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Aminomethylenediphosphonate: A Potent Type-Specific Inhibitor of Both Plant and Phototrophic Bacterial H^+-Pyrophosphatases[1]

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The suitability of different pyrophosphate (PPi) analogs as inhibitors of the vacuolar H^+-translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) of tonoplast vesicles isolated from etiolated hypocotyls of *Vigna radiata* was investigated. Five 1,1-diphosphonates and imidodiphosphate were tested for their effects on substrate hydrolysis by the V-PPase at a substrate concentration corresponding to the K_m of the enzyme. The order of inhibitory potency (apparent inhibition constants, K_i^{app} values, μM, in parentheses) of the compounds examined was aminomethylenediphosphonate (1.8) > hydroxymethylenediphosphonate (5.7) ≅ ethane-1-hydroxy-1,1-diphosphonate (6.5) > imidodiphosphate (12) > methylenediphosphonate (68) >> dichloromethylenediphosphonate (>500). The specificity of three of these compounds, aminomethylenediphosphonate, imidodiphosphate, and methylenediphosphonate, was determined by comparing their effects on the V-PPase and vacuolar H^+-ATPase from *Vigna*, plasma membrane H^+-ATPase from *Beta vulgaris*, H^+-PPi synthase of chromatophores prepared from *Rhodospirillum rubrum*, soluble PPase from *Saccharomyces cerevisiae*, alkaline phosphatase from bovine intestinal mucosa, and nonspecific monophosphoesterase from *Vigna* at a PPi concentration equivalent to 10 times the K_m of the V-PPase. Although all three PPi analogs inhibited the plant V-PPase and bacterial H^+-PPi synthase with qualitatively similar kinetics, whether substrate hydrolysis or PPi-dependent H^+-translocation was measured, neither the vacuolar H^+-ATPase nor plasma membrane H^+-ATPase nor any of the non-V-PPase-related PPi hydrolases were markedly inhibited under these conditions. It is concluded that 1,1-diphosphonates, in general, and aminomethylenediphosphonate, in particular, are potent type-specific inhibitors of the V-PPase and its putative bacterial homolog, the H^+-PPi synthase of *Rhodospirillum*.

It is now established that PPi is a major energy source for electrogenic H^+ translocation across the vacuolar membrane of plant cells (Rea et al., 1992b; Rea and Poole, 1993). The enzyme responsible, V-PPase (EC 3.6.1.1), is an abundant membrane constituent and capable of contributing substantially to the generation of an inside-acid, inside-positive transtonoplast H^+ electrochemical potential difference (Rea et al., 1992b; Rea and Poole, 1993).

Interest in the V-PPase derives not only from its exclusive use of PPi as energy source but also from its apparently unique evolutionary status among the various categories of primary ion translocases and PPi hydrolases. Data base searches of the deduced sequences of the polypeptides encoded by cDNAs corresponding to the major substrate-binding subunit of the V-PPase reveal no detectable homology between this pump and other sequenced ion translocases (Rea et al., 1992b; Sarafian et al., 1992). Similarly, close phylogenic links between the V-PPase and the soluble, non-energy-conserving PPases of both prokaryotes and eukaryotes are unlikely. All characterized soluble PPases have different subunit sizes from the V-PPase (Cooperman et al., 1992), and none of the known sequences for soluble PPases align with the deduced sequence of the V-PPase (Rea et al., 1992b). Instead, the V-PPase appears to trace its origins to another line of descent that it shares with the reversible H^+-PPi synthase located in the membranes bounding the chromatophores of the phototrophic, nonsulfur, purple bacterium *Rhodospirillum rubrum* (Rea et al., 1992b; Rea and Poole, 1993).

The existence of a H^+-PPi synthase on the energy-coupling membranes of *Rhodospirillum* has been known for some time (Baltscheffsky and Baltscheffsky, 1992), but only recently has it been shown that this translocase is an integral membrane protein with an apparent M_r of 56,000 (Nyren et al., 1991). Two features of the M_r 56,000 polypeptide are significant: (a) It is immunologically cross-reactive with the substrate-binding subunit of the V-PPase (Nore et al., 1991; Rea et al., 1992b), and (b) like the catalytic subunit of the V-PPase (Britten et al., 1992), it alone is capable of mediating PPi-dependent H^+-translocation (Nyren et al., 1991). Therefore, it has been proposed that the H^+-PPases of *Rhodospirillum* and plant vacuoles are structurally and functionally related (Rea and Poole, 1993).

