Evolutionary aspects of inorganic pyrophosphatase

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Abstract Based on the primary structure, soluble inorganic pyrophosphatases can be divided into two families which exhibit no sequence similarity to each other. Family I, comprising most of the known pyrophosphatase sequences, can be further divided into prokaryotic, plant and animal/fungal pyrophosphatases. Interestingly, plant pyrophosphatases bear a closer similarity to prokaryotic than to animal/fungal pyrophosphatases. Only 17 residues are conserved in all 37 pyrophosphatases of family I and remarkably, 15 of these residues are located at the active site. Subunit interface residues are conserved in animal/fungal but not in prokaryotic pyrophosphatases.

Key words: Pyrophosphatase; Evolution; Gene; Primary structure

1. Introduction

Inorganic pyrophosphatase (PPase, EC 3.6.1.1) catalyzes specifically the hydrolysis of inorganic pyrophosphate to orthophosphate. It is essential for life [1–3], providing a thermodynamic pull for biosynthetic reactions [4]. Two families of soluble PPases are known to date. Family I, including most of the currently known PPases, and family II, including PPase of *Bacillus subtilis* as well as putative PPases of four other bacterial strains. The two families do not show any sequence similarity to each other [5,6]. In addition to soluble PPases, plants and certain bacteria have a membrane-bound PPase, which works as a reversible proton pump. Membrane-bound PPases are much larger and do not have any sequence similarity to any of the two families of soluble PPases [7–9].

In the present study, 37 currently available family I PPase sequences were analyzed with special emphasis on the residues located at the active site and subunit interfaces. As a result of this analysis, family I PPases were classified into three subfamilies, of which plant PPases bear a closer similarity to prokaryotic than to animal and fungal PPases.

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Abbreviations: PPase, inorganic pyrophosphatase; E-PPase, *Escherichia coli* pyrophosphatase; Y-PPase, cytoplasmic pyrophosphatase of *Saccharomyces cerevisiae*

2. Materials and methods

Sequences were aligned by the program Clustal W with the BLOSUM amino acid substitution matrix, using gap penalties of 10.0 and 0.05 for gap opening and extension, respectively [10]. The resulting multiple alignment was refined, based on structural alignment of the three-dimensional (3D) structures of soluble PPases from *Saccharomyces cerevisiae* (Y-PPase), *Escherichia coli* (E-PPase) and *Thermus thermophilus*, available in the Brookhaven Protein Data Bank.

The phylogenetic tree was constructed by the neighbor-joining method and the confidence of the branching order was verified by making 1000 bootstrap replicates with the program Clustal W. Analysing the alignment with the programs Seqboot, Fitch and Protpars of the Phylip package [11] gave the same results in grouping the sequences into three subfamilies. The tree was drawn with the program Treeview [12].

The theoretical 3D model of *Arabidopsis thaliana* PPase was generated with the programs ProModII and Gromos96 available at the SWISS-MODEL server at the Glaxo Wellcome Experimental Research in Geneva [13]. The template structures used were those for E-PPase (PDB code 1ipw) and Y-PPase (1huk).

3. Results and discussion

3.1. Sequence alignment of family I PPases

There are currently 37 sequences of family I PPases in the GenBank (Fig. 1). Many of them have been directly shown to encode active PPase, the identity of others was deduced based on strong conservation of active site residues (see below). When alternative PPase sequences with minor differences were found in the GenBank, only the sequences having the greatest similarity to the overall set were chosen, but the general conclusions presented below are valid for the alternative sequences as well. For PPases of *S. cerevisiae* [14], thermophilic bacterium PS-3 [15], *T. thermophilus* [16] and *Bacillus stearothermophilus* [17], the primary structures have also been determined by protein sequencing and they are in good agreement with the gene-deduced amino acid sequences.

With respect to size and primary structure, the 37 PPases can be divided into three subfamilies, prokaryotic (Ia), plant (Ib) and animal/fungal (Ic) PPases, having 162–220, 211–216 and 280–292 amino acid residues per subunit, respectively (Fig. 1). Internal identities of the prokaryotic PPase sequences vary from 23% (*Haemophilus influenzae* versus *Chlamydia trachomatis*) to 99% (*B. strearothermophilus* versus PS-3), plant PPases vary from 74% (*Oryza sativa* versus *Hordeum vulgare*) to 90% (*Zea mays* versus *H. vulgare*) and animal/fungal PPases vary from 42% (*S. cerevisiae*, mitochondria versus *Caenorhabditis elegans*) to 95% (*Homo sapiens* versus *Bos taurus*).

