Pre-steady-state kinetics and solvent isotope effects support the “billiard-type” transport mechanism in Na⁺-translocating pyrophosphatase

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Abstract
Membrane-bound pyrophosphatase (mPPase) found in microbes and plants is a membrane H⁺ pump that transports the H⁺ ion generated in coupled pyrophosphate hydrolysis out of the cytoplasm. Certain bacterial and archaeal mPPases can in parallel transport Na⁺ via a hypothetical “billiard-type” mechanism, also involving the hydrolysis-generated proton. Here, we present the functional evidence supporting this coupling mechanism. Rapid-quench and pulse-chase measurements with [³²P]pyrophosphate indicated that the chemical step (pyrophosphate hydrolysis) is rate-limiting in mPPase catalysis and is preceded by a fast isomerization of the enzyme-substrate complex. Na⁺, whose binding is a prerequisite for the hydrolysis step, is not required for substrate binding. Replacement of H₂O with D₂O decreased the rates of pyrophosphate hydrolysis by both Na⁺- and H⁺-transporting bacterial mPPases, the effect being more significant than with a non-transporting soluble pyrophosphatase. We also show that the Na⁺-pumping mPPase of Thermotoga maritima resembles other dimeric mPPases in demonstrating negative kinetic cooperativity and the requirement for general acid catalysis. The findings point to a crucial role for the hydrolysis-generated proton both in H⁺-pumping and Na⁺-pumping by mPPases.

Abbreviations: AMDP, aminomethylene diphosphonate; Bv-mPPase, Bacteroides vulgatus mPPase; Da-mPPase, Desulfuromonas acetoxidans membrane pyrophosphatase; Dh-mPPase, Desulfobacterium hafniense membrane pyrophosphatase; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid; IMV, inverted membrane vesicles; Mm-mPPase, Methanosarcina mazei membrane pyrophosphatase; mPPase, membrane PPase; Pi, inorganic phosphate; PPase, pyrophosphatase; PPi, pyrophosphate; SIE, solvent isotope effect; TMA, tetramethylammonium; Tm-mPPase, Thermotoga maritima membrane pyrophosphatase.

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1 | INTRODUCTION

Membrane pyrophosphatases (mPPases, EC 7.1.3.1, formerly 3.6.1.1) are integral membrane proteins commonly found in the cytoplasmic membrane of diverse bacteria and archaea, the vacuolar membrane of plants, and the acidocalcisomal membrane of protozoa. mPPases hydrolyze PPi, a ubiquitous metabolic byproduct, to build up cation gradients in the cell, in contrast to soluble PPases, which dissipate PPi energy as heat. All initially described mPPases couple pyrophosphate (PPi) hydrolysis to H\(^{+}\) pumping (H\(^{+}\)-PPases),\(^1,2\) but later studies have identified an evolutionarily related prokaryotic subfamily that pumps Na\(^{+}\) (Na\(^{+}\)-PPases).\(^3,5\) Regardless of the subcellular localization, the direction of H\(^{+}\) and Na\(^{+}\) transport by mPase is away from the cytoplasm. Bacterial H\(^{+}\)-PPases are essential for growth in energy-limiting conditions.\(^6,7\) Accordingly, plants overexpressing H\(^{+}\)-PPase are more tolerant to various stresses, such as drought, salinity, and nutrient limitation.\(^8,9\) A similar biotechnological potential is expected for Na\(^{+}\)-PPase.\(^10\)

Membrane PPases bear no sequence homology to other protein families. Both transport and hydrolytic activities associate with a single polypeptide made of 600–800 amino acid residues\(^11,13\) that form 15–17 transmembrane helices.\(^14\) The polypeptide functions as a homodimer.\(^15–20\) whose constituent subunits exhibit kinetic and binding cooperativity.\(^21–23\)

Membrane PPases resemble all other PPases in being Mg\(^{2+}\)-dependent enzymes, but some H\(^{+}\)-PPases and all Na\(^{+}\)-PPases additionally require K\(^{+}\) for maximal activity.\(^24–27\) All K\(^{+}\)-dependent mPPases contain Ala in the last position of the GNXX(K/A) signature sequence.\(^21,28\) While K\(^{+}\) usually acts as a modulator in the K\(^{+}\)-dependent mPPase, Na\(^{+}\), the coupling ion, is absolutely required for the hydrolytic activity of Na\(^{+}\)-PPases.\(^3,29\) mPase is also similar to other PPases in that PPi hydrolysis proceeds as a direct attack of a water molecule on a phosphorous atom without the formation of a covalent intermediate.

