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Proton-Pumping Inorganic Pyrophosphatases in Some Archaea and Other Extremophilic Prokaryotes

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Comparative studies between the proton-pumping, membrane-bound inorganic pyrophosphatases (H^+-PPases) from hyperthermophilic and thermophilic prokaryotes and those from mesophilic organisms can now be performed because of very recent sequence data. Typical overall factors that contribute to protein thermostability are found in H^+-PPases from extremophiles; nevertheless, putative active site motifs of this class of enzymes may be identical over the whole range of average growth temperatures of the compared prokaryotes. Heterologous expression in yeast of H^+-PPases from organisms spanning a wide range of thermal habitats has allowed the biochemical comparison among these proteins within the same system, ensuring that differences observed are due to intrinsic characteristics of the proteins and not to their interactions with different cellular environments. On the other hand, the availability of H^+-PPase sequences from a variety of sources have permitted molecular phylogenetic studies of this class of proton pumps, thus providing information about their general structural and functional properties. A great step forward may be expected when one of the several groups now attempting crystallization and 3D structural determination of H^+-PPases will be successful.

KEY WORDS: Proton-pumping inorganic pyrophosphatases; protein thermostability; thermal adaptation; extremophile; hyperthermophile; thermophile; mesophile.

INTRODUCTION

Is the time ripe for a first attempt to obtain insight into various molecular properties of the proton-pumping, membrane-bound inorganic pyrophosphatases (H^+-PPases) of different extremophiles? Recently published amino acid sequences of H^+-PPases, as well as information from a number of ongoing or complete microbial and plant genome projects, add to the rapidly expanding list of primary structures of this enzyme family. They broaden the width of operational temperature range of its members in such a way as to make us accept the challenge of reviewing this incipient subject in this extremophile special issue, agreeing that it may well be worth our effort.

As will be seen in the following, we find it reasonable to make comparisons between three groups of prokaryotic H^+-PPases, with three selected examples in each group, where the groups are distinguished by the average temperatures for growth of the corresponding prokaryote in their natural habitats. The temperature ranges, which we arbitrarily established for these comparisons are under 35°C (for mesophiles), between 45 and 65°C (for thermophiles), and over 75°C (for hyperthermophiles).

BACKGROUND

Membrane-bound inorganic pyrophosphatases were discovered in the mid-1960s and were found to operate in isolated chromatophores from the purple, non-sulfur photosynthetic bacterium *Rhodospirillum rubrum*, in both

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the direction of uncoupler stimulated dark hydrolysis (Baltscheffsky, 1964; Baltscheffsky *et al.*, 1966b) and uncoupler inhibited light-induced synthesis (Baltscheffsky *et al.*, 1966a; Baltscheffsky and von Stedingk, 1966) of inorganic pyrophosphate (PP_i). This similarity with ATP synthases prompted the name PP_i synthase for the enzyme catalyzing this first and still only alternative system for biological electron transport coupled phosphorylation. The fact that oligomycin, the well-known inhibitor of ATP synthesis, did not inhibit the photophosphorylation of P_i to PP_i (Baltscheffsky and von Stedingk, 1966) was an early indication that PP_i formation in this system occurred at the pre-adenine nucleotide level. Thus, the synthesis of PP_i may have been the original biological electron transport coupled phosphorylation reaction, providing PP_i as a plausible early carrier of chemical energy between anabolism and catabolism in connection with the origin and early evolution of life on Earth (Baltscheffsky, 1967a).

Both light-induced proton movement (von Stedingk and Baltscheffsky, 1966) and proton-pumping activity of the H^+-PPase (Moyle *et al.*, 1972) were early demonstrated in bacterial chromatophores. PP_i was found to drive various energy-requiring reactions in the chromatophores (Baltscheffsky, 1967b; Baltscheffsky *et al.*, 1966b; Keister and Yike, 1967). The extremely tightly membrane-bound H^+-PPase was solubilized with retained activity (Nyrén *et al.*, 1984; Rao and Keister, 1978). In eukaryotes a membrane-bound PPase activity was first demonstrated in 1975 (Karlsson, 1975).

