Genetic, biochemical and physiological evidence of the widespread distribution of K^+-dependent and K^+-independent proton-translocating pyrophosphatases

José R. Pérez-Castiñeira, Rosa L. López-Marqués, Manuel Losada and Aurelio Serrano

Instituto de Bioquímica Vegetal y Fotosíntesis (Universidad de Sevilla-CSIC), Avda Americo Vespucio s/n, 41092 Sevilla, Spain

The phosphodiester bond (P-O-P) of inorganic pyrophosphate (PPi) is an energy-rich bond similar to that of ATP. Although PPi is still generally considered as a waste product of anabolism, nowadays there is an increasing body of evidence supporting that it should play an important role in cellular bioenergetics. Moreover, it has been suggested that PPi may have been the ancestor of ATP as "energy currency" in the early stages of evolution (Baltscheffsky *et al*, 1986). Membrane-bound proton-translocating inorganic pyrophosphatases (H^+-PPases, EC 3.6.1.1) belong to a recently identified category of proton pumps, distinct from F-, P- and V-ATPases, which utilize pyrophosphate hydrolysis as the driving force for H^+ movement across biological membranes (Rea *et al*, 1993). Until recently, H^+-PPases were identified in a limited number of organisms, including higher plants and some Rhodospirillaceae (non-sulfur purple bacteria), but their recent characterizations both at biochemical and genetic levels as well as its identification in some other bacteria and, for the first time, in an archaea reinforced the interest on these proton pumps (Baltscheffsky *et al*, 1999; Drozdowicz *et al*, 1999 and 2000; Kim *et al*, 1994; Nyrén *et al*, 1991). Moreover, little is known so far about their actual physiological roles. The work carried out on this subject in our laboratory can be summarized in three major points:

1) Systematic screening for H^+-PPases and their genes in protists and bacteria, with emphasis on photosynthetic organisms. Biochemical studies have demonstrated the

A. Serrano, ed.
Universidad de Sevilla-CSIC
2001. Printed in Spain

presence of plant-like H^+-PPases in acidocalcisomes of pathogenic parasitic protists such as *Trypanosoma cruzi* and *Leishmania donovanii* (Scott *et al*, 1998, Rodrigues *et al*, 1999). Recently, sequencing of the genome of *Plasmodium falciparum* has revealed the presence of H^+-PPase genes in this organism and data coming from some bacterial genome projects suggest that these proteins are more widespread than previously thought. In any case, the information concerning the occurrence of H^+-PPase genes in protists is rather scarce and unsystematic. H^+-PPases do not seem to be present in animals; therefore, studies on the possible implication of these proteins in the development of the diseases caused by pathogenic protozoa might be of great importance. Major steps towards this goal are to find out whether H^+-PPase genes are present in the genome of most protists -either free-living or parasitic, heterotrophic or photosynthetic-, the conditions under which they become expressed and the structural characteristics of the proteins they encode. Molecular approaches could give us valuable tools to accomplish these objectives. On the other hand, the occurence of H^+-PPases in a number of organisms such as the diverse and heterogeneous photosynthetic protists, and in soil and photosynthetic bacteria other than Rhodospirillaceae has not been studied in detail. This evidence could give us important clues about the evolution and physiological role of this unique type of proton pump.

By using polyclonal antibodies against conserved aminoacid regions (see Rea *et al.*, these Proceedings) we have found inmmunoreactive bands of the expected M_m in *Western* blots of membrane preparations from a number of evolutionary distant photosynthetic bacteria, like non-sulfur purple (alpha-proteo)bacteria, sulfur purple (gamma-proteo)bacteria, sulfur green bacteria and heliobacteria, as well as in those from a range of photosynthetic protists having simple and complex plastids (Chlorophyceae, Rhodophyceae, Euglenophyceae, Bacillariophyceae, Cryptophyceae, Glaucocystophyceae, photosynthetic heterokonts). On the other hand, by using a PCR approach with degenerate oligonucleotides designed from aminoacid 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, eukaryotic microalgae and soil bacteria relevant for plant biology (N_2-fixers and pathogenic strains). 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 have allowed us to analyze the sometimes surprising phylogenetic relationships amongst the H^+-PPases from evolutionary distant organisms.