The mechanism by which mPPases couple PPi hydrolysis to cation transport has been controversial. Critical issues are the sequence of the hydrolysis and ion translocation events and the role of the hydrogen ion generated during the water attack on PPi. The mechanism first suggested for H\(^{+}\)-Pase based on its crystal structure\(^19\) implies that the transported H\(^{+}\) ion is the one generated from the water nucleophile ("direct coupling") and, consequently, PPi hydrolysis precedes the transport event. An alternative proposal, solely grounded in the data measuring the generation of transmembrane electric potential difference upon substrate analog binding,\(^30,31\) was that H\(^{+}\) transport accompanies PPi binding and precedes PPi hydrolysis. However, this interpretation of the electrometric data was refuted later.\(^32\)

Even less is known about the coupling mechanism in Na\(^{+}\) transport. All Na\(^{+}\)-PPases can catalyze a parallel translocation of Na\(^{+}\) and H\(^{+}\) at sub-physiological Na\(^{+}\) concentrations (<5 mM),\(^33\) and some Na\(^{+}\)-PPases can simultaneously perform both transport functions even at excessive (up to 100 mM) Na\(^{+}\) concentrations.\(^34\) To explain the interplay between Na\(^{+}\) and H\(^{+}\) transport, we have suggested a "billiard-type" mechanism, in which the proton released from the nucleophilic water molecule pushes pre-bound Na\(^{+}\) through the ion conductance channel of Na\(^{+}\)-PPase.\(^26,32\) This speculative mechanism extends the "direct-coupling" H\(^{+}\)-PPase mechanism to Na\(^{+}\)-PPase.

This paper describes the first kinetic evidence favoring the billiard-type mechanism in Na\(^{+}\)-PPases. Specifically, we identify the chemical step as rate-limiting and Na\(^{+}\)-independent in PPi hydrolysis and show that this step involves proton transfer in both H\(^{+}\)-PPase and Na\(^{+}\)-PPases. The experiments involving a rapid mixing step, which imposed limitations on enzyme stability, were performed with a highly stable Thermotoga maritima mPase (Tm-mPPase). We also present relevant kinetic characteristics of this enzyme.

2 | RESULTS

2.1 | Steady-state kinetics of PPi hydrolysis by Tm-mPPase

Previous studies have detected negative kinetic cooperativity in PPi hydrolysis and H\(^{+}\) transport by different mesophytic mPPases.\(^21,23\) Specifically, substrate binding to one active site dramatically increased the Michaelis constant (K-type cooperativity) and moderately decreased the other site's catalytic constant (V-type cooperativity). These findings indicated that only one active site is predominantly operating in mPPase at any given time under in vivo conditions (substrate, metal cofactor, and H\(^{+}\).
concentrations). The steady-state kinetic measurements described below indicated similar behavior for Na\(^+\) transporting Tm-mPPase. This mPPase, originating from the most thermostable bacterium, was used in rapid kinetic studies because of its high stability during the associated manipulations.

The optimal temperature for Tm-mPPase is 70–75°C, but our studies were conducted at 40°C, the temperature at which it is still reasonably active (15% of the maximum activity).\(^{25}\) This temperature choice, dictated in part by the phase instability of substrate solutions at higher temperatures, necessitated a reassessment of the stability constants for the magnesium pyrophosphate complexes, required to maintain constant free Mg\(^{2+}\) concentration and thereby make substrate (Mg\(_2\)PP\(_i\)) concentration a single factor affecting catalysis. We used isothermal titration calorimetry to determine these constants at 40°C (Table A1) (Appendix A, Figure A1). We also estimated Mg\(_2\)PP\(_i\) solubility, which decreases with temperature and is another obstacle in kinetic measurements. Sedimentation analysis indicated that Mg\(_2\)PP\(_i\) remains soluble to 800 μM at 40°C during the enzyme assay (Appendix A).

