DOI: 10.1070/RCR4529

Inorganic pyrophosphatases: structural diversity serving the function

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The review is devoted to ubiquitous enzymes, inorganic pyrophosphatases, which are essential in all living organisms. Despite the long history of investigations, these enzymes continue to attract interest. The review focuses on the three-dimensional structures of various representatives of this class of proteins. The structural diversity, the relationship between the structure and some properties of pyrophosphatases and various mechanisms of enzyme action related to the structural diversity of these enzymes are discussed. Interactions of pyrophosphatase with other proteins and possible practical applications are considered.
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I. Introduction

Inorganic pyrophosphatases (PPases) are enzymes ubiquitous in all living organisms. They catalyze an essential reaction, the hydrolysis of the pyrophosphate ion $P_2O_7^{4-}$ to two phosphate ions. The significance of the action of these enzymes cannot be overemphasized since pyrophosphate is a side product of multiple biosynthetic reactions.[1] Pyrophosphatases play an important role in cell anabolism by shifting the equilibrium of such reactions as the protein, RNA and DNA synthesis.

Pyrophosphatases are divided into two large classes — soluble and membrane-bound. Soluble PPases are classified into three non-homologous families: I, II and III. Family I PPases have been known for more than 80 years and were studied in depth. Family II PPases were discovered in 1998 in bacteria and archaebacteria [2, 3] and were characterized in much less detail. Data on family III pyrophosphatases are scarce.[4, 5] Soluble pyrophosphatases dissipate energy released during pyrophosphate hydrolysis, while membrane-bound pyrophosphatases utilize this energy to drive proton or sodium ion transport across membranes. Membrane pyrophosphatases are less well studied. The membrane-bound form of pyrophosphatases was first discovered in photosynthetic bacteria. It was shown that this enzyme is able to synthesize pyrophosphate in amounts much larger than the equilibrium values for the pyrophosphate – phosphate system due to the coupling of this process to a change in the electrochemical potential of hydride ions.

Pyrophosphatases belong to metal-dependent enzymes. For most of pyrophosphatases, a magnesium ion is the most efficient and physiological activator.[6] Calcium, with rare exceptions, inhibits pyrophosphatases.[7] Pyrophosphatases most significantly differ in the interaction with other inhibitors, such as a fluoride ion and diphosphonates.[6] All these differences have a structural basis.

The ability of yeast pyrophosphatase (*Saccharomyces cerevisiae*) to crystallize was found for the first time in 1952.[8] Crystals which were used to collect the first X-ray diffraction data set suitable for the determination of the three-dimensional structure were grown at the A.V.Shubnikov Institute of Crystallography, Academy of Sciences of the USSR in 1980.[9] Extensive structural studies of pyro-

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Current research interests: X-ray diffraction analysis, protein crystallography, protein crystallization, cryocrystallography, protein – protein interactions, enzymes, metalloproteins.

Received 6 November 2014
Translation: T.N.Safonova

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phosphatases were continued in subsequent years, being often coupled to the development of the X-ray diffraction method.

Numerous studies were devoted to PPases from different families. Three reviews on this subject were published.[6, 10, 11] One of these reviews (Ref. 6) surveys key differences in the mechanism of action of family I and II pyrophosphatases and membrane-bound pyrophosphatases. The two other reviews [10, 11] consider exclusively membrane pyrophosphatases and hypotheses about their evolution and the mechanism of action as ion pumps. The present review summarizes studies on pyrophosphatases published in the past 15 years and focuses mainly on the specific structural features of different pyrophosphatases. These features are apparently related to the differences in the functioning associated with characteristic features of the organism, in which the protein is produced, and specific biological purposes. A rather long period of investigation allows one to trace the role of advancement of X-ray diffraction (XRD) methods in these studies. Some methodological approaches, which have been employed for the first time to study crystals of one of inorganic pyrophosphatases, extended the series of supplementary XRD techniques. These techniques are considered in special sections of the present review. Besides, the review discusses the following issues: the structural features of two classes of inorganic pyrophosphatases — soluble (including the structures of little-studied family III pyrophosphatases and monomeric family I PPases) and membrane-bound pyrophosphatases; the structure–function relationship for pyrophosphatases; the role of the quaternary structure of PPases, including in the protein crystallization; the structural methods of investigation of the mechanism of their action; the interactions of family I pyrophosphatase from *Escherichia coli* with other proteins.

II. Soluble pyrophosphatases

As mentioned above, soluble pyrophosphatases are classified into three families (I, II and III). Their representatives have no similarity in the amino acid sequences. Family I pyrophosphatases are divided into eukaryotic and prokaryotic. They are also nonhomologous, but they contain 20 conserved active site residues, which are responsible for the similar mechanism of the catalytic reaction. Yeast pyrophosphatase (Y-PPase) was the first eukaryotic pyrophosphatase, the structure of which was determined; *E. coli* pyrophosphatase (E-PPase) was the first structurally characterized prokaryotic pyrophosphatase.

II.1. Secondary structure of soluble pyrophosphatases

II.1.a. Family I and II pyrophosphatases

According to the classification of domains, family I inorganic pyrophosphatases belong to the $\alpha+\beta$ class. This structure comprises one domain composed of a five-stranded twisted β-barrel. Besides, the structure contains α-helices and additional β-strands. The number of helices varies from 15 to 20. The total number of β-strands varies from 28 to 36. Despite the fact that prokaryotic pyrophosphatases show low sequence similarity to eukaryotic enzymes (27% identity between Y-PPase and E-PPase), these enzymes have a very similar overall polypeptide chain fold (Fig. 1 *a*).[2]

The enzyme Y-PPase consisting of 286 amino acid residues contains one additional β-sheet and additional insertions at the N-terminus (26 residues) and C-terminus (53 residues) compared to E-PPase (175 residues) (see

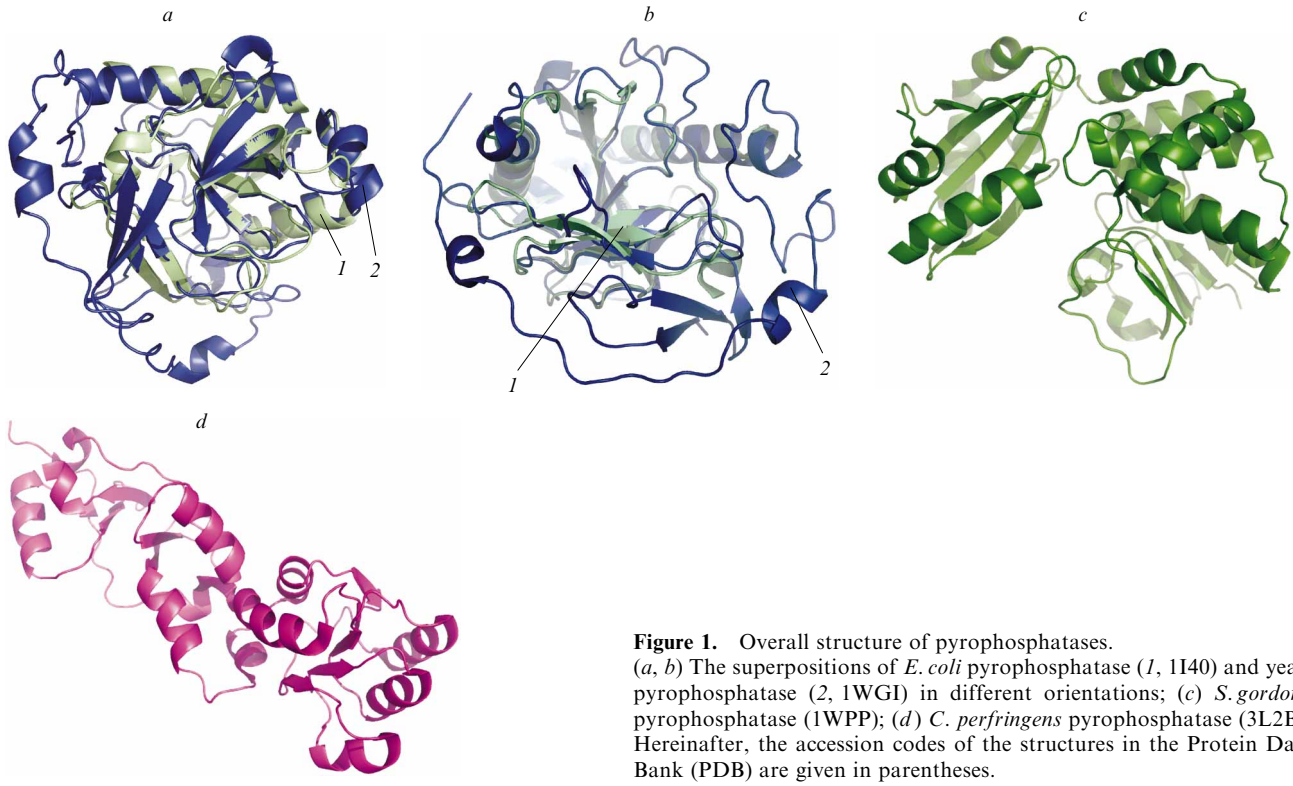


Figure 1. Overall structure of pyrophosphatases.
(*a*, *b*) The superpositions of *E. coli* pyrophosphatase (*1*, 1I40) and yeast pyrophosphatase (*2*, 1WGI) in different orientations; (*c*) *S. gordonii* pyrophosphatase (1WPP); (*d*) *C. perfringens* pyrophosphatase (3L2B). Hereinafter, the accession codes of the structures in the Protein Data Bank (PDB) are given in parentheses.

