

# Oxygen exchange reactions catalyzed by vacuolar H<sup>+</sup>-translocating pyrophosphatase

## Evidence for reversible formation of enzyme-bound pyrophosphate

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### Abstract

Vacuolar membrane-derived vesicles isolated from *Vigna radiata* catalyze oxygen exchange between medium phosphate and water. On the basis of the inhibitor sensitivity and cation requirements of the exchange activity, it is almost exclusively attributable to the vacuolar H<sup>+</sup>-pyrophosphatase (V-PPase). The invariance of the partition coefficient and the results of kinetic modeling indicate that exchange proceeds via a single reaction pathway and results from the reversal of enzyme-bound pyrophosphate synthesis. Comparison of the exchange reactions catalyzed by V-PPase and soluble PPases suggests that the two classes of enzyme mediate P<sub>i</sub>-HOH exchange by the same mechanism and that the intrinsic reversibility of the V-PPase is no greater than that of soluble PPases.

**Key words:** Inorganic pyrophosphatase; Oxygen exchange; Phosphate; Proton pump; Pyrophosphate; Vacuolar membrane

### 1. Introduction

Chemiosmotic coupling of energy-dependent solute transport across the vacuolar membrane of plant cells is contingent on the ability of two primary H<sup>+</sup> pumps, the V-PPase (EC 3.6.1.1) and V-ATPase (EC 3.6.1.3) [1,2], both of which mediate electrogenic H<sup>+</sup> translocation from the cytosol to vacuole lumen. Protein purification [3–6], covalent modification [3,5], molecular cloning [7–9] and heterologous expression [9] have demonstrated the abundance, high catalytic activity, novelty and structural simplicity of the V-PPase, and patch clamp analyses implicate the pump in the primary translocation of both H<sup>+</sup> and K<sup>+</sup> into the vacuole [10]. However, despite these advances, very little is understood of how the one polypeptide species constituting the V-PPase accomplishes catalysis.

Steady-state kinetic investigations have contributed towards identification of the ligands involved in V-PPase-mediated substrate hydrolysis. For instance, the pronounced Mg<sup>2+</sup>-dependence of PP<sub>i</sub> hydrolysis is explicable in terms of a three-state reaction scheme in which dimagnesium pyrophosphate (Mg<sub>2</sub>PP<sub>i</sub>) is the active substrate species and catalysis is performed by preformed

enzyme-Mg<sup>2+</sup> complex [11]. Steady-state rate measurements, however, suffer from one major shortcoming: they do not enable resolution of the catalytic steps following substrate binding. In contrast, oxygen exchange measurements through, for example, mass spectral or NMR analyses of the disappearance of <sup>18</sup>O from medium [<sup>18</sup>O]P<sub>i</sub> not only provide estimates of the rate of covalent bond formation/cleavage at the active site but also enable enumeration of the probability of bound P<sub>i</sub> undergoing the reaction leading to the exclusion of a water molecule vs. simple release of P<sub>i</sub> back into solution [12,13].

In this paper we report the results of a kinetic study of <sup>18</sup>O-exchange between medium P<sub>i</sub> and water ('medium exchange') and during PP<sub>i</sub> hydrolysis ('intermediate exchange') catalyzed by the V-PPase of vacuolar membrane vesicles isolated from *Vigna radiata* (mung bean). It is shown that the V-PPase has appreciable exchange activity and this is explicable in terms of the reversible formation of enzyme-bound PP<sub>i</sub>.

### 2. Materials and methods

Vacuolar membrane vesicles were purified from etiolated hypocotyls of *V. radiata* by differential and density gradient centrifugation [14], suspended in suspension medium (10% (w/v) glycerol, 1 mM ethylene glycol bis(b-aminoethyl ether)N,N'-tetraacetate (EGTA), 1 mM dithiothreitol, 5 mM Tris-MES, pH 7.2), frozen in liquid N<sub>2</sub> and stored at –85°C. Purified enzyme was prepared by gel filtration as described previously [11,15].

