

Differential sensitivity of membrane-associated pyrophosphatases to inhibition by diphosphonates and fluoride delineates two classes of enzyme

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Received 18 May 1993

1,1-Diphosphonate analogs of pyrophosphate, containing an amino or a hydroxyl group on the bridge carbon atom, are potent inhibitors of the H⁺-translocating pyrophosphatases of chromatophores prepared from the bacterium *Rhodospirillum rubrum* and vacuolar membrane vesicles prepared from the plant *Vigna radiata*. The inhibition constant for aminomethylenediphosphonate, which binds competitively with respect to substrate, is below 2 μM. Rat liver mitochondrial pyrophosphatase is two orders of magnitude less sensitive to this compound but extremely sensitive to imidodiphosphate. By contrast, fluoride is highly effective only against the mitochondrial pyrophosphatase. It is concluded that the mitochondrial pyrophosphatase and the H⁺-pyrophosphatases of chromatophores and vacuolar membranes belong to two different classes of enzyme.

Inorganic pyrophosphatase; Diphosphonate; Fluoride; Pyrophosphate analog

1. INTRODUCTION

Inorganic pyrophosphatases (PPases; EC 3.6.1.1) are ubiquitous enzymes which mediate the hydrolysis of pyrophosphate (PP_i) to orthophosphate (P_i). In many organisms the bulk of PPase activity is soluble, localized in the cytosol and serves to catalyze dissipative substrate hydrolysis. In other organisms, however, there are membrane-associated PPases which are capable of utilizing the energy contained in the phosphoanhydride bond of PP_i for the establishment of transmembrane ionic gradients. Notable examples of energy-transducing, or energy-conserving, PPases are the reversible H⁺-translocating PPase of chromatophores from the purple, non-sulfur bacterium *Rhodospirillum rubrum* [1], the vacuolar H⁺-translocating PPase of plant cells [2,3] and the membrane-associated PPase of animal and yeast mitochondria [4]. Whereas the capacity of the mitochondrial PPase for ion transport has yet to be demonstrated unequivocally, the enzymes from *R. rubrum* and plants are PP_i-linked ion translocases competent in the primary translocation of H⁺ [5] and H⁺ and/or K⁺

[3,6,7], respectively, and maintenance in this way of an energy buffer alternative to ATP [8].

Here, we compare the inhibitory sensitivity profiles of the bacterial, plant and mitochondrial PPases to show that whereas the first two enzymes have qualitatively identical profiles, their profiles differ markedly from the mitochondrial PPase. On the basis of these findings it is concluded that the mitochondrial PPase belongs to a distinct group of enzymes and that diphosphonates, particularly aminomethylenediphosphonate, are potent type-specific inhibitors of non-mitochondrial H⁺-PPases.

2. MATERIALS AND METHODS

Vacuolar membrane vesicles were isolated from etiolated hypocotyls of *V. radiata* (mung bean) as described by Rea et al. [9]. Chromatophores were prepared from *R. rubrum* according to Baltscheffsky [10]. Imidodiphosphate was synthesized as described previously [11,12] and the diphosphonates employed in this studies were kind gifts from Dr. S.V. Komissarenko (Institute of Biochemistry, Kiev, Ukraine) and Dr. B.S. Cooperman (University of Pennsylvania, Philadelphia, USA).

PPase activity was estimated from continuous recordings of the liberation of P_i from PP_i using an automated phosphate analyzer [13]. The reactions were performed in an initial volume of 5–25 ml containing 5 μM gramicidin-D, 40 μM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetate, 50 mM KCl, 1 mM MgCl₂, 0.12 M Tris-HCl (pH 7.5) to which 5–25 μl chromatophore or vacuolar membrane vesicle suspension were added. The assay media were preincubated for 1.5 min, reaction was initiated by the addition of 30 μM PP_i, and P_i liberation was monitored for 3 min at 25°C. Fluoride inhibition was

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Abbreviations: PPase, inorganic pyrophosphatase; PP_i, pyrophosphate; P_i, phosphate.

measured using 120 μM PP_i . The PPase activity of both types of vesicles was 0.2–0.3 μmol PP_i hydrolyzed per min per mg protein.

3. RESULTS

Five 1,1-diphosphonates and imidodiphosphate were tested for their capacity to inhibit the plant and bacterial PPase. To ensure effective competition and enable direct comparisons between these two enzymes and the mitochondrial PPase, a PP_i concentration equal to the Michaelis constant of the enzyme concerned (Table I) was employed throughout.

