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Inhibition of Family II Pyrophosphatases by Analogs of Pyrophosphate and Phosphate

A. B. Zyryanov[1], R. Lahti[2], and A. A. Baykov[1]*

[1]Belozersky Institute of Physico-Chemical Biology and Faculty of Chemistry, Lomonosov Moscow State University, 119899 Moscow, Russia; fax: (7-095) 939-3181; E-mail: baykov@genebee.msu.su
[2]Department of Biochemistry and Food Chemistry, University of Turku, Turku, FIN-20500, Finland; fax: (358-2) 633-6860; E-mail: reijo.lahti@utu.fi

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Abstract—Imidodiphosphate (the pyrophosphate analog containing a nitrogen atom in the bridge position instead of oxygen) is a potent inhibitor of family II pyrophosphatases from *Streptococcus mutans* and *Streptococcus gordonii* (inhibition constant $K_i \approx 10$ μM), which is slowly hydrolyzed by these enzymes with a catalytic constant of ≈1 min^{-1}. Diphosphonates with different substituents at the bridge carbon atom are much less effective (K_i = 1-6 mM). The value of K_i for sulfate (a phosphate analog) is only 12 mM. The inhibitory effect of the pyrophosphate analogs exhibits only a weak dependence on the nature of the metal ion (Mn, Mg, or Co) bound in the active site.

Key words: pyrophosphatase, diphosphonate, phosphate, inhibition, family II, *Streptococcus mutans*, *Streptococcus gordonii*

Pyrophosphatases (PPases) are universal cell components since they utilize pyrophosphate formed as a byproduct in essential cell processes. There are soluble and membrane PPases. Soluble PPases form two nonhomologous families, I and II. Family I has been known for more than 70 years and has been thoroughly studied. Family II was discovered only few years ago in bacteria and archaebacteria [1, 2], and has been scarcely investigated.

PPases of both families are only active in the presence of metal ion cofactor, which performs numerous functions in catalysis but differ in their catalytic properties and structure. Family II is 20-50 times more active and exhibits another specificity pattern towards the substrate and activating metal ions: it does not hydrolyze ATP [3], it is more active with Mn^{2+} and Co^{2+} instead of Mg^{2+} [1, 2, 4], and it is not inhibited by Ca^{2+} [5]. The three-dimensional structures of PPases belonging to families I and II are different, except for the active sites whose essential motifs match [6, 7]. Primarily this applies to the binuclear metal center, which is used for activation of a nucleophilic water molecule. This center is formed by two metal ions, one of which is bound with affinity typical for metalloenzymes [5, 8]. Due to this feature, it is possible to change the nature of the metal ion bound in the high affinity site while keeping Mg^{2+} in two other catalytically important binding sites (within the metal center and in a complex with the substrate). These mixed metal complexes are most probably the active enzyme forms *in vivo*, since bacteria containing family II PPases are able to accumulate the transition metals ions [9, 10].

The aim of this work was the determination of affinity of family II PPases towards structural analogs of pyrophosphate containing N and C atoms in the bridge position (Fig. 1). In practice, the substitution of the bridge atom does not affect the spatial orientation of PO_3^{2-} groups [11], which are the main anchor groups of the substrate. Substrate-like inhibitors of the enzyme are of interest both for fundamental research (e.g., for determination of three-dimensional structure in the complex with substrate) and practical aspects, since many bacteria containing family II PPases are pathogenic.

MATERIALS AND METHODS

Genes encoding *S. mutans* pyrophosphatase (*sm*PPase) and *S. gordonii* pyrophosphatase (*sg*PPase) were expressed in *Escherichia coli*, and the obtained enzymes were purified as described earlier [5]. Imidodiphosphate was synthesized according to the techniques described in [12, 13]. Diphosphonates were kindly provided by S. V. Komissarenko, B. P. Mischenko, and B. S. Cooperman.

* To whom correspondence should be addressed.

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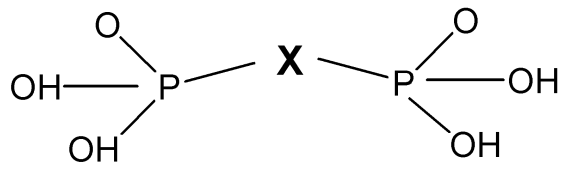

Compound	X =
I	CH_2
II	$C(OH)H$
III	$C(OH)CH_3$
IV	NH
V	$C(NH_2)H$
VI	$C(CH_2NH_2)H$
VII	$C(OH)(CH_2CH_2NH_2)$

Fig. 1. Chemical structures of the substrate analogs.

