On the chimeric nature of the soluble pyrophosphatases set from photosynthetic eukaryotes

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Photosynthetic eukaryotes -from unicellular microalgae to photosynthetic plant tissues- lack of a cytosolic inorganic pyrophosphatase (sPPase, EC 3.6.1.1) (1-3), the physiological role of this enzyme being presumably performed by a number of soluble and membrane-bound proteins involved in sugar phosphoylation and ion homeostasis, respectively. Virtually, all the sPPase activity is located in the cellular organelles, namely, plastids and mitochondria, of these organisms (2). We have purified to homogeneity and characterized the sPPases of photoautotrophic protists bearing plastids of different types, from primitive cyanobacterial-like cyanelles to plant-like (with 2 enfolding membranes) and complex (with >2 enfolding membranes) chloroplasts. All are monomeric proteins with apparent M_m in the range of 32-40 kDa, as determined by SDS-PAGE and FPLC gel permeation chromatography, and resemble fungal and animal eukaryotic-type sPPases in both protein structure and N-terminus sequences (1-3). Thus, the plastidic sPPases from the Glaucocystophycean flagellate (with cyanelles) *Cyanophora paradoxa* (M_m, 32 kDa), the thermoacidophilic rhodophyte *Cyanidium caldarium* (M_m, 40 kDa), the euglenoid *Euglena gracilis* (M_m, 38 kDa), the heterokont (cromophyte) *Ochromonas danica* (M_m, 38 kDa) and the chlorophycean microalga *Chlamydomonas reinhardtii* (M_m, 37 kDa) have been characterized, among others (2-3). Therefore, the plastidic sPPases are eukaryotic-type proteins clearly different to the homohexameric (20 kDa subunit) sPPases of cyanobacteria, the photoautotrophic bacteria which resemble the ancestral prokaryotic endosymbiont that gave rise to these organelles (1-3).

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A careful analysis of the sPPase preparations purified from the green alga *C. reinhardtii* revealed the presence of two polypeptides of slightly different Mm, both of them with PPase activity: a major enzyme of eukaryotic type named sPPase1 (37 kDa, SDS-PAGE) and a minor one named sPPase2 (32 kDa, SDS-PAGE) (2-3). 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 32-40 kDa polypeptide (corresponding to the plastidic sPPase) in crude extracts of other chlorophytes, rhodophytes, glaucocystophytes, euglenoids, heterokontophytes, diatoms and plant photosynthetic tissues, but not in roots tissues and bacteria. In agreement with biochemical data, this antibody also immnodetected the yeast citosolic sPPase. Subcellular fractionation with Percoll gradients and Western analysis localized the sPPase1 in the chloroplasts fraction and sPPase2 in the mitochondrial one (2-3).

We have identified by BLAST homology searches an *Arabidopsis thaliana* cDNA that encodes the precursor polypeptide of an eukayotic-type sPPase with a N-terminal chlroroplast transit peptide. This plant *ppa* gene has been heterologously overexpressed and the protein produced in *E. coli*, where it was processed to the mature active form that was efficiently inmunodetected by the antibody anti-sPPase1 of *C. reinhardtii*, both recombinant-plant and natural-algal mature proteins having virtually identical Mms (2-3). To our knowledge, this is the first *ppa* gene encoding a precursor polypeptide with a *chloroplast transit peptide* described so far. In agreement with its predicted cellular localization, *Northern* experiments showed that this gene is expressed in green tissues (leaves and shoots) but not in roots (2).

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. reinhartii*. Both algal *ppa* genes have been cloned and are expressed in photoautotrophic *C. reinhardtii* cells, from which cells the two sPPase proteins were purified. One of these cDNAs posseses a *chloroplast transit peptide* and encodes the precursor of an eukaryotic-type sPPase (the chloroplastic sPPase1); the other encodes a smaller bacterial-type sPPase

(presumably the mitochondrial sPPase2). Therefore, the sPPases set of *C. reinhardtii* is formed by two proteins of different molecular phylogeny (2-3).

The recently completed *A. thaliana* genome project 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 chloroplastic sPPase and a set of five highly-similar paralogous *ppa* genes located in different chromosomes encode a family of bacterial-type sPPases. One of these genes corresponds to a mitochondrial precursor and should be the 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 (4), so they should be expressed in non-photosynthetic tissues (roots). The high similarity found among the bacterial-type sPPases of *A. thaliana* suggests that they have probably originated from quite recent gene duplication events from a common ancestor, perhaps similar to the algal sPPase2 gene (2-3).

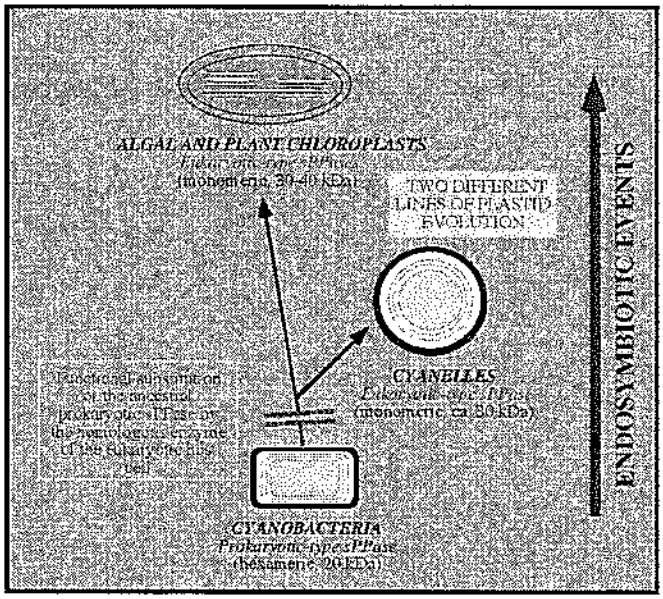

Fig. The evolutionary relationships between the sPPases of cyanobacteria and plastids

The results described above have clarified the molecular phylogeny of the sPPases of photosynthetic eukaryotes (algae and plants). All photosynthetic plastids contain a

nuclear-encoded eukaryotic-type sPPase, a finding that suggests that the homologous bacterial enzyme of the ancestral prokayotic 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 (see **Figure**). In contrast to this, the mitochondrial sPPases of algae and plants are nuclear-encoded bacterial-type proteins as should also be the case for cytosolic sPPases of non-photosynthetic tissues (2-4). The scenario found for the fungal and animal lineage is in this respect quite different, having eukaryotic-type cytosolic and mitochondrial sPPases (5). Therefore, photosynthetic eukaryotes seem to be the only group of organisms in which two sPPases with different molecular phylogeny and distinct cellular localization occur.

The gene (cDNA) sequences referred here have been submitted to databases and are under confidential status until publication. Supported by grants PB 97-1135 from DGICYT (MCYT; Spain) and Grupo PAI CVI-0261 (Junta de Andalucía).

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