Tris salts of [<sup>18</sup>O]-enriched P<sub>i</sub> (98.4%) and PP<sub>i</sub> (94.2%) were prepared as detailed by Hackney et al. [16]. The incubations with [<sup>18</sup>O]P<sub>i</sub> were

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**Abbreviations:** PPase, inorganic pyrophosphatase; P<sub>i</sub>, pyrophosphate; PP<sub>i</sub>, phosphate; V-ATPase, vacuolar H<sup>+</sup>-ATPase; V-PPase, vacuolar H<sup>+</sup>-pyrophosphatase.

performed in a total volume of 0.1 ml containing vacuolar membrane vesicles (0.28–0.35 mg protein), 4% (w/v) glycerol, 0.4 mM dithiothreitol, 40  $\mu$ M EGTA, 5  $\mu$ M gramicidin-D, 2 mM Tris-MES and the indicated concentrations of  $P_i$ ,  $MgCl_2$ , KCl and NaCl. The reaction media were incubated for 2–3 h at 25°C, and immediately applied to a column packed with Dowex 1  $\times$  4 (Cl-form, 100 mesh) for the separation of  $P_i$  [13]. The incubations with [ $^{18}O$ ]PP $_i$  were performed in a total volume of 1 ml containing vacuolar membrane vesicles (0.07 mg protein) or the equivalent activity of purified V-PPase, 0.5 mM PP $_i$ , 2 mM  $MgCl_2$ , 50 mM KCl, 5% (w/v) glycerol, 0.5 mM dithiothreitol, 50  $\mu$ M EGTA and 34 mM Tris-HCl, pH 7.5. Reaction was allowed to proceed for 10 min at 25°C, and the  $P_i$  was separated as described above. The activity of the V-PPase was unchanged during the course of the incubations and no more than 12–26% of the added PP $_i$  was hydrolyzed. The distribution of the  $^{18}O$  label in the  $P_i$  recovered from the incubations was analyzed by gas chromatography-mass spectrometry [13]. The values of the partition coefficient,  $P_c$ , and the exchange rate were calculated by the procedure of Hackney [12] and corrected for the [ $^{16}O$ ]PP $_i$  (approximately 0.2 mM) introduced into the  $P_i$ -HOH exchange media by the addition of vesicles. All data were fitted by non-linear regression analysis [17].

### 3. Results

Mass-spectral analysis of reaction media initially containing highly enriched [ $^{18}O$ ]PP $_i$  and unlabeled water demonstrated that vacuolar membrane vesicles isolated from etiolated hypocotyls of *V. radiata* catalyze oxygen exchange between medium  $P_i$  and water. Medium  $P_i$ -HOH exchange is selectively stimulated by KCl versus NaCl (Fig. 1) and has a near absolute requirement for  $Mg^{2+}$  (Fig. 2). The rate measured in the presence of 50 mM KCl was 2.2 times greater than with 50 mM NaCl (Fig. 1) and that measured in the absence of  $Mg^{2+}$  is only 1.4% of the rate estimated in the presence of 5–10 mM  $MgCl_2$  (Fig. 2). In conjunction with the inhibitor sensitivities of the reaction, these results clearly show that the V-PPase, the dominant phosphohydrolase activity associated with vacuolar membrane vesicles prepared from *V. radiata* [4,14], is responsible for the exchange reaction. First, medium  $P_i$ -HOH exchange exhibits a second-order dependence on  $P_i$  concentration (Fig. 2), indicating a requirement for the binding of two  $P_i$  molecules to the enzyme. This is consistent with the participation of PP $_i$

