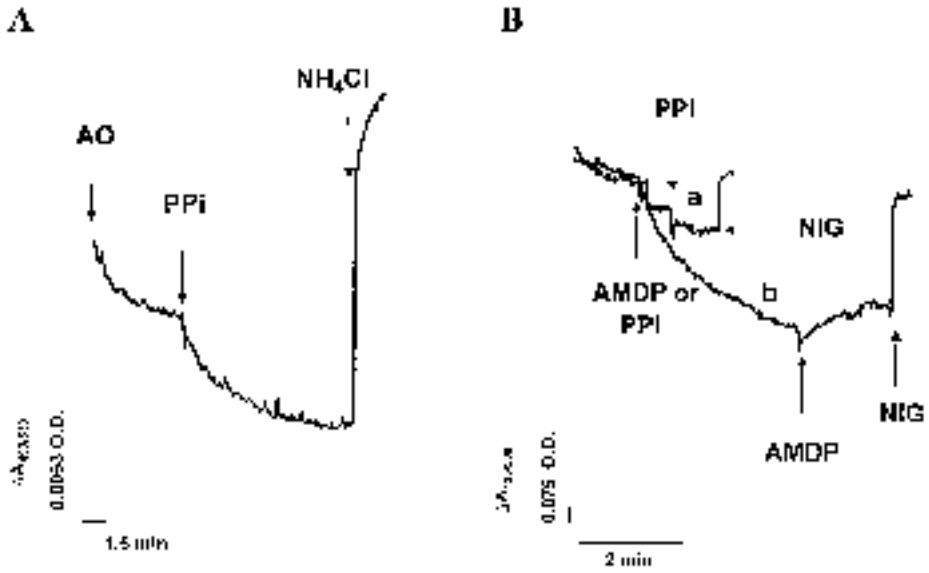

Fig. 1. Pyrophosphate-driven proton transport in trophozoite homogenates. Homogenates (60 μg protein/ml) were added to a buffer containing 130 mM KCl, 2 mM $MgSO_4$, 50 μM EGTA and 10 mM HEPES, pH 7.2, plus 3 μM acridine orange (B) in the absence (A, and trace b in B) or in the presence of 10 μM AMDP (B, trace a). Acridine orange (AO, 3 μM), 0.1 mM pyrophosphate (PPi), 10 mM NH_4Cl, 20 μM AMDP, and 1 μM nigericin (NIG) were added where indicated by the arrows.

ferred to nitrocellulose [17] using a Bio-Rad Transblot apparatus. Blots were blocked in 5% non-fat dry milk in PBS and kept overnight at 4°C. Polyclonal antisera raised against keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to the hydrophilic loops IV (antibody 324) and XII (antibody 326) of plant V-H^+-PPase [16] were used. A 1:1000 dilution of antiserum in blocking buffer was applied to blots at room temperature for 60 min. The nitrocellulose was washed three times for 15 min each with T-PBS buffer (Tween 20, 0.1% in PBS) before addition of a 1:10 000 dilution of goat anti-rabbit IgG in blocking buffer for 30 min. Immunoblots were visualized on radiographic film (Kodak) using the ECL chemiluminescence detection kit (Amersham Life Science).

For immunofluorescence microscopy, smears were made with cultures or with parasites released by the saponin treatment, fixed with acetone at −20°C for 30 min, blocked with ammonium chloride and 3% bovine serum albumin in PBS for 30 min and prepared for immunofluorescence using a 1:100 dilution of anti-V-H^+-PPase antibody or pre-immune serum in 5% bovine serum albumin-PBS and a FITC-coupled goat anti-rabbit IgG secondary antibody (1:160). Immunofluorescence images were obtained with an Olympus BX-60 fluorescence microscope and the image analysis system described previously [18].