The substrate saturation curve for Tm-mPPase measured at 40°C in the presence of 5 mM Mg\(^{2+}\) (Figure 1a) demonstrated inhibition by excess substrate, in agreement with the data reported by Goldman’s group\(^{22}\) but differed from them in demonstrating no sigmoidicity (positive substrate-binding cooperativity in terms of \(K_m\)) in the ascending part. We believe our data are more accurate at micromolar substrate concentrations due to the much greater sensitivity of our phosphate assay.\(^{35}\) A nearly identical substrate saturation curve was obtained at a 1 mM Mg\(^{2+}\) concentration.

These rate data were analyzed with Equation (1) derived for a homodimeric enzyme with two active sites\(^{25}\):

\[
v = \frac{(V_1 + V_2[S]/K_{m2})}{(1 + K_{m1}/[S] + [S]/K_{m2})},
\]

In this equation, \(S\) is the substrate (Mg\(_2\)PP\(_i\)), \(K_{m1}\) and \(K_{m2}\) are macroscopic Michaelis constants, and \(V_1\) and \(V_2\) are the specific activities of the mono- and di-substrate enzyme species. Table 1 lists the parameter values derived with Equation (1) from Figure 1a and similar data measured at a 1 mM Mg\(^{2+}\) concentration. The \(V_2\) value was indistinguishable from zero within the error of determination, which was relatively high because the limited substrate solubility did not permit substrate concentrations significantly exceeding \(K_{m2}\). To reiterate, homodimeric Tm-mPPase resembles other investigated mPPases in demonstrating increased \(K_m\) and decreased

![Figure 1](image-url)  
**FIGURE 1** Steady-state kinetics of Tm-mPPase. (a) Substrate concentration dependence of Tm-mPPase activity in IMVs at 40°C (0.1 M MOPS-KOH, pH 7.2, 5 mM Mg\(^{2+}\), 10 mM NaCl). The theoretical curve was calculated with Equation (1) using the parameter values found in Table 1. The inset with a linearly scaled abscissa shows the ascending part of the curve. (b) The pH-dependence of the maximal velocity for partially purified Tm-mPPase at 40°C, as estimated from the ascending part of the substrate concentration dependence (0.5–100 μM Mg\(_2\)PP\(_i\), range). The assay medium contained 20 mM Mg\(^{2+}\), 100 mM KCl, 0.2 mM KF, 0.1 mM EGTA, 3–100 mM NaCl, and 0.038 M each of MES, TES, TAPS, and CAPSO buffers adjusted to the desired pH with tetramethylammonium hydroxide. Bars refer to standard errors in the fitted \(V_1\) values. The theoretical curve was obtained with Equation (2) using the best-fit \(pK_a\) value of 7.8. (c) A typical titration of the active sites of Tm-mPPase in IMV. Steady-state rates of 8 μM PP\(_i\) hydrolysis were measured at 25°C in the presence of 100 mM NaCl (no KCl added) at a varied concentration of AMDP, a tightly bound PP\(_i\) analog. The total IMV protein concentration was 0.02 mg ml\(^{-1}\). The solid line shows the best fit of Equation (3) with \(K_{app} = 5 \pm 2\) nM and [E] = 10 ± 4 nM. AMDP,aminomethylene diphosphonate; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; IMV, inverted membrane vesicle; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; TAPS, N-[(tris(hydroxymethyl)-methyl]3-aminopropanesulfonic acid; TES, N-[(tris (hydroxymethyl)-methyl]2-aminoethanesulfonic acid; TMA, tetramethylammonium
The true binding constant for AMDP was as low as 1.5 nM, allowing the titration of the Tm-mPPase active sites by measuring enzyme activity at similar enzyme and AMDP concentrations at a non-saturating substrate concentration. The titrations were performed at 25°C without K+ to decrease enzyme activity, thereby allowing a higher enzyme concentration in the assay.

A typical dose-dependence curve is shown in Figure 1c. The inhibition data were analyzed with Equation (3) derived by solving the quadratic mass-balance equation. Here, \( v_1 \) and \( v_0 \) are the inhibited and non-inhibited rates, respectively, and \( |I| \) is the AMDP concentration. Fitting Equation (3) to the dependence of the rate of the mPPase reaction on \( |I| \) allowed the estimation of the apparent inhibition constant for AMDP (\( K_{i\text{app}} \)) and binding site concentration (\( |E| \)) treated as adjustable parameters. The determined \( |E| \) value in the stock IMV (8–10 μM in terms of the dimer, depending on the IMV preparation) was sufficient to assess pre-steady-state kinetics with the quenched-flow method. The error in \( |E| \) was relatively high because the competing substrate, present in the assay medium, increased the inhibition constant, \( K_{i\text{app}} \), to ~5 nM.