The first cloning of a H^+-PPase gene, from the higher plant *Arabidopsis thaliana* (Sarafian *et al.*, 1992) led to a good explanation for the strong membrane binding of its vacuolar enzyme, as hydropathy plots appeared to indicate the existence of 13 transmembrane α-helix segments (today 15–16 putative transmembrane segments are usually proposed for H^+-PPases from different sources) and to show that about half of this enzyme resided as transmembrane segments within the membranes. The first cloned bacterial gene for a H^+-PPase, the H^+-PPase/PP_i synthase from *Rhodospirillum rubrum* (Baltscheffsky *et al.*, 1998, 1999), gave clear homology of the enzyme with plant vacuolar H^+-PPases, which had been found to be extremely similar to each other. These plant and bacterial H^+-PPases are, respectively, the best-studied members of the two biochemically distinct subfamilies into which this protein group is divided, namely, the K^+-stimulated and K^+-independent H^+-PPases. The fact that the complete amino acid sequence of the *R. rubrum* protein was only less than 40% identical to the vacuolar ortholog opened the way to a closer look at essential parts of the amino acid sequences, giving three cytosolic loops with particularly pronounced sequence preservation (Baltscheffsky *et al.*, 1999). The first cloned archaeal gene for a H^+-PPase, that from the aerobic euryarchaeon *Pyrobaculum aerophilum* (Drozdowicz *et al.*, 1999), gave added impetus to attempts to unravel functionally significant regions of the H^+-PPases. Besides, two other bacterial H^+-PPases relevant for this study have been recently characterized: the hyperthermophilic protein from the primitive bacterium *Thermotoga maritima*, which was the first prokaryotic K^+-stimulated H^+-PPase described (Pérez-Castiñeira *et al.*, 2001b), and the thermophilic K^+-independent H^+-PPase of the photosynthetic bacterium *Chloroflexus aurantiacus*, that is able to functionally complement a cytosolic sPPase-lacking yeast mutant (Pérez-Castiñeira *et al.*, 2002b).

Life can thrive in extreme environments (temperatures $<10^\circ$C and $>100^\circ$C; pH values 1–13; high salt; high pressures; low nutrients). The extremophiles, mostly prokaryotes (bacteria and archaea), have evolved specific strategies to growth and survival under these harsh conditions. The biochemistry of hyperthermophilic proteins is, however, very similar to that of their mesophilic homologs, the stability apparently being the result of very subtle synergistic intra- and intermolecular interactions, or of extrinsic protectants. Some factors relevant for protein thermostability include (i) increased number of hydrogen bonds and extension of the ion-pair networks, (ii) increased number of charged amino acids (acidic amino acids replace the corresponding amides), also in transmembrane helices of integral membrane proteins, and (iii) decreased length of "superficial" loops and increased protein compactness (Cambillau and Claverie, 2000; Jaenicke, 1991; Schneider *et al.*, 2002; Vieille and Zeikus, 2001). Evidence is presented below supporting that (hyper)thermophilic H^+-PPases also follow some of these rules.

RESULTS AND DISCUSSION

Selection Based on Optimal Growth Temperatures

Today we know several amino acid sequences of H^+-PPases from both archaea and bacteria, with elevated growth temperatures, up to those of extremophiles. With optimal temperature for growth as indicator, we shall treat here three different groups expecting to exhibit different protein thermostability, as was mentioned in the introduction.

Protein sequences of H^+-PPases from extremophilic prokaryotes were retrieved by similarity searching using PSI-BLAST database search (Altschul *et al.*, 1997) on the nonredundant protein database or on the microbial genomes websites of The Joint Genome

Institute (JGI, USA; www.jgi.doe.gov/JGI_microbial/html/) and The Institute for Genomic Research (TIGR, USA; www.tigr.org/tdb/mdb/mdb.html). Six full-length thermophilic/hyperthermophilic H^+-PPase sequences from bacteria and archaea are in this way available to date: those of the moderate thermophiles *Chlorobium tepidum* (a green sulfur photosynthetic bacterium) (Wahlund *et al.*, 1991), *Chloroflexus aurantiacus* (a green non-sulfur photosynthetic bacterium) (Pierson and Castenholz, 1974), and *Thermobifida fusca* (a soil cellulose-degrading actinobacterium) (Zhang *et al.*, 1998), and the hyperthermophiles *Thermoanaerobacter tengcongensis* (an obligately anaerobic firmicute) (Xue *et al.*, 2001), *Thermotoga maritima* (a primitive marine bacterium) (Huber *et al.*, 1986), and *Pyrobaculum aerophilum* (a thermoproteal euryarchaeon) (Volki *et al.*, 1993). They have been compared with three selected H^+-PPase sequences of the mesophilic bacteria *Rhodospirillum rubrum* (a photosynthetic α-proteobacterium), *Streptomyces coelicolor* (a gram-positive soil bacterium), and the facultative psychrotroph *Novosphingobium aromaticivorans* (a deep-subsurface α-proteobacterium isolated from North Atlantic coastal sediments) (Takeuchi *et al.*, 2001). Figure 1 shows the distribution of the nine selected H^+-PPase containing prokaryotes clustered in the three above-defined temperature ranges.