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Abbreviations: ΔpH, transmembrane pH difference; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; P-ATPase, plasma membrane H^+-translocating ATPase; V-ATPase, vacuolar H^+-translocating ATPase; V-PPase, vacuolar H^+-translocating inorganic pyrophosphatase.

Phosphoanhydride-energized H^+ pumps have been classified into three categories: F, P, and V, based on subunit organization and mechanism. Each class of H^+-phosphohydrolase, the V-PPase excepted, can be readily distinguished from the others on the basis of its susceptibility to inhibition by type-specific inhibitors. Thus, the F-ATPases of energy-coupling membranes are selectively inhibited by azide, the P-ATPases of plasma membranes and endomembranes are specifically inhibited by the transition state analog orthovanadate, and the V-ATPase is strongly and preferentially inhibited by the bafilomycins (Bowman et al., 1988). To date, however, no potent type-specific inhibitor of the V-PPase has been identified. Strict criteria for the identification of V-PPase activity in uncharacterized membrane fractions, other than through the measurement of azide-, orthovanadate-, and bafilomycin-insensitive, K^+-stimulated, PPi-dependent H^+ translocation (Rea and Turner, 1990), are therefore lacking.

Here we remedy this deficiency to show that 1,1-diphosphonates, when used under appropriate conditions, are potent V-PPase-specific inhibitors with little or no activity against other phosphohydrolases, whether they are ion-translocating or soluble, and that aminomethylenediphosphonate is the most potent inhibitor of the V-PPase yet identified, causing appreciable inhibition at submicromolar concentrations. The remarkable sensitivity of both the plant V-PPase and H^+-PPi synthase of *Rhodospirillum* to inhibition by aminomethylenediphosphonate, hand in hand with the near total lack of inhibition of other phosphohydrolases under the conditions employed, demonstrates the applicability of this diphosphonate as a potent type-specific inhibitor of both plant and phototrophic bacterial H^+-pyrophosphatases.

MATERIALS AND METHODS

Materials

Seeds of mung bean (*Vigna radiata* cv Berken) were surface sterilized, allowed to imbibe in tap water for 48 h, and grown in a 1:1 mixture of vermiculite and perlite in the dark at 20 to 25°C. Etiolated hypocotyls were harvested 3 to 4 d after planting. Fresh red beets (*Beta vulgaris* L.) were purchased commercially. Cells of *Rhodospirillum rubrum* (strain S1) were cultured in a medium containing 0.3% (w/v) peptone, 0.3% (w/v) yeast extract, 1.6 mM $MgCl_2$, and 1.0 mM $CaCl_2$ (pH 7.0) at 30°C at a light intensity of 20 $\mu E\ s^{-1}\ m^{-2}$. Cells were harvested after 2 to 3 d of growth. Crude preparations of nonspecific phosphomonoesterase from *Vigna* were obtained from the supernatant fraction remaining after the sedimentation of microsomes at 100,000*g*. HPLC-purified soluble PPase from *Saccharomyces cerevisiae* and alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1; type VII-T) were purchased from Sigma.

Preparation of Membranes

Tonoplast vesicles were isolated from etiolated hypocotyls of *Vigna* as described previously (Rea et al., 1992a), plasma membrane-enriched vesicles were purified from *Beta* storage root by Suc density gradient centrifugation of microsomes (Rea et al., 1987), and chromatophores were purified from *Rhodospirillum* as described by Atta-Asafo-Adjei and Daldal (1991). To ensure direct comparability between the chromatophore, tonoplast, and plasma membrane vesicle preparations, the former were diluted into standard vesicle suspension medium (10% [v/v] glycerol, 1 mM Tris-EGTA, 2 mM DTT, 10 mM Tris-Mes, pH 7.2) and pelleted at 100,000*g* for 40 min. The pellet was resuspended in the same medium, frozen in liquid nitrogen, and stored at −85°C.

