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REVIEW ARTICLE

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Enzymatic systems of inorganic pyrophosphate bioenergetics in photosynthetic and heterotrophic protists: remnants or metabolic cornerstones?

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Abstract An increasing body of biochemical and genetic evidence suggests that inorganic pyrophosphate (PPi) plays an important role in protist bioenergetics. In these organisms, two types of inorganic pyrophosphatases [EC 3.6.1.1, namely soluble PPases (sPPases) and proton-translocating PPases (H^+-PPases)] that hydrolyse the PPi generated by cell anabolism, thereby replenishing the orthophosphate pool needed for phosphorylation reactions, are present in different cellular compartments. Photosynthetic and heterotrophic protists possess sPPases located in cellular organelles (plastids and mitochondria), where many anabolic and biosynthetic reactions take place, in addition to H^+-PPases, which are integral membrane proteins of the vacuolysosomal membranes and use the chemical energy of PPi to generate an electrochemical proton gradient useful in cell bioenergetics. This last category of proton pumps was considered to be restricted to higher plants and some primitive photosynthetic bacteria, but it has been found recently in many protists (microalgae and protozoa) and bacteria, thus indicating that H^+-PPases are much more widespread than previously thought. No cytosolic sPPase (in bacteria, fungi and animal cells) has been shown to occur in these lower eukaryotes. The widespread occurrence of these key enzymes of PPi metabolism among evolutionarily divergent protists strongly supports the ancestral character of the bioenergetics based on this simple energy-rich compound, which may play an important role in survival under different biotic and abiotic stress conditions.

Keywords Inorganic pyrophosphate · Soluble inorganic pyrophophatase · Proton-translocating pyrophosphatase · Photosynthetic protists · Parasitic protists

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Introduction

Inorganic pyrophosphate (PPi) is a simple molecule composed of two metaphosphate groups ($\sim PO_3^-$) linked by an oxygen anion (O_2^-), thus forming the P-O-P structure, also known as a "pyrophosphate bridge". P-O-P is a chemical group that stores readily usable energy for biochemical reactions and it can be found not only in PPi but also in ATP, the well-known "energy currency" of living cells. PPi is produced in large amounts by a variety of vital biosynthetic reactions, such as the synthesis of biopolymers (polysaccharides, proteins, nucleic acids, lipids). PPi hydrolysis is important to pull these anabolic reactions (most of which are reversible) in the direction of biosynthesis. Moreover, an efficient PPi hydrolysis is also essential to replenish the orthophosphate (Pi) needed for phosphorylation [2] (Fig. 1).

Although PPi was until recently considered a waste product of anabolism, an increasing body of evidence indicates that it can play a relevant role in cellular bioenergetics. Moreover, it has been suggested that PPi may have been the ancestor of ATP as the "energy currency" during the early stages of biochemical evolution [4]. Two major types of PPi-hydrolysing enzymes, known as inorganic pyrophosphatases (PPases, EC 3.6.1.1), have been characterized to date: soluble and membrane-embedded. Soluble PPases (sPPases) are ubiquitous proteins whose role is the removal of the PPi produced by anabolic reactions, so that they can efficiently proceed in the correct (biosynthetic) direction [20]. Membrane-bound, proton-translocating, inorganic pyrophosphatases (H^+-PPases) belong to a recently identified category

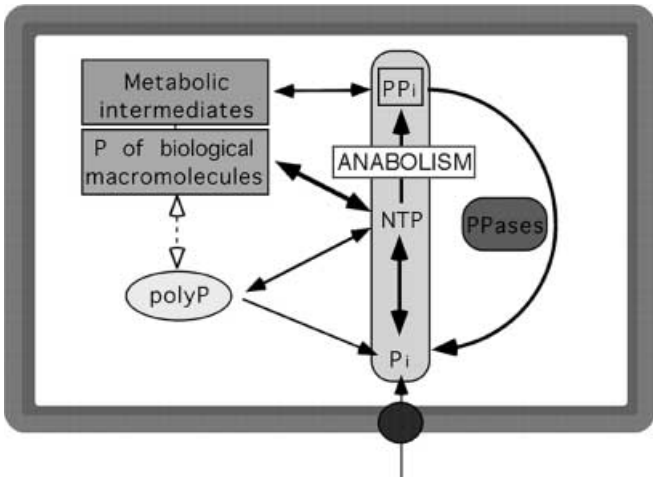

Fig. 1 Metabolic scheme showing the intracellular phosphate cycle of energization–deenergization in which inorganic pyrophosphatases (*PPases*) play a key role. The metabolic scenario represents a prokaryotic cell but may be applied also to protist cellular organelles. *NTP* Nucleosides triphosphate, *PolyP* inorganic polyphosphates