Of the six PP_i analogs examined, aminomethylenediphosphonate was the most effective against the vacuolar enzyme. Hydroxy derivatives were markedly more effective than dichloromethylenediphosphonate but only aminomethylenediphosphonate caused a significant decrease in the rate of hydrolysis at submicromolar concentrations (Fig. 1 and Table I). The inhibition of the vacuolar PPase by aminomethylenediphosphonate was competitive with respect to PP_i (data not shown).

Apparent inhibition constants for all substrate analogs were computed from the equation $v = v_0 / (1 + [I] / K_i^{\text{app}})$, where v and v_0 are hydrolysis rates at inhibitor concentrations of zero and $[I]$, respectively, and are summarized in Table I. The values of K_i^{app} follow the sequence dichloromethylenediphosphonate \gg methylenediphosphonate $>$ imidodiphosphate $>$ ethane-1-hydroxy-1,1-diphosphonate \approx hydroxymethylenediphosphonate $>$ aminomethylenediphosphonate.

Qualitatively similar results were obtained when the same range of inhibitors was tested against the PPase from *R. rubrum*. While minor differences in the K_i^{app} values were found, the same basic pattern of inhibitor sensitivity and selectivity was established. Thus, the K_i^{app} values for this enzyme also followed the above mentioned sequence of PP_i analogs.

In striking contrast to the vacuolar and chromatophore PPases, rat liver mitochondrial PPase is relatively insensitive to all 1,1-diphosphonates and only markedly

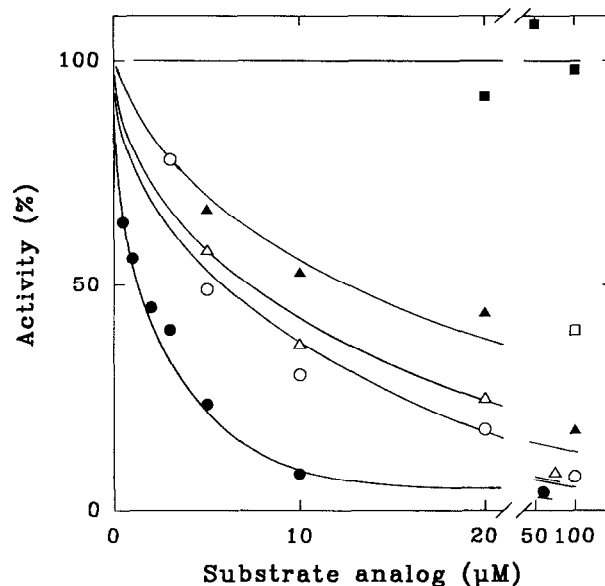


Fig. 1. The effects of aminomethylenediphosphonate (●), hydroxymethylenediphosphonate (○), ethane-1-hydroxy-1,1-diphosphonate (△), imidodiphosphate (▲), methylenediphosphonate (□) and dichloromethylenediphosphonate (■) on hydrolytic activity of the PPase of vacuolar membrane vesicles isolated from *V. radata*.

sensitive to imidodiphosphate [14]. The mitochondrial enzyme was 38-fold more sensitive to imidodiphosphate and 125- and 20-fold less sensitive to aminomethylenediphosphonate and hydroxymethylenediphosphonate, respectively, vs. the vacuolar and chromatophore enzymes (Table I).

Independent confirmation of the functional division of the vacuolar and chromatophore PPases from the mitochondrial PPase was provided by the effect of fluoride. The values of K_i^{app} for the bacterial and vacuolar PPase, 4.8 and 3.4 mM, respectively (Fig. 2), were two orders of magnitude greater than the corresponding value for the mitochondrial PPase [15].

Table I
Apparent inhibition constants (μM) for a series of pyrophosphate analogs with the structure $\text{O}_3\text{P-R-PO}_3$

Compound	R	Enzyme source		
		Chromatophores	Vacuoles	Mitochondria ^b
Methylenediphosphonate	-CH ₂ -	41	68	190
Aminomethylenediphosphonate	-CH(NH ₂)-	1.2	1.8	150
Hydroxymethylenediphosphonate	-CH(OH)-	1.9	5.7	37
Ethane-1-hydroxy-1,1-diphosphonate	-C(CH ₃ (OH))-	25	6.5	870
Dichloromethylenediphosphonate	-CCl ₂ -	>1,000	>500	3,000
Imidodiphosphate	-NH-	30	12	0.8
Pyrophosphate ^a	-O-	36	30	2

^a Apparent Michaelis constant in the presence of 1 mM Mg^{2+}

^b 0.05 M Tris-HCl (pH 7.2), 25°C, 1 μM PP_i [14].