\[
\frac{v_1}{v_0} = 1 - \left\{ \frac{|E| + |I| + K_{i\text{app}} - \sqrt{(|E| + |I| + K_{i\text{app}})^2 - 4|E||I|}}{2|E|} \right\},
\]

Based on Tm-mPPase subunit mass of 77 kDa and assuming one tight binding site for AMDP per enzyme dimer, Tm-mPPase accounted for 7% of the total IMV protein. Notably, this approach could not be used to determine the Mm-mPPase active-site concentration because Mm-mPPase binds AMDP much weaker.

### 2.2 Transient kinetics of PP\(_i\) hydrolysis

The first catalytic cycle of Tm-mPPase was investigated by mixing the enzyme with nearly saturating concentrations of Mg\(_2\)PP\(_i\) and Mg\(^{2+}\), quenching the enzymatic reaction after a short time, and determining the product \( P_i \). Notably, the \( P_i \) amount measured in these experiments refers to the sum of enzyme-bound and medium \( P_i \) because the acid-quenching step releases enzyme-bound \( P_i \) into the solution. The measured time-course of \( P_i \) accumulation was linear and extrapolated to origin in the presence of either Na\(^+\) as the sole alkali metal activator or both Na\(^+\) and K\(^+\) (Figure 2). These findings identified \( P_i \) cleavage as the rate-limiting step in catalysis in both cases. Rate-limiting product release or conformational
change in the enzyme-substrate complex would result in a product burst (up to 4.3 μM Pi hydrolyzed) or lag, respectively.

The slopes in Figure 2 yield turnover numbers of 5.3 and 12 s⁻¹ for Na⁺- and Na⁺, K⁺-activated Tm-mPPase, respectively. There was no significant product formation in the absence of Na⁺ (Figure 2), suggesting that Na⁺ binds before the chemical step in catalysis.

2.3 Pulse-chase measurements of PPᵢ binding and its Na⁺ requirement

Substrate binding to Tm-mPPase in the absence of added Na⁺ ions was measured under single turnover conditions. The enzyme was mixed with a 2.5-fold excess of ³²Pᵢ in buffer containing only Mg²⁺ or both Mg²⁺ and K⁺ as metal cofactors in this experiment. The ³²Pᵢ-binding reaction was allowed to proceed for a varied time before diluting with a large excess of nonlabeled Pᵢ solution, which contained Na⁺ (20 mM final concentration) to allow bound Pᵢ hydrolysis (Na⁺ is absolutely required for Tm-mPPase activity). The mixture was further incubated for 1 s to complete the first reaction cycle, and the amount of bound ³²Pᵢ hydrolyzed was estimated by measuring the amount of ³²Pᵢ formed. This ³²Pᵢ predominantly arose from enzyme-bound ³²Pᵢ, considering the negligible probability of medium ³²Pᵢ binding and hydrolysis after extensive dilution with nonlabeled Pᵢ. Based on the data in Figure 2, one can calculate the percentage of medium Pᵢ conversion of <0.7% during the 1 s chase step. However, such hydrolysis of the medium ³²Pᵢ was evident with longer incubation times with nonlabeled Pᵢ and eventually resulted in a complete conversion of ³²Pᵢ into ³²Pᵢ. One should also keep in mind that some enzyme-bound ³²Pᵢ could be released intact as a reversal of the binding reaction during the chase step, causing an underestimation of the amount of the bound Pᵢ. In contrast, partial hydrolysis of the bound Pᵢ during the preceding incubation step would result in an overestimation of bound Pᵢ, but such hydrolysis does not occur in the absence of Na⁺, according to Figure 2.

One significant finding of these experiments was that ³²Pᵢ formation and, hence, ³²Pᵢ binding were observed in the absence of Na⁺ during the first incubation step (Figure 3), indicating that this cation is not required for substrate binding. The other significant finding was that the time courses shown in Figure 3 were biphasic, with the first phase unresolved in time by the current technique. Notably, the slower phase in Figure 3 terminated before all Pᵢ was processed and therefore was not associated with an irreversible conversion of Pᵢ into Pᵢ, insofar as such conversion does not occur in the absence of Na⁺, according to Figure 2.