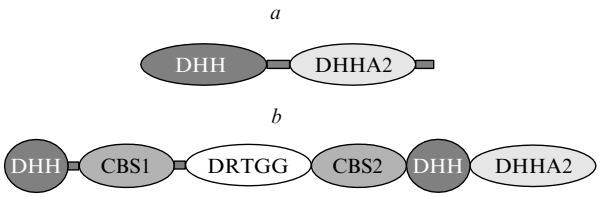


Figure 2. Scheme of the domain structure of family II pyrophosphatases.[15]
(*a*) Usual pyrophosphatases; (*b*) *C. perfringens* CBS-pyrophosphatase.

Fig. 1 *b*). The insertion at the C-terminus 'embraces' the globule and interacts with the additional insertion at the N-terminus. The largest number of structures are known for prokaryotic pyrophosphatases (28 structures on February 2016).

Family II pyrophosphatases occur almost exclusively in bacteria and archaea, including several human pathogens.[11] They have no structural similarity to family I pyrophosphatases (see Fig. 1 *c*,*d*). Only a few three-dimensional structures of family II pyrophosphatases are known: from *Streptococcus mutans* (1I74),[12] *Streptococcus gordonii* (1K20), *Bacillus subtilis* (1K23)[13] and *Streptococcus agalactiae* (2ENX).[14] Family II pyrophosphatases are composed of two domains connected by a flexible linker — the N-terminal DHH domain and the C-terminal DHHA2 domain (see Figs 1 *c* and 2 *a*). Some of these pyrophosphatases, for example, *Clostridium perfringens* pyrophosphatase,[15] contain an insertion of 250 residues in the N-terminal domain. The insertion consists of the DRTGG domain (about 120 residues) and two CBS domains (see Figs 1 *d* and 2 *b*). These pyrophosphatases are called CBS-pyrophosphatases. The CBS domains are named after cystathionine β-synthase, in which they were identified for the first time. Since CBS domains bind adenine nucleotides with varying affinities, they act as sensors of cellular energy status.[16]

II.2. Family III pyrophosphatases

Family III soluble pyrophosphatases, which were discovered as late as in 2009, are multifunctional enzymes, because they belong to the dehalogenase (haloacid dehalogenase) superfamily. The first representative of this family — *Thermococcus onnureneus* PPase[4] — has low catalytic activity. The structure of *Pirococcus horikoshii* pyrophosphatase, which is the closest homologue of this enzyme (60% protein sequence identity), was determined (PDB code 2OM6). Family III pyrophosphatases also belong to the α + β class and, like family II pyrophosphatases, contain two domains.[5] They do not reveal structural similarity with other soluble pyrophosphatases and are composed of the catalytic Rossmann-fold domain (six-stranded parallel β-sheet flanked by six helices) and a domain consisting of four helices located between the first β-strand and the first helix (Fig. 3 *a*). A comparison of the structure of *Bacteroides thetaiotaomicron* pyrophosphatase BT2127 with the structure of *P. horikoshii* pyrophosphatase shows the similarity of the domains (see Fig. 3 *b*). Most likely, the substrate is bound at the interface between the domains, as in family II PPases. Presumably, the active site in family III pyrophosphatases is less conserved compared to the active sites in family I and II pyrophosphatases.[5]

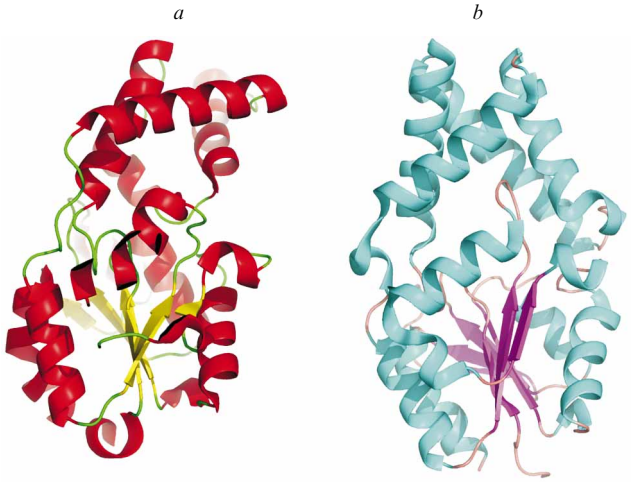


Figure 3. Secondary structures of family III pyrophosphatases from *B. thetaiotaomicron* (3QUQ) (*a*) and *P. horikoshii* (2OM6) (*b*).

III. Membrane pyrophosphatases

Membrane-bound pyrophosphatases utilize the energy that is released during hydrolysis of pyrophosphate to drive H^+ or Na^+ ion transport across membranes. Therefore, these enzymes maintain a transmembrane concentration gradient of these ions — an important energy source in the cell. In 2007 it was found[17] that three membrane-bound PPases provide transport of Na^+ ions rather than protons across membranes. Sodium ion-translocating PPases are homologous to H^+-translocating enzymes, *i.e.*, they have similar primary structures. Membrane pyrophosphatases stand alone in the large class of pyrophosphatases. Although the first membrane pyrophosphatase was discovered in the 1960s,[18] the first structure of membrane pyrophosphatase was published in 2012.[19,20] The determination of this structure has been made possible due to methods for the purification and crystallization of membrane-bound proteins developed in the last decade. Membrane-bound pyrophosphatases are divided into K^+-independent and



Figure 4. Membrane-bound Na^+-pyrophosphatase from *Thermotoga maritima* (4AV6).

K^+-dependent H^+-pyrophosphatases, Na^+-pyrophosphatases and Na^+,H^+-pyrophosphatases and consist of 15 – 17 transmembrane (TM) helices (Fig. 4), *i.e.*, they belong to another structure class as compared to soluble pyrophosphatases.

IV. Role of the quaternary structure of pyrophosphatases

IV.1. Multimeric pyrophosphatases

Most of family I soluble pyrophosphatases are either dimers or hexamers. Experiments with Y-PPase and E-PPase demonstrated that, by means of site-directed mutagenesis or changes in the physicochemical characteristics of the medium, these enzymes can be converted to monomers (in the case of Y-PPase and E-PPase [21 – 24]) or trimers and dimers (in the case of E-PPase),[21] which retain the ability to catalyze hydrolysis, though to a lesser extent compared to high-oligomeric forms (Table 1). In the case of yeast pyrophosphatase, the dimerization is favourable for substrate binding.[24] Dimeric forms of pyrophosphatases from eukaryotes and family II pyrophosphatases and hexameric forms of family I pyrophosphatases from prokaryotes (except for some organisms, which will be considered separately) (Fig. 5) prevail in the nature. Family II PPases are also more active in the dimeric form than in the monomeric one (see Table 1).[26]

Apparently, the quaternary structure of pyrophosphatases makes a contribution to thermal stability of the soluble enzymes. All family I pyrophosphatases are thermally stable and this property is associated not only with the compact form of the monomer but also, most likely, with oligomerization. Hexamers formed by prokaryotic family I PPases are leaders with respect to thermal stability. There is a direct relationship between the resistance to high temperatures and the degree of compactness of the hexamer. It was shown that a chimeric (fusion) protein consisting of *Thermus thermophilus* PPase (residues 1 – 135) and E-PPase (residues 136 – 173) is thermally more stable.[27] This is due to the compact structure of the hexamer because the fragment of the fusion protein sequence contains simultaneously key residues essential for the oligomerization, *viz.*, Thr138 and Ala141 in T-PPase and His140 and Asp143 in E-PPase, resulting in an increase in the monomer interaction surface area in the oligomer. Pyrophosphatases isolated from extremophiles serve as a model for investigation of thermal stability. A magnesium ion provides additional stability to the hexamer of *E. coli* pyrophosphatase. This ion binds with high affinity not in the active site but at the interface between the trimers and coordinates amino acid residues from different trimers through water molecules.[28]

An analysis of the structure of hexameric hyperthermophilic *P. horikoshi* pyrophosphatase (Pho-PPase), which has the optimum growth temperature of 88 °C, demonstrated [29] that the structure contains a larger number of arginine residues (which tend to form ion pairs) and proline residues (which decrease the flexibility of the loops) compared to other thermally stable PPases. In addition, the amino acid sequence of the protein includes a larger number of hydrophobic and aromatic residues, whereas two loops near the active site of Pho-PPase are absent. On the whole, the hexamer of Pho-PPase is more tightly packed due to a larger number of intratrimeric contacts and contacts between the trimers.[29] It should be noted that enzymes resistant to extreme temperatures are of interest for potential applications in biotechnology.