Comparison of Whole H^+-PPase Sequences

Since protein amino acid composition has long been thought to be correlated with thermostability (Cambillau and Claverie, 2000; Jaenicke, 1991; Vieille and Zeikus, 2001), a statistical analysis has been done using the whole sequences of the selected mesophilic, and thermophilic H^+-PPases. The ratio (E+D+K)/(Q+H+C) has been used as an indicator of adaptative changes in amino acid composition to lifestyles, since it was reported that protein sequences of hyperthermophiles include more charged (E, D, K) and fewer uncharged polar residues (Q, N, C, H, S, T) than their mesophilic counterparts (Jaenicke, 1991; Vieille and Zeikus, 2001). This is due to the fact that reactive groups of amino acid side chains readily undergo chemical modifications—i.e., deamination, sulfhydryl oxidation—at high temperature. Moreover, charged residues are involved in ion pairs and networks that may contribute to thermal stabilization. Thermostability of membrane proteins also seems to follow these rules (Schneider *et al.*, 2002). As shown in Fig. 2, the membrane-bound H^+-PPases adhere in general to these rules—hyperthermophilic orthologs clearly exhibit an increased proportion of charged residues at the expense of uncharged polar residues—although considerable variation exists also within the chosen groups. As was expected, the highest ratio value corresponds to the protein of the hyperthermophlic archaeon *P. aerophilum*. To our knowledge the *P. aerophilum* protein is so far the only H^+-PPase that lacks cysteine residues.

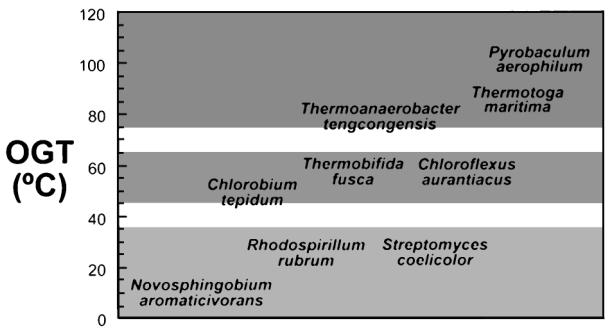

Fig. 1. Selected representative prokaryotes that according to biochemical and/or genomic data possess H^+-PPases are clustered considering the average temperature of their natural habitats in three groups corresponding to mesophiles —*Novosphingobium aromaticivorans* (facultative psychrotroph), *Rhodospirillum rubrum*, *Streptomyces coelicolor*; moderate thermophiles —*Chlorobium tepidum*, *Chloroflexus aurantiacus*, *Thermobifida fusca*; and hyperthermophiles —*Thermoanaerobacter tengcongensis*, *Thermotoga maritima*, *Pyrobaculum aerophilum*. Only the proteins of *R. rubrum*, *C. aurantiacus*, *T. maritima*, and *P. aerophilum* have been biochemically characterized; others are putative proteins deduced from the corresponding genes. OGT, organism growth temperature.

Figure 3 shows the temperature dependence of PP_i hydrolysis activity by H^+-PPases, all of them being heterologously expressed in yeast, from organisms adapted to very different thermal habitats. The normophlic K^+-stimulated AVP1 isoform of the plant *A. thaliana* is the only one with considerable activity in the 30–40°C range whereas the H^+-PPases of the thermophilic bacterium *C. aurantiacus* (K^+-insensitive) and the hyperthermophilic prokaryotes *T. maritima* (K^+-stimulated) and *P. aerophilum* (K^+-insensitive) exhibit higher optimal temperature values at ca. 65, 70, and 80°C, respectively, which are consistent with the growth temperatures reported for these microorganisms. Interestingly, the dramatic differences in temperature dependence activity observed among these H^+-PPases—which are naturally adapted to different temperature habitats—occur despite all of them being proteins heterologously expressed in yeast. This suggests that these differences are due to intrinsic characteristics of the proteins and not to interactions with other cell components in their homologous systems.