of proton pumps, distinct from F-, P- and V-ATPases, which utilize PPi hydrolysis as the driving force for H^+ movement across biological membranes [28]. H^+-PPases have been identified and characterized both at biochemical and genetic levels in higher plants, some photosynthetic bacteria and, more recently, in many bacteria, archaea and some parasitic protists [5, 9, 10, 12, 16, 18, 23, 25, 30, 31, 33]. However, they seem to be absent from animals, fungi and several types of bacteria, including enterobacteria.

The sPPases of protists are located in cell organelles (plastids, mitochondria) and have diverse molecular phylogeny

So far, cytosolic sPPases have been found and well characterized in bacteria, fungi and animal cells. In these eukaryotes, a mitochondrial sPPase was also found as an extrinsic protein of the inner membrane and was found to be essential for the function of this organelle [22]. In yeast, both proteins have the same molecular phylogeny, being eukaryote-like sPPases. The metabolic scenario concerning sPPases is completely different in photosynthetic protists (Fig. 2). Protists [both photosynthetic (microalgae) and heterotrophic (protozoa)] lack cytosolic sPPase, the physiological role of this enzyme presumably being performed by a number of soluble and membrane-bound proteins involved either in sugar phosphorylation or in ion transport and homeostasis, respectively [13, 14, 15, 29]. Virtually all sPPase activity is located in the cellular organelles – namely, plastids and mitochondria – of these lower eukaryotes (Fig. 2), as is also the case in higher plant photosynthetic tissues [13, 14, 15, Gómez R (2001) PhD thesis, University of Seville]. We have purified to homogeneity and characterized the sPPases of photoautotrophic protists bearing plastids of different types, from primitive cyanobacteria-like cyanelles to plant-like chloroplasts (with two enfolding membranes) and complex chloroplasts (with more than two enfolding membranes). All are monomeric proteins with apparent molecular masses in the range 32–40 kDa, as determined by SDS-PAGE and fast-performance gel permeation chromatography; and they resemble fungal and animal eukaryote-like sPPases, both in protein structure and N-terminus sequences [13, 14, 15, Gómez R et al., in prep.]. Among others, the plastid sPPases from the glaucocystophycean flagellate (with cyanelles) *Cyanophora paradoxa* (32 kDa), the thermoacidophilic rhodophycean microalga *Cyanidium caldarium* (40 kDa), the euglenoid *Euglena gracilis* (38 kDa), the photosynthetic heterokont (chromophyte) *Ochromonas danica* (38 kDa) and the chlorophycean microalga *Chlamydomonas reinhardtii* (37 kDa) have been characterized. The plastid sPPases are therefore eukaryote-like proteins clearly different from the homohexameric (20 kDa subunit) sPPases of cyanobacteria, which are photoautotrophic bacteria resembling the ancestral prokaryotic endosymbiont that gave rise to these organelles [13, 14, 15, Gómez R et al., in prep.].

A careful analysis of the sPPase preparations purified from the chlorophycean microalga *C. reinhardtii* revealed the presence of two polypeptides of slightly different molecular mass, both of them with PPase activity: a major eukaryote-like enzyme named sPPase1 (37 kDa, SDS-PAGE) and a minor one named sPPase2 (32 kDa, SDS–PAGE) [14, 15, Gómez R et al., in prep.]. Monospecific polyclonal antibodies raised in rabbits against these two proteins did not cross-react, indicating that they should be structurally different proteins. *Western blot* analyses with the anti-sPPase1 antibody immunodetected a single polypeptide (32–40 kDa), which corresponded to the plastid sPPase in cell-free crude extracts of other Chlorophyceae, euglenoids, Glaucocystophyceae, Rhodophyceae, photosynthetic heterokonts (Chromophyceae, diatoms) and plant photosynthetic tissues (Table 1), but not to that in plant root tissues and bacteria. Note that, in agreement with biochemical data, this antibody also immunodetected yeast cytosolic sPPase. Subcellular fractionation using Percoll gradient VCX analysis localized sPPase1 in the chloroplast fraction and sPPase2 in the mitochondrial one [15, Gómez R (2001) PhD thesis, University of Seville].