PPase and ATPase Assays

For the experiments requiring the measurement of PPi hydrolysis at low substrate concentrations (Fig. 1 and Table I), the liberation of Pi from PPi was monitored continuously at 25°C using an automated Pi analyzer (Baykov and Avaeva, 1981). The reaction media contained 1 mM $MgCl_2$, 50 mM KCl, 40 μM EGTA, 5 μM gramicidin-D, 60 mM Tris-HCl (pH 7.5), and the indicated concentrations of Tris-PPi. Diphosphonates or imidodiphosphate were added as their 1:1 magnesium complexes. In all other experiments, PPase or ATPase activity was estimated discontinuously as described by Rea and Turner (1990). PPase activity was assayed in reaction media containing 300 μM Tris-PPi, 1.3 mM $MgSO_4$, 50 mM KCl, 5 μM gramicidin-D, 30 mM Tris-Mes (pH 8.0), and the indicated concentrations of diphosphonate or imidodiphosphate. V-ATPase and plasma membrane H^+-ATPase activity were measured at pH 8.0 and pH 6.5, respectively, in the same medium containing 3 mM Tris-ATP and 3 mM $MgSO_4$ in place of 300 μM Tris-PPi and 1.3 mM $MgSO_4$. Two hundred micromolar molybdate and 1 mM sodium azide were added to the plasma membrane H^+-ATPase assay media to minimize interference from contaminating phosphomonoesterase and mitochondrial F-H^+-ATPase, respectively (Rea and Turner, 1990).

Measurement of PPi- and ATP-Dependent H^+-Translocation

PPi- and ATP-dependent H^+-translocation by tonoplast vesicles isolated from *Vigna* and chromatophores from *Rhodospirillum* were assayed fluorimetrically at 25°C using the monoamine dye acridine orange as ΔpH indicator (Rea and Turner, 1990). Fluorescence was measured at excitation and emission wavelengths of 495 and 540 nM, respectively, at a slit width of 5 nM for both emission and excitation. The assay media contained 300 μM Tris-PPi (or 3 mM Tris-ATP), 50 mM KCl, 2.5 μM acridine orange, 5 mM Tris-Mes (pH 8.0), and the indicated concentrations of PPi analogs. Intravesicular acidification was initiated by the addition of 1.3 mM (or 3.0 mM) $MgSO_4$ and terminated by the addition of protonophore (2.5 μM FCCP).

Protein was estimated by the semimicro method of Peterson (1977).

Chemicals

The diphosphonates employed in this study were kind gifts from Dr. S.V. Komissarenko (Institute of Biochemistry, Kiev, Russia) and Dr. B.S. Cooperman (University of Pennsylvania,

Philadelphia). All of the general reagents were purchased from Sigma, Fisher Scientific (Pittsburgh, PA), or Research Organics, Inc. (Cleveland, OH).

RESULTS

Competitive Inhibition of V-PPase by PPi Analogs

Five 1,1-diphosphonates and imidodiphosphate were tested for their capacity to inhibit the V-PPase of tonoplast vesicles isolated from *V. radiata*. To ensure effective competition and to enable the enumeration of apparent inhibition constants, a PPi concentration equal to the Michaelis constant of the enzyme for total PPi (30 μM; Table I) was employed at the outset.

In all cases, the inhibitor concentration-dependence data approximated the relationship:

$$v = v_o/(1 + [I]/2K_i^{app}) \quad (1)$$

where v_o and v are the rates of PPi hydrolysis at inhibitor concentrations of zero and [I], respectively. The values of the apparent inhibition constant (K_i^{app}) for each of the PPi analogs examined are summarized in Table I.

Aminomethylenediphosphonate was the most active inhibitor tested. Hydroxydiphosphonates and imidodiphosphate were markedly more effective than dichloromethylenediphosphonate and methylenediphosphonate, but aminomethylenediphosphonate was the most potent PPi analog, causing a significant decrease in the rate of substrate hydrolysis at submicromolar concentrations. The calculated K_i^{app} values for the V-PPase followed the sequence dichloromethylenediphosphonate (>500 μM) >> methylenediphosphonate (68 μM) >> imidodiphosphate (12 μM) > ethane-1-hydroxy-1,1-diphosphonate (6.5 μM) ≅ hydroxymethylenediphosphonate (5.7 μM) > aminomethylenediphosphonate (1.8 μM) (Table I).

Inhibition of the V-PPase by aminomethylenediphosphonate was competitive with respect to PPi (Fig. 1), and the true inhibition constant (K_i) calculated by nonlinear least-squares analysis was 1.0 μM.