All known membrane pyrophosphatases are homodimers. Presumably, the dimerization of H^+-PPases may be essential for the transport of H^+ and Na^+ ions.[11]

IV.2. Monomeric pyrophosphatases

Family I pyrophosphatases that function as monomers were found several years ago in the microalgae *Chlamydomonas reinhardtii*, *Cyanidium caldarium*, *Ochromonas danica* and *Euglena gracilis* and in the chloroplasts *Spinacea oleracea*.[25] Chloroplast pyrophosphatases are structurally more similar

Table 1. Kinetic characteristics of oligomeric pyrophosphatases.[a]

PPase	Oligomeric form	K_m /μmol litre^{-1}	k_{cat} /s^{-1}	k_{cat}/K_m /μmol^{-1} litre s^{-1}	Ref.
		Family I			
E-PPase	Dimer	0.8	76.0	95	22
	Hexamer	3.3	330.0	100	23
	Trimer	766	230.0	0.3	23
E-PPase Y77D/Q80E/H136Q/H140Q	Monomer	1500	0.15	0.0001	23
Y-PPase	Dimer	1.5	304	202.6	24
Y-PPase W52S	Monomer	17	111	6.5	24
Cr-sPPase1	Monomer	10.5	314.6	30.0	25
Cr-sPPase2	Monomer	65.2	334.6	5.1	25
		Family II			
B-PPase	Dimer	19	160	8.4	26
	Monomer	170	4.4	0.026	26
Sg-PPase	Dimer	17	400	23.5	26
	Monomer	320	9.3	0.029	26
Sm-PPase	Dimer	10.2	110	10.8	26
	Monomer	200	4.7	0.023	26

[a] K_m is the Michaelis constant; k_{cat} is the catalytic constant, which characterizes the efficiency of the substrate conversion in the active site of the enzyme.

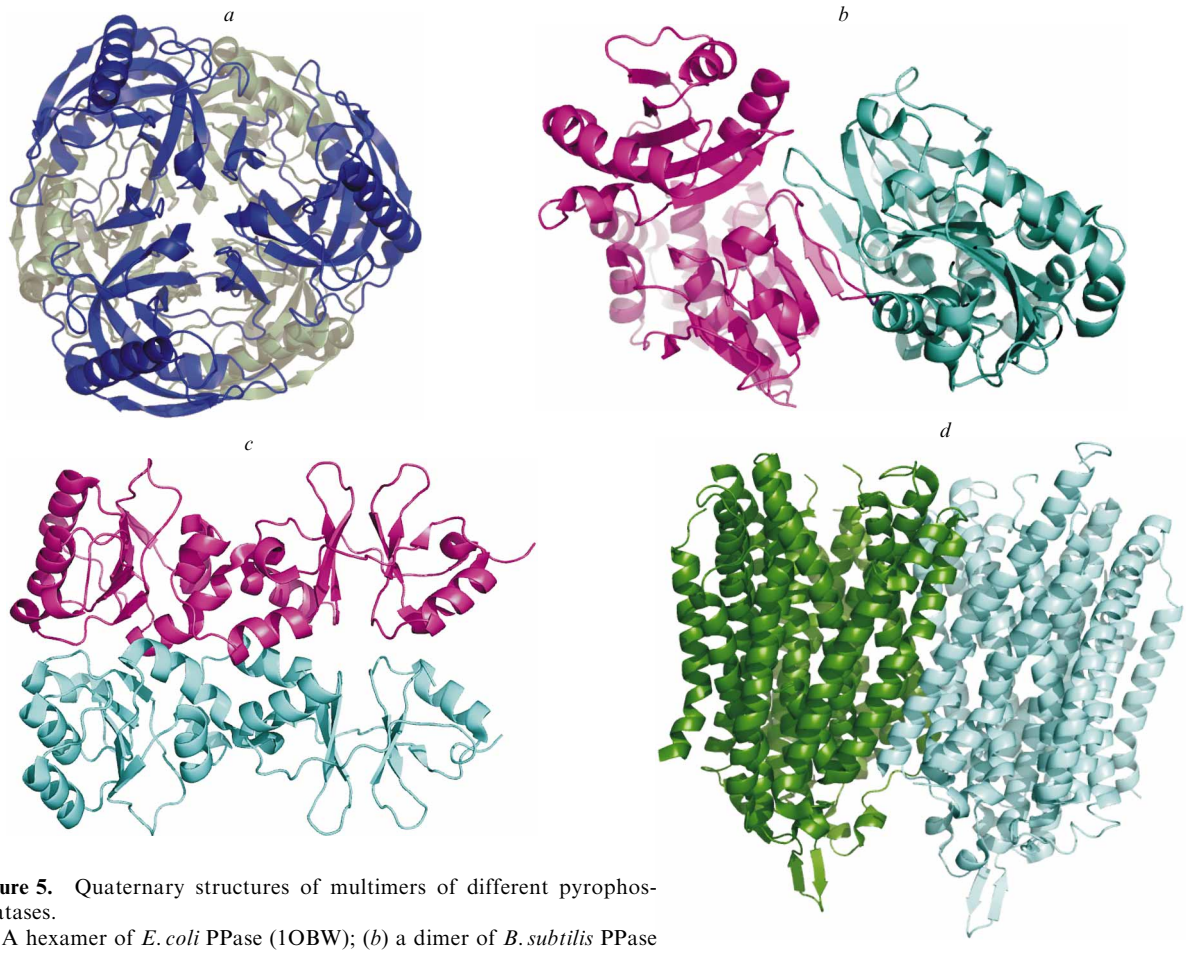


Figure 5. Quaternary structures of multimers of different pyrophosphatases.
(*a*) A hexamer of *E. coli* PPase (1OBW); (*b*) a dimer of *B. subtilis* PPase (1K23); (*c*) a dimer of the regulatory fragment of *C. perfringens* CBS-PPase (3L2B); (*d*) a dimer of *T. maritima* PPase (4AV3).

to eukaryotic soluble pyrophosphatases than to PPases from cyanobacteria, although cyanobacteria-like ancestral symbionts are believed to be evolutionary ancestors of chloroplasts.

Let us consider in more detail the unique pyrophosphatase from the microalgae *C. reinhardtii*,[25] which is a photosynthetic eukaryotic organism. This organism contains two family I pyrophosphatases. One of them (Cr-sPPase1) with a molecular mass of 30 kDa is homologous to eukaryotic pyrophosphatases and is present in a fraction of chloroplasts. Another pyrophosphatase (Cr-sPPase2, 24 kDa) is homologous to prokaryotic pyrophosphatases and is present in mitochondria.

In order to elucidate the structural basis of this form, the three-dimensional structures of the enzymes were modelled by the author of this review using the I-TASSER server.[30] Of five models calculated for each enzyme, the models with the highest C-score were selected.[30] These models were compared with the three-dimensional structures of yeast pyrophosphatase (1E9G) and *E. coli* pyrophosphatase (1I40) (Fig. 6). The superposition of the structures based on the secondary structure elements was performed using the Coot program.[31] Pyrophosphatase Cr-sPPase1 is homologous to yeast pyrophosphatase. A comparison of the structures revealed that Cr-sPPase1 contains additional 44 residues at the N-terminus. The monomeric form is explained by rather large differences in the regions essential for the dimerization (see Fig. 6 *a*,*b*). Pyrophosphatase Cr-sPPase1 lacks 40 residues at the C-terminus, which 'embrace' the globule of yeast pyrophosphatase and include the zone of intradimeric contacts. The loop 82–87, which contains Phe84, Pro85, His86 and His87 involved in the intersubunit contact in Y-PPase, are also absent in Cr-sPPase1.

The enzyme Cr-sPPase2 related to bacterial pyrophosphatases shares a high degree of secondary structure similarity to *E. coli* pyrophosphatase. The exceptions are the longer C-terminus, the helix at which has an additional turn followed by a flexible loop comprising 9 residues, and an additional flexible loop composed of 11 residues at the N-terminus. The latter difference may be a key factor responsible for the monomeric form of Cr-sPPase2, since the considerable length of this region sterically hinders the close arrangement of this molecule and two other monomers, which could form a trimer.

