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SHORT REPORT

Family II pyrophosphatases from photosynthetic bacteria can hydrolyze free pyrophosphate

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Abstract: The hydrolytic activity from three partially purified family II pyrophosphatases from the photosynthetic bacteria *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodovulum sulfidophilum*, as well as the recombinant cytoplasmic pyrophosphatase from *Rba. sphaeroides*, was tested with Mg^{2+}–PPi, Mn^{2+}–PPi, and PPi^{4-}. Unlike family I pyrophosphatases that hydrolyze only the Mg^{2+}–PPi complex like those from *Rhodospirillum rubrum*, all family II enzymes tested showed hydrolytic activity with Mg^{2+}–PPi, Mn^{2+}–PPi, and PPi^{4-} without cation. The activity without added cation remained the same, even under exhaustive dialysis or after desalting the enzyme through a Sephadex G-25 column. However, this activity disappeared upon the addition of ethylenediaminetetraacetic acid and could not be restored by adding Mg^{2+}. Moreover, the enzyme inactivation was not related to dissociation into lower molecular subunits as in other family II enzymes. This is the first report on pyrophosphatases that can hydrolyze pyrophosphate without a divalent cation added and that presumably contain a tightly bound divalent cation in their structure.

Keywords: cytoplasmic pyrophosphatases, metal cofactors, inorganic pyrophosphate, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*

Introduction

Soluble inorganic pyrophosphatases (EC 3.6.1.1; sPPase) are enzymes that specifically catalyze the rupture of pyrophosphate (PPi) into two molecules of orthophosphate (Pi). This reaction is essential for cell metabolism, as it provides thermodynamic pull for many biosynthetic reactions.[1] Two families of sPPases (family I and family II), which are structurally different, genetically distant,[2,3] and with similar catalytic mechanisms,[4] have been found to date, and despite the great difference in sequence identity, both families have similar structures in the arrangement of their active site ligands and residues.

In prokaryotes, family I sPPases are homohexameric enzymes with a monomeric molecular mass of ~20 kDa and a highly conserved cuplike active site structure, formed by 14–16 aa residues and three to four Mg^{2+} ions. By contrast, family II sPPases are homodimeric enzymes formed by large subunits of ~34 kDa; every subunit folds into two domains in which the active site is located at the interface. In addition, all family II sPPases have the "DHH" aa signature in their active sites, similar to those of phosphoesterases.[5]

In order to be able to hydrolyze, both sPPase families require divalent metal cations (M) that mediate the protein–PPi interactions.[6] First, divalent cations form a complex that activates the enzyme (M–E), and then, divalent cations form a complex with pyrophosphate that forms the substrate (M–PPi). In family I sPPases, Mg^{2+} is

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preferred as the enzyme activator in contrast to family II, in which Mn^{2+} is preferred over Mg^{2+}.[7] It has been suggested that the pattern of catalysis for these enzymes is similar,[5] and it consists of five steps: first, the formation of the activated complexes E–M and M–PPi; second, the binding of the substrate to the preformed enzyme-activated complex; third, isomerization of the resulting complex; fourth, breakdown of the phosphoanhydride bound by a water nucleophile, and finally, the dissociation of two Pi molecules.

Despite that enzymes from both families share the same hydrolytic pattern, their functional properties are notably different. In this article, we report that family II pyrophosphatases from the photosynthetic bacteria *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodovulum sulfidophilum* can hydrolyze substrates that do not preform an M–PPi complex, but this is not the case for the *Rhodospirillum rubrum* family I pyrophosphatase that needs to preform an M–PPi complex in order to hydrolyze its substrate.

Materials and methods

The chemicals used in these experiments were purchased from Sigma-Aldrich Co. and J.T. Baker. All chemicals were of analytical grade or higher.

Photosynthetic bacterial cultures

The photosynthetic strains of *Rba. sphaeroides* 2.4.1, *Rba. capsulatus* DSM 1710, *Rdv. sulfidophilum DSM* 1374, and *Rsp. rubrum* ATCC 11170 were grown anaerobically, under a tungsten lamp of 40 W at 30 cm in the medium, which was previously described by Cohen-Bazire and Kunisawa.[8] Once bacterial cultures were in the late exponential phase, the cells were harvested and washed with 50 mM 3-(*N*-morpholino) propanesulfonic acid and 50 mM KCl, pH 7.5. Liquid medium was removed by centrifugation, and the pellet obtained (wet weight) was stored at −80°C.