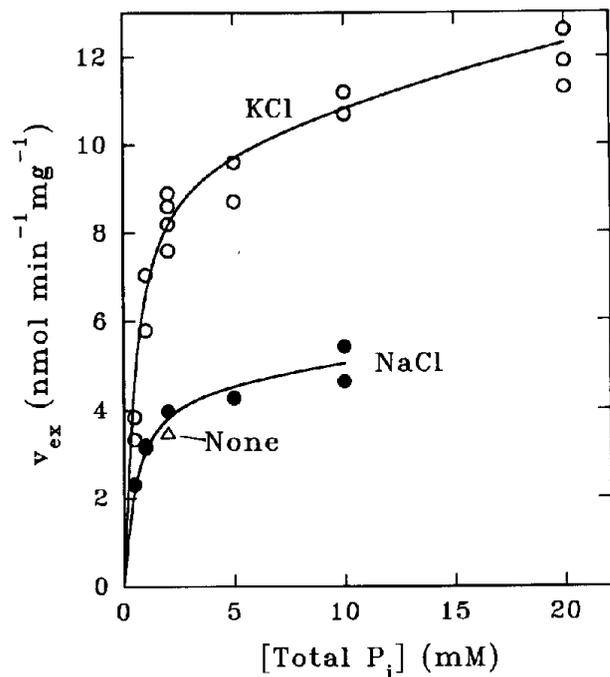


Fig. 1. Influence of alkali metal ions on  $P_i$  concentration-dependence of rate of medium  $P_i$ -HOH oxygen exchange by vacuolar membrane vesicles. The rates of exchange ( $v_{ex}$ ) were measured in media containing no alkali metal ions ( $\Delta$ ), 50 mM KCl ( $\circ$ ) or 50 mM NaCl ( $\bullet$ ) in the presence of 20 mM  $MgCl_2$ . The upper line was computed for Scheme 1 using Eq. (1).

as an intermediate in the exchange reaction. Second, the pronounced  $Mg^{2+}$  concentration dependence is in agreement with what is known of the substrate hydrolytic activity of the V-PPase. Since the hydrolysis of PP $_i$  requires the formation of an  $Mg_2PP_i$  complex [11], it is reasonable that binding of two  $MgP_i$  molecules will be required for the reaction resulting in medium  $P_i$ -HOH oxygen exchange. Third, unlike soluble PPases, whose activity is not specifically modulated by monovalent cations, the V-PPase is strongly stimulated by these ions in the sequence  $K^+ > Na^+ > \text{none}$ . The order of potency is the same whether PP $_i$  hydrolysis, PP $_i$ -dependent  $H^+$  translocation or, as shown here, oxygen exchange is measured. Fourth, agents which are known to inhibit substrate hydrolysis by the V-PPase inhibit oxygen exchange. Potassium fluoride and  $Ca^{2+}$ , both of which are potent inhibitors of the V-PPase [18–20], inhibit medium  $P_i$ -HOH oxygen exchange by more than 90% whereas addition of the type-specific V-ATPase inhibitor bafilomycin  $A_1$  [21] causes 50% stimulation (Table 1). Likewise, pretreatment of vesicles with *N*-ethylmaleimide in the presence of both  $Mg^{2+}$  and PP $_i$  [3], which irreversibly inhibits only 6% of the total PP $_i$  hydrolytic activity but 65% of the total ATP hydrolytic activity, diminishes the rate of exchange by only 27% when the F-ATPase inhibitor,  $NaN_3$ , is included in the reaction medium (Table 1). Consequently, any contribution from phosphohydro-

Table 1  
Effects of inhibitors on rate of and  $P_c$  value for medium  $P_i$ -HOH oxygen exchange by vacuolar membrane vesicles

Inhibitor	Exchange rate, $nmol \cdot min^{-1} \cdot mg^{-1}$	$P_c$
None	3.57	0.14
KF (20 mM)	0.10	
$CaCl_2$ (0.5 mM)	0.32	
Aminomethylenediphosphonate (0.5 mM)	1.90	0.16
Bafilomycin $A_1$ (0.1 mM)	5.40	0.14
<i>N</i> -Ethylmaleimide (50 $\mu$ M) + $NaN_3$ (1 mM)	2.75	0.13

All reactions were performed in the presence of 2 mM  $P_i$  and 1 mM  $MgCl_2$ .