To obtain manganese-, magnesium-, and cobalt-containing forms of pyrophosphatases (Mn-PPase, Mg-PPase, and Co-PPase) containing the corresponding metal ion in the high affinity site, the enzymes were converted into apo-form by treatment with EDTA and then incubated with equimolar amounts of $MnCl_2$ and $CoCl_2$ or 5 mM $MgCl_2$ [8]. The concentration of PPase solutions estimated per monomer was determined spectrophotometrically using molecular weight of 33.5 kD and specific absorption coefficient $\varepsilon_{280}^{1\%}$ 3.07 and 2.62, accordingly [5, 8].

Initial rates of PP_i hydrolysis were determined by a continuous method using an automatic phosphate analyzer [14]. The reaction mixture with total volume of 5 ml contained (unless stated otherwise) 5 μM PP_i, 5 mM Mg^{2+}, 10 μM $MnCl_2$, 83 mM Tes/KOH buffer (pH 7.2), and 17 mM KCl. Together with diphosphonates, equimolar amounts of $MgCl_2$ were added to prevent decrease in the concentration of Mg^{2+} free form due to the formation of diphosphonate–magnesium complex. The reaction was started by adding the enzyme and performed for 3-4 min at 25°C.

The rate of imidodiphosphate hydrolysis was also determined from the rate of P_i accumulation; however, a manual technique was used [15]. The reaction was performed in 0.3 ml volume. Aliquots of the reaction mixture (50 μl) were collected every minute, the reaction was stopped by adding 5 μl of 5 M trifluoroacetic acid, then the aliquots were diluted with water to the final volume 0.8 ml, 0.2 ml of phosphate reagent added, and absorbance measured at 660 nm. The phosphate reagent was a mixture of 6% ammonium molybdate solution, 5 M sulfuric acid, and 0.3% of Malachite green dye in proportion of 15 : 18 : 25 v/v [15]. In calculating the rate of the enzymatic reaction, it was taken into account that despite the fact that during imidodiphosphate hydrolysis one phosphate molecule is formed, the second product (phosphamide) is rapidly converted into phosphate in strongly acidic medium in the course of phosphate determination [16].

The effective inhibition constant K_i^{eff} was determined using the equation $A = A_0/(1 + [PXP]/K_i^{eff})$, where A_0 and A are activities in the absence and presence of the inhibitor (PXP), respectively, and [PXP] is inhibitor concentration.

RESULTS AND DISCUSSION

Binding strength between the substrate analogs and family II PPases was evaluated according to their inhibition of PP_i hydrolysis (Fig. 2). Values of K_i^{eff} presented in Table 1 illustrate the influence of the nature of the bridge moiety on inhibition of *sm*PPase (manganese form). In these experiments, the active enzyme–substrate complex contained Mn^{2+} in the high affinity site of the enzyme, and Mg^{2+} in other catalytically important sites. Since the reaction mixture contained 5 mM Mg^{2+}, the major amount of substrate analog (>90%) was present in the form of magnesium complex [17], which apparently was the active form of the inhibitor. For comparison, Table 1 shows similar data for family I pyrophosphatases from baker's yeast and *Escherichia coli*.