3. Results

When acridine orange was added to cell homogenates of *P. falciparum* trophozoites some dye was accumulated and retained in the absence of added energy sources (Fig. 1A). Once a steady state of acridine orange accumulation was reached, addition of 0.1 mM PPi led to further dye uptake. This indicated the establishment of a proton gradient (ΔpH) across the membrane of a subcellular compartment, and increasing organelle acidity. This gradient collapsed completely after the addition of 10 mM NH_4Cl (Fig. 1A). Addition of 10 μM of the pyrophosphate analog and specific inhibitor of plant vacuolar pyrophosphatases AMDP [14] prevented acridine orange accumulation (Fig. 1B, trace a). AMPD (10 μM) released acridine orange when added after acidification had started (Fig. 1B, trace b). The vesicle pH was neutralized and acridine orange released after addition of 1 μM nigericin (Fig. 1B). Pyrophosphatase was also assayed in membrane preparations by inorganic phosphate detection [7–9]. The effects of monovalent cations on AMDP-inhibitable pyrophosphate hydrolysis are shown in Table 1. Replacing 130 mM KCl with 250 mM sucrose in the buffer resulted in lower pyrophosphatase activity that was further reduced by replacement of 130 mM KCl with 130 mM NaCl. Use of a buffer containing equimolar concentrations of NaCl (65 mM) and KCl (65 mM) resulted in lower pyrophosphate hydrolysis than in the presence of 130 mM KCl or 65 mM KCl/125 mM sucrose. These results suggest that K^+ was necessary for this activity, whilst Na^+ was inhibitory. Together, these results agree with those obtained with plant [11], trypanosomatid [7–9], and *T. gondii* (Rodrigues et al., submitted) V-H^+-PPases, which are K^+-dependent and inhibited by Na^+.

Immunoblotting of total lysates allowed immunological detection of *P. falciparum* V-H^+-PPase with antibodies developed against the plant enzyme. A single protein band with a molecular mass of approximately 76.5 kDa was detected (Fig.

Table 1
Effect of buffer composition on pyrophosphatase activity in *P. falciparum* trophozoites

Experimental conditions	Pyrophosphate hydrolysis (% of control)[a]
130 mM KCl	100 ± 9.3
65 mM KCl/125 mM sucrose	86 ± 13
65 mM KCl/65 mM NaCl	53 ± 12.0
130 mM NaCl	36 ± 5.8
250 mM sucrose	51 ± 8.0

Rates are relative (%) to the 130 mM KCl buffer. All buffers contained, in addition, 2 mM $MgSO_4$, 10 mM HEPES and 50 μM EGTA, and were adjusted to pH 7.2 with KOH, NaOH, or Tris base for KCl, NaCl, and sucrose buffers, respectively. Values are means ± S.D. of triplicate samples. Control activity was 1.96 ± 0.1 Δ_{360}/min/mg protein.
[a]Rates were corrected by subtraction of non-specific activity in the presence of 20 μM AMDP.

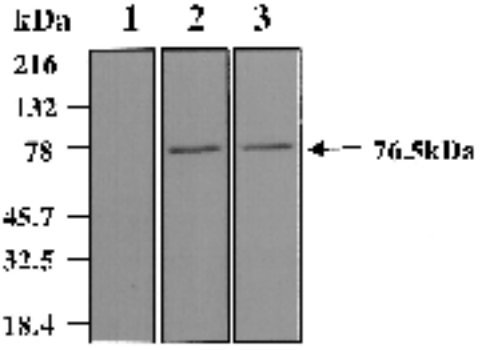

Fig. 2. Western blot analysis of the V-H$^+$-PPase. Panels show the detection of the V-H$^+$-PPase by immunoblot, using antibodies specific for the plant enzyme. *P. falciparum* proteins (10 μg) were separated by SDS-PAGE and transferred to nitrocellulose. Lane 1, immunoblot probed with normal rabbit serum. Lanes 2 and 3 show immunoblots probed with antibody 324 and 326, respectively. The V-H$^+$-PPase antibody recognized a polypeptide with an apparent molecular mass of 76.5 kDa.

2, lanes 2 and 3) indicating cross reaction of plant antibodies with the *P. falciparum* protein. No background staining was observed when normal serum was used as a control (Fig. 2, lane 1). Immunofluorescence of culture cells using the same antibodies (Fig. 3B,C) showed a general fluorescence over the whole parasites and intracellular bright spots suggesting a vesicular and plasma membrane localization. No fluorescence was observed in control cultures incubated only with pre-immune serum and secondary fluorescein-labeled goat anti-rabbit IgG (Fig. 3A) or in non-infected erythrocytes (Fig. 3B,C). A similar labeling pattern was obtained with isolated trophozoites (data not shown).

4. Discussion

In this study, we have identified a H$^+$-translocating pyrophosphatase activity in homogenates of *P. falciparum*. Acridine orange uptake in the presence of pyrophosphate was reversed by the K$^+$/H$^+$ exchanger, nigericin. As occurs with plant [11] and trypanosomatid [7–9] vacuolar pyrophosphatases, pyrophosphate-driven proton transport was blocked by the pyrophosphate analog AMDP and the pyrophosphatase activity was stimulated by potassium and inhibited by sodium ions.