Using this molecular genetics strategy we have found H^+-PPases genes (very similar to those of human parasitic trypanosomes) in a range of non-pathogenic parasitic trypanosomatids of insects and other animals (*Leptomonas*, *Herpetomonas*, *Endotrypanum*) and plants (*Phytomonas*), and in free-living strains (*Crithidia*), thus indicating that the presence of this protein is not necessarily associated to the pathogenic or even the parasitic character of these protozoa. It is interesting at this respect that we have also identified H^+-PPase genes in a number of free-living protozoa of other phylogenetic groups: ciliates, both peritrichous (*Tetrahymena*, *Paramecium*, *Vorticella*) and hypotrichous (*Histrio*) strains (having the peculiar features for genes of this group, namely, in-frame stop codons coding for Gln and/or very short 20-30 bp-long introns), as well as in heterotrophic euglenoids (*Astasia longa*). In agrement with immunochemical data, our group has identified and cloned H^+-PPase genes from representative strains of the main phylogenetic groups of photosynthetic protists: Chlorophyceae (*Chlorella, Chlamydomonas, Tetraselmis, Nannochloris*), Rhodophyceae (*Cyanidium*), photosynthetic heterokonts (Chromophyceae, *Ochromonas*; Eustigmatophyceae, *Nannochloropsis*), Bacillariophyceae or diatoms (*Phaeodactylum*, *Skeletomonas*), Cryptophyceae (*Cryptomonas*, *Rhodomonas*), Haptophyceae (*Isochrysis*) and Dynophyceae (*Heterocapsa*). We are currently performing molecular physiology studies with two model photosynthetic microorganisms whose H^+-PPase genes we cloned: the chlorophyceae *Chlamydomonas reinhardtii* and *Chlorella fusca*, that due to their very versatile metabolisms and susceptibility to genetic manipulation are ideal systems to clarify the physiological role(s) of this proton pump. Also in agreement with immunochemical data H^+-PPase genes were found in most

photosynthetic prokayotes we have tested so far: *Rps. viridis*, *Rps. palustris* (non-sulfur purple bacteria), *Chro. vinosum* (sulfur purple bacteria), *Chloroflexus aurantiacus* (non-sulfur green bacteria), non thermophilic *Chlorobium* spp. (sulfur green bacteria) and, interestingly, in *Heliobacterium chlorum* (heliobacteria, Gram+ photosynthetic bacteria). Halophilic photosynthetic bacteria, like *Rhodothalasium*, also have H^+-PPase genes. On the other hand, we have found H^+-PPase genes in a range of N_2-fixing non-photosynthetic soil bacteria of agronomical interest, both free-living (*Azospirillum brasiliensis*) and plant symbiotic (*Frankia* spp. and rhizobia) species, as well as in the plant pathogenic strain *Agrobacterium tumefaciens*. Interestingly, *A. tumefaciens* has two H^+-PPase genes, as it is also the case of the green non-sulfur bacterium *Dehalococcoides ethenogenes*. One of them was assigned in phylogenetic trees to the group of eukaryotic potassium-dependent H^+-PPases together with some other bacterial H^+-PPases, like the *Thermotoga* protein -the first prokaryotic potassium-dependent H^+-PPase functionally characterized so far (Pérez-Castiñeira *et al*, 2001; see below)- whereas the other clustered with the typical potassium-independent H^+-PPases of alpha proteobacteria.