An *Arabidopsis thaliana* cDNA that encodes the precursor polypeptide of an eukaryote-like sPPase with a N-terminal chloroplast transit peptide has been identified by BLAST homology searches. This plant *ppa* gene has been cloned (accession number AJ252210) and heterologously overexpressed in *Escherichia coli*, where the protein was processed to the mature active form; and it was efficiently inmunodetected by the antibody anti-sPPase1 of *C. reinhardtii*, both recombinant-plant and natural-alga mature proteins having virtually identical

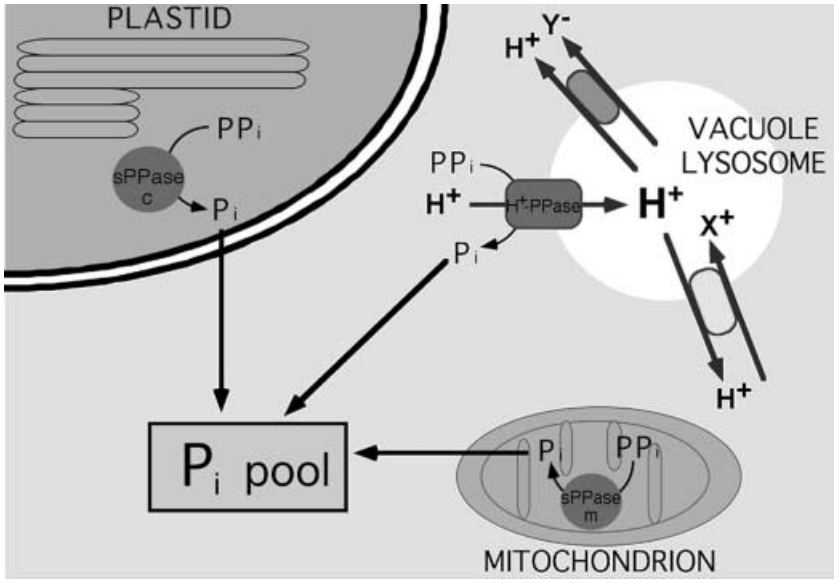

Fig. 2 Schematic representation of the metabolic scenario concerning the three PPases that occur in different cell compartments of photosynthetic protists and their relationships with the cytosolic orthophosphate (*Pi*) pool. Cellular organelles, plastids and mitochodria, contain respectively the soluble PPases (*sPPases*) *c* and *m* that hydrolyse the inorganic pyrophosphate (*PPi*) generated in anabolic reactions, whereas the proton-translocating PPase (H^+-*PPase*) of the intracellular membranes of vacuoles or lysosomes do the same job in the cytosol. In this case, however, the chemical energy of the phosphate bond is used to generate an electrochemical proton gradient that is used to drive a number of symport and antiport membrane systems. A similar scenario is proposed for protozoa, except that no photosynthetic plastids are present in this case

molecular masses. To our knowledge, this is the first *ppa* gene encoding a precursor polypeptide with a "chloroplast transit peptide" described so far [15, Gómez R et al., in prep.]. In agreement with its predicted cellular localization, *Northern blot* experiments showed that this gene is expressed in green tissues (leaves, shoots), but not in roots.

In accordance with the biochemical data, BLAST homology searches on EST databases allowed us to identify two potentially encoding sPPase cDNAs of the microalga *C. reinhardtii*. Both algal *ppa* genes have been cloned (accession numbers AJ298231 for *ppaI* gene coding for sPPase1, AJ298232 for *ppaII* gene coding for sPPase2) and found to be expressed in photoautotrophic *C. reinhardtii* cells, from which the two sPPase proteins were purified. One of the cDNAs possesses a chloroplast transit peptide and encodes the polypeptide precursor of an eukaryote-like sPPase (the chloroplast sPPase1); and the other encodes a smaller bacterial-like sPPase (presumably the mitochondrial sPPase2). Therefore, the sPPases set of *C. reinhardtii* is formed by two proteins of distinct molecular phylogeny that are located in different cellular organelles [14, 15, Gómez R et al., in prep.].