Figure 2 Pre-steady-state kinetics of Tm-mPPase. The enzyme was rapidly mixed with ³²Pᵢ to yield 8.6 mg ml⁻¹ IMV protein (4.3 μM Tm-mPPase dimer) and 79 μM Pᵢ final concentrations. The hydrolysis reaction was allowed to proceed at 40°C for the time indicated on the abscissa, the mixture was quenched with acid, and ³²Pi in the system was measured. The curve labels refer to the alkali cations present (none, K⁺ only, Na⁺ only, or both Na⁺ and K⁺). Typical results are shown. IMV, inverted membrane vesicle.

Figure 3 Pulse-chase measurements of ³²Pᵢ binding to Tm-mPPase in the absence of Na⁺. The enzyme was rapidly mixed with ³²Pᵢ to yield 0.8 μM Tm-mPPase and 2.5 μM Pᵢ final concentrations, and the binding reaction was allowed to proceed at 40°C for the indicated time before it was arrested by adding an excess of nonlabeled Pᵢ (450 μM final concentration) and NaCl. After 1 s, the mixture was finally quenched with acid, and the amount of ³²Pᵢ in the system was measured. The curve labels indicate whether 50 mM K⁺ was present during the initial binding step. Typical results are shown.
occur in the absence of Na\(^+\) (Figure 2). Fitting a simple exponent to the slower phase in Figure 3 yielded similar apparent rate constants of 46 ± 10 and 36 ± 10 s\(^{-1}\) and different ordinate intercepts—0.33 ± 0.03 and 0.10 ± 0.02 μM for the reaction in the absence and presence of K\(^+\), respectively. The rate constants exceeded the respective turnover numbers of 5.3 and 12 s\(^{-1}\) derived above from Figure 2. The slower phase may thus refer to a relatively rapid conformational change preceding hydrolysis.

K\(^+\) decreased the amount of \(^{32}\)P\(_i\) formed (Figure 3). This effect could result from decreased \(^{32}\)PP\(_i\) binding, consistent with the earlier finding that K\(^+\) increases the Michaelis constant for the substrate in several mPPases.\(^{21,36}\)

### 2.4 Solvent isotope effects on catalysis

If the rate-limiting step of an enzyme-catalyzed reaction involves H\(^+\) transfer, replacing H\(_2\)O with D\(_2\)O as a solvent decreases the observed reaction rate.\(^{40}\) Since the effects are generally moderate, the conditions for solvent isotope effect (SIE) measurements with mPPase were chosen to minimize the nonspecific effects of D\(_2\)O on enzyme activity. First, the PP\(_i\) concentrations used substantially exceeded the respective K\(_{m1}\) values but were less than K\(_{m2}\), permitting saturation of only one active site per dimer (“unisite” catalysis). Notably, the values of K\(_{m1}\) and the Na\(^+\)-binding constant governing mPPase activation did not vary significantly between H\(_2\)O and D\(_2\)O. Second, Mg\(^{2+}\) and alkali metal ions were used at their saturating concentrations. Finally, since pD = pH\(_{\text{read}}\) + 0.4,\(^{41}\) where pH\(_{\text{read}}\) is the pH meter reading, SIE analysis was performed at a fixed pH\(_{\text{read}}\) value slightly above the lower boundary of the range in which the rate of the hydrolytic reaction (V\(_1\)) was insensitive to pH\(_{\text{read}}\) in either H\(_2\)O or D\(_2\)O. This setup eliminated complications from an equilibrium isotope effect on a catalytic residue or buffer pK\(_a\). For instance, measurements with Mm-mPPase were performed at pH\(_{\text{read}}\) 7.2 because its activity did not vary in the pH\(_{\text{read}}\) range 6.8–8.0. The pH dependencies of V\(_1\) for other mPPases are shifted to lower or higher pH values; therefore, experiments with them were conducted at different pH\(_{\text{read}}\) values.

