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Oxygen exchange reactions catalyzed by vacuolar H^+-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^+-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_i–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^+ 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^+ 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^+ and K^+ 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^{2+}-dependence of PP_i hydrolysis is explicable in terms of a three-state reaction scheme in which dimagnesium pyrophosphate (Mg_2PP_i) is the active substrate species and catalysis is performed by preformed enzyme–Mg^{2+} 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 ^{18}O from medium [^{18}O]P_i 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_i undergoing the reaction leading to the exclusion of a water molecule vs. simple release of P_i back into solution [12,13].

In this paper we report the results of a kinetic study of ^{18}O-exchange between medium P_i and water ('medium exchange') and during PP_i 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_i.

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_2 and stored at −85°C. Purified enzyme was prepared by gel filtration as described previously [11,15].

Tris salts of ^{18}O-enriched P_i (98.4%) and PP_i (94.2%) were prepared as detailed by Hackney et al. [16]. The incubations with [^{18}O]P_i were

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Abbreviations: PPase, inorganic pyrophosphatase; P_i, pyrophosphate; PP_i, phosphate; V-ATPase, vacuolar H^+-ATPase; V-PPase, vacuolar H^+-pyrophosphatase.

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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 μM EGTA, 5 μM 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 × 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 μM 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]P_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]P_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 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 $K^+ > Na^+ >$ 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-

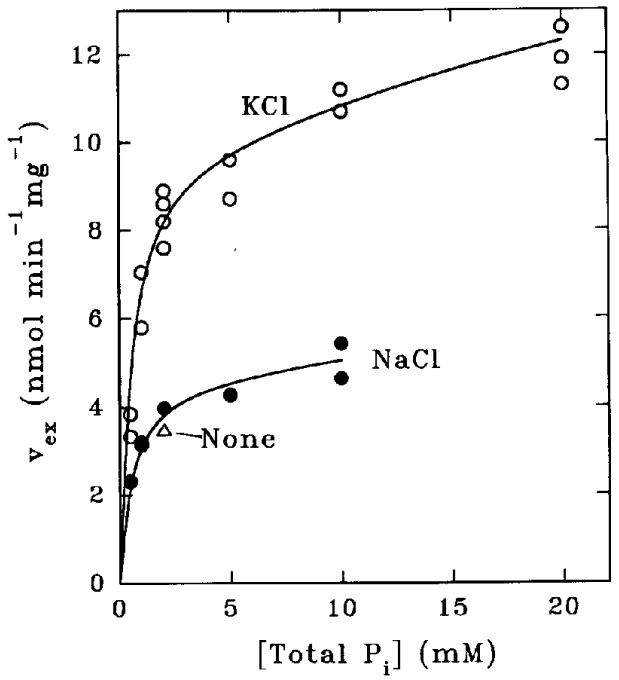

Fig. 1. Influence of alkali metal ions on P_i concentration-dependence of rate of medium P_i HOH oxygen exchange by vacuolar membrane vesicles. The rates of exchange (v_{ex}) were measured in media containing no alkali metal ions (△), 50 mM KCl (○) or 50 mM NaCl (●) in the presence of 20 mM $MgCl_2$.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

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·min^{-1}·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
N-Ethylmaleimide (50 μM) + NaN_3 (1 mM)	2.75	0.13

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

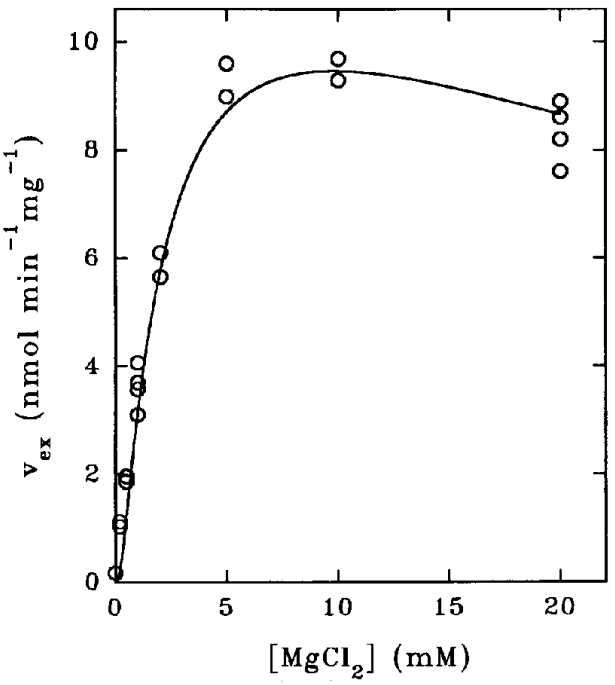

Fig. 2. Dependence of the rate of medium P_i–HOH oxygen exchange by vacuolar membrane vesicles on the concentration of $MgCl_2$. All reactions were performed in the presence of 2 mM P_i and 50 mM KCl. The line shown was computed using Eq. (1).

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 μM, 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 ± S.E.) are 0.17 ± 0.02 (n = 4), 0.15 ± 0.01 (n = 33) and 0.18 ± 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:

$$E \overset{K_a}{\rightleftharpoons} EM \overset{K_1}{\rightleftharpoons} EMP \overset{K_2}{\rightleftharpoons} EM(MP_2) \overset{k_{ex}}{\rightarrow} \underset{EM(MP)_2}{\overset{K_a'}{}}$$

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 ± 1.4 nmol/min per 1 mg protein) is the catalytic constant and K_a (0.26 mM), K_a' (31 ± 9), K_1 (0.10 ± 0.05 mM) and K_2 (0.57 ± 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[a]	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), $EM(MP_2)$	52
6	E, EM, EMP, $EM(MP_2)$	19
7	E, EM, EM(MP), $EM(MP)_2$	29
8	E, EM, EM(MP), $EM(MP)_2$, $EM(MP_2)$	51
9	E, EM, EM(MP), $EM(MP_2)$, $EM(MP)_2$	29
10	E, EM, EMP, $EM(MP)_2$, $EM(MP_2)$	9.6
11	E, EM, EMP, $EM(MP_2)$, $EM(MP)_2$	19
12	E, EM, EMP, EM(MP), $EM(MP_2)$	29
13	E, EM, EMP, EM(MP), $EM(MP)_2$	18

[a] 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 ± 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·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·P-P formation to the sum of this constant and the rate constant for P_i release from E·2P_i (enzyme with two non-covalently bound phosphates). P_c is therefore a measure of the capacity of the V-PPase for E·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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