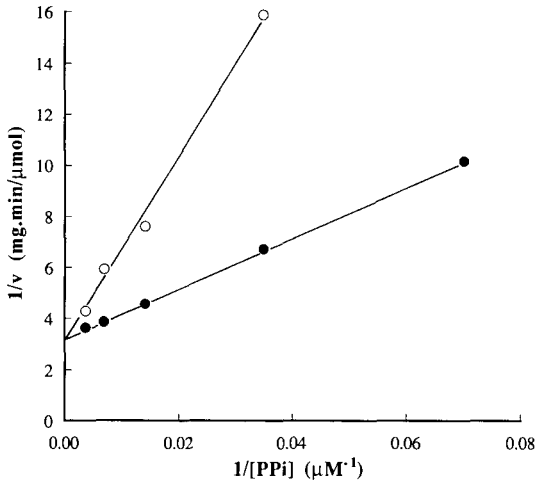

Figure 1. Double-reciprocal plot for PPi hydrolysis by the V-PPase of tonoplast vesicles isolated from *V. radiata* in the absence (●) and presence (○) of 3 μM aminomethylenediphosphonate. The liberation of Pi from PPi was monitored continuously using an automated phosphate analyzer (Baykov and Avaeva, 1981). The reaction media contained 50 mM KCl, 40 μM EGTA, 1 mM $MgCl_2$, 5 μM gramicidin-D, 60 mM Tris-HCl, and the indicated concentrations of PPi.

Aminomethylenediphosphonate as a Type-Specific Inhibitor

With the objective of determining their suitability as type-specific inhibitors of the V-PPase, three PPi analogs–methylenediphosphonate, an unsubstituted diphosphonate, aminomethylenediphosphonate, the most effective diphosphonate, and imidodiphosphate, the most commonly employed competitive inhibitor of the V-PPase–were investigated further. Three primary criteria were applied: (a) Inhibition was examined at a PPi concentration (300 μM) corresponding to 10 times the K_m of the V-PPase for PPi, the usual concentration used to assay the enzyme in vitro (Rea and Turner, 1990) and the prevailing cytosolic concentration of this compound in plants (Weiner et al., 1987; Takeshige and Tazawa, 1989), to assess the potential of the PPi analogs as inhibitors both in vitro and in vivo. (b) A range of plant ion translocases (V-PPase, V-ATPase, and plasma membrane H^+-ATPase) and PPi hydrolases (soluble PPase, alkaline phosphatase, and nonspecific phosphomonoesterase) were tested for their susceptibility to inhibition to determine the specificity of the compounds concerned. (c) The inhibitor

Table I. *Apparent inhibition constants of PPi analogs with the general structure O_3P-R-PO_3*

The assays were performed as described in the legend to Figure 1 and the K_i^{app} values were estimated using Equation 1.

Compound	R	K_i^{app}		
		V-PPase (*Vigna*)	Soluble PPase (rat liver)[a]	Soluble PPase (*Saccharomyces*)[a]
			μM	
Methylenediphosphonate	-CH_2-	68	70	1100
Aminomethylenediphosphonate	-$CH(NH_2)$-	1.8	11	20
Hydroxymethylenediphosphonate	-CH(OH)-	5.7	8	50
Ethane-1-hydroxy-1,1-diphosphonate	-$C(CH_3)OH$-	6.5	7	>3000
Dichloromethylenediphosphonate	-$C(Cl_2)$-	>500	180	>3000
Imidodiphosphate	-NH-	12	2.2	15
Pyrophosphate[b]	-O-	30	1.0	0.9

[a] Values from Smirnova et al. (1988). [b] Apparent Michaelis constant for total PPi in the presence of 1 mM Mg^{2+}.

sensitivities of PPi hydrolysis and PPi-dependent H^+ translocation by the plant V-PPase and H^+-PPi synthase of chromatophores from *R. rubrum*, which are suspected to be homologous, were compared to define the potential of the inhibitors for the (provisional) identification of "V-like PPases" in membranes from disparate sources.

Figures 2, 3, and 4 summarize the results of these analyses. All of the analogs inhibited substrate hydrolysis by the V-PPase of *Vigna* and H^+-PPi synthase of *Rhodospirillum* to similar degrees and in the same rank order. The K_i^{app} values for inhibition by aminomethylenediphosphonate, imidodiphosphate, and methylenediphosphonate were 1.7, 11, and 54 μM for the V-PPase and 1, 8, and 18 μM for the H^+-PPi synthase (Fig. 2, A and B). Accordingly, aminomethylenediphosphonate strongly inhibited both the initial rate and extent of PPi-dependent ΔpH formation by tonoplast vesicles from *Vigna* and chromatophores from *Rhodospirillum* to yield K_i^{app} values of 3.6 and 4.0 μM, respectively (Figs. 3 and 4).

