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Regulation of divalent cations of the membrane-bound pyrophosphatase of *Rhodospirillum rubrum*, as shown by the hydrolysis of tripositive-pyrophosphate complexes

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Tripositive-pyrophosphate [M(III)–PPi] complexes were used to investigate the role of free divalent cations on the membrane-bound pyrophosphatase. Divalent cations remain free and the M(III)–PPi complexes were employed as substrates. Formation of a La–PPi complex was studied by fluorescence, and the fact that Zn^{2+} and Mg^{2+} remain free in the solution was validated. Hydrolysis of La–PPi is stimulated by the presence of fixed concentrations of free Mg^{2+} or Zn^{2+} and this stimulation depends on the concentration of the cations when the La–PPi complex is fixed. The divalent cation stimulation order is $Zn^{2+} > Co^{2+} > Mg^{2+} > Mn^{2+} > Ca^{2+}$ (at 0.5 mM of free cation). With different M(III)–PPi complexes, Zn^{2+} produces the same K_m for all the complexes and Mg^{2+} stimulates with a different K_m. The results suggest that both Mg^{2+} and Zn^{2+} activate the membrane-bound pyrophosphatase but through different mechanisms.

Keywords: membrane-bound pyrophosphatase, *Rhodospirillum rubrum*, regulation, tripositive-pyrophosphate complexes

Introduction

Chromatophores of *Rhodospirillum rubrum* contain a membrane-bound pyrophosphatase (EC 3.6.1.1) that catalyzes the synthesis and hydrolysis of pyrophosphate coupled to the electrogenic translocation of protons in a fully reversible process (Baltscheffsky *et al*. 1966, Baltscheffsky 1978). Like most inorganic pyrophosphatases, either cytoplasmics or membrane-bound, this pyrophosphatase requires Mg^{2+} to form the real Mg–PPi^{2-} substrate for the hydrolysis and Mg–Pi for the synthesis (Lahti 1983). It has been suggested that Mg^{2+} exerts a regulatory action on the catalytic properties of membrane-bound pyrophosphatase (Randahl 1979, Celis *et al*. 1985). Celis & Romero (1987) suggested that the enzyme could have a site for free Mg^{2+}, which could also be used for other divalent cations (Zn^{2+}, Co^{2+}) inducing changes in the kinetic properties of the enzyme.

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Recent data demonstrate that free Mg^{2+} is an essential activator of the hydrolytic reaction and increased concentrations of Mg^{2+} produce a higher affinity for the substrate (Sosa *et al*. 1992).

In this work, we use a tripositive metal ion–pyrophosphate complex [M(III)–PPi] as the enzyme substrate, which has a higher stability constant than Mg–PPi or Zn–PPi complexes; hence, the divalent cations remain in free form, allowing us to substantiate the role of free divalent cations upon the enzyme. The data show that free divalent cations, such as Mg^{2+} or Zn^{2+}, exert an activator effect on the enzyme.

Materials and methods

Wild-type *R. rubrum* (ATCC 11170) were grown anerobically under light (tungsten lamps of 40 W at 30 cm) at 30 °C in the medium described by Cohen-Bazire *et al*. (1957). Bacteria were harvested in the late exponential phase.

Chromatophores were prepared by sonication of the bacteria and centrifugation as previously described (Celis & Romero 1987). The chromatophore preparation was kept at 4 °C and used for the experiments within the next 3

days. Protein content was determined by the method of Lowry *et al.* (1951).

The hydrolytic reaction was determined in the dark at 30 °C in a green safety light under the conditions described under Results. The reactions were arrested by the addition of 10% trichloroacetic acid (final concentration). Phosphate was determined in the supernatant by the method of Avron (1966) for concentrations of Pi in the nanomolar range and with the method of Sumner (1944) in the micromolar range.

The concentrations of free metal ions, ligands and complexes were calculated using a PC program that solves the simultaneous equations that describe the multiple equilibria present in the solution, using the association constants previously reported (Martell & Sillén 1971, Smith & Martell 1976). The program is available upon request.

Since PPi can precipitate in the presence of cations in all our experimental conditions, we determine the free Pi concentration remaining in solution after centrifugation at $1000 \times g$ for 10 min. For this purpose, we used the colorimetric method described by Heinonen *et al.* (1981). The concentrations in the La–PPi complex experiments were corrected for this factor.