Interestingly, plant PPases have the same type of deletions as prokaryotic PPases and resemble them more closely than animal/fungal PPases (Fig. 1). In the phylogenetic tree, they

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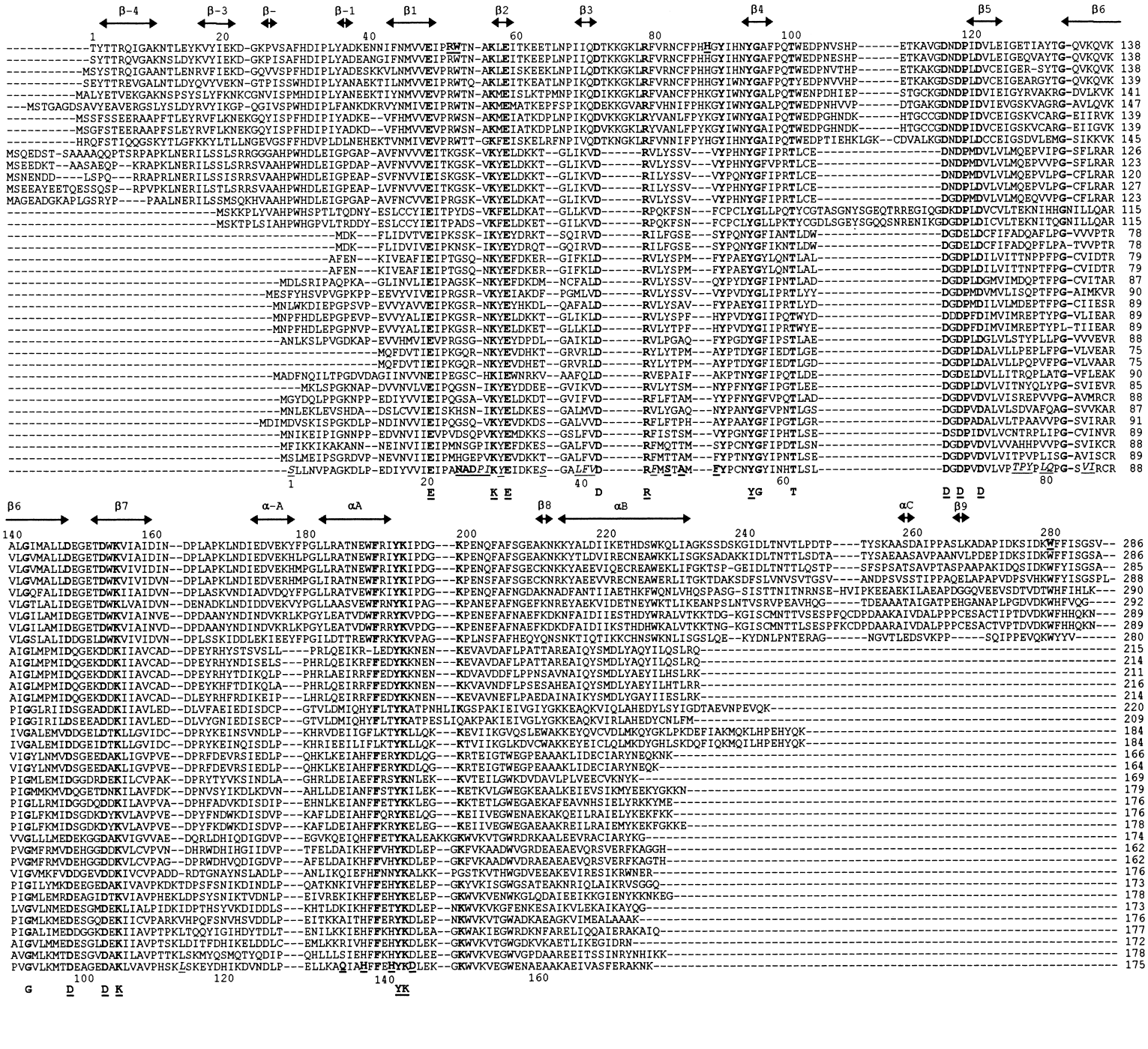
β-4
β-3
β-
β-1
β1
β2
β3
β4
β5
β6
S. cerevisiae
K. lactis
P. pastoris
S. pombe
D. melanogaster
C. elegans
B. taurus
H. sapiens
S. cerevisiae mit.
H. vulgare
Z. mays
S. tuberosum
A. thaliana
O. sativa
C. pneumoniae
C. trachomatis
M. pneumoniae
M. genitalium
PS-3
B. stearothermophilus
Synechocystis PCC6803
T. acidophilum
M. thermoautotrophicum
T. litoralis
P. horikoshii
T. thermophilus
M. leprae
M. tuberculosis
H. influenzae
S. acidocaldarius
A. aeolicus
H. pylori
G. suboxydans
B. bacilliformis
R. prowakezii
L. pneumophila
E. coli
β6
β7
α-A
αA
β8
αB
αC
β9