The results shown in Figure 2, C and D, and Figure 4 confirm the specificity of the inhibitors examined when a PPi concentration of 300 μM is employed for the PPi hydrolase assays and an ATP concentration of 3 mM is employed for the ATPase assays. Neither aminomethylenediphosphonate nor methylenediphosphonate inhibited the soluble PPase from *Saccharomyces*, phosphomonoesterase from *Vigna*, alkaline phosphatase from bovine intestinal mucosa, V-ATPase from *Vigna*, or plasma membrane H^+-ATPase from *Beta* (Figs. 2, C and D, and 4). Imidodiphosphate was similarly inactive with all of these enzymes except for the soluble PPase, which showed slight inhibition at high analog concentrations (Fig. 2C). In no case was appreciable hydrolysis of the PPi analogs detected during the course of the assays.

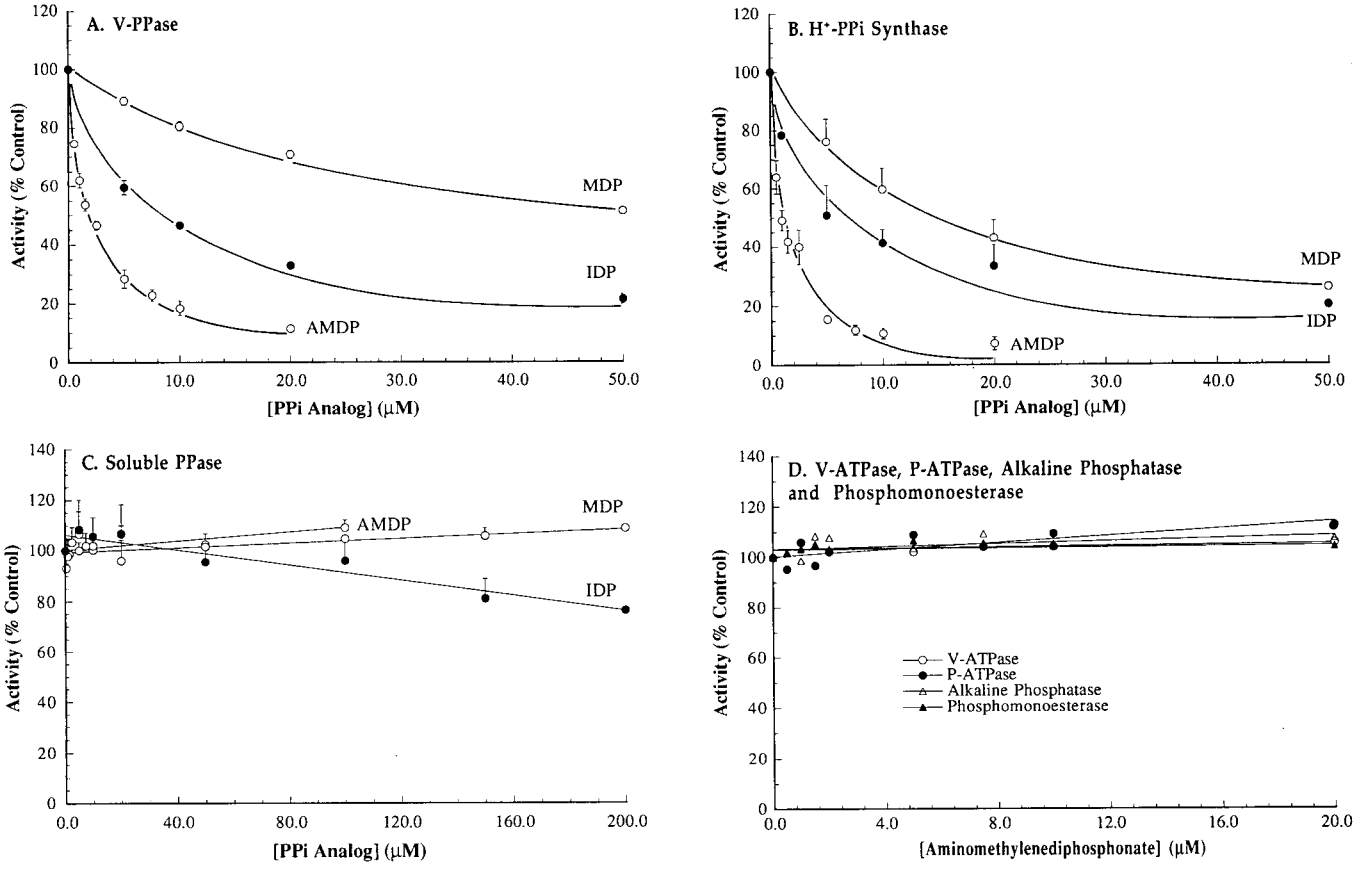

Figure 2. Comparison of effects of different PPi analogs on substrate hydrolysis by V-PPase of tonoplast vesicles isolated from *Vigna* (A), H^+-PPi synthase of chromatophores isolated from *R. rubrum* (B), purified soluble PPase from *S. cerevisiae* (C), and V-ATPase, plasma membrane