The fluorescence measurements of the La–PPi complex were made with a Hitachi fluorescence spectrophotometer (Model 650-15). The excitation wavelength used was 280 nm and the emission wavelength was 365 nm according to the fluorescence characteristics obtained for this complex in this work.

Results and discussion

The La–PPi complex was used as a substrate model of M(III)–PPi complexes for membrane-bound pyrophosphatase of *R. rubrum*. The hydrolytic activity with this complex alone is very low (Figure 1A). The K_m is 0.35 mM and V_{max} was 7.65 nmol Pi min^{-1} mg protein^{-1} (Figure 1B), indicating that the La–PPi complex can be hydrolyzed by the enzyme although with a very low activity. When Mg^{2+} or Zn^{2+} were added, stimulation of the activity was obtained (Figure 1A), being larger with Zn^{2+} than with Mg^{2+}. The K_m of the La–PPi complex was also lowered by the addition of the divalent cations, and V_{max} was stimulated 4.5 times by Mg^{2+} and 13 times by Zn^{2+} (La–PPi plus Mg^{2+}: $K_m = 0.096$ mM, $V_{max} = 34.59$ nmol Pi min^{-1} mg protein^{-1}; La–PPi plus Zn^{2+}: $K_m = 0.052$ mM, $V_{max} = 99.66$ nmol Pi min^{-1} mg protein^{-1}).

K_m and V_{max} values for Mg–PPi as substrate and Mg^{2+} as activator (Sosa *et al.* 1992) are 0.6 mM and 515 nmol Pi min^{-1} mg protein^{-1}. Comparing these kinetic parameters with those obtained in this work, it is apparent that the V_{max}/K_m is higher for the natural substrate (858) than for the La–PPi substrate (360.3); indicating, as expected, a better specificity for the natural substrate.

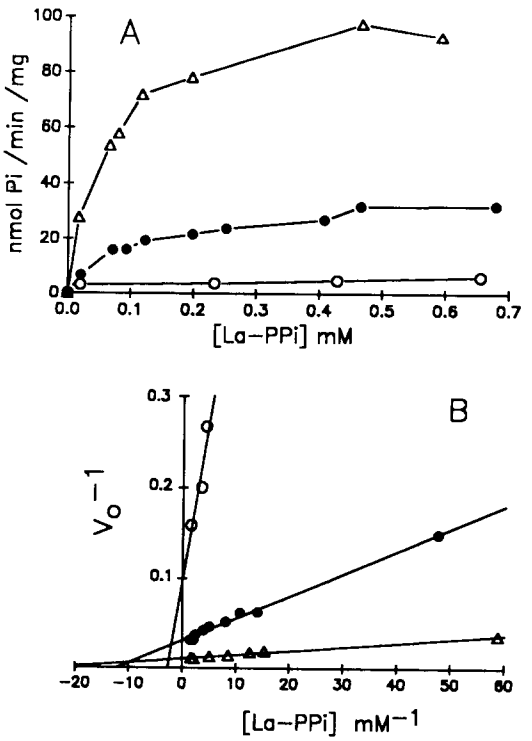

Figure 1. Effect of free Mg^{2+} or Zn^{2+} on the hydrolysis of La–PPi complex by membrane-bound pyrophosphatase. (**A**) The incubation media contains 50 mM Tris–maleate, pH 6.0, 1 mg of protein of chromatophores, the concentrations of $LaCl_3$ and NaPPi were varied equimolecularly. The concentrations were corrected for the precipitation of the complex as indicated under Materials and methods. Hydrolysis of La–PPi complex (○), with 1.0 mM $MgCl_2$ (●) and with 0.5 mM $ZnCl_2$ (△). Incubation time was 1 min at 30 °C. (**B**) Lineweaver–Burk plot of the same data.

From the experiment depicted in Figure 1, two possibilities arise. One is that Mg^{2+} or Zn^{2+} are substituting La^{3+} in the La–PPi complex and the hydrolyzed complex could be Mg–PPi or Zn–PPi. The second is that Mg^{2+} or Zn^{2+} remains free in the experiment. To differentiate between these possibilities, the calculated concentrations of the possible formed complexes and free ions were plotted with the hydrolytic activity obtained in Figure 1. This comparison is depicted in Figure 2; panel A clearly shows a parallel rise in La–PPi concentration with the hydrolytic activity, whereas free Mg^{2+} remains constant throughout the experiment. The Mg–PPi concentration also rises but in a very low range. A parallel experiment with these concentrations of Mg–PPi and free Mg^{2+}, but without the La–PPi complex was performed and no measurable activity