This is the first report of the presence of a V-H$^+$-PPase activity in *P. falciparum*. A *P. falciparum* gene with homology with genes for V-H$^+$-PPases is listed in GenBank (AF115766), but the sequence has not been published and it is not known whether it encodes a functional V-H$^+$-PPase. A gene encoding a protein with homology with inorganic pyrophosphatases has also been reported recently in *P. falciparum* [19]. The apparent lack of a V-H$^+$-PPase in mammalian cells makes this enzyme a potential target for specific chemotherapy. V-H$^+$-PPases had been described before in plants, phototrophic bacteria, yeasts [11], and trypanosomatids [7–9]. In plants, V-H$^+$-PPases are present in the vacuole membrane (tonoplast) [11] and also in the plasma membrane [20,21]. Antibodies to conserved regions of the plant V-H$^+$-PPase [14] cross reacted with the enzyme from *T. cruzi* and suggested an intracellular and plasma membrane localization in this parasite [7]. The same antibodies used in this study reacted with a *P. falciparum* polypeptide (Fig. 2) and also suggested an intracellular and plasma membrane localization of the V-H$^+$-PPase (Fig. 3).

Inorganic pyrophosphate is the substrate for the pyrophosphate-dependent phosphofructokinase found in several api-

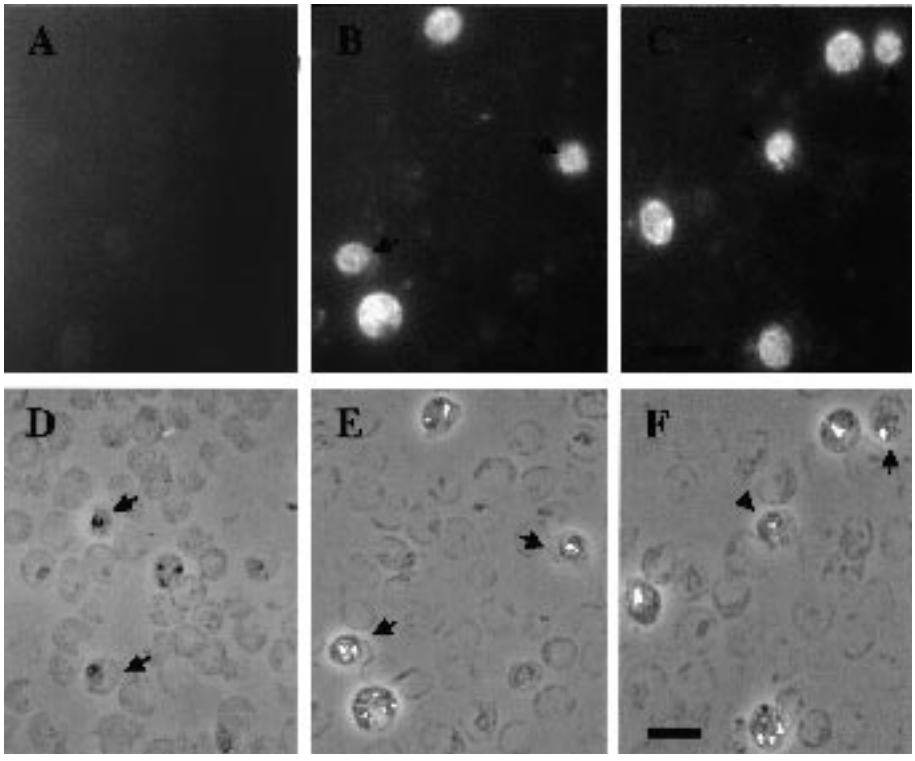

Fig. 3. Indirect immunofluorescence analysis of V-H$^+$-PPase in trophozoites of *P. falciparum*. Fluorescence (A, B, C) or bright field (D, E, F) images of infected erythrocytes: A,D, with preimmune serum; B,E, with antibody 324; C,F, with antibody 326. The images show intense labeling over the whole parasites and bright, intracellular spots. Arrows show trophozoite stages of the parasite. Bars = 10 μm.

complexan parasites including *T. gondii* [22,23], *Cryptosporidium parvum* [23], and *Eimeria tenella* [23]. However, little is known about pyrophosphate metabolism in malaria parasites. Our detection of a proton translocating pyrophosphatase activity in *P. falciparum* suggests that pyrophosphate could play an important role in the metabolism of these parasites.