Alignment of the amino acid sequences of thermophilic and hyperthermophilic H^+-PPases with selected mesophilic orthologs found in public databases and genome projects allowed to establish the phylogenetic relationships of these extremophilic proteins with the H^+-PPases of other bacteria, archaea, protists, and

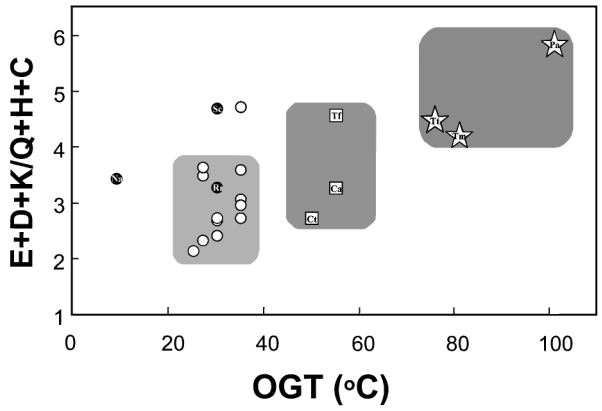

Fig. 2. E+D+K/Q+H+C values for the H^+-PPases from organisms of different degree of thermal adaptation versus average growth temperature (OGT) of their natural habitats. The ratio includes percent values with respect to the full-protein sequence of selected amino acids that were reported to increase (E, D, K) or decrease (Q, H, C) in thermophilic proteins. Three clusters could be defined corresponding to H^+-PPases of mesophilic organisms (bacteria, protozoa, and plants) (○), thermophilic bacteria (□), and hyperthermophilic prokaryotes (two bacteria and one archaeon) (stars). Several exceptions to this rule are found among mesophilic H^+-PPases. The three protein sequences from nonthermophilic organisms selected for comparative studies are indicated as ●. Symbols corresponding to the nine selected H^+-PPases used in this study are further indicated by the organism name initials. The H^+-PPase sequences —deduced from selected full-length genes of bacteria, archaea, and eukaryotes from public databases and genome projects— are from the following: (i) normophilic organisms: the higher plant *A. thaliana* (K^+-stimulated V-PPase AVP1, AB015138, and K^+-insensitive V-PPase AVP2, AF182813), the protozoa *Trypanosoma cruzi* (AF159881) and *Plasmodium falciparum* PVP1 (AAD17215), and the bacteria *Novosphingobium aromaticivorans* (Na) (ZP_00095038), *Rhodospirillum rubrum* (Rr) (AAC38615.1), *Streptomyces coelicolor* (Sc) (CAB38484), *Magnetospirillum magnetotacticum* (gi|23005032), *Brucella melitensis* (NP_540102), and *Caulobacter crescentus* (gi|16124256); (ii) thermophilic bacteria: *Chlorobium tepidum* (Ct) (gi|21672841), *Chloroflexus aurantiacus* (Ca) (gi|22970992), and *Thermobifida fusca* (Tf) (ZP_00057642); and (iii) hyperthermophilic prokaryotes: *Thermoanaerobacter tengcongensis* (Tt) (NP_62197), *Thermotoga maritima* (Tm) (AAD35267), and *Pyrobaculum aerophilum* (Pa) (AF182812).