H^+-ATPase, alkaline phosphatase, and phosphomonoesterase of *Vigna* tonoplast vesicles, *Beta* plasma membrane vesicles, bovine intestinal mucosa, and *Vigna* soluble fraction, respectively (D). PPase, alkaline phosphatase, and phosphomonoesterase activity were assayed in a reaction medium containing 300 μM Tris-PPi, 1.3 mM $MgSO_4$, 50 mM KCl, 5 μM gramicidin-D, 30 mM Tris-Mes buffer (pH 8.0), and the indicated concentrations of PPi analogs. V-ATPase and plasma membrane H^+-ATPase activity were assayed at pH 8.0 and pH 6.5, respectively, in the same medium containing 3 mM Tris-ATP and 3 mM $MgSO_4$. The specific (control) activities of the enzyme preparations employed were: V-PPase, 0.6 μmol mg^{-1} min^{-1}; H^+-PPi synthase, 0.3 μmol mg^{-1} min^{-1}; soluble PPase, 81.7 μmol mg^{-1} min^{-1}; V-ATPase, 0.4 μmol mg^{-1} min^{-1}; plasma membrane H^+-ATPase, 0.8 μmol mg^{-1} min^{-1}; alkaline phosphatase, 20.8 μmol mg^{-1} min^{-1}; phosphomonoesterase, 0.5 μmol mg^{-1} min^{-1}. Values shown are the mean ± SE for four replicates. AMDP, Aminomethylenediphosphonate; IDP, imidodiphosphate; MDP, methylenediphosphonate.