Fig. 1. Alignment of 37 sequences of family I PPases. The 17 residues conserved in all sequences are bold-faced and shown by the letter below. The 13 conserved, functionally important active site residues are further emphasized by underlining. The residues conserved in ≥34 sequences are also bold-faced. The residues located at the subunit interface of Y-PPase are underlined and bold-faced. The residues located at the inter- and intratrimeric subunit interfaces of E-PPase are underlined with bold facing and italics, respectively. The positions of the consensus secondary structure elements in E- and Y-PPase are marked as α and β, numbered sequentially from the N-terminus. Secondary structure elements found in Y-PPase but not in E-PPase are marked with negative numbers (e.g. β-4) if they occur N-terminal to the conserved core. Residue numbering is shown both for Y-PPase and E-PPase. The numbers at the end of each row indicate the total number of residues. The sequences derived from complete genes are shown in all cases, except for the mitochondrial PPase of *S. cerevisiae* (*S. cerevisiae* mit.) lacking the signal peptide sequence [2]. Accession numbers for the sequences follow: *S. cerevisiae*, 2781300; *Kluyveromyces lactis*, P13998; *Pichia pastoris*, O13505; *Schizosaccharomyces pombe*, P19117; *Drosophila melanogaster*, O77460; *C. elegans*, CAA93107; *B. taurus*, P37980; *H. sapiens*, A. Salminen et al., manuscript in preparation; the mitochondria of *S. cerevisiae*, P28239; *H. vulgare*, O23979; *Z. mays*, O48556; *S. tuberosum*, O43187; *A. thaliana*, AAC33503; *O. sativa*, AAC78101; *Chlamydia pneumoniae*, AAD19056; *C. trachomatis*, O84777; *Mycoplasma pneumoniae*, P75250; *Mycoplasma genitalium*, P47593; *Bacillus* sp. PS-3, P19514; *B. stearothermophilus*, BAA19837; *Synechocystis* PCC6803, P80507; *T. acidophilum*, P37981; *M. thermoautotrophicum*, O26363; *T. litoralis*, P77992; *Pyrococcus horikoshii*, O59570; *T. thermophilus*, P38576; *Mycobacterium leprae*, O69540; *Mycobacterium tuberculosis*, CAB08851; *H. influenzae*, 1170585; *S. acidocaldarius*, P50308; *Aquifex aeolicus*, O67501; *Helicobacter pylori*, P56153; *Gluconobacter suboxydans*, O05545; *B. bacilliformis*, P51064; *Rickettsia prowazekii*, CAA15034; *Legionella pneumophila*, O34955; *E. coli*, P17288.
←

represent a separate group, close to prokaryotic PPases (Fig. 2). Internal identities of plant versus prokaryotic PPases range from 27% (*C. trachomatis* versus *H. vulgare*) to 49% (*Methanobacterium thermoautotrophicum* versus *O. sativa*), whereas internal identities of plant versus animal/fungal PPases range from 20% (*H. vulgare* versus *S. cerevisiae*, mitochondria) to 29% (*Solanum tuberosum* versus *B. taurus*).

Typical for animal/fungal PPases are four insertions located between residues 71/78, 84/88, 102/115 and 175/179 (hereafter, all numbering is for Y-PPase, unless otherwise indicated). The 102/115 insertion is located beside the highly conserved region including three functionally important active site residues (D-115, D-117 and D-120) [18–21] and has an extra four residues in mitochondrial PPase (Fig. 1). These extra residues are suggested to have some role in loosely linking the enzyme to the membrane [22] and may be useful in screening for mitochondrial PPase genes. The human PPase sequence shown in Fig. 1 is based on the sequence of a gene that we have expressed in *E. coli* (A. Salminen et al., manuscript in preparation). Very recently, another human PPase sequence was released in the GenBank (accession number AAD24964). These two sequences differ from each other at four sites. The latter is devoid of the N-terminal MSGF and the C-terminal QKN sequences (which are also present in *B. taurus* PPase) and has A instead of P in the position corresponding to Y-PPase K-10.