Acknowledgements: We thank Philip A. Rea for the gift of AMDP and polyclonal antibodies against V-H^+-PPase and Daniel Goldberg for help to start the *P. falciparum* cultures in our laboratory. This work was supported by a Burroughs Wellcome New Initiatives in Malaria Research Award to R.D. S.N.J.M. is a Burroughs Wellcome New Investigator in Molecular Parasitology.

References

[1] Roberts, F., Roberts, C.W., Johnson, J.J., Kyle, D.E., Krell, T., Coggins, J.R., Coombs, G.H., Milhous, W.K., Tzipori, S., Ferguson, D.J.P., Chakrabarti, D. and McLeod, R. (1998) Nature 393, 801–805.
[2] Wilson, R.J.M., Denny, P.W., Preiser, P.R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D.J., Moore, P.W. and Williamson, D.H. (1996) J. Mol. Biol. 261, 155–172.
[3] McFadden, G.I., Reith, M.E., Muholland, J. and Lang-Unnasch, N. (1996) Nature 381, 482.
[4] Köhler, S., Delwiche, C.F., Denny, P.W., Tilney, L.G., Webster, P., Wilson, R.J.M., Palmer, J.D. and Roos, D.S. (1997) Science 275, 1485–1489.
[5] Fichera, M.E. and Roos, D.S. (1998) Nature 390, 407–409.
[6] McConkey, G.A., Rogers, M.J. and McCutchan, T.F. (1997) J. Biol. Chem. 272, 2046–2049.
[7] Scott, D.A., de Souza, W., Benchimol, M., Zhong, L., Lu, H.G., Moreno, S.N.J. and Docampo, R. (1998) J. Biol. Chem. 273, 22151–22158.
[8] Rodrigues, C.O., Scott, D.A. and Docampo, R. (1999) Biochem. J. 340, 759–766.
[9] Rodrigues, C.O., Scott, D.A. and Docampo, R. (1999) Mol. Cell. Biol. (in press).
[10] Ikeda, M., Rahman, M.D., Moritani, C., Umami, K., Tanimura, Y., Akagi, R., Tanaka, Y., Maeshima, M. and Watanabe, Y. (1999) J. Exp. Bot. 50, 139–140.
[11] Rea, P.A. and Poole, R.J. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 157–180.
[12] Brightman, A.O., Navas, P., Minnifield, N.N. and Morré, D.J. (1992) Biochim. Biophys. Acta 1104, 188–194.
[13] Trager, W. and Jensen, J.B. (1976) Science 193, 673–675.
[14] Dluzewski, A.R., Ling, I.T., Rangachari, K., Bates, P.A. and Wilson, R.J.M. (1984) Trans. R. Soc. Trop. Med. Hyg. 78, 622–624.
[15] Zhen, R.G., Baykov, A.A., Bakuleva, N.P. and Rea, P.A. (1994) Plant Physiol. 104, 153–159.
[16] Serafian, V., Kim, Y., Poole, R.J. and Rea, P.A. (1992) Proc. Natl. Acad. Sci. USA 89, 1775–1779.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Lu, H.G., Zhong, L., de Souza, W., Benchimol, M., Moreno, S.N.J. and Docampo, R. (1998) Mol. Cell. Biol. 18, 2309–2323.
[19] Bowman, S., Lawson, D., Basham, D., Brown, D., Chillingworth, T., Churcher, C.M., Craig, A., Davies, R.M., Devlin, K., Feltwell, T., Gentles, S., Gwilliam, R., Hamlin, N., Farris, D., Holroyd, S., Hornsby, T., Horrocks, P., Jagels, K., Jassal, B., Kyes, S., McLean, J., Moule, S., Mungall, K., Murphy, L., Oliver, K., Quail, M.A., Rajandream, M.-A., Rutter, S., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Whitehead, S., Woodward, J.R., Newbold, C. and Barrell, B.G. (1999) Nature 400, 532–538.
[20] Long, A.R., Williams, L.E., Nelson, S.J. and Hall, J.L. (1995) J. Plant Physiol. 146, 629–638.
[21] Robinson, D.G., Haschke, H.P., Hinz, G., Hoh, B., Maeshima, M. and Marty, F. (1996) Planta 198, 95–103.
[22] Peng, Z.Y. and Mansour, T.E. (1992) Mol. Biochem. Parasitol. 54, 223–230.
[23] Denton, H., Brown, S.M.A., Roberts, C.W., Alexander, J., McDonald, V., Thong, K.W. and Coombs, G.H. (1996) Mol. Biochem. Parasitol. 76, 23–29.