Extraction and partial purification of cytoplasmic pyrophosphatases

Rsp. rubrum family I pyrophosphatase was partially purified as previously described by Romero et al.[9] *Rba. sphaeroides*, *Rba. capsulatus*, and *Rdv. sulfidophillus* family II pyrophosphatases were partially purified as reported by Celis et al.[10]

Construction of an *Escherichia coli* strain that overproduces His_6-sPPase from *Rba. sphaeroides*

In order to obtain the recombinant His_6-sPPase from *Rba. sphaeroides*, the corresponding 305 aa from the DNA region encoding the PPase was amplified by polymerase chain reaction using the following primers: Rspp Fw 5′ *ctg aat tca tga tca agg tat tcg gcc aca* 3′ and Rspp Rv 5′ *gca agc ttt cag agc ttg agg acc gga atg* 3′. The amplified region was inserted between the restriction sites *Eco*RI–*Hin*dIII into the pBAD/HisB plasmid. Then, a strain of *Escherichia coli* Rosetta was transformed by electroporation with the mentioned plasmid. Recombinant His_6-sPPase protein was overproduced as described by Suaste-Olmos et al.[11]

Purification of His_6-sPPase

The transformed Rosetta/pFSO/pPIRL strain was grown in an Luria-Bertani broth (LB) LB medium with antibiotics (100 μg ampicillin·mL^{-1} and 25 μg chloramphenicol·mL^{-1}) at 37°C until the bacterial culture reached an OD_{600} of 0.6. Then, arabinose was added at 0.2% v/v final concentration. After 3 hours of incubation (37°C and shaken at 250 rpm), the cells were centrifuged at 5,524× *g* for 15 min, and the resulting pellet was resuspended in 50 mM Tris−HCl (pH 8.6), 10 mM $MgCl_2$ (buffer A), and 10 mM imidazole. Later, the cells were lysed with 200 μg lysozyme·mL^{-1} for 30 min and were disrupted by sonication (Branson Sonifier 250) with an output of 40% (80 W) applying five pulses of 10 s. Then, they were centrifuged to remove cellular debris at 15,200× *g* for 20 min. The supernatant fraction was mixed with Ni-NTA beads (Quiagen NV) and placed into a polypropylene column. Then, the mixture was washed 15 times with buffer A plus 20 mM imidazole, and finally, the recombinant protein was eluted with buffer A plus 200 mM of imidazole.

Pyrophosphatase assay

PPase assays were carried out at 37°C in a reaction medium containing 50 mM Tris–HCl (pH 8.6), 2 mM Na_4PPi (pH 8.6), and 3 mM $MgCl_2$ (modifications of these conditions are indicated in the figure legends). The reactions were initiated by adding the enzyme and arrested by the addition of trichloroacetic acid to a final concentration of 6% v/v. The hydrolyzed pyrophosphate was determined according to Sumner.[12] Proteins were measured by the method of Lowry et al,[13] using bovine serum albumin as a standard. All experiments were repeated at least three times.

Native polyacrylamide gel electrophoresis

Gel electrophoresis was performed as in Laemmli,[14] without sodium dodecyl sulfate and dithiothreitol; then, proteins were silver stained according to Bloom et al.[15]

Results and discussion

Divalent-cation requirement for PPase activity

It is well known that family II pyrophosphatases need divalent metal ions as necessary cofactors for hydrolysis, expressing their maximal hydrolytic rate with Mn^{2+} and Mg^{2+}. However, the cytoplasmic family II pyrophosphatase from the photosynthetic bacterium *Rba. sphaeroides* (Rs-PPase) can hydrolyze substrate without the addition of divalent cations to the reaction medium (Table 1). Family I pyrophosphatases from photosynthetic bacteria like *Rsp. rubrum* cannot hydrolyze pyrophosphate with Mn^{2+}, expressing their maximal hydrolytic rate with Mg^{2+}, and they certainly cannot function if the reaction medium does not contain divalent cations.[10]