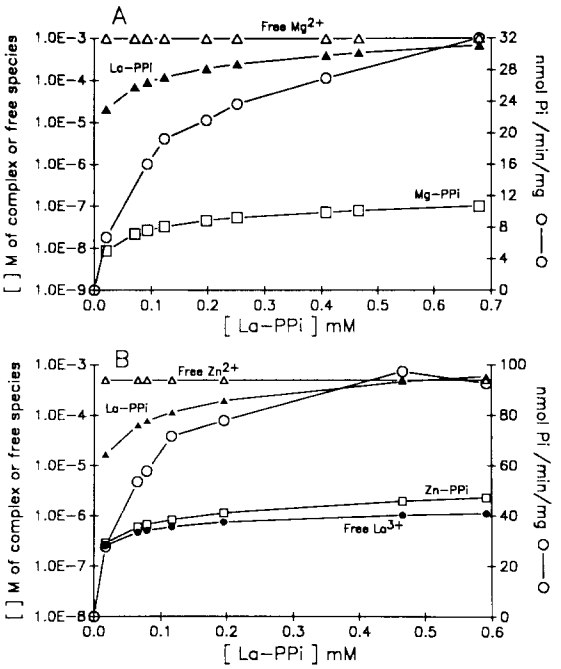

Figure 2. Dependance of the hydrolytic reaction of Figure 1 on the concentrations of complexes and free metal ions. (**A**) Analysis for Mg^{2+} activation. (**B**) Analysis for Zn^{2+} activation. Data for hydrolytic activity are taken from Figure 1. Calculated concentration of La–PPi, Mg–PPi, Zn–PPi and free cations under the same conditions of the experiments are also plotted.

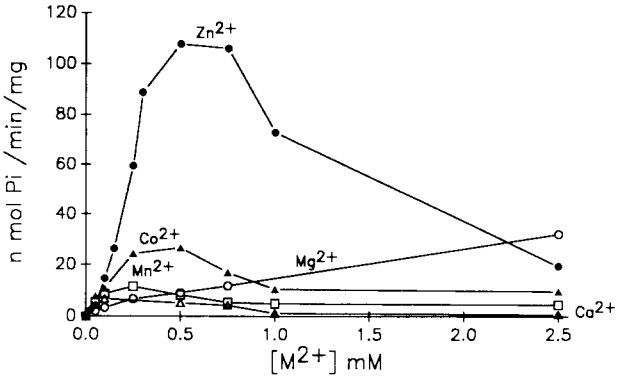

Figure 3. Effect of the concentration of divalent cations on the hydrolysis of the La–PPi complex by membrane-bound pyrophosphatase. Experimental conditions were as in Figure 1, but the media contained 0.5 mM NaPPi, 0.5 mM $LaCl_3$ and the concentrations of divalent cations were varied as indicated. Mg^{2+} (○), Zn^{2+} (●), Ca^{2+} (△), Co^{2+} (▲) and Mn^{2+} (□). The concentration of soluble La–PPi complex, corrected after precipitation, was about 0.25 mM in the presence of all divalent cations, except for Mn^{2+}, which at its highest concentration was 0.05 mM.

was obtained. The same type of calculations were made for the experiment with added Zn^{2+} (Figure 2B) and the results were similar to those obtained with Mg^{2+}, except that the Zn–PPi complex concentration is of the order of 10^{-7} to 10^{-6} M. It is important to note that the concentrations depicted in Figure 1 and the analysis made in Figure 2 were corrected to account for the PPi precipitations with the ions present.

These experiments clearly suggest that free Mg^{2+} or Zn^{2+} stimulates the hydrolysis of the La–PPi complex by membrane-bound pyrophosphatase.

Ting & Dunaway-Mariano (1984) used M(III)–PPi complexes as substrates for yeast inorganic cytoplasmic pyrophosphatase and also obtained hydrolysis of these complexes with free Mg^{2+}.