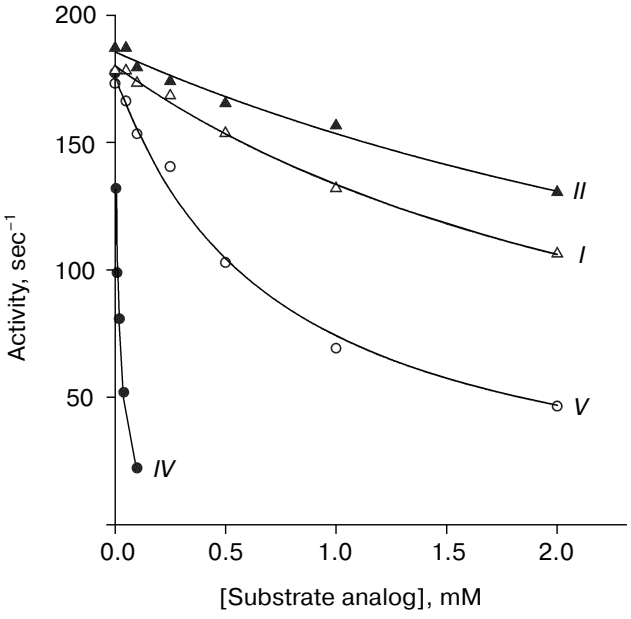

Fig. 2. Inhibitory effect of imidodiphosphate and three diphosphonates on PP_i hydrolysis by Mn-*sm*PPase in the presence of 5 mM Mg^{2+}. Curves are marked with the number of the compound according to Fig. 1.

Table 1. Effective inhibition constants of *sm*PPase and family I PPases by substrate analogs

Substrate analog*	Analog concentration, mM	K_i^{eff}, mM		
		*sm*PPase**	yeast PPase***	*E. coli* PPase***
I	0-2****	3.0 ± 0.2	1.1	0.33
II	0-2****	4.1 ± 0.6	0.05	0.015
III	0-6****	6 ± 1	>3	0.24
IV	0-0.1	0.016 ± 0.001	0.015	0.005
V	0-2****	1.0 ± 0.3	0.02	0.07
VI	0-0.5****	1.9 ± 0.4		
VII	0-0.25****	1.7 ± 0.4		
SO_4^{2-}	0-20	12 ± 1		

* Compounds I-VII are shown in Fig. 1.
** Mn-form of the enzyme; activity was measured in the presence of 10 μM Mn^{2+} and 5 mM Mg^{2+}.
*** Measured earlier at pH 7.2 in the presence of 1 μM PP_i and 1 mM Mg^{2+} [18].
**** The upper boundary is determined by diphosphonate solubility.

Table 2. Effect of the nature of metal ion bound in the high affinity site on the inhibition of *sg*PPase by substrate analogs

Metal ion	K_i^{eff}, mM		K_m, μM**	K_s, mM***
	X = $C(OH)CH_3$ (III)*	X = NH (IV)*		
Mn^{2+}	1.7 ± 0.1	0.012 ± 0.001	40	0.18
Mg^{2+}	1.8 ± 0.3	0.009 ± 0.001	8	0.060
Co^{2+}	1.9 ± 0.1	0.017 ± 0.003	11	0.17

* Compounds III and IV are shown in Fig. 1.
** Michaelis constant for pyrophosphate [8].
*** Dissociation constant for enzyme–pyrophosphate complex [8].

As seen from the presented data, imidodiphosphate (compound IV) inhibits *sm*PPase much more strongly than diphosphonates, equally strongly as family I PPases. Diphosphonates are much less efficient towards *sm*PPase than towards family I PPases. Moreover, the nature of substituent at the bridge atom in diphosphonate has less influence on the inhibitory ability, but the presence of an amino group slightly increases the inhibition (compare compound I with V and VI), whereas the presence of a hydroxyl group decreases it (compare compound I with II and III). Modeling of enzyme–substrate complex based on X-ray data indicates the possibility of hydrogen bond formation between the bridge oxygen atom in PP_i and the nitrogen atom in a histidine residue in family II PPases [7]. This bond is also possible in the case of imidodiphosphate but not in the case of diphosphonates, which perhaps explains their low affinity to *sm*PPase.

It should be noted that the examined compounds are competitive inhibitors (derived from the fact that inhibition degree decreased with increase in inhibitor concentration and also knowing that these compounds are structurally very close to PP_i [11]), and the efficiency of competitive inhibitor depends on the ratio between Michaelis constant and substrate concentration. This dependence is described by the equation:

$$K_i^{eff} = K_i (1 + [S]/K_m),$$

where K_i is the true inhibition constant, K_m is the Michaelis constant, and [S] is substrate concentration.

The value of K_m for Mn-*sm*PPase under our experiment conditions was 21 ± 3 μM. Since the substrate concentration in the inhibition experiments was 5 μM, the values of K_i^{eff} in Table 1 are insignificantly different from the true inhibitor binding constants. This also refers to family I PPases [18].