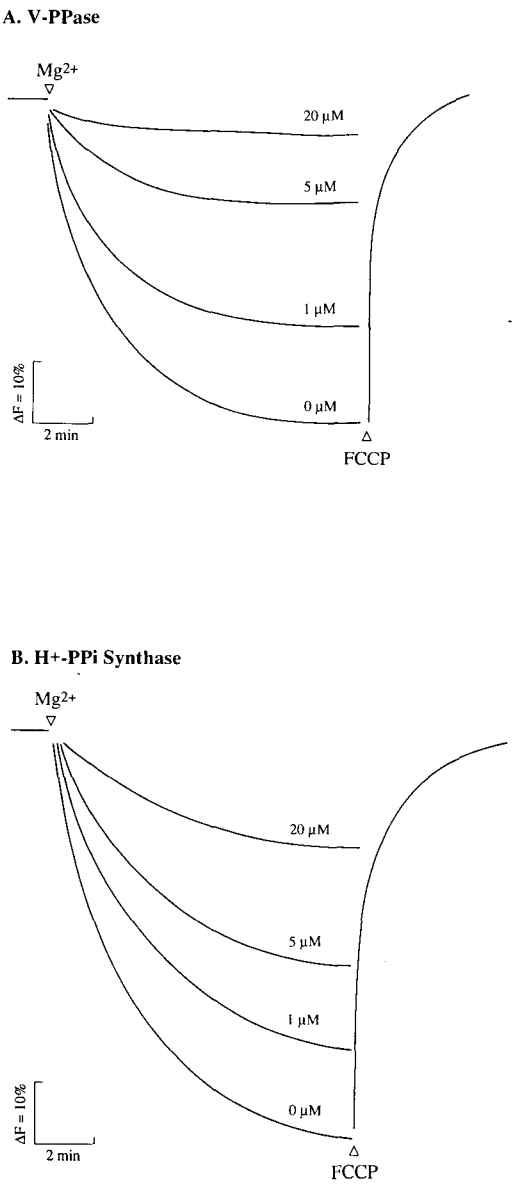

Figure 3. Inhibition of PPi-dependent H^+-translocation by aminomethylenediphosphonate. A, V-PPase-mediated H^+-translocation into tonoplast vesicles (20 μg/mL) isolated from *V. radiata* in the presence of 0, 1, 5, and 20 μM aminomethylenediphosphonate. B, H^+-PPi synthase-mediated H^+-translocation into chromatophores (100 μg/mL) isolated from *R. rubrum* in the presence of 0, 1, 5, and 20 μM aminomethylenediphosphonate. Intravesicular acidification (ΔpH formation) was initiated by the addition of 1.3 mM $MgSO_4$ to assay media containing 300 μM Tris-PPi, 50 mM KCl, and 5 mM Tris-Mes (pH 8.0). Uncoupler (2.5 μM FCCP) was added at the times indicated.

CONCLUSIONS

The studies described demonstrate that the 1,1-diphosphonate aminomethylenediphosphonate is 6- to 38-fold more active as an inhibitor than the two PPi analogs most commonly employed for investigations of the V-PPase, methylenediphosphonate and imidodiphosphate (e.g. Chanson and Pilet, 1987; Table I). As such, aminomethylenediphosphonate is an inhibitor of unsurpassed potency that should prove invaluable as a diagnostic tool for the identification of V-PPase activity and membrane fractions with which the enzyme is associated.

Two factors contribute to the exquisite selectivity of aminomethylenediphosphonate as an inhibitor of the V-PPase when the assays are performed at 300 μM PPi: (a) the 6- to 12-fold greater susceptibility of the V-PPase to inhibition by this 1,1-diphosphonate by comparison with other PPases (Table I); and (b) the high K_m of the V-PPase for substrate (Table I). All PPases (with the exception of the H^+-PPi synthase) have K_m values that are at least 1 order of magnitude smaller than that of the V-PPase (Table I). From the expression for simple competitive inhibition:

$$v = \frac{V_{max}\,[S]}{K_m\,\{1 + ([I]/K_i)\} + [S]} \qquad (2)$$

therefore, it follows that even if all of the enzymes concerned had similar inhibitor profiles, the V-PPase would be the most strongly inhibited. For an enzyme with a K_m of 1 μM (e.g. a soluble PPase), an inhibitor (I) with a K_i of 2 μM would inhibit activity by only about 3% when [I] = 20 μM and [PPi] = 300 μM. For an enzyme with a K_m of 30 μM (e.g. the V-PPase), a similar concentration of inhibitor would cause approximately 50% inhibition under the same conditions. Thus, the K_m factor, together with the greater intrinsic sensitivity of the V-PPase to aminomethylenediphosphonate, confer on this agent a high degree of specificity.

The type specificity of aminomethylenediphosphonate under the assay conditions employed is substantiated by its

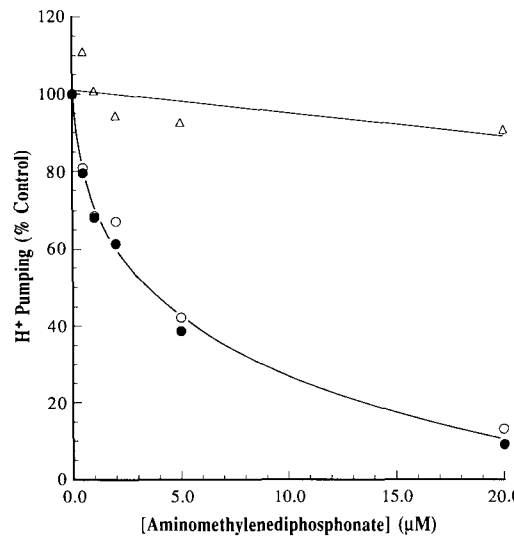

Figure 4. Relationship between initial rate of H^+ translocation and aminomethylenediphosphonate concentration for V-PPase (*Vigna*) (●), H^+-PPi synthase (*Rhodospirillum*) (○), and V-ATPase (*Vigna*) (△). PPi-dependent intravesicular acidification (V-PPase and H^+-PPi synthase) was assayed as described in Figure 3 and ATP-dependent acidification was assayed in the same medium except that 3.0 mM Tris-ATP and 3.0 mM $MgSO_4$ replaced the 300 μM Tris-PPi and 1.3 mM $MgSO_4$ employed in the PPase assays.