The recently completed *A. thaliana* genome project has added new interesting information that confirmed our results. Thus, a single *ppa* gene located in chromosome 5 (the same one we cloned and experimentally validated) encodes a chloroplast eukaryote-like sPPase; and a set of five paralogous, highly-similar *ppa* genes located in different chromosomes encode a family of bacteria-like sPPases. One of these genes corresponds to a mitochondrial polypeptide precursor and should be equivalent to the sPPase2 of *C. reinhardtii*. The other four bacterial sPPase genes exhibit a very high homology, even at the DNA level and are clearly equivalent to the orthologous *ppa* gene that encodes the cytosolic sPPase found in potato tuber [11], so they should be expressed in non-photosynthetic tissues (roots). The high similarity found among the bacteria-like sPPases of *A. thaliana* suggests that they probably originated by quite recent gene duplication events from a common ancestor, perhaps similar to the microalgal sPPase2 gene [15, Gómez R (2001) PhD thesis, University of Seville].

The results described above have clarified the molecular phylogeny of the sPPases of photosynthetic eukaryotes (microalgae, plants). All photosynthetic plastids contain a nuclear-encoded eukaryote-like sPPase; and this finding suggests that the homologous bacterial enzyme of the ancestral prokaryotic endosymbiont was lost very early during the evolutionary processes that gave rise to photosynthetic plastids and was functionally substituted by the nuclear-encoded sPPase of the eukaryotic host cell (Fig. 3). In contrast, the mitochondrial sPPases of microalgae and plants are nuclear-encoded bacteria-like proteins, as should also be the case for the cytosolic sPPases of non-photosynthetic tissues [13, 14, 15, Gómez R et al., in prep.]. The scenario found for the fungal and animal lineages is in this respect quite different, since they have eukaryote-like cytosolic and mitochondrial sPPases [22]. Recent data suggest that this may also be the case for the organellar (mitochondrial, apicoplast, kinetoplast) sPPases of a number of protozoan strains (trypanosomatids, apicomplexa) that seem to be eukaryote-like sPPases (see Table 1; Gómez R (2001) PhD thesis, University of

Table 1 Protists in which soluble pyrophosphatase (sPPase) proteins located in cellular organelles (plastids, mitochondria, apicoplasts) and/or genes encoding their precursors have been identified. Evidence from experiments (in the authors' laboratory, either by protein purification, subcellular fractionation, Western blot analysis, or gene cloning) and/or from bioinformatic analyses of genome databases (*g*). *M* Mitochondrial sPPase, *M*/*A* mitochondrial or apicoplast sPPase, *P* plastid sPPase, *p* only partial sequences with no N-terminal regions are known and/or no cellular localization data are yet available. The two sPPase genes of *Chlamydomonas reinhardtii* and the one of *Leishmania major* have been cloned and experimentally validated

Heterotrophic protists (protozoa)	Photosynthetic protists (microalgae)
Trypanosomatidae	Chlorophyceae
Leishmania major (M, g)	*Chlamydomonas reinhardtii* (M, P, g)
Trypanosoma brucei (M, g)	*Chlorella fusca* (P)
	Dunaliella salina (P)
Apicomplexa	*Monoraphidium braunii* (P)
Plasmodium falciparum (M/A, g)	
P. berghei (p, g)	Rhodophyceae
Cryptosporidium parvum (p, g)	*Cyanidium caldarium* (P)
Heterotrophic euglenoids	Euglenophyceae
Astasia longa (p)	*Euglena gracilis* (P)
Entamoebidae	Glaucocystophyceae
Entamoeba histolytica (p, g)	*Cyanophora paradoxa* (P)
Dictyosteliida	Photosynthetic heterokonts (Stramenopiles)
Dictyostelium discoideum (p, g)	Chrysophyceae
	Ochromonas danica (P)
Non-photosynthetic heterokonts (Stramenopiles)	Bacillariophyceae (diatoms)
Oomycetes	*Navicula pelliculosa* (P)
	Phaeodactylum tricornutum (P)
Phytophthora sojae (p, g)	

Seville). Summarizing, although many protists have sPPases only in cellular organelles (plastids, mitochondria), the photosynthetic eukaryotes (microalgae, plants) are, so far, the only group of organisms in which two sPPases with different molecular phylogeny and distinct cellular localization occur.