The nature of the activating metal ion bound in the high affinity site of the enzyme did not affect the inhibition constant of *sg*PPase by the two substrate analogs (Table 2). The remaining catalytically important sites were in these cases occupied by Mg^{2+} present in the reaction mixture. To prevent the possibility of replacement of Mn^{2+} and Co^{2+} by Mg^{2+}, the reaction mixture was supplemented with 10 μM Mn^{2+} or Co^{2+} [5, 8]. Using K_i^{eff} and K_m values it can be calculated that the enzyme affinity towards the inhibitors characterized by K_i value changes only twofold depending on the metal ion. This result also indicates that differences in K_i^{eff} values between *sm*PPase and family I PPases (Table 1) are not associated with the fact that they had different metal ions in the high affinity site (Mn^{2+} in *sm*PPase and Mg^{2+} in family I).

Values of K_i^{eff} (and K_i) are comparable with Michaelis constants for PP_i hydrolysis (Table 2). However, the Michaelis constant is a complex combination of elementary step rate constants and is not a characteristic of enzyme affinity towards the substrate. The *sg*PPase is the only enzyme from family II PPases for which the dissociation constants for complex with PP_i (K_s) are known (also presented in Table 2). It can be seen that K_s value is less dependant on the nature of metal ion and (what is important) by an order of magnitude greater than K_i^{eff} (and K_i) values for imidodiphosphate. In case of family I PPase from yeast, the K_s value for imidodiphosphate [19] is lower than K_i by 1-2 orders of magnitude.

Family I PPases slowly but with measurable rate hydrolyze imidodiphosphate [15]. A similar result was also obtained for family II PPases. The curve of imidodiphosphate hydrolysis by Mg-*sm*PPase is presented in Fig. 3. Based on these data the catalytic constant of hydrolysis is 0.85 min^{-1}, which is 20,000 times lower than for pyrophosphate hydrolysis. No noticeable diphosphonate hydrolysis occurred under similar conditions. Imidodiphosphate hydrolysis was significantly inhibited by a specific inhibitor of soluble PPases, fluoride (nine times in the presence of 0.1 mM NaF), which confirmed the catalytic role of the PPase. Hydrolysis rate did not depend on Mg^{2+} concentration in the 1-5 mM range. The absence of an initial peak of accumulation of the reaction product comparable in amplitude with the enzyme concentration (10 μM) in Fig. 3 indicates that the reaction rate is limited by imidodiphosphate hydrolysis in the active site and not by the dissociation and removal of the reaction products.

The effective constant for Mn-*sm*PPase inhibition by sulfate was determined in the same way as for the substrate analogs and was equal to 12 ± 1 mM, which corresponds to K_i = 8.4 mM. The dissociation constant for the complex of this enzyme with phosphate measured under similar conditions was 2.5 mM [3]. Relatively high affinity towards sulfate can explain that the enzyme crystallized from ammonium sulfate contains one or two sulfate ions in the active site [6, 7]. Sulfate was also found in *E. coli* PPase structure [20].

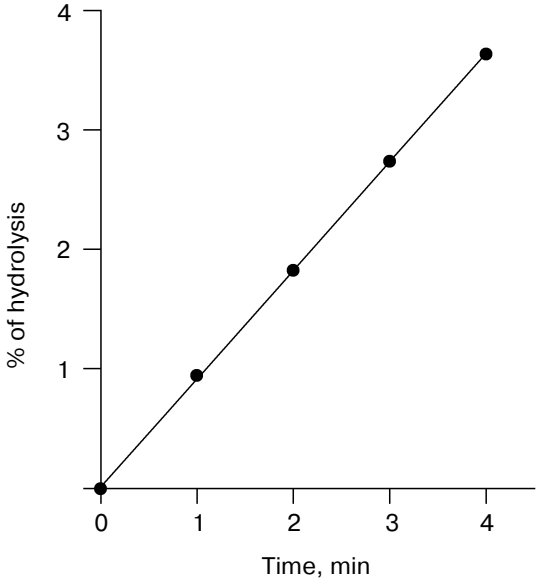

Fig. 3. Kinetics of imidodiphosphate (1 mM) hydrolysis by *sm*PPase in the presence of 5 mM Mg^{2+}. The enzyme concentration was 10 μM.

Hence, family I and II PPases differ in affinity towards diphosphonate analogs of their natural substrate, pyrophosphate. Imidodiphosphate is the best inhibitor among the substrate analogs, which binds to family II PPases more strongly than pyrophosphate.

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