The N-terminal loop is of functional importance because it is present in all known PPases from algae and in part of plant PPases and, apparently, acts as a signal peptide for their transport to chloroplasts. In Cr-sPPase2 (as opposed to Cr-sPPase1), this region unambiguously hinders the oligomerization of the enzyme. Besides, all monomeric PPases have substitutions in six positions, which are occupied by residues involved in the stabilization of a hexamer of E-PPase (Asn24, Tyr77, Glu80, His136, His140 and

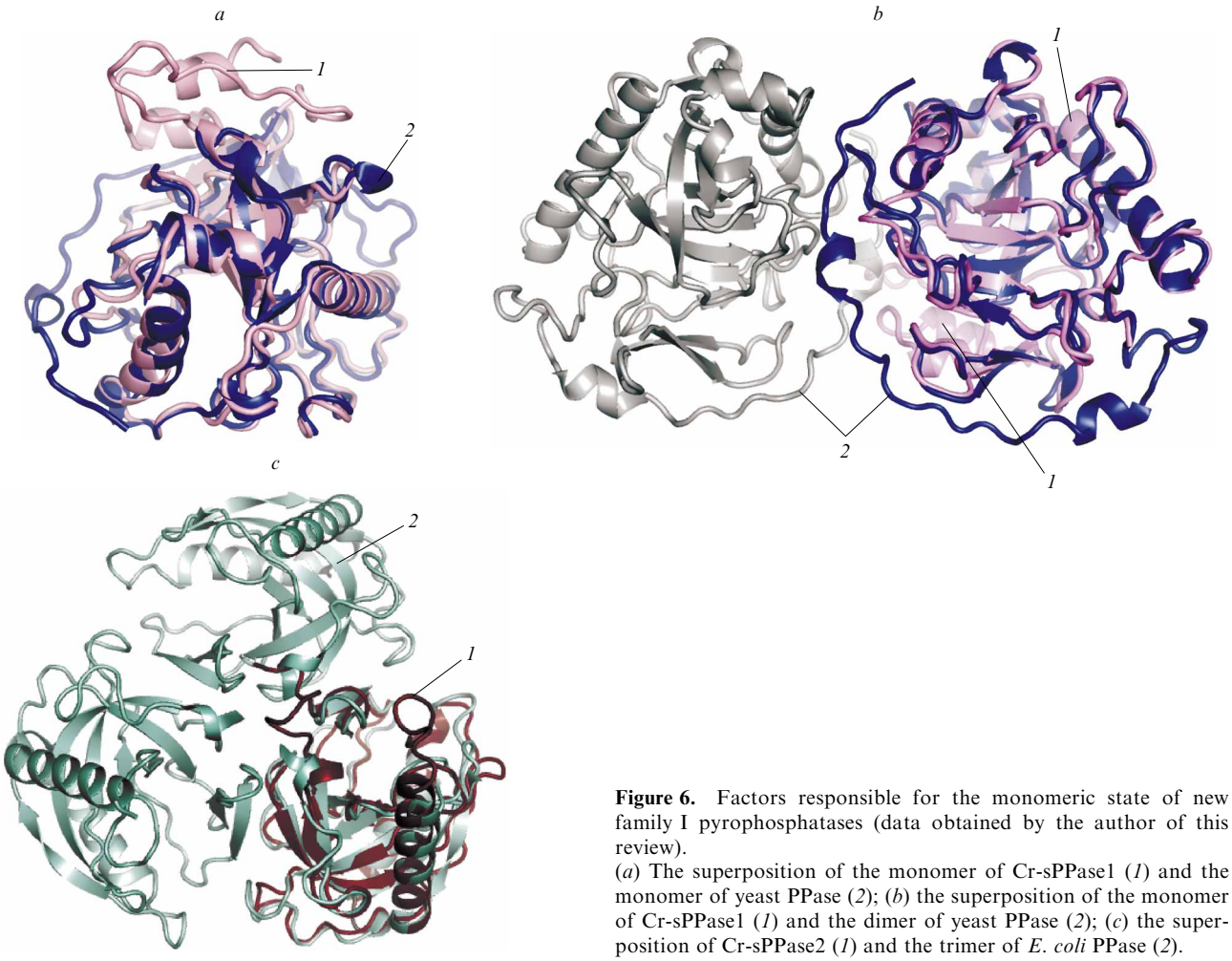


Figure 6. Factors responsible for the monomeric state of new family I pyrophosphatases (data obtained by the author of this review).
(*a*) The superposition of the monomer of Cr-sPPase1 (*1*) and the monomer of yeast PPase (*2*); (*b*) the superposition of the monomer of Cr-sPPase1 (*1*) and the dimer of yeast PPase (*2*); (*c*) the superposition of Cr-sPPase2 (*1*) and the trimer of *E. coli* PPase (*2*).

Asp143). Probably, it is these substitutions in plant proteins lacking an additional loop at the N-terminus that play an essential role in the formation of the quaternary structure.

The monomeric form of Cr-sPPase1 and Cr-sPPase2 may be a factor responsible for lower catalytic activity of these enzymes compared to yeast and *E. coli* pyrophosphatases (see Table 1) since it is known that the dimerization in the case of, for example, Y-PPase improves substrate binding.

All known family III pyrophosphatases also exist as monomers.[5]

IV.3. High crystallizability of family I pyrophosphatases

Currently, the PDB contains more than 90 crystal structures of family I pyrophosphatases determined at resolution from 3 to 0.99 Å. The tendency of pyrophosphatases to crystallize was found in 1951 for the yeast enzyme, when a method was developed for its isolation and purification, resulting in the formation of crystals in the final step.[8] The use of modern high-throughput techniques and robotic systems for crystallization and the employment of 10 commercial kits, each containing 96 solutions for the initial screening of crystallization conditions, resulted in obtaining crystals of *Vibrio cholerae* pyrophosphatase in half of trials. The examination of the crystals with a size of 0.05–0.15 μm using synchrotron radiation at the Max-Planck beamline BW6 (Hamburg, Germany) at 100 K showed that crystals diffracted to a resolution of 1.5 Å, particularly in the presence of high concentrations of sulfate and phosphate ions (unpublished data of the author of this review). Photographs of the crystals of different eukaryotic family I PPases are shown in Fig. 7.

A medium resolution is typical only of the structures of family I inorganic pyrophosphatases deposited in the PDB before 1999; the structures determined later are characterized by higher resolution. This is mainly due not to the crystal quality but to the rapid progress in X-ray diffraction methods in the 20th century and the widespread use of cryotemperatures for the collection of X-ray diffraction data, the improvement of synchrotron sources and the design of new detectors. The improvement of the quality of X-ray diffraction data made it possible to gain a deeper insight into the mechanism of enzyme action. High tendency to form crystals is apparently attributed to the compact structure of these enzymes. Family I pyrophosphatases consist of one domain. The molecule is symmetrical, which is favourable for crystallization.[32] There are almost no disordered regions, the flexible loops between the helices and β-strands are short, and the loops at the N- and C-termini (Y-PPase) are involved in oligomeric contacts.

Hexameric pyrophosphatases contain a rigid α-helix at the C-terminus, and the N-terminus generally contains an extended loop with a short 3d10 helix at its end (or bearing the latter close to the end). In some cases, the loop includes

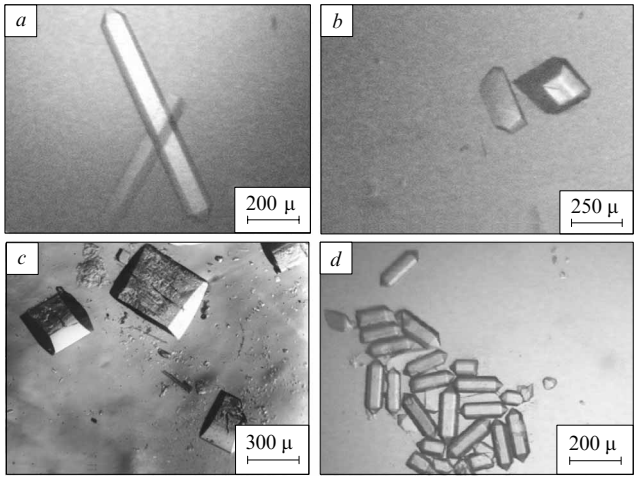


Figure 7. Crystals of different family I pyrophosphatases (data obtained by the author of this review).
(*a*, *b*) Crystals of *M. tuberculosis* PPase; (*c*) crystals of *E. coli* PPase; (*d*) crystals of *V. cholerae* PPase.

a β-strand (for example, *Coxiella burnetii* PPase), but the N-terminus, although being located on the surface of the hexamer, is always held in place by contacts with the surrounding structural elements and points inward the trimer toward a threefold axis (see Fig. 5 *a*).

Oligomeric forms of eukaryotic and prokaryotic pyrophosphatases, dimers and hexamers, have a compact structure, resulting in that they are stable in a wide range of conditions.[24]

Due to high crystallizability of PPases I, it was possible to obtain crystals of pyrophosphatases from *S. cerevisiae* and *Thermococcus thioreducens* (Tt-PPase) suitable for neutron diffraction studies. This method requires large crystals; the sizes of the crystals of Tt-PPase [33] were up to 5 mm^3.