H^+-PPases occur in endocellular membranes (vacuoles, lysosomes, acidocalcisomes) of a broad range of evolutionarily diverse protists

The membrane-bound H^+-PPase was first described in chromatophores from the phototrophic bacterium *Rhodospirillum rubrum* and shown to be able to both synthesize and hydrolyse PPi [1, 3]. Eukaryotic H^+-PPases were originally identified in higher plants and reported to be located in the vacuolar membrane (tonoplast), catalysing electrogenic H^+-translocation from the cytosol to the vacuole lumen, thus generating an electrochemical gradient, which can be utilized to couple diverse secondary transport processes (Fig. 2). This electrochemical gradient has similar or greater magnitude than that created by the other proton pump present in the tonoplast: the vacuolar H^+-ATPase [28]. Vacuolar H^+-PPases have a subunit size of about 66 kDa and radiation inactivation analyses have shown that the protein seems to be a dimer in vivo [17].

The biochemical characterization of H^+-PPases from different sources has suggested the occurrence of two types of protein, depending on their requirement for

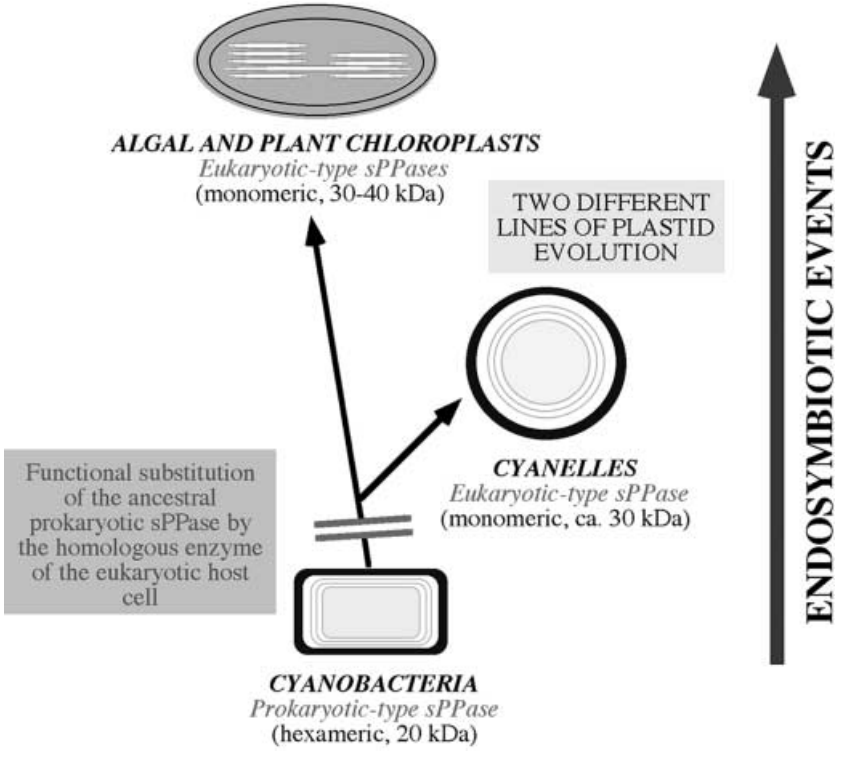

Fig. 3 The evolutionary relationships between the sPPases of cyanobacteria and plastids. Cyanelles are primitive plastids, very similar to cyanobacterial cells, found in glaucocystophycean protists. They have eukaryote-like sPPases, suggesting that the functional substitution of the ancestral enzyme of the cyanobacteria-like endosymbiont was an early event during plastid evolution