To explore further this phenomenon, we fixed the La–PPi concentration and increased the concentration of several divalent cations. Figure 3 shows that stimulation depends on the concentrations of the divalent cations having different efficiencies (at cation 0.5 mM: $Zn^{2+} > Co^{2+} > Mg^{2+} > Mn^{2+} > Ca^{2+}$). The effect of Zn^{2+}, Co^{2+} and Mn^{2+} upon the hydrolytic activity has a biphasic behavior; stimulatory at low concentrations and inhibitory at high concentrations. The inhibition produced by high concentrations of divalent cations in membrane-bound pyrophosphatase of *R. rubrum* has previously been reported and is due to the free species of cations (Celis & Romero 1987). The fact that Zn^{2+} has a biphasic behavior and Mg^{2+} has a linear dependance on stimulation probably suggests different mechanisms of action.

Figure 4 presents the analysis of the experiment depicted in Figure 3. For both panels (A and B), the concentration of La–PPi remains constant whereas the concentration of free Mg^{2+} or Zn^{2+} rises concomitantly with the hydrolytic activity of the enzyme. This, again, suggests that free divalent cations stimulate the hydrolytic activity of the enzyme. In the case of Zn^{2+}, as mentioned above, the activity is inhibited at high concentrations.

The analysis employed depends on the accuracy of the stability constants in our experimental conditions. The experiments shown in Figure 5 were performed to determine whether the predicted complexes and free ions were present in our particular conditions. We took advantage of the fact that the La–PPi complex fluoresces. Figure 5(A) shows the fluorescence of this complex; this fact has not been previously reported (Moeller 1972). Figure 5(B) shows the displacement of Mg^{2+} and Zn^{2+} from previously formed Mg–PPi or Zn–PPi complexes, when increasing concentrations of La^{3+} were added to the solution, as shown by the increment of fluorescence. As a control, the Fe–PPi complex was

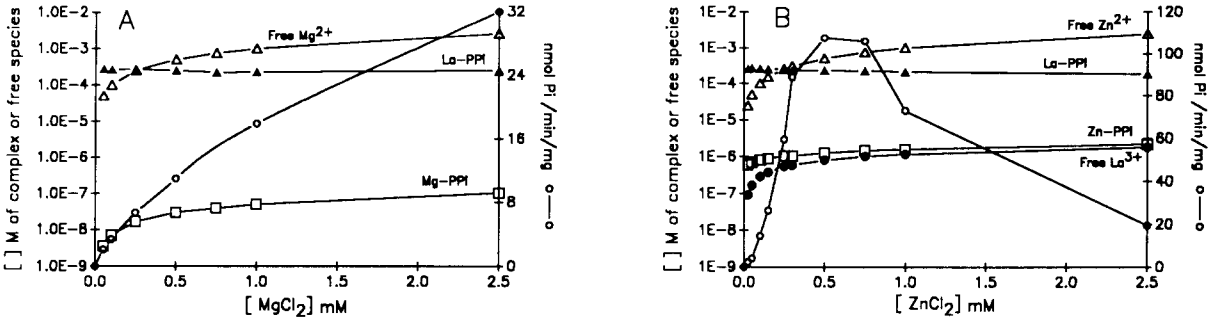

Figure 4. Dependance of the hydrolytic reaction of Figure 3 on the concentrations of complexes and free ions. (**A**) For Mg^{2+} activation. (**B**) For Zn^{2+} activation. The hydrolytic activity was taken from Figure 3. Calculated concentrations of different species are also plotted.