Crystals of family I PPases are stable and tolerant to various handling procedures. Crystals of hexameric PPases contain a small amount of the solvent (~40%). This property made it possible to develop two procedures for *E. coli* PPase. One of them allows the improvement of the resolution of the already grown crystals.[34] Another procedure (based on the former one) is suitable for investigation of intermediate catalytic states of the enzyme in the crystal. It was found that crystals of *E. coli* pyrophosphatase withstand repeated flash-freezing. As opposed to the widely applied annealing technique, which utilizes brief thawing and rapid freezing cycles of the crystal with a blocking of the liquid nitrogen stream (under which the crystal is flash-frozen) for a few seconds, it was proposed to use a controlled increase in the concentration of sodium chloride (which served as a precipitate for crystallization) by the repeated transfer of the crystal from the cold nitrogen-gas stream to cryosolutions with increasing NaCl concentrations. This technique made it possible to achieve the reproducible improvement of the resolution for the crystals from 1.8 to 1.1 Å. Apparently, crystallization conditions for the direct preparation of protein crystals diffracting to atomic resolution can be found by performing extensive screening experiments. However, the above-described technique saved the time for the collection of high-resolution X-ray diffraction data. Later, this technique was used to improve the quality of diffraction data for some other proteins.[35–39] This technique also served as a basis for the procedure employed to detect intermediate states of the catalytic reaction of *E. coli* PPase (see below).

V. Characteristic features of hydrolysis and structural plasticity of pyrophosphatases

V.1. Characteristic features of hydrolysis by various pyrophosphatases

The detailed scheme of hydrolysis based on the structural data was proposed for the first time for family I soluble pyrophosphatases (Y-PPase [40] and E-PPase [41]). Soluble pyrophosphatases require 3–4 divalent metal ions for activity. Family I and III pyrophosphatases and membrane-bound PPases require a magnesium ion. Family II pyrophosphatases exhibit the highest activity in the presence of a manganese or cobalt ion.

Family I enzymes already contain two metal ions, M1 and M2, in the active site (activating metals) before substrate binding. These ions provide the conformation of the active site residues favourable for substrate binding and also coordinate a water molecule, which acts as a nucleophile and is one of the key participants of the reaction. The residue Asp67 (numbering for E-PPase) additionally assists in activating the nucleophile. Other one or two ions (M3 and M4) are bound simultaneously with the substrate (Fig. 8 *a*). Then the nucleophile attacks the electrophilic phosphate group of pyrophosphate coordinated by metal ions. The phosphate leaving group is coordinated by amino acid residues, in particular, by arginine. The hydrolysis and removal of two phosphate groups proceed at comparable rates.[42, 43]

The hydrolysis catalyzed by family II pyrophosphatases has specific features.[13] The mechanism of water activation involves three rather than two metal ions. The difference in the nature of the effector was considered above. High affinity of the enzyme for manganese and cobalt ions is attributed to the fact that two metal ions bound at the M1 and M2 sites are coordinated by histidine residues instead of aspartate, which serves this function in family I PPases (see Fig. 8 *b*). In addition, the coordination of the ion M2 changes during the catalysis, which is more easily achieved with transition metal ions than with Mg^{2+}. The position of the ion M3 also coincides with the position of the substrate metal ion bound at the M3 site in family I pyrophosphatases. However, it seems more appropriate to denote the additional metal ion, which has been labelled as M4,[13] in a somewhat different way (in Fig. 8 *b*, this ion is labeled as M*) because it forms one coordination bond with the substrate and one coordination bond with the nucleophile, whereas the ion M4 in other soluble and membrane-bound pyrophosphatases is bound only to the substrate, which acts as a bidentate ligand (see Fig. 8 *a*,*c*). There is also another type of bond between the phosphate leaving group of the substrate and the guanidine group of arginine (see Fig. 8 *a*,*b*). It is interesting that one of family I pyrophosphatases (from *Mycobacterium tuberculosis*) contains histidine residues in the active site, but these residues do not coordinate metal ions. One of these residues, His86, coordinates the phosphate leaving group at pH 5.0.[44] A magnesium ion is also more efficient than a manganese ion as an activator for this pyrophosphatase, although the difference in their efficiency is rather small compared with other

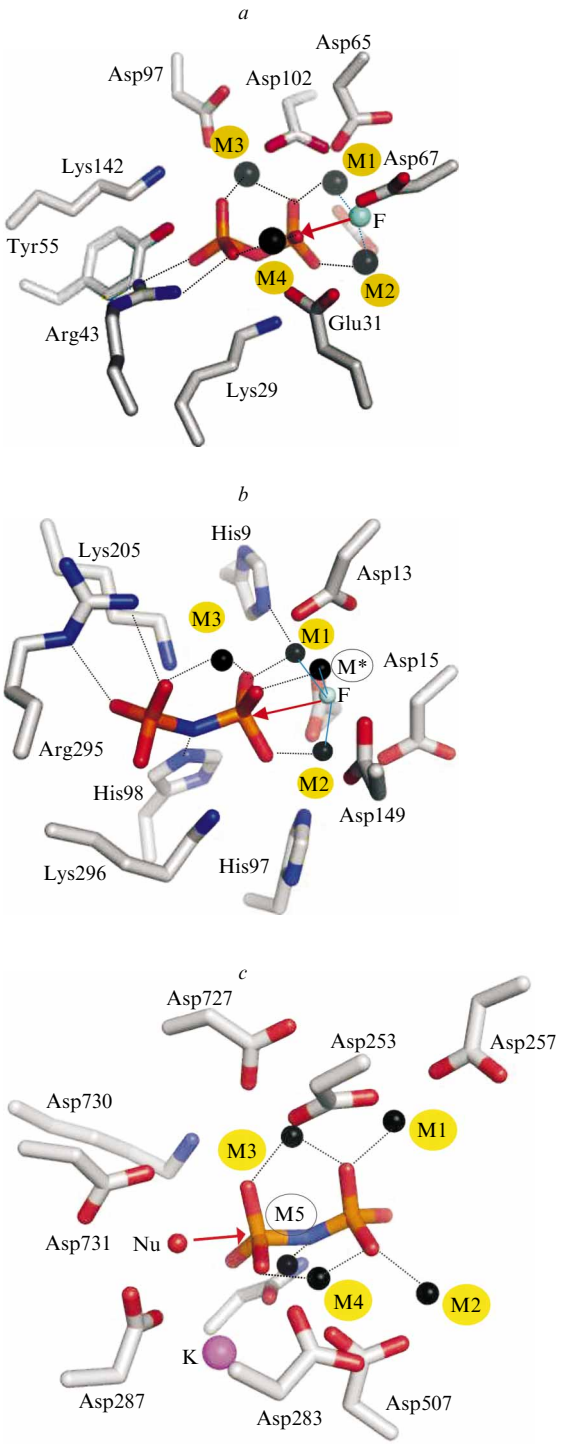


Figure 8. Active sites of soluble and membrane-bound pyrophosphatases (see the text).
(*a*) *E. coli* PPase (2AUU); (*b*) *B. subtilis* PPase (2HAW); (*c*) membrane-bound VrPPase (4A01). Substrates: pyrophosphate (*a*), imidodiphosphate (*b*, *c*).

representatives of the family. However, the catalytic activity of this enzyme was found to be lower (2.5 times lower than that of E-PPase), and the substrate specificity was found to be independent of the nature of the metal activator.[45] The residue His86 plays the same role as His98 in family II pyrophosphatase from *B. subtilis*. Therefore, *M. tuberculosis* PPase is an intermediate unit between family I and II pyrophosphatases.

Since the nucleophile is coordinated by metal ions, the fluoride ion, which replaces very easily the nucleophile, is a very efficient inhibitor. The exceptions are family II pyrophosphatases. In the latter enzymes, one of the metal ions is manganese or cobalt that weakly interacts with fluoride (see Fig. 8 *b*). Apparently, family II pyrophosphatases deprotonate water more efficiently than family I PPases due to the coordination of water simultaneously by three metal ions. The residue His98 can serve as a proton donor for the phosphate leaving group. Some distortion of the substrate structure and the above-described findings may be evidence of the dissociative mechanism.[46]

The mechanisms of action of family I and II PPases share common traits, whereas the mechanism of hydrolysis by membrane pyrophosphatases is substantially different. The catalytic site of *Vigna radiata* PPase (VrPPase) contains 5 metal ions: two activating ions, M1 and M2, two substrate metal ions and an additional fifth ion, which was called an inhibitory ion (see Fig. 8 *c*). The latter ion is present in the structure due to a high magnesium concentration in the crystallization solution.