complete inactivity against the V-ATPase, plasma membrane H^+-ATPase, alkaline phosphatase, and phosphomonoesterase. Its general applicability, on the other hand, is suggested by its marked inhibition of the H^+-PPi synthase of *Rhodospirillum*. In accord with their proposed common membership in the same category of ion translocase (Rea and Poole, 1993), both the plant V-PPase and bacterial H^+-PPi synthase, unlike all other characterized translocases and PPi hydrolases, exhibit similar inhibitor profiles and are strongly inhibited by aminomethylenediphosphonate. It is probable, therefore, that the use of aminomethylenediphosphonate as a type-specific inhibitor of the V-PPase is not restricted to plant systems but also extends to nonplant cells containing "V-PPase" homologs.

It is not known why aminomethylenediphosphonate is such a potent inhibitor of the V-PPase, but it is perhaps significant that this 1,1-diphosphonate is unique among the compounds screened because it contains an amino group that carries a positive charge at pH 8.0. Its high activity toward the V-PPase may therefore be indicative of the presence of a carboxylate group proximal to the substrate-binding site of the enzyme to which the protonated amino function of aminomethylenediphosphonate binds, thereby increasing the stability of the inhibitor-enzyme complex.

Although the results of these in vitro inhibitor studies demonstrate the utility of aminomethylenediphosphonate as a diagnostic probe, a cautionary note is warranted concerning their extension to intact cells. The suitability of 1,1-diphosphonates and other PPi analogs as V-PPase-specific inhibitors in vivo will be critically dependent on the distribution of PPi and target PPi hydrolases between compartments within the cell. Neuhaus and Stitt (1991) describe the administration of imidodiphosphate to detached leaves via the transpiration stream and show that this is accompanied by the inhibition of Suc synthesis concomitant with the depletion of cellular UDP-Glc and accumulation of PPi, hexose phosphates, and Fru-1,6-bisP.

On the basis of the observation that soluble PPase activity is largely absent from the cytosol of photosynthetic tissues and almost exclusively located in the chloroplast stroma (Weiner et al., 1987), the V-PPase has been deduced to be the sole enzyme responsible for the disposal of the (cytosolic) PPi generated by UDP-Glc pyrophosphorylase (Weiner et al., 1987). By inference, the inhibition of Suc synthesis and the elevation of cytosolic PPi seen during the administration of imidodiphosphate has been attributed to selective inhibition of the V-PPase and retardation of the UDP-Glc pyrophosphorylase reaction through end-product (PPi) accumulation (Quick et al., 1989; Neuhaus and Stitt, 1991). The validity of this conclusion, however, is contingent on the relative contributions made by the cytosol itself and the chloroplast stroma to total cellular PPi turnover and the PPi concentrations prevailing in these compartments. Specifically, if stromal PPi concentrations are less than 1 μM, as indicated by the results of intracellular perfusion studies of *Chara* (Takeshige and Tazawa, 1989), and the bulk of soluble PPase activity is restricted to the chloroplast stroma (Weiner et al., 1987), imidodiphosphate would be expected to inhibit not only the V-PPase but also the stromal PPase. The K_i values for inhibition of soluble PPases by imidodiphosphate fall in the range 2 to 15 μM (Smirnova et al., 1988; Table I); the corresponding value for the V-PPase is 12 μM (Table I). Therefore, it is evident from Equation 2 that if the stromal PPase has a K_m of 1 to 5 μM PPi (Table I) and the V-PPase has a K_m of 30 μM, the former will be considerably more sensitive to inhibition by imidodiphosphate than the latter in vivo if imidodiphosphate readily equilibrates between the cytosol and stroma.

The predicted high sensitivity of the stromal enzyme in vivo is simply a reflection of the observation that cytosolic PPi concentrations exceed the K_m of the V-PPase by about 10-fold, whereas stromal PPi content is approximately equal to the K_m of the soluble PPase. Thus, although PPi analogs such as imidodiphosphate, but more particularly aminomethylenediphosphonate, can be used as type-specific inhibitors of the V-PPase in vitro, where PPi concentration can be controlled and set at a value far in excess of the K_m values of other PPi hydrolases, the use of such compounds as type-specific inhibitors in vivo is limited by the effects of compartmental substrate concentration on inhibitor efficacy.

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