As Figure 4 highlights, 96% D\(_2\)O decreased V\(_1\) for Tm-mPPase and Desulfuromonas acetoxidans mPPase (Da-mPPase) by 32 and 35%, respectively, suggesting that a step involving proton transfer determines the overall catalytic rate. Furthermore, the linearity of the rate dependence on the D\(_2\)O molar fraction (n) suggested a single proton transfer.\(^{42}\) Fitting a modified Gross-Butler equation for one-proton inventory (Equation 4) to these dependencies yielded isotopic fractionation factors φ of 0.66 and 0.64 for Na\(^+\), K\(^-\)-activated Tm-mPPase and Da-mPPase, respectively. Similar φ values were obtained for three other mPPases of different transport specificities (Table 2), indicating that the formation of the rate-determining transition state involves proton transfer in all mPPase types.

\[
\frac{V_D}{V_H} = 1 + n(\phi - 1),
\]

TABLE 2 Kinetic solvent isotope effects in PP\(_i\) hydrolysis by mPPases

| Enzyme       | Transport specificity | Assay conditions | V\(_{1H}\), nmol min\(^{-1}\) mg\(^{-1}\) | φ       |
|-------------|----------------------|-----------------|--------------------------------------|--------|
| Tm-mPPase   | Na\(^+\)             | pH\(_{\text{read}}\) 6.2, 50 mM K\(^+\), 10 mM Na\(^+\) | 320 ± 10 | 0.66 ± 0.02 |
| Da-mPPase   | Na\(^+\)             | pH\(_{\text{read}}\) 8.5, 100 mM K\(^+\), 10 mM Na\(^+\) | 360 ± 10 | 0.64 ± 0.01 |
| Mm-mPPase   | Na\(^+\)             | pH\(_{\text{read}}\) 7.2, 100 mM K\(^+\), 10 mM Na\(^+\) | 1,400 ± 20 | 0.66 ± 0.01 |
| Bv-mPPase   | Na\(^+\) and H\(^+\) | pH\(_{\text{read}}\) 6.7, 50 mM K\(^+\), 10 mM Na\(^+\) | 108 ± 1 | 0.59 ± 0.03 |
| Dh-mPPase   | H\(^+\)              | pH\(_{\text{read}}\) 6.7, 50 mM K\(^+\) | 950 ± 10 | 0.67 ± 0.01 |
DISCUSSION

One must answer three key questions to formulate the mPPase transport mechanism: (a) At which step of the hydrolysis reaction is PPi energy input to drive ion transfer against its electrochemical potential gradient in the membrane? (b) What is the origin of the transported H+ ion in H+-PPase and Na+-PPases acting as H+ transporters at low (<5 mM) Na+ concentrations? (c) How is the transport specificity of Na+-PPase modulated by Na+? The results of this study provide the answer to the first question and allow informed speculations concerning the remaining questions.

3.1 The energy-coupling step in Na+-transporting and H+-transporting mPPases

One of the hypothetical mechanisms proposed for mPPase (“binding-change” mechanism) posits that cation transport uses substrate-binding energy.20,30,31 If so, cation binding should precede substrate binding, and Na+ as the transported cation offers the possibility to test this prediction, which is inaccessible with a proton whose binding is not easily controllable. The data in Figure 3 provide direct evidence that Na+ is not required for substrate binding, which means that Na+ and substrate bind independently or Na+ binding follows substrate binding. Furthermore, Figure 2 indicates that hydrolysis in the enzyme–substrate complex is arrested without Na+. Random-order binding of Mg2PPi and the activating Na+ ion was evident from steady-state kinetics of PPi hydrolysis by Mm-mPPase,36 although it was unclear whether the activating Na+ ion is the one that is transported. These findings demonstrated that Na+ is required for the catalytic, not binding step and supported the transport model implying energy coupling at the rate-limiting PPi hydrolysis step. The possibility that energy coupling occurs at the product release steps seems less likely because they are not rate-limiting and, hence, require less energy to occur.

Several lines of evidence suggest that the chemical (hydrolysis) step is likewise the rate-limiting and coupling step in H+-pumping mPPase, in accord with the notion that H+ transfer and PPi hydrolysis occur synchronously in this transporter.32 First, similar kinetic isotope effects in Na+-PPases and H+-PPases also point to identical rate-limiting steps. Second, the active sites of the H+-pumping and Na+-pumping mPPases are very similar and contain identical catalytic residues involved in hydrolysis (Figure 5). Finally, the substrate-binding regions are also similar in mPPase and soluble PPases, suggesting equally high product release rates. In both PPase types, the principal PPi-binding ligands are three to five bridging metal ions,43 consistent with similar (micromolar) Km values.