The M3 and M4 sites, as well as the M1 site, are similar to those in E-PPase. The M2 site is rather largely shifted compared to the position of the activating metal M2 in the active site of the enzyme E-PPase. The distance between M1 and M2 in VrPPase is 1.8 Å longer than that in E-PPase. Therefore, these ions cannot bind a nucleophile (Nu). Presumably, the nucleophile is coordinated by two aspartates, Asp287 and Asp731, and is oriented *in line* with the phosphate group. These two aspartates are conserved in H^+-PPases, but they are replaced with Arg43 and Tyr141 in E-PPase. The F^- ion cannot displace a water molecule from this site due to the repulsion from the negatively charged aspartates. This accounts for the weaker inhibition by fluoride compared to soluble pyrophosphatases.

In addition, the active site contains a K^+ ion, which coordinates the electrophilic phosphate group and can partially neutralize its charge. The data on an increase in the affinity of Na^+- and Na^+,H^+-PPases for Na^+ ions in the presence of K^+ ions are indicative of the possible regulatory role of this metal.[17,47] Therefore, the role of metals in the hydrolysis catalyzed by membrane PPases is much smaller than in the hydrolysis catalyzed by related soluble enzymes. Their role is apparently to bind and position the substrate and to neutralize the charge of pyrophosphate.

The role of hydrolysis in the mechanism of cation transport is unclear. Three different mechanisms of transport were proposed; however, the available data are insufficient to unambiguously decide between them.[11] This is the subject of further structural investigations of membrane pyrophosphatases.

The mechanism of hydrolysis by family III soluble pyrophosphatases is poorly understood. However, according to preliminary data, this mechanism is somewhat different from the mechanism of action of other soluble pyrophosphatases.[5] The first discovered representative of family III, *Thermococcus onnurineus* pyrophosphatase, is activated by nickel ions,[4] while magnesium and cobalt ions are less efficient. An insignificant activity is observed in the presence of other divalent metal ions (Cu^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}). A pyrophosphate ion and Mg^{2+} were modelled in

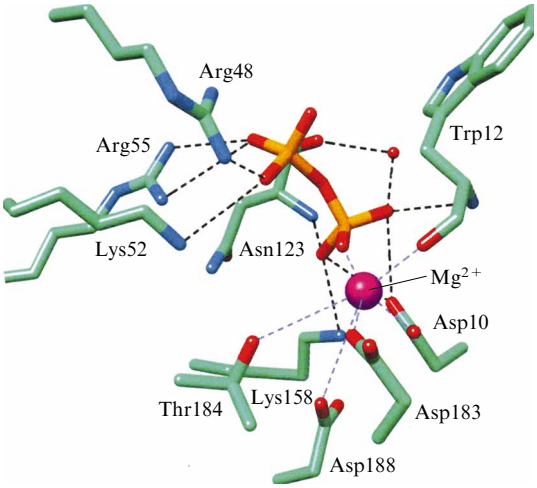


Figure 9. Active site of *B. thetaiotaomicron* PPase (pyrophosphate was modelled).[5]

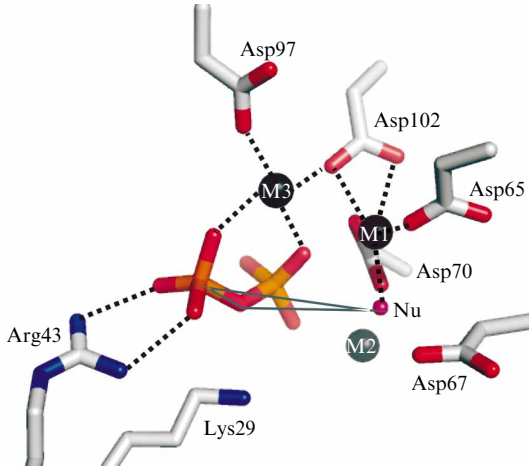


Figure 10. Active site of *E. coli* PPase (1I6T) with potassium pyrophosphate.

the active site (Fig. 9) by superimposing the structure of *B. thetaiotaomicron* PPase BT2127 and the structure of the complex of *P. horikoshii* PPase.[5] An analysis of this model suggests that the mechanism of hydrolysis shares common traits with the mechanism of hydrolysis by family I and II soluble PPases. In three structures of family III PPases, a calcium or magnesium ion occupies the position similar to the M1 site. In the model of pyrophosphatase BT2127, this metal ion is coordinated by carboxyl groups of three aspartic acid residues — Asp10, Asp183 and Asp188. This metal ion is additionally bound to Thr184 and the main-chain oxygen atom of Trp12. The phosphate leaving group is coordinated by two arginine residues (Arg55 and Arg48) and Lys52. One of the arginine residues can play the same role as in the active site of family I and II pyrophosphatases. Thus, it assists in releasing the phosphate ion from the active site. The electrophilic phosphate group is coordinated by Asn123 and Lys158. Data on the number of additional metal ions required for the hydrolysis and the type of their binding are lacking.

V.2. Combination of structural and physicochemical methods for investigation of the mechanism of action of soluble pyrophosphatases

The main drawback of X-ray diffraction methods is that they give a static image of the active site, which researchers can obtain in experiments with enzymes. Enzymatic reactions involving proteins proceed at a very high rate, and it is difficult, if at all possible, to detect the substrate binding or conversion in the crystal. The soaking is the most rapid method used for the preparation of a protein–substrate complex; however, it requires at least a few seconds, which is often a too long period. Standard procedures for the determination of the position of the substrate in the active site are based on mutations or the use of analogues of the substrate or inhibitors, which do not hinder substrate binding but prevent further conversions. The latter approach can be employed to elucidate the mechanism of inhibition of enzymes, which is of scientific interest by itself and often has practical applications. Two main pyrophosphatase inhibitors — a calcium ion and a fluoride ion — were used in studies of the enzymatic reaction, as well as for the determination of the mechanism of inhibition of PPases by X-ray crystallography.

A calcium ion is bound in the active site by the same ligands as metal activators. The X-ray diffraction study of the complex of *E. coli* pyrophosphatase with calcium ions and a pyrophosphate ion showed that the active site of the enzyme binds 3 calcium ions and pyrophosphate.[41] Due to a large size of calcium ions and the bidentate coordination of the ion M1 by the residue Asp102, pyrophosphate is forced to occupy the position such that the nucleophilic attack is sterically impossible (Fig. 10).[41,47]

Another inhibitor of PPases, a fluoride ion, allowed the researchers to analyze the substrate binding step.[40,48,49] Due to the replacement of the nucleophile by a fluoride ion, the arrangement of the substrate and metal ions is the same as that required for the hydrolysis. Nonhydrolyzable analogues of pyrophosphate mimic the substrate, but the scope of their application for investigation of the reaction mechanism is limited. The advantage of the use of a fluoride ion is the reversibility of its inhibitory action. This property was employed to obtain data on intermediates steps of the catalytic reaction using one crystal of E-PPase.[49] An X-ray diffraction data set was collected at 100 K from a crystal of the complex of *E. coli* PPase with pyrophosphate, manganese ions and a fluoride ion. Then the same crystal was placed in a cryosolution, which did not contain fluoride ions. This led to the washing out of fluoride ions from the crystal and the replacement of these ions with the nucleophile, thus triggering the hydrolysis. After a brief soak, the crystal was again flash-frozen and the second X-ray diffraction set was collected, and then the procedure was repeated once again. As a result, the crystal structures provide snapshots of the active site at three distinct steps: the substrate binding (Fig. 11 *a*), the intermediate state, in which a part of pyrophosphate is hydrolyzed (see Fig. 11 *b*), and the final structure with the electrophilic phosphate group remaining in the active site (Fig. 11 *c*). According to the proposed mechanism of pyrophosphate cleavage, the attacking nucleophile activated by the metals M1 and M2 is oriented *in line* with the phosphorus atom of pyrophosphate. In the transition state, the phosphorus atom that is attacked has a trigonal-bipyramidal coordination with the