Within the prokaryotic PPases, there is a subgroup (eight PPases, from *E. coli* to *S. acidocaldarius*) including a unique two residue insertion with a conserved lysine (K-112-L-113 in E-PPase). This subgroup further includes four bacterial strains (from *E. coli* to *Bartonella bacilliformis*) having a specific insertion of one proline (P-27 in E-PPase) close to the N-terminus (Fig. 1). Interestingly, the sequences of *B. strearothermophilus* and PS-3 PPases are almost identical. PS-3 PPase just has two more residues (NK) at the C-terminus and P instead of T in one position [17]. These two enzymes are very similar, but PS-3 PPase is somewhat less thermostable [23]. The two urkingdoms of the prokaryotes, eubacteria and archaebacteria, cannot be clearly separated, even though archaeal PPases (from *Thermococcus litoralis* to *Thermoplasma acidophilum*), except for *Sulfolobus acidocaldarius* PPase, are closely clustered within the prokaryotic subfamily in one branch of the phylogenetic tree (Fig. 2).

Chlamydia PPases are quite unusual members of the prokaryotic subfamily (Fig. 2). Their unique features include an insertion in the same region as animal/fungal PPases have the 102/115 insertion, an AHPWH sequence found in all plant PPases close to the N-terminus, an insertion of one asparagine residue next to the conserved glycine (G-82 in E-PPase) and an insertion in the region where *T. thermophilus* PPase has a KK insertion close to the C-terminus. Because of these insertions together with the short insertion both at the N- and C-terminus, *Chlamydia* PPases with 209–220 amino acids/subunit are of the same size as plant PPases and they are longer than the other prokaryotic PPases having 162–184 residues/subunit (Fig. 1).

3.2. Conservation of active site residues

Only 17 residues are conserved in all the 37 PPase sequences, 13 of which are functionally important active site residues (Y-PPase/E-PPase numbering: E-48/20, K-56/29, E-58/31, R-78/43, Y-93/55, D-115/65, D-117/67, D-120/70, D-147/97, D-152/102, K-154/104, Y-192/141 and K-193/142) (Fig. 1) [20,21] and belonging to the group of 17 polar Y-PPase active site

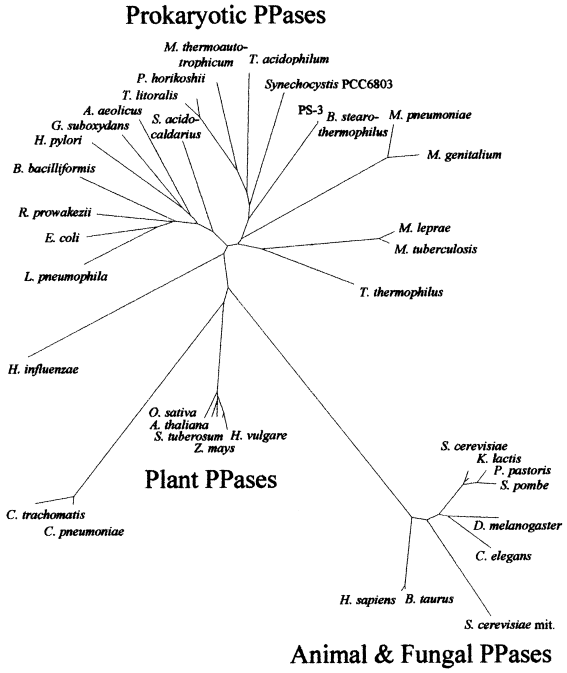

Fig. 2. Phylogenetic tree of family I PPases based on the sequence alignment shown in Fig. 1.