The amount of hydrolyzed pyrophosphate by the partially purified Rs-sPPase when divalent cations were not added to the reaction medium (1,080.56 nmol $Pi^{-1}\cdot min^{-1}$ [mg of protein]$^{-1}$) was equivalent to the amount obtained when Mg^{2+} was added (1,100.5 nmol $Pi^{-1}\cdot min^{-1}$ [mg of protein]$^{-1}$; Table 1); this suggests the existence of divalent cations bound to the enzyme that may form the metal–substrate complex. Based on the above assumption, another two partially purified family II pyrophosphatases from the photosynthetic bacteria *Rba. capsulatus* (Rc-PPase) and *Rdv. sulfidophilum* (Rv-PPase) were depleted from free cations desalting the enzymes three times through a Sephadex G-25 filtration column. Then, the hydrolytic activities of the enzymes were tested with and without metal cofactors (Table 1). Despite the lack of externally added free divalent cations, all FII pyrophosphatases tested (Rs-PPase, Rc-PPase, and Rv-PPase) can hydrolyze pyrophosphate. Moreover, we know that if a PPi–Mg complex is not formed, the protein–PPi interactions are not being replaced by monovalent cations from the complex Na_4PPi; this is because the substrates K_4PPi, Li_4PPi, and Tris–PPi are hydrolyzed at the same rate (data not shown).

Table 1 Specific activity of family I (*Rsp. rubrum*) and family II (*Rba. sphaeroides*, *Rba. capsulatus*, *Rdv. sulfidophilum*) PPases, with and without divalent cation added at the hydrolysis reaction medium: 50 mM Tris–HCl (pH 8.6), 2 mM Na4PPi, and 3 mM of divalent cation

Bacteria		nmol $Pi\cdot min^{-1}$ (mg of protein)$^{-1}$	
		Control	Desalted with Sephadex G-25
Rsp. rubrum	–	0.00	
	$MgCl_2$	127.46	
	$MnCl_2$	0.00	
Rba. sphaeroides	–	1,080.56	1,400.8
	$MgCl_2$	1,100.5	1,002.1
	$MnCl_2$	1,400.1	1,500.2
Rba. capsulatus	–	1,320.8	920.46
	$MgCl_2$	1,410.1	1,460.0
	$MnCl_2$	1,010.3	1,006.8
Rdv. sulfidophilum	–	650.22	390.605
	$MgCl_2$	1,403.28	1,403.439
	$MnCl_2$	1,070.24	1,003.99

Note: The symbol "–" means that no cations were added at the reaction medium.
Abbreviations: *Rsp. rubrum*, *Rhodospirillum rubrum*; *Rba. sphaeroides*, *Rhodobacter sphaeroides*; *Rba. capsulatus*, *Rhodobacter capsulatus*; *Rdv. sulfidophilum*, *Rhodovulum sulfidophilum*.

Effect of ethylenediaminetetraacetic acid on the enzyme activity

Family II pyrophosphatases were cleaned from free divalent cations after running the enzyme three times through a Sephadex G-25 column. The hydrolytic activity of these cleaned enzymes is inhibited by ethylenediaminetetraacetic acid (EDTA) at low concentrations (Figure 1). All Rs-PPase, Rc-PPase, and Rv-PPase decrease their hydrolytic activity by 50% with 5 μM of EDTA, and with 100 μM of EDTA, the activity is almost completely lost. To discard the possibility that even a family I pyrophosphatase hydrolytic activity using Mg^{2+}–PPi as a substrate could be inhibited by small concentrations of EDTA, the *Rsp. rubrum* family I pyrophosphatase (Rr-PPase) was tested with increasing concentrations of EDTA. The enzyme was not inhibited even with a fivefold concentration to the one needed for the complete inhibition of the family II desalted enzyme. Owing to the hydrolytic activity without an added cation had not changed the three