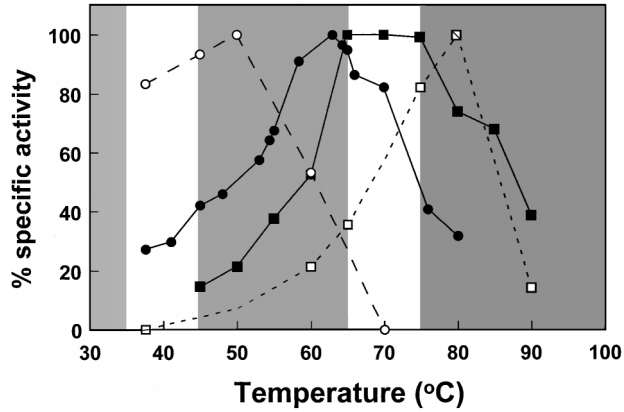

Fig. 3. Temperature dependence of PP_i hydrolysis activity by the H^+-PPases from the higher plant *A. thaliana* (AVP1 isoform, ○), the thermophilic bacterium *C. aurantiacus* (●), and the hyperthermophilic prokaryotes *T. maritima* (■) and *P. aerophilum* (□) heterologously expressed in yeast. Activities are expressed as percentages of the maximal activity obtained for each protein in the heterologous system. Data corresponding to *A. thaliana, P. aerophilum* , *C. aurantiacus*, and *T. maritima* H^+-PPases were obtained from Drozdowicz *et al.* (1999) and Pérez-Castiñeira *et al.* (2001b, 2002b).

plants. A distance phylogenetic tree was thus generated by the CLUSTAL X program using a neighbor-joining algorithm (Thompson *et al.*, 1997) (Fig. 4). Interestingly, all thermophilic and hyperthermophilic H^+-PPases rooted very deep in the tree, in accordance with the ancestral character of the corresponding prokaryotes. Moreover, they appear clustered in the two catalytically different protein families (K^+-stimulated or K^+-insensitive) found among H^+-PPases, the *T. maritima* protein being the only extremophilic K^+-stimulated H^+-PPase described so far. Since recent evidence support a broad, although somewhat sporadic, occurrence of H^+-PPases among very diverse microorganisms both prokaryotes and protists (Pérez-Castiñeira *et al.*, 2001a, 2002a), other extremophilic H^+-PPases are expected to be found in the next future (see below).

Comparison at the Putative Active Site Loop 6

In plant vacuolar H^+-PPases there is an unusually high degree of overall amino acid sequence identity (around 90%). With the first prokaryotic sequences obtained identities decreased to less than 40% (Baltscheffsky *et al.*, 1998, 1999; Drozdowicz *et al.*, 1999). However, a very strong similarity has been retained in certain regions, specially in a few loops, on the cytosolic side, between the 15 and 16 usually predicted transmembrane α–helix segments, as derived by hydropathy plots in the over 50 currently known full-length H^+-PPases sequences.

A striking example is given from the putative active site loop 6 of the *R. rubrum* H^+-PPase (between transmembrane segments 5 and 6) (Baltscheffsky *et al.*, 1999, 2001). As Table I shows, seven out of nine sequences considered in this presentation (over the whole temperature scale) are identical in the motif which corresponds to the P-loop of the active site β subunit in F-ATPases (Saraste *et al.*, 1990; Walker *et al.*, 1982) whereas one, the hyperthermophile *T. maritima*, shows variation in two of the eight amino acid residues and the moderate thermophile