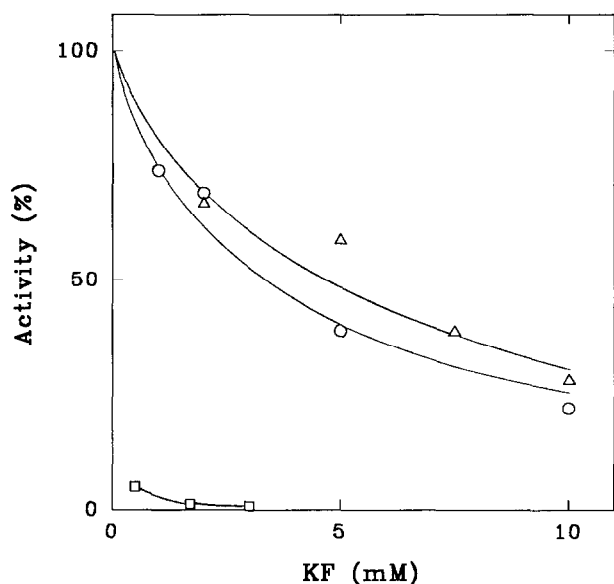


Fig. 2. The effects of potassium fluoride on the hydrolytic activity of the PPases of *R. rubrum* chromatophores (Δ) and *V. radiata* vacuolar membrane vesicles (\circ). The data shown for rat liver mitochondrial PPase (\square) were taken from Baykov et al. [15].

4. DISCUSSION

Two important conclusions derive from these studies: (i) Aminomethylenediphosphonate is an extremely potent competitive inhibitor of the bacterial and plant membrane-associated PPases. This compound is 7- to 38-fold more effective as an inhibitor than the two P_i analogs, methylenediphosphonate and imidodiphosphate, most commonly employed for investigations of these two enzymes (e.g. [16,17]). 1,1-Diphosphonates, especially the amino and hydroxy derivatives, are therefore selective inhibitors of unsurpassed potency for functional studies of both the chromatophore and vacuolar PPases. (ii) Membrane-associated PPases can be subdivided into two categories on the basis of their inhibitor sensitivities. One category consists of the bacterial and plant PPases, which have a relatively low affinity for substrate ($K_m = 30\text{--}36 \mu\text{M}$), are strongly inhibited by aminomethylenediphosphonate and hydroxymethylenediphosphonate and only weakly inhibited by imidodiphosphate and fluoride. The other category consists of the mitochondrial PPase, which has a high affinity for substrate ($K_m = 2 \mu\text{M}$), is strongly inhibited by imidodiphosphate and fluoride and only weakly inhibited by diphosphonates.

On the basis of inhibitor sensitivity and structural considerations, the mitochondrial PPase can be classified as 'soluble PPase-like'. Soluble PPases are very sensitive to fluoride [15,18] and imidodiphosphate [19], moderately sensitive to diphosphonates [19] and have high affinity for substrate [20]. Although all known soluble PPases have subunit sizes different from the chro-

matophore and vacuolar PPases, 20 kDa for the soluble PPases from prokaryotes and 32 kDa for the enzyme from eukaryotes [21], the peripheral catalytic subunit of the mitochondrial PPase has a molecular mass of 28–35 kDa [22–24] and, in *Saccharomyces*, is 49% identical to the soluble PPase from the same source [24]. The mitochondrial PPase from *Saccharomyces* shows no sequence identities with the vacuolar enzyme [2,25,26], and the corresponding enzyme from rat liver mitochondria does not react with antibody raised against the vacuolar PPase [27]. Thus, the soluble and mitochondrial PPases have the characteristics of closely related enzymes, divergent from the chromatophore and vacuolar PPases.

The latter enzymes, on the other hand, appear to trace their origins to another line of descent [2,3]. In addition to their near-identical inhibitor profiles, they exhibit pronounced structural similarities. The bacterial and vacuolar PPases are immunologically cross-reactive [9,28], and, like the vacuolar PPase, the bacterial enzyme appears to consist of a single hydrophobic 53–73 kDa subunit [3,29] capable of catalyzing both PP_i hydrolysis and H^+ translocation [6,29]. It is therefore concluded that the membrane-associated PPases of *R. rubrum* and plant vacuoles represent a category of PPases, distinct from the 'soluble PPase-like' mitochondrial enzyme.

Acknowledgements: This work was in part supported by Grant DE-FG02-91ER20055 from the United States Department of Energy awarded to P.A.R.

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