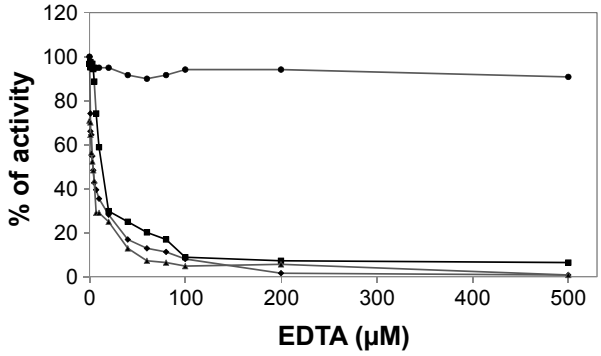

Figure 1 EDTA effect on the hydrolysis of pyrophosphatase.
Notes: Reaction conditions were 50 mM Tris–HCl (pH 8.6) and EDTA at the concentrations indicated on the abscissa. Different complexes were used as substrates: Mg–PPi (3 mM $MgCl_2$ and 2 mM Na_4PPi) in *Rsp. rubrum* (●) family I sPPase and PPi 2 mM without divalent cation added in *Rba. capsulatus* (■), *Rba. sphaeroides* (♦), and *Rdv. sulfidophilum* (▲) family II sPPases. For family I pyrophosphatase, 100% is equivalent to 127 nmol $Pi\cdot min^{-1}$ (mg of protein)$^{-1}$, and for family II pyrophosphatases, 100% is equivalent to 1,240 nmol $Pi\cdot min^{-1}$ (mg of protein)$^{-1}$.
Abbreviations: EDTA, ethylenediaminetetraacetic acid; *Rsp. rubrum*, *Rhodospirillum rubrum*; *Rba. capsulatus*, *Rhodobacter capsulatus*; *Rba. sphaeroides*, *Rhodobacter sphaeroides*; *Rdv. sulfidophilum*, *Rhodovulum sulfidophilum*.

times dialyzed and desalted enzyme (1,110 nmol Pi·min^{-1} [mg protein]$^{-1}$ at the beginning to 950 nmol Pi·min^{-1} [mg protein]$^{-1}$ in the end) in which we calculated that only 1.2×10^{-18} M of Mg^{2+} remains; it is possible that inhibition by EDTA is caused by chelation of a tightly bound divalent cation, available from the enzyme structure (not the media), that serves as a cofactor for the enzyme or as a part of the substrate inside the active site. To know whether the cation may reassociate with the enzyme under further dialysis of the chelate, the recombinant *Rba. sphaeroides* (rRs-PPase) was treated with EDTA and subsequently dialyzed. It is worth noting that once inhibited, the rRs-PPase by the chelate, the hydrolytic activity cannot be restored either by EDTA dialysis or by adding Mg^{2+} (Figure 2). In this case, the irreversible loss of activity may lay in the fact that in some reports of family II pyrophosphatases such as *Basillus subtillis*, *Streptococcus gordonii*, and *Streptococcus mutans*, in the absence of metal ions (2 mM EDTA present), the sedimentation velocity of the enzymes decreased markedly, indicating dissociation into lower molecular mass species.[16]

However, regardless of the amount of EDTA added at the recombinant *Rba. sphaeroides* family II pyrophosphatase, the dissociation into lower molecular mass subunits does not occur (Figure 3), as judged by the native electrophoresis, suggesting that the quaternary integrity of the enzyme is maintained even after inactivation with EDTA and cation unavailability.

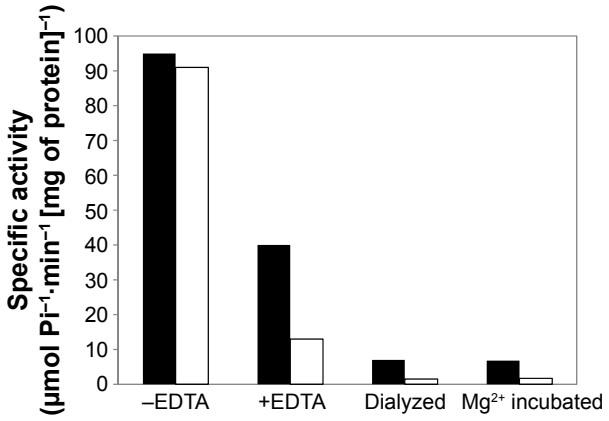

Figure 2 EDTA effect on the *Rba. sphaeroides* recombinant sPPiase activity.
Notes: Black bars represent reactions with 3 mM $MgCl_2$ added, and white bars represent reactions in which no divalent cation was added. –EDTA represents hydrolysis reaction without EDTA. +EDTA represents hydrolysis reaction with 100 mM EDTA-preincubated enzyme for 15 min at room temperature. Dialyzed represents EDTA-preincubated enzyme dialyzed against 20 mM Tris–HCl, pH 8.6, and 0.1 mM $CoCl_2$. Mg^{2+} incubated represents dialyzed enzyme, incubated with 7 mM $MgCl_2$ for 7 days at 8°C. Reaction conditions were 50 mM Tris–HCl (pH 8.6), 2 mM Na4PPi, and EDTA. Reaction incubation temperature was 37°C.
Abbreviations: EDTA, ethylenediaminetetraacetic acid; *Rba. sphaeroides*, *Rhodobacter sphaeroides*.