Notably, the structures suggest a much lower efficiency of mPPases at the chemical step because of a different mode of nucleophilic water activation. In soluble PPases, the nucleophile is activated by coordination to two or three divalent metal ions, which convert the water to highly reactive hydroxide.44,45 In mPPase, the activating metal ions are replaced in this role by two Asp residues (Figure 5), which polarize the water molecule less efficiently. The slow chemical step clearly explains the 1–
3 orders of magnitude lower catalytic constant in mPPases than in soluble PPases.

### 3.2 | Proton role in Na\(^+\) transport

As already mentioned, the nucleophilic water molecule is activated by different mechanisms in membrane PPases (Figure 5) and soluble PPases.\(^{38,44}\) There are two main reasons for this. First, although magnesium ions polarize the water molecule more efficiently, their insertion into the charged membrane would involve an energy cost. Second, a milder activation of the water nucleophile by carboxylates, without its conversion to hydroxide, permits proton generation in the catalytic reaction in the right place for its subsequent transfer across the membrane. The water-borne proton creates high local acidity at the entrance to the ion-conducting channel, which drives the proton against the gradient of its electrochemical potential. That the transported proton belongs to the water molecule, not the coordinating aspartate before the chemical step is evidenced by the mode of their coordination in the V. radiata mPPase complex with IDP.\(^{19}\) (Figure 5b). To add, the program PropKa, version 3.4\(^{46}\) predicted the respective pK\(_a\) values of 3.6–3.7 and 0.5–1.2 for Asp287 and Asp731 carboxylates in this complex, indicating that they are ionized under physiological conditions. The Asp287 oxygen atom may initially accept the water-born proton and, following tautomerization, direct it to the ion-conducting channel.

Although the above analysis refers primarily to H\(^+\)-PPase, the overall structural and functional similarity suggests that it is, at least, in part relevant to Na\(^+\)-PPases, which transport H\(^+\) in parallel to Na\(^+\).\(^{33,34}\) The ion-conducting channels are very similar in H\(^+\)-PPases and Na\(^+\)-PPases, suggesting a common H\(^+\) transport mechanism. The principal structural difference between the two mPPase types is that the gate-forming glutamate residue of Na\(^+\)-PPases is one helix turn closer to the hydrolytic center than in H\(^+\)-PPases.\(^{20}\) The crystal structure of Tm-mPPase (Figure 5a) indicates that this residue forms a Na\(^+\)-binding site that can also bind H\(^+\) on its way through the channel in Na\(^+\)-PPase.

Because the sodium ion is not a reaction product, it is transported by a different mechanism. The “billiard” hypothesis\(^{36}\) unifies the H\(^+\) and Na\(^+\) transport activities by placing the proton originating from the nucleophilic water molecule as the common driver. This assertion is supported by similar kinetic isotope effects in Na\(^+\)-PPases and H\(^+\)-PPases (Table 2). The reactions in D\(_2\)O are typically slower because of its lower vibrational zero-point energy, and hence, a higher activation energy is required to break the O–D bond than the O–H bond.

Noteworthily, all the Na\(^+\)-PPases used in the isotope effect measurements catalyze the transmembrane transfer of Na\(^+\) but not H\(^+\) at the 10 mM Na\(^+\) concentration used.\(^{33}\) The isotopic fractionation factor \(\phi\) for both types of mPPase is larger than that for soluble yeast PPase\(^{47}\) despite similar reaction chemistry. Moreover, the isotope effect in the soluble PPase primarily results from a different step, that is, product release.\(^{47}\) Our findings thus emphasize the crucial role of the proton-generating step in both mPPase types.