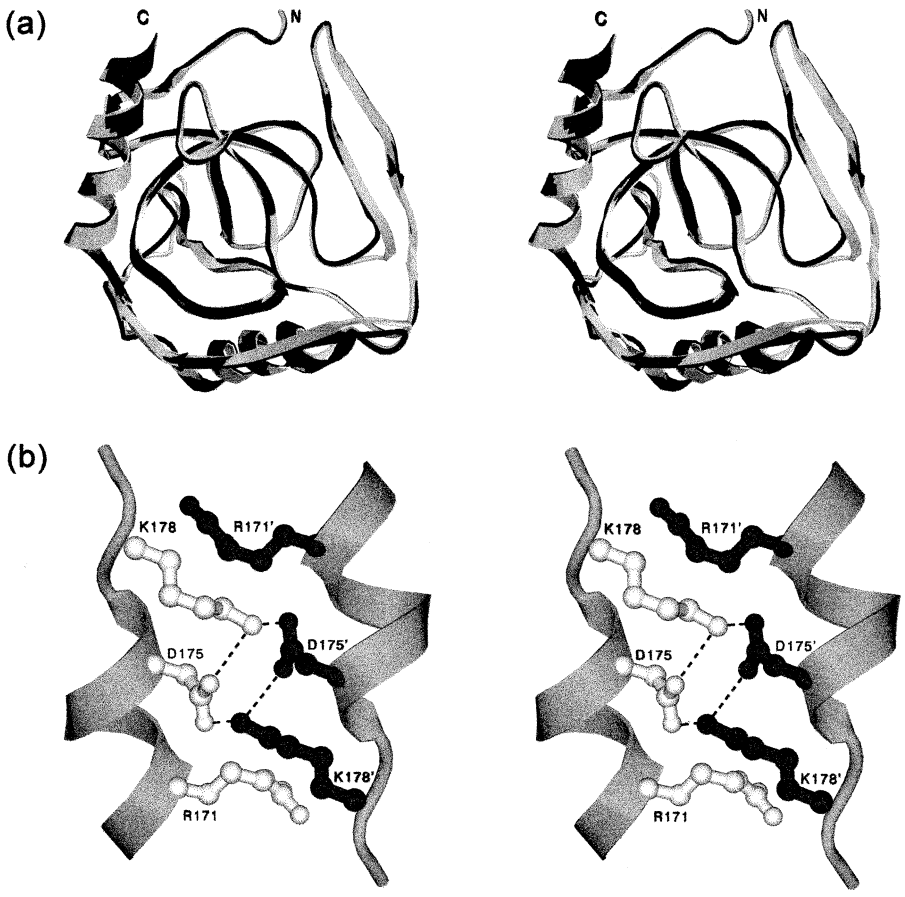

Fig. 3. (a) A stereo view of the predicted 3D structure of *A. thaliana* PPase (gray) superposed on the structure of E-PPase (black). Residues 1–38 at the N-terminus and 207–216 at the C-terminus of *A. thaliana* PPase are not shown as they have no analogs in E-PPase. (b) Possible subunit contact in *A. thaliana* PPase. Unprimed and primed residues refer to different subunits. Hydrogen bonds are shown with dashed lines. Residues R-171, D-175 and K-178 correspond to H-136, H-140 and D-143 of E-PPase in the sequence alignment (Fig. 1). The figure was created with the program Swiss-PdbViewer [40].

residues originally identified by X-ray crystallographic analysis [24]. The four other conserved residues are D-71/42, G-94/56, T-99/61 and G-141/91, two of which are also located at the active site: G-94/56 is beside the functionally important Y-93/55 [18–21], whereas D-42 points directly to the active site cavity of E-PPase beside K-29 and R-43 [25], which, like their Y-PPase counterparts (K-56 and R-78), are important for substrate-binding [20,21]. D-42 has recently been shown to be important for substrate-binding in E-PPase [26], even though in Y-PPase, its equivalent, D-71, does not seem to be important [27]. A previous comparison of eight PPase sequences revealed 24 conserved residues in total, 15 of which are belonging to the group of the 17 polar active site residues mentioned above [28].

Y-89, P-118, G-132, F-189 and K-198 are conserved in $\geq$34 sequences (Fig. 1). Of these, Y-89 and K-198 belong to the group of the 17 Y-PPase active site residues identified by Terzyan et al. [24]. However, mutational and functional analyses have indicated that these two residues are not essential for catalysis [21,27], even though structural studies have shown Y-89 to be involved in the binding of the electrophilic phosphoryl group [18]. P-118 is very important for the structure of the active site cavity and is located in the highly conserved region including the catalytically essential residues D-115, D-117 and D-120 [18,21]. Two of the 17 polar active site residues identified by Terzyan et al. [24] are somewhat less well-conserved (E-148 and E-150 are conserved in 17/37 and 22/37 sequences, respectively) and mutational analysis has shown these residues to be non-essential for catalysis [27].