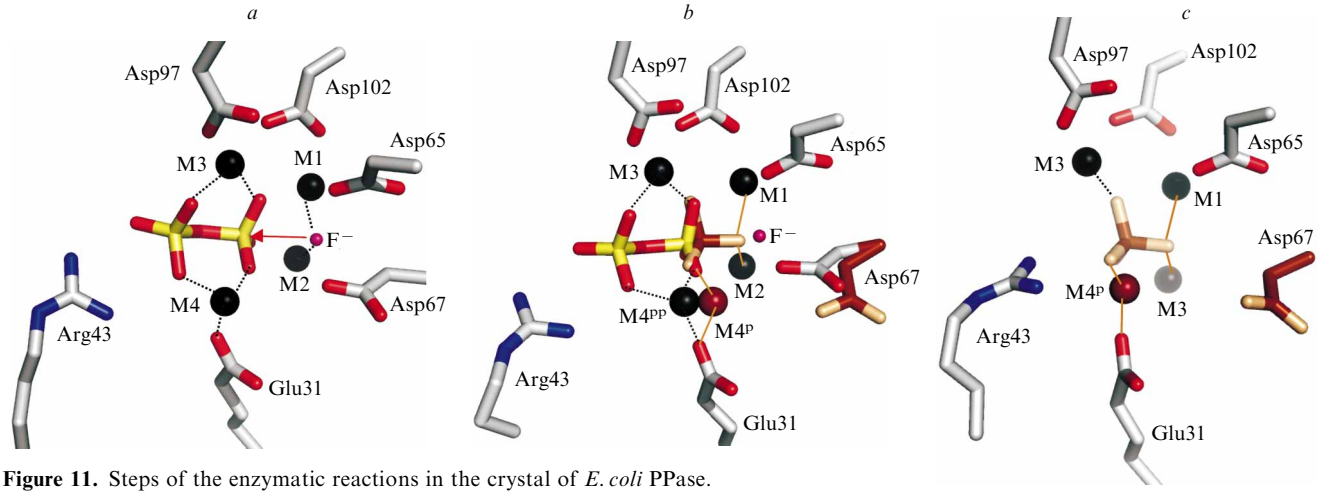


Figure 11. Steps of the enzymatic reactions in the crystal of *E. coli* PPase.
(*a*) The MnPPi substrate (100%) and a fluoride ion (2AU9); (*b*) partially hydrolyzed MnPPi, a fluoride ion and a phosphate ion (2AU6) (the M4 metal sites corresponding to the binding of pyrophosphate and phosphate are labelled as $M4^{pp}$ and $M4^{p}$, respectively); (*c*) the product (2AU7).

bridging oxygen atom and the nucleophile in apical positions. Then the oxygen atom from the attacking nucleophile enters the electrophilic phosphate group, which has a reverse configuration compared to that in pyrophosphate.

The near-atomic resolution of the second structure provides a detailed information about the changes in the active site upon substrate cleavage (see Fig. 11 *b*). Initially, the active site contains 100% of pyrophosphate and four manganese ions with full occupancy. The structure corresponding to the intermediate state of the enzyme contains 45% of pyrophosphate and the electrophilic phosphate group with occupancy of 55%. This is direct evidence that the phosphate leaving group is the first to release from the active site. An analysis of the active site and the occupancies of the ion M4 disordered over two positions showed that the substrate cleavage is accompanied by the shift of the metal M4, the bond with the phosphate group being retained. This also leads to a change in the position of Asp67, which coordinates the nucleophile or fluoride that replaces the nucleophile, such that this residue is linked to the oxygen atom of the electrophilic phosphate group through a water molecule. The third structure in this series characterizes the hydrolysis step, at which pyrophosphate is completely cleaved. The active site contains a phosphate ion and four metal ions. The occupancies of the M1 – M4 sites and the binding site for the phosphate leaving group are 85%. The occupancy of M4 is 60%, and the position of this atom is the same as in the structure containing a mixture of the substrate and the product. The phosphate group remains to be linked through an H_2O molecule to Asp67, which also changes its position.

The above-considered study has been made possible due to the unique stability of pyrophosphatase crystals to various handling conditions and it also demonstrated that PPases are catalytically active in the crystal.

The use of a fluoride ion as an inhibitor made it possible to obtain structures corresponding to the step of substrate binding for Y-PPase.[40] An analysis of these structures not only elucidated the role of essential active site residues in the mechanism of hydrolysis but also revealed the difference in the behaviour of large structural elements, which contain the catalytically important conserved residues, during the process.

V.3. Structural plasticity of pyrophosphatases for the mechanism of hydrolysis

Events taking place at the active site of the enzyme are accompanied by the reorientation of some active site residues. The latter occurs not only due to small movements of the residues but also often due to the movement of structural elements or their parts. It seems likely that the structural diversity of pyrophosphatases is responsible for different types of structural changes associated with the altered mechanism of hydrolysis. The substrate binding is the step that causes the most significant changes.

In family I pyrophosphatases, the residues Asp67, Asp42 and Arg43 undergo reorientation and the flexible loops 64 – 70 and 96 – 102 (the numbering of residues for E-PPase) containing the catalytically important residues Asp70 and Asp102 are shifted by almost 1 Å (Fig. 12 *a*) so that the metal ions M1 and M2 come close to each other (due to the shift of M1) and simultaneously coordinate the nucleophile, as well as the substrate.[40, 41, 49]

Family II pyrophosphatases undergo a more substantial rearrangement. The active site of these two-domain enzymes is located at the interface between the domains. Upon substrate binding, the domains come close to each other, and the conformation changes from open to closed (see Fig. 12 *b*). This is accompanied by the rotation by 70° around the flexible linker between two domains serving as a hinge, resulting in that the end of the C-terminal domain is displaced by ~37 Å towards the N-terminal domain.[46] Despite the fact that the structures of family III pyrophosphatases with pyrophosphate or its analogue are unknown, the analysis of the structures of *B. thetaiotaomicron* pyrophosphatase BT2127, including its complex with the product, and the modelling of the substrate suggested[5] that the hydrolysis causes the domains to come closer to each other (see Fig. 12 *c*), as in the case of family II enzymes, although this movement is much smaller. It was shown that, upon the superposition of the structure with the metal ion and the structure containing the product, the displacements of some secondary structure elements are as large as 3.6 Å.