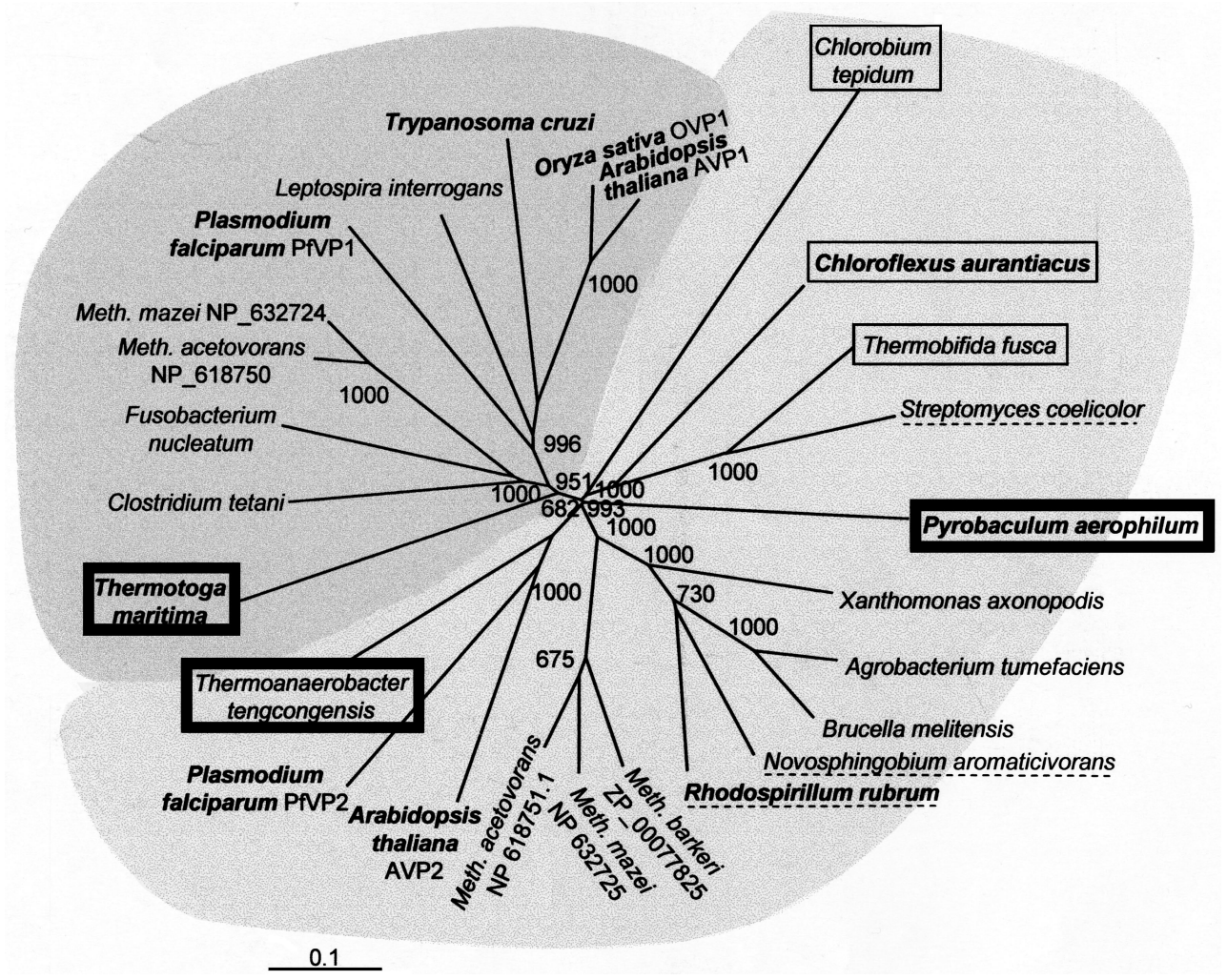

Fig. 4. Molecular phylogenetic analysis of H^+-PPases of organisms with different degree of thermal adaptation. Amino acid sequences derived from selected full-length genes of bacteria, archaea, and eukaryotes from public databases and genome projects were aligned using CLUSTAL X (Thompson *et al.*, 1997), and then subjected to phylogenetic analysis by the distance (neighbor-joining) method. The resulting tree showing sequence-based phylogenies is shown. Numbers in nodes indicate the statistical support (bootstrap values from 1000 replicates) of selected associated groups. Bar represents amino acid substitutions per site. The H^+-PPase sequences are from higher plants (*A. thaliana* AVP1, AB015138, and AVP2, AF182813); protozoa (*Leishmania major*, contig 93A1.TF; *Trypanosoma brucei*, AY043295; *Trypanosoma cruzi*, AF159881; *Toxoplasma gondii*, AAK38077;*Plasmodium falciparum* K^+-stimulated V-PPase PVP1, AAD17215, and K^+-insensitive PVP2, NP_701702); archaea (*Pyrobaculum aerophilum*, AF182812; *Methanosarcina acetivorans*, NP_618750 and NP_618751; *Methanosarcina mazei*, NP_632724 and NP_632725); and bacteria (*Rhodospirillum rubrum*, AAC38615.1; *Streptomyces coelicolor*, CAB38484; *Novosphingobium aromaticivorans*, ZP_00095038; *Brucella melitensis*, NP_540102; *Agrobacterium tumefaciens*, NP_359162; *Xanthomonas axonopodis*, NP_643747; *Clostridium tetani*, NP_781083; *Caulobacter crescentus*, gi|16124256; *Leptospira interogans*, NP_711652; *Fusobacterium nucleatum*, NP_602816; *Chloroflexus aurantiacus*, gi|22970992; *Chlorobium tepidum*, gi|21672841; *Thermobifida fusca*, ZP_00057642; *Thermoanaerobacter tengcongensis*, NP_62197; and *Thermotoga maritima*, AAD35267). Nonannotated sequences were derived from putative PPase genes found by BLAST searches (Altschul *et al.*, 1997) in the corresponding microbial genome project websites (JGI and TIGR). Proteins that have been biochemically characterized are indicated in bold. Thermophilic (light boxed) and hyperthermophilic (heavy boxed) H^+-PPases rooted very deep in the tree, and appear in the two catalytically different (K^+-stimulated or K^+-insensitive) protein families (heavy and light grey shadowed areas, respectively) found among the H^+-PPases; remarkably, the *T. maritima* protein is the only extremophilic K^+-stimulated H^+-PPase known so far. The three mesophilic bacteria selected for comparisons are dashed underlined. Note the existence of paralogous genes encoding H^+-PPases in the plant *A. thaliana*, the parasitic protozoan *P. falciparum* and the archaea *Methanosarcina* spp., and the occurrence of very similar genes in *A. tumefaciens* and *B. melitensis* —parasitic bacteria of plants and animals, respectively— suggesting common evolutionary events.