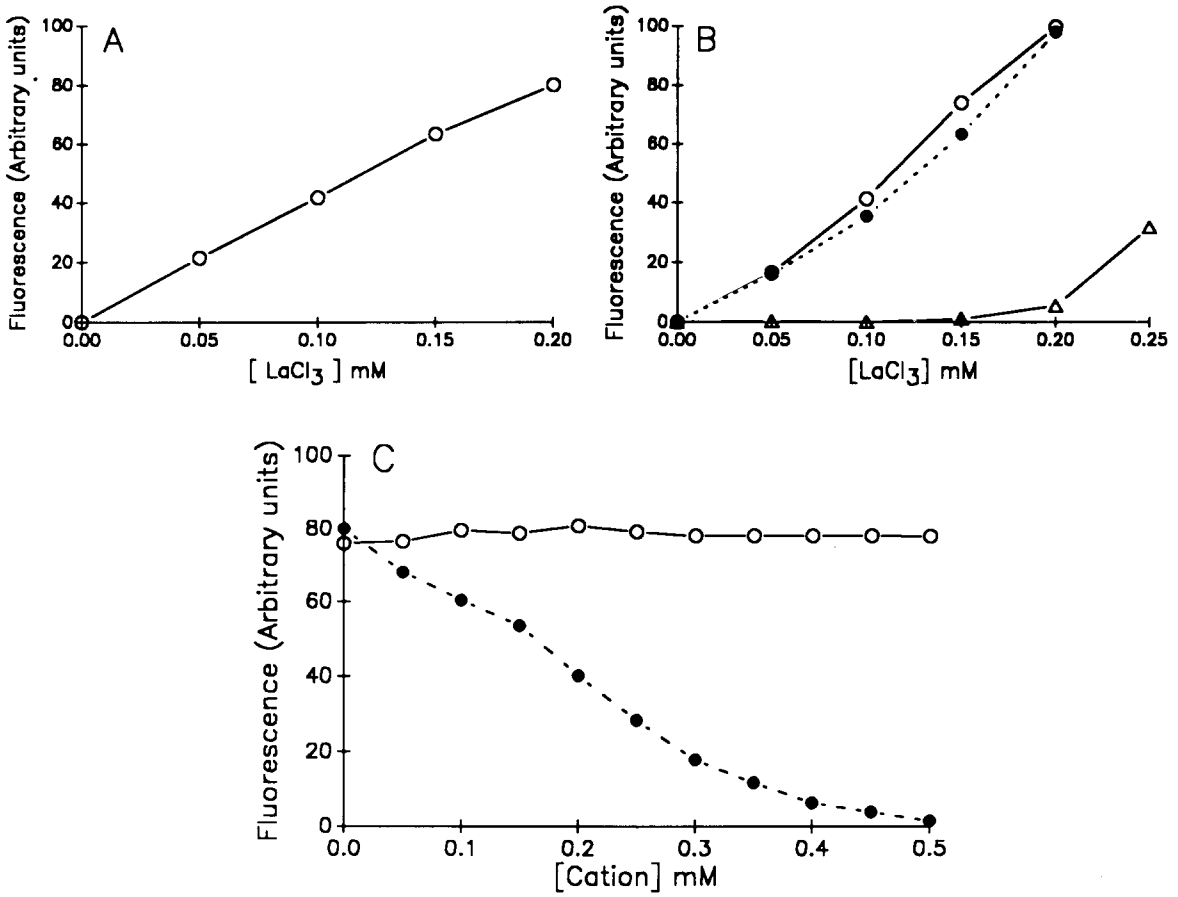

Figure 5. Fluorescence of the La–PPi complex under different experimental conditions. (**A**) Fluorescence due to the formation of the La–PPi complex. The medium contained 0.2 mM NaPPi and the indicated concentrations of $LaCl_3$. (**B**) Effect of Zn^{2+}, Mg^{2+} and Fe^{3+} ions upon the fluorescence signal of the La–PPi complex. The medium contained 0.2 mM of NaPPi, 0.2 mM of the corresponding cation in chloride: Mg^{2+} (○), Zn^{2+} (●) or Fe^{3+} (△) and the indicated concentration of $LaCl_3$. (**C**) Effect of the concentration of cations on the fluorescence of the La–PPi complex. The medium contained 0.2 mM NaPPi, 0.2 mM $LaCl_3$ and the correspondent concentration of the tested cations. For Mg^{2+} and Zn^{2+} (○), and for Fe^{3+} (●).

analyzed; this complex is not fluorescent. As can be seen in Figure 5(B), La^{3+} displaces Fe^{3+} from the Fe–PPi complex only at high concentrations; this is expected since Fe^{3+} has a 3.5 orders of magnitude higher stability constant for PPi than La^{3+} (Martell & Sillén 1971). On the other hand, Figure 5(C) depicts the La–PPi complex previously formed; neither Mg^{2+} nor Zn^{2+} displace La^{3+} from the complex, but Fe^{3+} completely displaces La^{3+} from the complex, quenching the fluorescence. These experiments completely validate the analysis and stability constants used in our experiments of Figures 2 and 4, and strongly support the conclusion that both Mg^{2+} and Zn^{2+} exert a stimulatory effect of membrane-bound pyrophosphatase of *R. rubrum*.