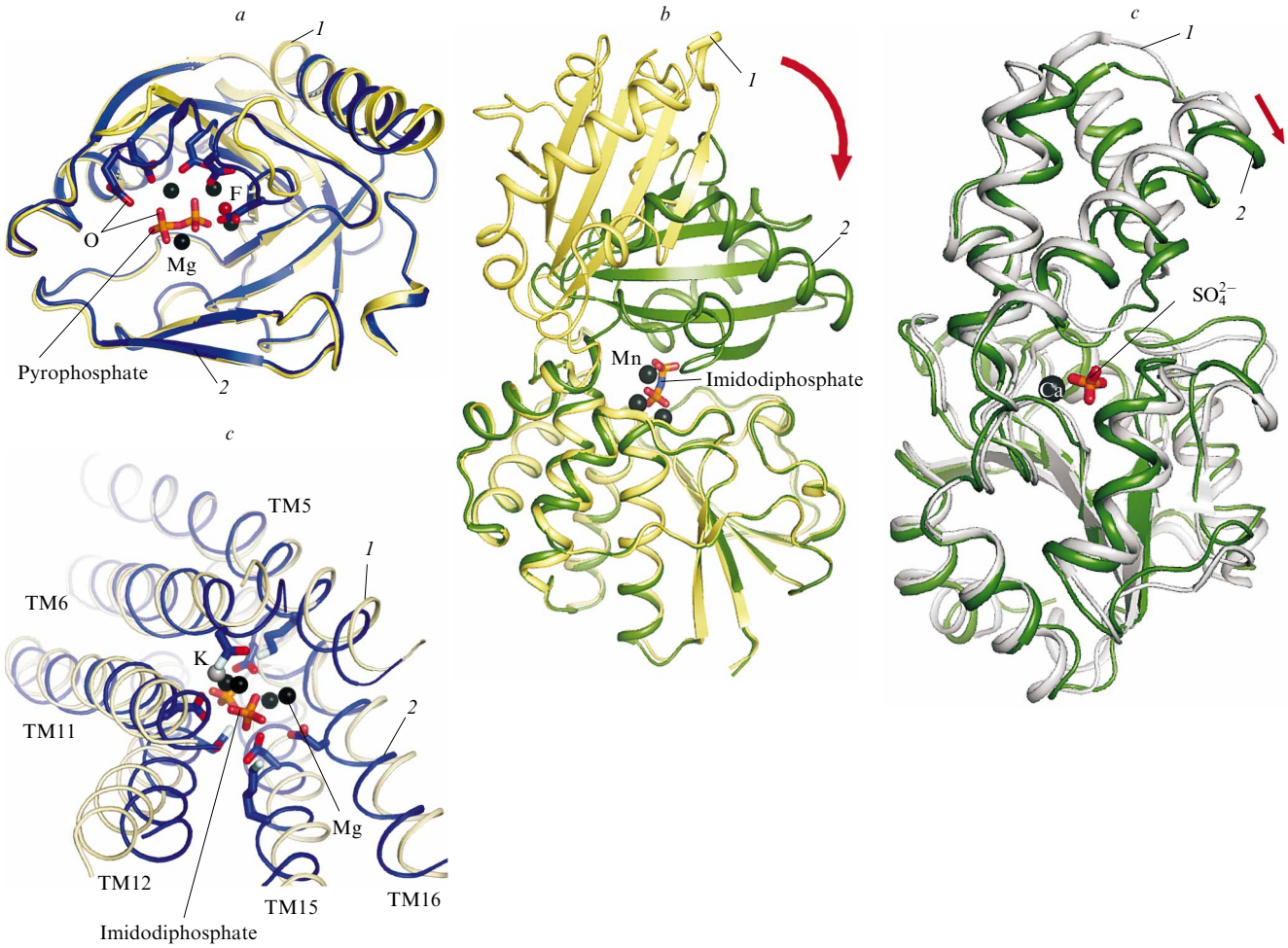


Figure 12. Mobility of structural elements of soluble PPases of different families and membrane-bound PPases upon the binding of the substrate (product) and its analogues. The superposition of the structures of the apo or holo enzymes and the structures of the enzyme–substrate (product) complexes.
(*a*) *E. coli* PPase: the holo form (1I40) (*1*) and the protein backbone as a ribbon diagram for the complex with magnesium pyrophosphate and fluoride (2AUU) (*2*); (*b*) *B. subtilis* PPase: the apo form (1K23) (*1*) and the protein backbone as a ribbon diagram for the complex with mangenese and imidodiphosphate (2HAW) (*2*); (*c*) *B. thetaiotaomicron* PPase: the open conformation of the holo enzyme (3QUQ) (*1*) and the protein backbone as a ribbon diagram for the closed conformation of the complex with an analogue of the product (3QU7) (*2*); (*d*) membrane-bound pyrophosphatases: the holo form of TmPPase (4AV3) (*1*) and the the protein backbone as a ribbon diagram for the complex of VrPPase with magnesium, potassium and imidodiphosphate (4A01) (*2*); the main active site residues that interact with the substrate analogue and metal ions are shown.

The secondary structure of membrane-bound pyrophosphatases essentially differs from that of soluble PPases. The substrate binding is accompanied by the ordering of the loop between the fifth and sixth transmembrane helices and a change in the positions of the transmembrane helices which contain catalytically important residues (see Fig. 12 *d*). The fragments TM5, TM6, TM11 and TM12 move toward the active site cavity, resulting in that it becomes more compact. The upper part of TM8 undergoes the largest displacement (by 5 Å). Due to these changes induced by substrate binding, membrane pyrophosphatases become more resistant to proteolysis and modification by *N*-ethylmaleimide.[20] Presumably, the conformational changes induced by the binding of pyrophosphate by membrane pyrophosphatases are closely related to the mechanism of cation transport.[7]

VI. Interactions of family I soluble pyrophosphatases with other proteins

The formation of binary complexes of PPase *E. coli* with other proteins has been described recently.[50] Protein–protein interactions are functional events in biological systems and they play a key role in most of cellular processes. Generally, the complexation leads to mutual modifications of the functions of interacting partners. Therefore, the investigation of these modifications is important for an understating of the cell functioning as a complex dynamic system.

Since the pyrophosphatase activity is essential for cell viability, this enzyme is a possible participant of protein–protein interactions. The interactions of *E. coli* PPase with three proteins — fructose-1,6-bisphosphate aldolase I (FbaB), L-glutamate decarboxylase (GAD) and 5-keto-4-deoxyuronate isomerase (KduI) — were found by the pull-

down assay with the identification of the proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and were confirmed by the sedimentation velocity analysis and dynamic light scattering.[50] The formation of complexes was also confirmed by calorimetry. All three proteins are involved in carbohydrate and amino acid metabolism. The enzyme FbaB is a lyase that catalyzes the reversible conversion of fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Potassium ions have a beneficial effect on the interaction of FbaB with PPase, whereas MgATP (ATP is adenosine triphosphate) exerts an adverse effect. The enzyme KduI is involved in pectin catabolism by catalyzing the isomerization of 5-5-keto-4-deoxyuronate to 2,3-diketo-3-deoxygluconate. This is the second, after FbaB, enzyme of sugar metabolism identified as a partner of PPase. The enzyme GAD is a pyridoxal phosphate-dependent enzyme that catalyzes the conversion of L-glutamate into 4-aminobutyrate and carbon dioxide. This enzyme is utilized by one of stress regulatory systems.

In principle, it would be expected that pyrophosphatases will form protein–protein complexes with enzymes that catalyze reactions giving pyrophosphate as a side product. However, such PPase complexes have not been found. Pyrophosphatases can be involved in sugar metabolism through transmembrane transport. A large fraction of sugars is transferred in the cell in the form of phosphates. Pyrophosphatase producing phosphate can be indirectly related to such transport, which involves the chain of reactions, providing the substrate for central metabolism enzymes. Another possible role of PPases in the complexes with FbaB, KduI and GAD is to stabilize these enzymes and protect them against unfolding and subsequent proteolytic degradation. This is confirmed by high thermal stability of the PPase–FbaB complex and a broader pH stability range of the GAD–PPase complex compared to the individual proteins. Since proteins are involved in the stress regulatory system, this suggestions seems highly probable.[50]

It cannot be ruled out that this stabilization is favourable for the compact quaternary structure of *E. coli* PPase typical of all bacterial PPases, as discussed in Section IV.

VII. Possible practical applications of pyrophosphatases

Due to the unique structure of family I soluble pyrophosphatases responsible for their thermal stability, as well as owing to an essential role in the regulation of many cellular processes, these enzymes are used in biotechnology. In numerous biosynthetic reactions driven by hydrolysis of nucleoside triphosphate to nucleoside monophosphate and pyrophosphate, the PPase activity is essential for the removal of the pyrophosphate that formed. Thermally stable family I pyrophosphatase from *P. horikoshii* is active at high temperatures (70–95° C). The activity of this protein produced by the recombinant technology is used in the polymerase chain reaction (PCR) owing to its ability to shift the chemical equilibrium toward the DNA synthesis by removing pyrophosphate from the reaction.[51] Therefore, the application of thermally stable PPase may help in overcoming limitations associated with the fact that DNA polymerization in PCR is thermodynamically unfavourable and, consequently, larger amounts of the product can be obtained. The calorimetric study using molybdate and oxidizing agents showed that family I pyrophosphatase from *P. horikoshii* substantially increases the PCR product yield compared to a mixture which does not contain pyrophosphatase after the same number of cycles. Due to stability of *E. coli* pyrophosphatase and the possibility to detect this enzyme with high sensitivity, it can be employed as a marker in enzyme immunoassay.[52]

Based on the results of investigations of plant pyrophosphatases, it was suggested that these enzymes can be applied in agricultural biotechnology.[53, 54] In the agricultural production, phosphorus is one of the most limited microelements. In the soil, orthophosphate exists mostly as insoluble calcium, iron and aluminium salts inaccessible to plants. The overexpression of *Arabidopsis thaliana* H^+-pyrophosphatase in rice and tomatoes leads to enhanced phosphorus uptake in plants.[53] Rice is the staple food for more than half the world's population, the demands for the rice production being steadily increasing. The genetically modified plant line with overexpression of *Arabidopsis* H^+-pyrophosphatase exhibited enhanced sprout growth under phosphorus deficiency conditions compared to the control. The overexpression of the E229D mutant of *Arabidopsis* H^+-pyrophosphatase led to the development of a more robust root system both under phosphorus deficiency conditions and in the absence of this deficiency. Roots and sprouts of plants with overexpression of the E229D mutant of *Arabidopsis* H^+-pyrophosphatase were larger under phosphorus deficiency conditions compared to control plants.[53] Besides, the increased expression of the *Arabidopsis* vacuolar pyrophosphatase gene was found to lead to a larger root system for transgenic cotton. As a result, these roots more efficiently absorb water under drought and saline conditions.[54] The main mechanism apparently relies on an increase in the proton pump activity, which energizes the vacuolar membrane and thus accelerates sodium transport to avoid accumulation of sodium ions to the toxic level in the cytoplasm.[54] The results of these studies are important for the development of climate-resilient crops.

The discovery of manganese-dependent psychrophilic *Shewanella* sp. AS-11 pyrophosphatase, which is not prone to cold denaturation and is highly active at low temperatures, may be of practical value for food industry, where it is essential to prevent modifications of heat-sensitive substrates and finished products.[55]

The overexpression of human pyrophosphatase is associated with the migration and, probably, gastric cancer cell invasion. This enzyme was found in cells of patients with metastatic lymph nodes, as well as in late stages and the recurrent disease. Therefore, PPase is a potential biomarker for poor prognosis in patients with this type of cancer.[56]

* * *

Despite a rather long period of investigation of pyrophosphatases and extensive data on these enzymes, the findings in the past decade (discovery of a new family of PPases, as well as of new representatives of known families; the X-ray crystallography of membrane-bound PPases) suggest that investigations of these enzymes will be extensively developed. The potential of the application of pyrophosphatases in biotechnology and medicine is also still not fully understood. Family II pyrophosphatases and membrane-bound pyrophosphatases are of interest as potential targets for drug therapy, because many bacteria containing these enzymes belong to pathogens.[6, 10] Apparently, further structural studies of this class of enzymes combined with avail-

able data on the mechanism of their action, modern biochemical methods and genetic engineering techniques hold great promise for practical applications of pyrophosphatases.

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