potassium for full activity. Thus, higher plant H^+-PPases were shown to have a near-obligatory requirement for millimolar concentrations of potassium for activity [6], whereas *R. rubrum* H^+-PPase was reported to be insensitive to monovalent cations [25]. More recently, the gene encoding the *A. thaliana* H^+-PPase was isolated and sequenced; and the protein was shown to be a potassium-dependent proton pump, by expressing the gene in the yeast *Saccharomyces cerevisiae* (which only has soluble PPases) and characterizing the PPi-hydrolysis activity and the PPi-dependent H^+-translocation activity associated with a vacuolar membrane-enriched fraction of the transformed yeast cells [18]. This approach was subsequently followed by different groups, thus characterizing a number of H^+-PPases [4, 9, 16]. Initially, potassium-sensitive proteins were found only in eukaryotes, namely higher plants [18, 28] and parasitic protists [16, 23, 30, 31, 33], whereas H^+-PPases from prokaryotic sources were insensitive to monovalent cations [5, 25]. However, during the course of the *A. thaliana* genome project, another gene coding for a putative H^+-PPase was identified; this gene showed a higher homology with bacterial genes than with the other *A. thaliana* H^+-PPase gene identified at that time. Consistently, the new gene was shown to code for a potassium-insensitive H^+-PPase by heterologous expression in *S. cerevisiae* and was also reported to be expressed in several plant tissues [10]. The presence of both types of H^+-PPase in *A. thaliana* suggests different physiological roles and even different subcellular localization for the two types of protein, however, these aspects remain to be studied. This evidence also demonstrated that the occurrence of the two types of H^+-PPase was far from clear. The situation has received a new twist with the work carried out in our laboratory with the H^+-PPase from the hyperthermophilic eubacterium, *Thermotoga maritima*. We have biochemically characterized the heterologously expressed protein in yeast and found it to be stimulated more than six-fold by potassium [26]. This was the first report of a potassium-sensitive H^+-PPase in a prokaryote. Moreover, since *T. maritima* is a member of the order Thermotogales, one of the deepest and most slowly evolving lineages in bacteria, this finding also has an important evolutionary significance. Thus, potassium-stimulated H^+-PPases seem to be as ancient as their potassium-insensitive counterparts, which, as mentioned before, are distributed among eukaryotes (including protists), archaea and bacteria.

Although, as described above, plenty of evidence is already available about H^+-PPases, many important issues about these proteins remain to be solved. These issues can be summarized in three major points: (a) the occurrence of membrane-bound H^+-PPases, (b) their physiological role and (c) the reason for the existence of proteins with different sensitivities to monovalent cations.

Acidocalcisomes are acidic, calcium-storage organelles found in several protists, although they were first defined in trypanosomatids [7, 32]. Since acidocalcisomes were initially thought to be related to the vacuoles of plants, the presence of a H^+-PPase in these organelles was investigated and found in *Leishmania donovani* [30], *Trypanosoma brucei* [31, 34], *T. cruzi* [32, 33], *Plasmodium berghei*, *Plasmodium falciparum* and *Toxoplasma gondii* [8, 30]. The genes coding for these proteins in *P. falciparum* [8, genome project], *T. cruzi* [16] and *T. gondii* [8] have been cloned and sequenced.

As far as photosynthetic protists are concerned, the situation is less clear. Thus far, H^+-PPases have only been identified in the macroscopic unicellular alga *Acetabularia mediterranea* and in the tallophytic alga *Chara corallina* [24]. In any case, the information concerning the occurrence of PPase genes in protists is scarce and non-systematic. H^+-PPases do not seem to be present in animals and, thus, they might be potential targets for vaccines and drugs against parasitic protists. Therefore, studies on the possible implication of these proteins in the development of the diseases caused by these organisms might be of great importance. Major steps towards this goal are to find out: (a) whether H^+-PPase genes are present in the genome of other parasitic protists, (b) the conditions under which they become expressed and (c) the structural characteristics of the proteins they encode. A molecular approach could give us valuable tools to accomplish these objectives. In addition, the study of the occurrence of the different H^+-PPase genes in such a heterogeneous group of organisms will give most valuable information about the evolution and phylogeny of this unique class of proton pumps and their physiological roles in living cells.

By using polyclonal antibodies against conserved amino acid regions [9, 10, 28], we have found immunoreactive bands of the expected molecular mass in *Western blots* of membrane preparations from a number of photosynthetic protists having simple and complex plastids (Bacillariophyceae, Chlorophyceae, Chromophyceae, Cryptophyceae, Euglenophyceae, Glaucocystophyceae, Rhodophyceae, other photosynthetic heterokonts; [27], Pérez-Castiñeira et al., in prep.; Table 2). Also, by using a PCR approach with degenerate oligonucleotides designed from amino acid domains common to H^+-PPases of higher plants and *Rhodospirillum rubrum*, we have performed a systematic search for genes coding for H^+-PPases in parasitic and free-living protozoa and eukaryotic microalgae (Table 2). We have usually amplified a DNA fragment (ca. 0.6 kb) of the 3′ region of the gene (from the cytosolic loop V to the C-terminus region), corresponding to ca. 25–30% of the total coding region that was demonstrated to be a good molecular marker for phylogenetic studies. *Southern blot* analysis further confirmed the presence of these genes in the respective genomes of the organisms tested. Computer analysis of the sequences obtained allowed us to analyse the sometimes surprising phylogenetic relationships amongst the H^+-PPases from evolutionarily distant organisms.