To explore further the previously suggested different mechanisms of stimulation of Mg^{2+} and Zn^{2+}, we performed experiments with other trivalent cations forming a complex with PPi. In Figure 6(A), we show the hydrolysis supported by Nd–PPi, Fe–PPi, La–PPi and Tb–PPi as substrates, stimulated by free Mg^{2+}. As can be seen, the K_ms were different for each complex (Figure 6B and Table 1);

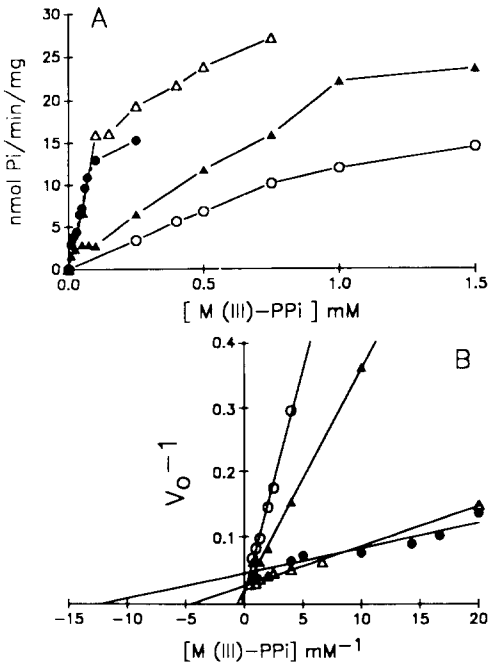

Figure 6. Effect of different M(III)–PPi complexes as substrates of membrane-bound pyrophosphatase activated by Mg^{2+}. (**A**) The incubation media contained: 50 mM Tris–maleate, pH 6.0, 1 mg of protein of chromatophores; the concentrations of the trivalent cations and NaPPi were varied equimolecularly, as indicated, and 1 mM $MgCl_2$ was added. Tb–PPi (○), Nd–PPi (●), La–PPi (△) and Fe–PPi (▲). (**B**) Lineweaver–Burk plot of the same data.

however, when Zn^{2+} was used as the activator for the same M(III)–PPi complexes (Figure 7A) the K_ms are practically the same (Figure 7B and Table 1). This suggests that Zn^{2+} makes the enzyme very unspecific and activates through a different mechanism from the activation exerted by Mg^{2+}. At present, we cannot offer a complete explanation for

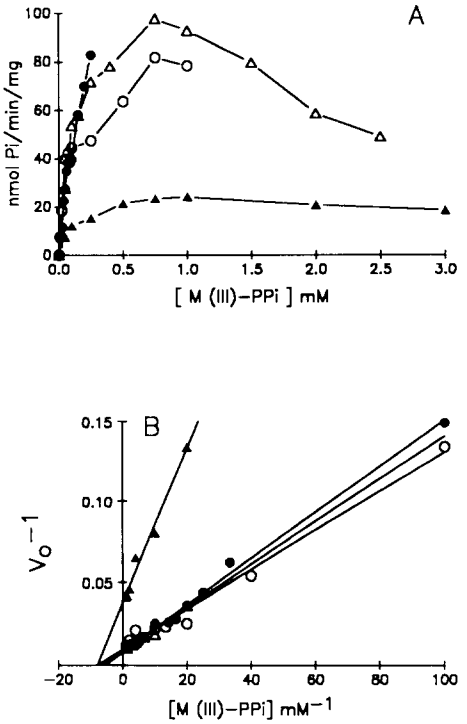

Figure 7. Effect of different M(III)–PPi complexes as substrates of membrane-bound pyrophosphatase activated by Zn^{2+}. (**A**) The incubation media were the same as in Figure 6, but 0.5 mM $ZnCl_2$ was added instead of $MgCl_2$. Tb–PPi (○), Nd–PPi (●), La–PPi (△) and Fe–PPi (▲). (**B**) Lineweaver–Burk plot of the same data.

Table 1. K_m and V_{max} values for M(III)–PPi complexes in the presence of free Mg^{2+} or Zn^{2+}

	K_m (mM)	V_{max} (nm Pi min^{-1} mg^{-1})
With 1.0 mM free Mg^{2+}		
Nd–PPi	0.068	20.15
La–PPi	0.20	37.05
Fe–PPi	1.5	47.40
Tb–PPi	3.3	48.10
With 0.5 mM free Zn^{2+}		
Nd–PPi	0.179	126.9
La–PPi	0.125	120.0
Fe–PPi	0.121	25.6
Tb–PPi	0.104	112.4

this behavior; more work with the enzyme in the membrane or in its purified forms must be done. Figures 6 and 7 were not corrected for precipitation; however, when they were corrected, the K_ms were lower, as expected because there is less substrate; nevertheless, the phenomenon shows the same pattern.

Acknowledgments

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