3.3. Conservation of subunit interface residues

The subunits of prokaryotic PPases are significantly smaller than those of animal/fungal PPases. Furthermore, prokaryotic PPases of family I are generally homohexamers arranged as dimer of trimers [25,29–31], whereas animal/fungal PPases are homodimers [5,18,32]. Accordingly, the subunit interfaces of prokaryotic and animal/fungal PPases are completely different. However, it should be recognized that the oligomeric structure of plant PPases has not been studied in detail. The interface residues of animal/fungal PPases are very well conserved: R-51, W-52 and W-279 are found in all nine sequences, whereas H-87 is conserved in 5/9 cases (in the remaining four sequences, H is replaced by K) (Fig. 1), supporting the idea that all these nine subfamily Ic PPases are dimers.

The principal intertrimeric interaction of E-PPase involves a three center ionic, hydrogen-bonding interaction among the residues H-136-H-140-D-143 [33–35] (E-PPase numbering). H-125, a counterpart of E-PPase H-136, is important for the oligomeric structure of PS-3 PPase [36]. H-136, H-140 and D-143 are conserved in 17, 8 and 10 of the 23 prokaryotic se-

quences, respectively. All three residues are found in six sequences and in seven more cases, one of them (H-140 or D-143) is conservatively substituted (by R and E, respectively).

Another important trimer-trimer interaction occurs in E-PPase at three cavities per hexamer, each formed by N-24-A-25-D-26 from a pair of subunits and containing a Mg^{2+} ion, which is important for the hexamer stability [37–39]. These residues are poorly conserved among the 23 prokaryotic PPases, suggesting that the interface metal ion is unique to E-PPase. Supporting this, structural studies of PPases of *T. thermophilus* [29] and *S. acidocaldarius* [30] have indicated no interface metal ion-binding site.

The intratrimeric contacts of E-PPase involve several hydrophilic and hydrophobic residues [33], which are poorly conserved. The exceptions are Y-30, V-41, V-84 and I-85 (E-PPase numbering) conserved in 20/23 (in two cases, Y is replaced by F and in one by I), 13/23 (in the remaining 10 sequences, V is replaced by L), 14/23 (in eight cases, V is replaced by either I or L) and 12/23 (in six cases, I is replaced by V and in three by L) cases, respectively, suggesting that hydrophobic interactions are important for intratrimeric contacts in prokaryotic PPases (Fig. 1).

Although the subunit interface residues of prokaryotic and animal/fungal PPases are not conserved in plant PPases, they exhibit some similarity with prokaryotic PPases in the region corresponding to the H-136-H-140-D-143 triad of E-PPase. The triad becomes R-D-K in all plant PPases, i.e. also contains two basic and one acidic residues, and may therefore form a similar ionic, hydrogen-bonding interaction between subunits (see below).

3.4. Predicted tertiary structure of A. thaliana PPase

As no tertiary structure of a plant PPase has been yet determined, we used the sequence alignment shown in Fig. 1 for homologous modeling of the tertiary structure of *A. thaliana* PPase. The structures of Y-PPase and E-PPase determined at 2.2–2.3 Å resolution were used as templates. The program used [13] calculates positions only for equivalent residues in two proteins (169 and 204 residues in the prediction based on E-PPase and Y-PPase, respectively). As expected, the major differences between the predicted and template structures were observed in the regions where the *A. thaliana* PPase sequence has gaps or insertions (Fig. 3a). If these regions are not considered, both predicted structures resemble the template structures quite closely (rmsd < 0.8 Å per C_α). However, the superposition of the 17 conserved active site residues gave a significantly lower rmsd value (0.31 Å versus 0.87 Å per C_α) for the model based on E-PPase. Thus, in terms of the active site structure, *A. thaliana* PPase also appears to be closer to E-PPase than to Y-PPase.

Interestingly, when two monomers of *A. thaliana* PPase were placed side by side by directly using the transformation matrix contained in the PDB file for E-PPase (1ipw), pairs of D-175 and K-178 from two monomers were found to be close enough to form two bridging ionic pairs (Fig. 3b). Besides, the two residues form a hydrogen bond within each monomer, like H-140 and D-143 in E-PPase [33,34]. Monomer-monomer contacts in plant and prokaryotic PPases may thus be similar also.

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