Table I. Similarities Between the P-Loop of the β Subunit of F-ATPases (ATP Synthases) and a Partially Identical "P-Loop Region" of H^+-PPases

P-loop of H^+-F ATPase β subunit	GGAGVGKT
"P-loop region" of "active site loop 6"	
-in 7 of the 9 H^+-PPases compared here	GADLVGKV
-in *Thermotoga maritima*	**A**ADLVGK**T**
-in *Chlorobium tepidum*	**GS**DL**M**-K**I**

Note. An eight amino acid sequence is presented corresponding to a highly conserved putative PP_i-binding motif of H^+-PPases located at the hydrophilic loop between the predicted transmembrane α-helices 5 and 6 (Baltscheffsky *et al.*, 1999). Nonconserved residues in the motif among the selected H^+-PPases are indicated in bold.

C. tepidum only has four identities and one deletion. In fact, this sequence is the least similar to all the more than 50 full-length H^+-PPase sequences (mostly putatives, deduced from the corresponding DNA coding sequences) from prokaryotes, protists, and plants known so far. No protein expression has been heterologously obtained with this H^+-PPase although its transcript certainly is synthesized in yeast (Pérez-Castiñeira, J. R., and Serrano, A., unpublished), and in *C. tepidum* it is generally considered to be doubtful that an active form of this enzyme can be formed physiologically.

The sequence GADLVGKV of Table I shares seven of its eight amino acids with the above-mentioned sequence DVGADLVGK. In the *R. rubrum* H^+-PPase (and the six other identical "P-loop regions") the four identities with the P-loop could well embrace the PP_i molecule in a way similar to that demonstrated for the P-loop and the two terminal phosphate residues of ATP in the ATP synthase (Abrahams *et al.*, 1994). Inspection of the whole loop 6, with its particularly discussed sequences GGG, DVGADLVGK, and DNVGDNVGD (Baltscheffsky *et al.*, 1998, 2001), in *R. rubrum*, *T. maritima*, and *P. aerophilum*, adds to the general picture obtained.

There is no doubt that crystallization and structural determination of extremophilic H^+-PPases are essential to definitively identify the thermostabilization determinants of these membrane proteins. Unfortunately, to our knowledge this remains to be done, mainly because of the fact that in the case of membrane proteins, generation of crystals and, hence, determination of high-resolution structures is much more difficult to achieve than in soluble proteins. We hope that by using new techniques, like 2D crystals and atomic force microscopy, some of the several groups involved in this task will be able soon to produce detailed H^+-PPase structures, an achievement that may also decisively contribute to the knowledge of the biological energy conversion processes at the molecular level.

Special Considerations

There are indications on the presence of H^+-PPases in extremophiles other than thermophilic prokaryotes. Genetic evidence was recently obtained by PCR experiments for the presence of H^+-PPases in halophilic microorganisms, namely, photosynthetic non-sulfur purple bacteria (*Rhodovibrio salinarum*, *Rhodothalassium salexigens*) and green microalgae (*Dunaliella salina*), as well as in the thermoacidophilic photosynthetic protist *Cyanidium caldarium* (Pérez-Castiñeira *et al.*, 2001a; Serrano, 2001). The biochemical characterization of these proteins will shed new light on the adaptative molecular strategies that allow them to function in such harsh environments.

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