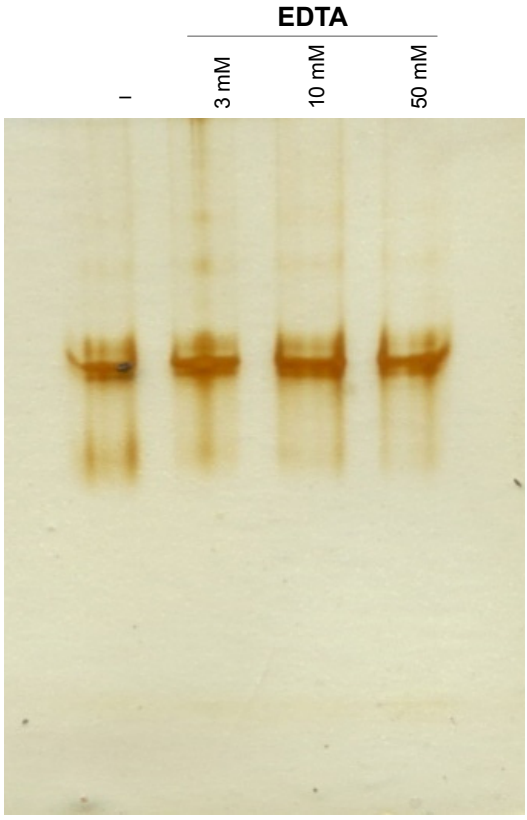

Figure 3 Silver-stained native PAGE of the recombinant *Rba. sphaeroides* sPPase preincubated with different concentrations of EDTA.
Abbreviations: PAGE, polyacrylamide gel electrophoresis; *Rba. sphaeroides*, *Rhodobacter sphaeroides*; EDTA, ethylenediaminetetraacetic acid.

Finally, it is well known that EDTA chelates divalent cation and its use in reports in which the role of divalent cations is discussed are plenty. Since the first characterization of a family II enzyme from *B. subtillis*[17] was stated that EDTA causes disruption of subunits and subsequently loss of activity, this is the case for another two family II pyrophosphatases mentioned earlier, *S. gordonii* and *S. mutans*, that shows the same behavior as *B. subtillis*, including the fact that inhibition caused by EDTA can be reverted.

We do not have detailed mechanistic explanation about how the pyrophosphate is hydrolyzed without added divalent cations, why the quaternary structure of our enzyme is maintained in the presence of EDTA or why the loss of activity cannot be restored even with exhaustive dialysis and divalent cation supplied. Only solving the crystal structures of these enzymes with and without divalent cations may reveal if they can hydrolyze free pyrophosphate using a structural tightly bound cation that shields the charge from this highly electronegative molecule. What we already know is that another structural cation is also present in the photosynthetic bacterium family II pyrophosphatase from *Rba. capsulatus*;[18]

proton-induced X-ray emission analysis showed that three Co^{2+} ions are coupled in the enzyme, but no localization in the structure is determined. We think, based on non-published data, that Co^{2+} plays a protective role against chelation with EDTA, and this is why we are currently working on solving the crystal structures from this enzyme.

Conclusion

Although the structure of family II pyrophosphatases differs greatly from that of family I, yet they catalyze the same hydrolysis reaction with apparently the same mechanism. However, unlike family I PPases, the *Rba. sphaeroides* family II PPase, as well as other photosynthetic bacterium pyrophosphatases belonging to the same family, can hydrolyze PPi presumably with a tightly bound divalent cation in its structure, without preforming a PPi–divalent cation substrate complex.

Apparently, the tightly bound divalent cation from *Rba. sphaeroides* family II PPases is not related to the enzyme quaternary structure stability, since its dissociation into lower molecular subunits was not observed in the presence of EDTA, as in other non-photosynthetic bacterium family II pyrophosphatases.

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Disclosure

The authors report no conflicts of interest in this work.

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