2) The wide distribution of <u>both</u> potassium-dependent and potassium-independent H^+-PPases. The biochemical information currently available suggests the occurence of two types of H^+-PPases depending on their requirement of potassium for full activity. Thus, potassium-stimulated H^+-PPases have been found to date only in eukaryotes (Kim *et al*, 1994; Rodrigues *et al*, 1999; Scott *et al*, 1998) whereas potassium-independent H^+-PPases have been identified both in eukaryotes (Drozdowicz *et al*, 2000) and prokaryotes (Drozdowicz *et al*, 2000; Nyrén *et al*, 1991). The presence of both types of H^+-PPases in *Arabidopsis thaliana* might suggest different physiological roles and even different subcellular localization for the two types of protein, however, these aspects remain to be clarified. At this respect it is interesting to note that the PCR strategy described above allowed us to identify <u>both</u> potassium-dependent and potassium-independent H^+-PPase genes in all main groups of photosynthetic protists (Chlorophyceae, Rhodophyceae, Cryptophyceae, Heterokonts, Haptophyceae, Bacillariophyceae and Dynophyceae) -most of

which do not belong to the plant evolutionary lineage- as well as in free-living protozoa (ciliates and heterotrophic flagellates). Interestingly, phylogenetic studies show that the putative eukaryotic potassium-independent H^+-PPases appear to be polyphyletic: some of them cluster with AVP2 in a sort of eukayotic sub-group of potassium-independent H^+-PPases whereas others (from Cryptophyceae, Haptophyceae, Dynophyceae, Euglenoids) are much more related to the proteobacterial proteins, suggesting different evolutionary histories and/or horizontal gene transfer events.

Molecular phylogeny studies indicate that the vice-versa situation, that is, the existence of bacterial genes encoding potassium-dependent H^+-PPases, is also possible. Thus, sequences of H^+-PPases from *Heliobacterium chlorum*, *Agrobacterium tumefaciones*, *Thermotoga maritima* and *Dehalococcoides ethenogenes* were assigned in phylogenetic trees to one group deeply rooted in the potassium-dependent H^+-PPases branch together with the H^+-PPases from *Acetabularia mediterranea* and those from photosynthetic protists representatives of some groups of that are not in the evolutionary lineage of plants, namely, Bacillariophyceae, Cryptophyceae, Haptophyceae and Dynophyceae (all of them with plastids from secondary endosymbiosis). This proposal has been supported by the biochemical characterization of the *Thermotoga* H^+-PPase which appears deeply rooted in this group (see below).

Very recently, a potassium-insensitive H^+-PPase from the hyperthermophilic archaeon *Pyrobaculum aerophilum* was identified and characterized (Drozdowicz *et al*, 1999). This was the only description of a H^+-PPase sequence from a non-photosynthetic organism with the biochemical characterization of the clone reported so far. Previous reports had shown the presence of a possible H^+-PPase gene in the genome of the hyperthermophilic anaerobic bacterium *Thermotoga maritima*, however, the full sequence has been available only recently with the completion of the genome project of this organism. We have engineered the full-length gene coding for the H^+-PPase of *Thermotoga* (TVP) so that the functional characterization of the heterologously-expressed protein in yeast could be accomplished. As expected, TVP has an optimal temperature similar to that

of the *Pyrobaculum* PPase (PVP); however, unlike the latter it is potassium-stimulated. To our knowledge, this is the first report of a potassium-sensitive H^+-PPase in a prokaryote. The *Thermotoga* protein seems to be resistant to the yeast proteases and its thermostability seemingly depends on the binding of magnesium ions to the protein. Phylogenetic studies performed with sequences of H^+-PPases from different organisms indicate that *Thermotoga* H^+-PPase should be an ancestral member of the potassium-dependent H^+-PPase family (Pérez-Castiñeira *et al*, 2001).