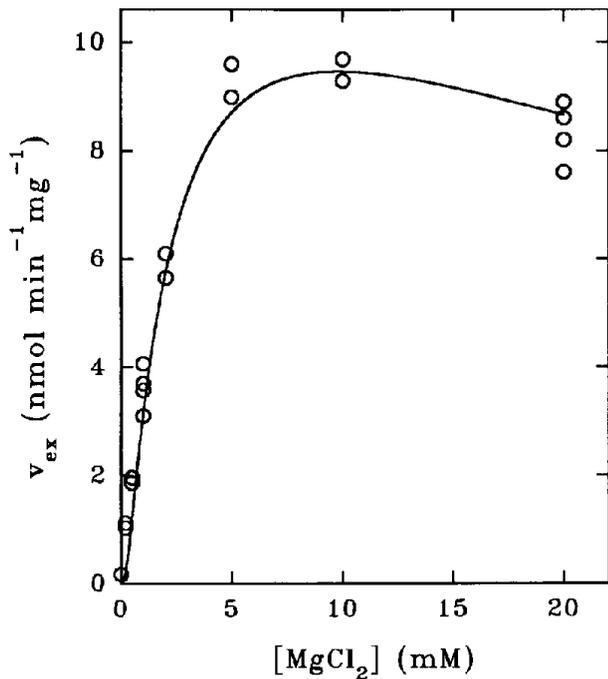


Fig. 2. Dependence of the rate of medium  $P_i$ -HOH oxygen exchange by vacuolar membrane vesicles on the concentration of  $MgCl_2$ . All reactions were performed in the presence of 2 mM  $P_i$  and 50 mM KCl. The line shown was computed using Eq. (1).

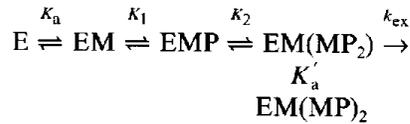
lases other than the V-PPase is negligible. Although aminomethylenediphosphonate, a potent type-specific inhibitor of the V-PPase [18,22], partially inhibits the exchange reaction its effect is much less pronounced than would be expected from its  $K_i$  of 1  $\mu M$ , estimated from steady-state  $PP_i$  hydrolysis measurements [18,22], because of precipitation of its magnesium salt during the first few minutes of the incubation under the conditions employed.

By comparison with the exchange rates, the partition coefficient,  $P_c$ , does not exhibit significant dependence on alkali metal,  $P_i$  or  $Mg^{2+}$  ion concentration. The values of  $P_c$  (mean  $\pm$  S.E.) are  $0.17 \pm 0.02$  ( $n = 4$ ),  $0.15 \pm 0.01$  ( $n = 33$ ) and  $0.18 \pm 0.02$  ( $n = 11$ ) in the absence of added alkali metal ions and in the presence of 50 mM KCl and 50 mM NaCl, respectively. The predicted distributions approximated the measured ones in all cases, and the average sum of the deviations for the five  $P_i$  species ( $P^{18}O_0$ ,  $P^{18}O_1$ ,  $P^{18}O_2$ ,  $P^{18}O_3$ ,  $P^{18}O_4$ ) is only 1.15%, indicative of a uniform reaction scheme with a single catalytic pathway. Analogously, the activities remaining after treatment of the membrane vesicles with inhibitors are attributed to the same reaction pathway since the  $P_c$  values obtained after inhibition are similar to those of untreated controls (Table 1).

In order to provisionally identify the enzyme species participating in medium  $P_i$ -HOH exchange, the  $P_i$  and  $MgCl_2$  concentration-dependence data (Figs. 1 and 2)

were fitted to the kinetic models listed in Table 2. In all cases, the enzyme (E) was assumed to be active as its  $Mg^{2+}$  complex (EM) [12] and the rate equations were expressed with respect to the concentrations of free  $Mg^{2+}$  (M) and  $MgP_i$  (MP) [23]. The goodness of fit was enumerated as the minimum sum of the squares of the residuals for 30 independent rate measurements.

Of the 13 model examined, model 10:



Scheme 1.

gave the best fit. This model assumes that medium  $P_i$ -HOH exchange depends on the binding of both  $P_i$  and  $MgP_i$  to EM such that the exchange reaction has a second order dependence on  $P_i$  concentration.