Using this molecular genetics strategy, we found plant-like H^+-PPases genes (very similar to those of human parasitic trypanosomes) in a range of parasitic, mostly non-pathogenic trypanosomatids of insects and

Table 2 Protists in which H^+-PPase proteins or their genes have been identified. Evidence from experiments (either Western blots of membrane preparations or PCR amplification and sequencing of gene regions, using degenerate oligonucleotides) and/or from bioinformatic searches on genome databases (*g*). Unpublished DNA sequences obtained in the authors' laboratory have been submitted to databases and most of them are under confidential status until publication

Heterotrophic protists (protozoa)	Photosynthetic protists (microalgae)
Trypanosomatidae	Chlorophyceae
Chritidia fasciculata	*Chlamydomonas reinhardtii* (g)
Herpetomonas muscarum	*C. euriale*
Endotrypanum schaudinni	*Chlorella fusca*
Leptomonas ctenocephali	*C. salina*
Phytomonas sp.	*Dunaliella salina*
Leishmania major	*Monoraphidium braunii*
L. donovani	*Tetraselmis chuii*
Trypanosoma cruzi	*T. baltica*
T. brucei (g)	
	Rhodophyceae
Heterotrophic euglenoids	*Cyanidium caldarium*
Astasia longa	*Porphyridium purpureum*
	Euglenophyceae
Dictyosteliida	*Euglena gracilis*
Dictyostelium discoideum	Glaucocystophyceae
Alveolates	*Cyanophora paradoxa*
Apicomplexa	Cryptophyceae
Plasmodium falciparum (g)	*Cryptomonas* sp.
P. berghei	*Rhodomonas baltica*
Plasmodium sp.	
Toxoplasma gondii (g)	Haptophyceae
Ciliophora (ciliates)	*Isochrysis galbana*
Hymenostomatids	
Paramecium tetraurelia	Alveolates
Tetrahymena pyriformis	Dynophyceae
Peritrichous	*Heterocapsa* sp.
Vorticella microstoma	
Hypotrichous	Photosynthetic heterokonts (Stramenopiles)
Histriculus cavicola	Eustigmatophyceae
Non-photosynthetic heterokonts (Stramenopiles)	*Nannochloropsis gaditana*
	N. oculata
Oomycetes	Chrysophyceae
Phytophthora infestans (g)	*Ochromonas danica*
	Bacillariophyceae (diatoms)
	Navicula pelliculosa
	Phaeodactylum tricornutum
	Skeletomonas costata

other lower animals (*Crithidia*, *Endotrypanum*, *Herpetomonas*, *Leptomonas*) and plants (*Phytomonas*), thus indicating that the presence of this protein is not necessarily associated with the pathogenic character of these protozoa. It is interesting in this respect that we have also identified plant-like H^+-PPase genes in a number of free-living, non-parasitic protozoa of other phylogenetic groups: both in ciliates [such as hymenostomatids (*Tetrahymena*, *Paramecium*), peritrichous (*Vorticella*) and hypotrichous (*Histriculus*) strains (having the peculiar features for genes of this group, namely in-frame stop codons coding for Gln and, depending of the strain, very short, 20–30 bp-long introns)] and in heterotrophic euglenoids (*Astasia longa*). In addition, in agreement with immunochemical data, our group has identified and cloned H^+-PPase genes from representative strains of the main phylogenetic groups of photosynthetic protists: Chlorophyceae (*Chlamydomonas*, *Chlorella*, *Nannochloris*, *Tetraselmis*), Rhodophyceae (*Cyanidium*), photosynthetic heterokonts [*Ochromonas* (Chromophyceae), *Nannochloropsis* (Eustigmatophyceae), *Phaeodactylum*, *Skeletomonas* (Bacillariophyceae, diatoms)], Cryptophyceae (*Cryptomonas*, *Rhodomonas*), Haptophyceae (*Isochrysis*) and Dynophyceae (*Heterocapsa*; Table 2; [27] Pérez-Castiñeira et al., in prep.).