Summarizing, the broad distribution of both potassium-stimulated and potassium-independent H^+-PPases among bacteria and protists -either photosynthetic or heterotrophic, parasitic or free-living- and the occurrence of diverse evolutionary lineages in both protein classes suggest an ancestral origin of this category of proton pums and/or the occurrence of horizontal gene transfer events between phylogenetic diverse species. It may be relevant at this respect our finding of plant-like potassium-dependent H^+-PPase genes in plant endoparasitic microorganisms, both eukaryotic (the trypanosomatid *Phytomonas*) and prokaryotic (*Agrobacterium tumefaciens*).

3) Functional complementation of an engineered yeast sPPase mutant with foreign sPPase and V-PPase genes. It has been known for some time that the soluble pyrophosphatase present in the cytoplasm of the yeast *Saccharomyces cerevisiae* is essential for the viability of the cell, therefore, it is not straightforward to knock out the gene in haploid cells. We have circumvented this problem by transforming a haploid wild type cell of *S. cerevisiae* with a multicopy autonomous plasmid containing the gene coding for the soluble PPase (*IPP*1) under the control of the galactose-inducible glucose-repressable promoter *GAL1*; then, the chromosomal gene was disrupted by deleting a portion of the allele with the simultaneous insertion of an *HIS*3 expression cassette. Transformants were selected in medium containing galactose without histidine. Most of the recombination presumably occured within the plasmid yielding transformants with no apparent phenotype; however, after extensive selection procedures we were able to isolate a clone that could grow in galactose but not in glucose. Subsequent biochemical analyses showed that the cytoplasmic soluble pyrophosphatase activity of this clone dropped to

negligible levels after transferring the cells from galactose to glucose. Moreover, genetic analyses by *Southern blot* and PCR demonstrated that the chromosomal allele was indeed interrupted by the *HIS*3 cassette. *Western* and *Northern blot* analyses have been performed in order to further characterized the mutant. We have demonstrated that many sPPases from photosynthetic bacteria and eukaryotes are able to efficiently complement the mutant. Moreover, some H^+-PPases under the control of a constitutive promoter can support growth of this mutant in glucose, thereby suggesting that membrane-bound PPases might be able to substitute for the soluble PPases. To our knowledge this is the first demonstration that H^+-PPases indeed act as PPi-hydrolysing enzymes *in vivo*.

Gene sequences referred here have been submitted to databases and most of them are under confidential status until publication. Several publications on the topics described here are in preparation.

Supported by grants PB 97-1135 from DGICYT (MCYT; Spain) and Grupo PAI CVI-0261 (Junta de Andalucía).

References

Baltscheffsky, H., Lundin, M., Luxemburg, C., Nyrén, P. and Baltscheffsky, M. (1986) Chemica Scripta 26B, 259-262.

Baltcheffsky, M., Nadanaciva, S. and Schultz, A. (1998) Biochim. Biophys. Acta 1364, 301-306.

Drozdowicz, Y., Kissinger J.C. and Rea P.A. (2000) Plant Physiol. 123, 353-362.

Drozdowicz, Y., Lu, Y.-P., Patel, V., Fitz-Gibbon, S., Miller, J.H. and Rea P.A. (1999) FEBS Lett. 460, 505-512.

Kim, E.J., Zheng, R.-G., Rea, P.A. (1994) Proc. Natl. Acad. Sci. USA 91, 6128-6132.

Nyrén, P., Nore, B.F. and Strid Å. (1991) Biochemistry 30, 2883-2887.

Pérez-Castiñeira, J.R., Lopéz-Marqués, R.L., Losada, M. and Serrano, A. (2001) FEBS Letters (in press) (Available online at **www.elsevier.nl/febs/show**). Pérez-Castiñeira, J.R., Lopéz-Marqués, R.L., Losada, M. and Serrano, A. (2001) Internatl. Microbiol. (in press).

Rea, P.A. and Poole, R.J. (1993) Ann. Rev. Plant Physiol. Plant Mol. Biol. 44, 157-180

Rodrigues, C., Scott, D.A. and Docampo, R. (1999) Biochem. J. 340, 759-766.

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.