The rate equation for model 10 is:

$$v_{ex} = k_{ex}[E]_0 / (1 + [M]/K'_a + K_2/[MP] + K_1K_2/[MP]^2 + K_1K_2K_a/[MP]^2[M]) \quad (1)$$

in which  $[E]_0$ ,  $[M]$  and  $[MP]$  are the concentrations of total enzyme, free  $Mg^{2+}$  and  $MgP_i$ , respectively,  $k_{ex}$  ( $16.5 \pm 1.4$  nmol/min per 1 mg protein) is the catalytic constant and  $K_a$  (0.26 mM),  $K'_a$  ( $31 \pm 9$ ),  $K_1$  ( $0.10 \pm 0.05$  mM) and  $K_2$  ( $0.57 \pm 0.09$  mM) are dissociation constants.  $K_a$ , which has only a small effect on the fits and parameter values at the high  $Mg^{2+}$  concentrations employed in these studies, was derived from the kinetics of substrate hydrolysis [11].

The close correspondence between the theoretical data computed from Eq. (1) and the experimental results can

Table 2  
Kinetic models for medium  $P_i$ -HOH oxygen exchange by vacuolar membrane vesicles

Model	Enzyme species <sup>a</sup>	Sum of the squares of residuals
1	E, EM, EMP	298
2	E, EM, EM(MP)	33
3	E, EM, EMP, EM(MP)	33
4	E, EM, EM(MP), EMP	35
5	E, EM, EM(MP), EM(MP) <sub>2</sub>	52
6	E, EM, EMP, EM(MP) <sub>2</sub>	19
7	E, EM, EM(MP), EM(MP) <sub>2</sub>	29
8	E, EM, EM(MP), EM(MP) <sub>2</sub> , EM(MP) <sub>2</sub>	51
9	E, EM, EM(MP), EM(MP) <sub>2</sub> , EM(MP) <sub>2</sub>	29
10	E, EM, EMP, EM(MP) <sub>2</sub> , EM(MP) <sub>2</sub>	9.6
11	E, EM, EMP, EM(MP) <sub>2</sub> , EM(MP) <sub>2</sub>	19
12	E, EM, EMP, EM(MP), EM(MP) <sub>2</sub>	29
13	E, EM, EMP, EM(MP), EM(MP) <sub>2</sub>	18

<sup>a</sup>The rightmost species is considered to be the reactive one. E, enzyme; M, magnesium ion; P, phosphate.

be seen in Figs. 1 and 2. Models 1–4, which assume the binding of one  $P_i$  molecule, by comparison, give poor fit and systematic deviations and, while models 3, 6, 11–13 incorporating a preference for free  $P_i$  at the first and  $MgP_i$  at the second binding step, are an improvement on the former, none yields as good a fit as model 10.

The distribution of  $P_i$  species containing from zero to four  $^{18}O$  atoms was also measured during  $[^{18}O]PP_i$  hydrolysis ('intermediate exchange') catalyzed by native vacuolar membrane vesicles, vesicles permeabilized with detergent (CHAPS, 3-[(3-chloraminopropyl)-dimethylammonio]-1-propanesulfonate) and purified V-PPase. In all cases, the  $P_c$  value ( $0.07 \pm 0.01$ ) was approximately two times smaller than that determined for medium  $P_i$ -HOH exchange. It should, however, be appreciated that because the calculation procedure for intermediate exchange involves subtraction of half of the  $P_i$  that is not exchanged during hydrolysis [12], it is more prone to systematic errors.