Note that the PCR strategy described above allowed us to identify both potassium-stimulated and potassium-independent H^+-PPase genes in all of the main groups of photosynthetic protists: Chlorophyceae, Cryptophyceae, Dynophyceae, Euglenophyceae, Haptophyceae, Heterokonts (Bacillariophyceae, Eustigmatophyceae) and Rhodophyceae – most of which do not belong to the plant evolutionary lineage – and in free-living protozoa (ciliates, euglenoids). Interestingly, phylogenetic studies including these sequence data and other sequences published or identified by searching in microbial genome databases (NCBI, TIGR websites, see Tables 1, 2) show that eukaryotic H^+-PPases appear to be polyphyletic. Two evolutionary lineages were found for both potassium-stimulated and potassium-independent H^+-PPases: one of them corresponds to the prototypic V-PPase set of plants and is found in apicomplexans, ciliates, green algae, trypanosomatids and some heterokonts (both photosynthetic and heterotrophic), whereas another set of H^+-PPases more related to proteobacterial proteins (and therefore more primitive) is found mostly in phototrophic protists with complex plastids (Cryptophyceae, Dynophyceae, Haptophyceae, diatoms, some euglenoids) and in some marine (red, green) algae. These results suggest different evolutionary histories and/or horizontal gene transfer events [27, Pérez-Castiñeira et al., in prep.].

Summarizing, the broad distribution of both potassium-stimulated and potassium-independent H^+-PPases among bacteria and protists – either photosynthetic/heterotrophic, parasitic or free-living – suggests an ancestral origin and the occurrence of different evolutionary lineages of these proton pumps and/or the occurrence of horizontal gene transfer events between phylogenetically diverse species. In this respect, it may be relevant that we found plant-like, potassium-dependent H^+-PPase genes in plant endoparasitic microorganisms, both eukaryotic (the trypanosomatid *Phytomonas*) and prokaryotic (*Agrobacterium tumefaciens*) [27, Pérez-Castiñeira et al., in prep.]. Finally, a possible photosynthetic ancestry of the potassium-stimulated, plant-like H^+-PPases of parasitic trypanosomatids and free-living ciliates deserves consideration, since it has been recently reported that several enzymes of the oxidative pentose phosphate pathway of *Trypanosoma brucei* have cyanobacterial and/or plant phylogenies [19], suggesting that the trypanosomatid lineage may be secondarily non-photosynthetic. Note that many Euglenoids – a sister group phylogenetically very close to

trypanosomatids – are photosynthetic, having plastids of secondary symbiotic origin; and some parasitic protists, most notably the apicomplexans (that, like ciliates, belong to the alveolates), are known to be secondarily non-photosynthetic, although they still possess a plastid, the apicoplast (both groups have plant-like H^+-PPases, see Table 2). The possibility that the ancestral trypanosomatid lineage had plastids that were subsequently lost deserves consideration, since several groups of non-photosynthetic eukaryotes are believed to have lost their plastids secondarily, most notably the oomycetes, a group of mostly parasitic fungus-like heterokonts that share many biochemical and structural characteristics with plants and also possess a plant-like H^+-PPase (see Table 2). Therefore, the H^+-PPases may be useful markers to elucidate the evolutionary past of the different eukaryotic lineages and perhaps also to clarify the intriguing relationships between photosynthesis and parasitism in the protist world.

We are currently performing molecular physiology studies with the Chlorophyceae *Chlamydomonas reinhardtii* and *Chlorella fusca*, two model photosynthetic protists whose H^+-PPase genes have been cloned in our laboratory (accession numbers AJ304836 and AJ251470, respectively). Due to their very versatile metabolisms and susceptibility to genetic manipulation, these protists are ideal systems to clarify the physiological role(s) of this proton pump. Both mRNA and protein level analyses show a clear induction of the microalgal H^+-PPase under a number of ionic and osmotic stress conditions [21]. These levels are also affected in response to changes in trophic conditions (photo-, mixo-, heterotrophy). It seems, therefore, that protist H^+-PPases are tightly regulated in response to a number of environmental conditions (trophic level, stress) that presumably may affect the energetics status of the cell [21, López-Marqués et al., in prep.]. The fine regulation of the genes encoding these ionic pumps, also found in photosynthetic bacteria [21], suggests that they must play an important physiological role in the adaptive responses of all these organisms.

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