#### 4. Discussion

The results presented indicate that the V-PPase of plant vacuolar membranes readily catalyzes  $P_i$ -HOH oxygen exchange in the presence of  $Mg^{2+}$ . The cation sensitivity and inhibitor sensitivity profiles of medium  $P_i$ -HOH exchange and the constancy of the  $P_c$  value implicate direct participation of the V-PPase. Oxygen exchange is not only dependent on the provision of  $P_i$  but, like the  $PP_i$  hydrolytic and  $H^+$ -translocation activities of the V-PPase, also exhibits an absolute requirement for  $Mg^{2+}$ , stimulation by  $K^+$  and inhibition by  $Ca^{2+}$  and  $F^-$ . In contrast, acid phosphatase, which is present in large amounts in the initial homogenate and has the potential to contaminate the vesicle preparations, neither requires  $Mg^{2+}$  for activity nor is stimulated by  $K^+$  (Baykov, A.A., Bakuleva, N.P. and Rea, P.A., unpublished). Since addition of the type-specific V-ATPase inhibitor, bafilomycin  $A_1$  and F-ATPase inhibitor,  $NaN_3$ , to the reaction medium does not inhibit the exchange reaction, any contribution from these enzymes is also unlikely.

The medium  $P_i$ -HOH oxygen exchange catalyzed by the V-PPase could, in principle, proceed through one of two alternative pathways: the formation of a phosphoryl-enzyme intermediate ( $E-P_i$ ) [24] or reversible generation of non-covalently-bound  $PP_i$  ( $E \cdot P-P$ ) [25]. Of the two alternatives, the second is the more probable on two counts: (i) the requirement for the binding of two  $P_i$  molecules for medium exchange is difficult to reconcile with  $E-P_i$  formation which demands direct participation of only one  $P_i$  molecule, unless multiple cooperative phosphorylation sites are invoked. (ii) The V-PPase does not appear to undergo autophosphorylation during its catalytic cycle: all attempts to phosphorylate the V-

PPase through the provision of  $[^{32}P]PP_i$  have been unsuccessful (Britten, C.J. and Rea, P.A., unpublished).

If the mechanism involving enzyme-bound  $PP_i$  generation is indeed applicable to the V-PPase, three corollaries may follow: (i)  $P_c$  is the ratio of the rate constant for  $E \cdot P-P$  formation to the sum of this constant and the rate constant for  $P_i$  release from  $E \cdot 2P_i$  (enzyme with two non-covalently bound phosphates).  $P_c$  is therefore a measure of the capacity of the V-PPase for  $E \cdot P-P$  formation and thence reversal through the exclusion of water. (ii) The independence of the value of  $P_c$  from  $P_i$  concentration implies that of the two  $P_i$  molecules involved, the one containing the electrophilic center for attack by water is released from the complex first [26]. (iii) The exchange reaction cannot be limited by the rate of release of the second  $P_i$  into solution [12, 26]. Modulation of medium exchange by monovalent cations during the substrate hydrolytic cycle must necessarily correspond to  $PP_i$  hydrolysis and/or release of the first  $P_i$  molecule.

In conclusion, the medium  $P_i$ -HOH exchange catalyzed by plant vacuolar membranes is deduced to result from the reversible formation of V-PPase-bound  $PP_i$  from  $P_i$  without release of the bound  $PP_i$  to the medium. Given the apparent mechanistic conformity of the V-PPase and soluble PPases in that in both cases catalysis entails the direct participation of three  $Mg^{2+}/PP_i$  hydrolyzed [11,27], the finding that the  $P_c$  value of the V-PPase for medium  $P_i$ -HOH exchange (0.13–0.18) approximates the values obtained for the soluble PPases from *Escherichia coli* and *Saccharomyces cerevisiae* [12,26,28,29] indicates that the  $PP_i$ - $P_i$  interconversion mechanisms of the two classes of enzyme are remarkably alike. The rate of medium  $P_i$ -HOH exchange by the two classes of enzyme exhibits a pronounced requirement for  $Mg^{2+}$  and  $P_i$ , does not necessitate the formation of a phosphoryl-enzyme intermediate and the value of the  $P_c$  is relatively constant. Thus, despite the marked differences in the gross organization of their constituent polypeptides, the congruence between the  $P_c$  values of the V-PPase and soluble PPases demonstrates that the two classes of enzyme catalyze the formation of enzyme-bound  $PP_i$  from  $P_i$  with similar efficiencies.

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