The available data suggest the minimal H\(^+\) and Na\(^+\) transport mechanism in Na\(^+\)-PPase illustrated in Figure 6. The cation-binding site formed by the Glu residue near the gate contains a Na\(^+\) or H\(^+\) ion in the resting state according to the principle of the local electroneutrality of stable intermediates.\(^{48}\) PropKa predicts a pK\(_a\) of 7.0–7.2 for the gate Glu residue (Glu246) of Tm-mPPase, suggesting its appreciable protonation in the resting state. Functional studies indicated the presence of an additional, Na\(^+\)-specific, high-affinity site (marked by “C” in Figure 6), whose occupancy by Na\(^+\) is required for PP\(_i\) hydrolysis and H\(^+\)/Na\(^+\) transport.\(^{29,33}\) This site appears to be permanently occupied at physiological Na\(^+\) concentrations. The whole transport reaction at high Na\(^+\) concentrations (H\(^+\) transport arrested) may involve five steps in Na\(^+\)-PPase. (Step 1) The substrate binds, closing the channel on the cytoplasmic side. (Step 2) The hydrolysis reaction commences, releasing a proton at the channel entrance. (Step 3) The proton reaches the gate through linked water molecules (Grotthuss mechanism), forcing the Glu-bound Na\(^+\) or H\(^+\) ion to enter the exit channel and finally be released to the periplasm. (Step 4) Products diffuse from the
active site; the Glu residue again becomes protonated. (Step 5) The cytoplasmic Na\(^+\) partially or wholly replaces the Glu-bound proton through the open active site, preparing the protein for the next hydrolysis/transport cycle. The kinetic limitation of Na\(^+\) delivery to its binding site does not seem likely as it takes <20 ms for Na\(^+\) to cross membrane-integral Na\(^+\)-rhodopsin, another Na\(^+\) transporter.\(^{39}\)

This tentative mechanism provides a simple explanation for Na\(^+\) modulation of transport specificity. For this mechanism, the ratio of the Na\(^+\) and H\(^+\) transport rates depends on their gate site occupancies, which are determined by their binding affinities and cytoplasmic concentrations. Na\(^+\) fully occupies the gate site at high concentrations and becomes the only transported cation, redirecting the “chemical” proton to the cytoplasm. At which Na\(^+\) concentration will the gate Glu bind equal amounts of Na\(^+\) and H\(^+\)? As already mentioned, the predicted pK\(_a\) of the gate Glu246 in Tm-mPPase is 7.0–7.2, indicating a 300–500 times lower K\(_a\) value than expected for the Glu carboxylate in an aqueous medium. The dissociation constant of sodium acetate in water is 1.5–1.8 M\(^{30,51}\) but will become approximately 7 mM in mPPase if the environment changes the carboxylate affinities for proton and Na\(^+\) equally. In accord with this calculated value, switching between H\(^+\) and Na\(^+\) transport occurs at low millimolar Na\(^+\) concentrations in most Na\(^+\)-PPases.\(^{33}\) Assuming simple competition between Na\(^+\) and H\(^+\) for binding to the gate Glu carboxylate, the ability of some Na\(^+\)-PPases to transport H\(^+\) at a 100 mM Na\(^+\) concentration may result from a higher H\(^+\)-binding or lower Na\(^+\)-binding affinity of the gate site.

### 3.3 Kinetic evidence for a conformational change during substrate binding

The biphasic kinetics of substrate binding to mPPase suggests that the enzyme undergoes a conformational change (isomerization) before the hydrolysis step. The rapid binding phase in the kinetic curves in Figure 3 appears to be diffusion-controlled, based on the value of its rate constant of >10\(^9\) M\(^{-1}\) s\(^{-1}\) estimated from Figure 3, and can be assigned to the second-order reaction of PP\(_i\) binding. The presumed isomerization reaction can be associated with the slower phase. Notably, the effects seen in Figure 3 do not refer to bound PP\(_i\) hydrolysis, which does not occur without Na\(^+\).

The pulse-chase data can be interpreted in terms of two kinetic models assuming that the isomerization reaction involves substrate-free enzyme (Scheme 1a) or the enzyme–substrate complex (Scheme 1b). In model A, there is a preexisting equilibrium between two conformations with essentially different affinities to substrate in the resting enzyme, and this equilibrium is slowly shifted upon rapid substrate binding, producing more enzyme in the binding-competent conformation. In model B, the isomerization of the enzyme–substrate complex may be a required step of the complete hydrolysis reaction, or a side process, depending on which enzyme–substrate complex, EPP, or EPP*, leads to products. In both models, the isomerization reaction increases the amount of the PP\(_i\)-bound enzyme, in accordance with the data in Figure 3.

In principle, the presumed isomerization step should cause deviations of the product formation curves from a linear form by causing a product burst. However, large deviations are not expected because the rate constant for the isomerization step, as estimated from Figure 3, exceeds k\(_{\text{cat}}\). A more detailed study is, however, needed to determine the isomerization rate constant in the presence of Na\(^+\) for a direct comparison with k\(_{\text{cat}}\).

A plethora of data obtained by single-molecule fluorescence measurements,\(^{52}\) kinetic analysis of protection against proteolysis and chemical modification,\(^{36}\) X-ray crystallography,\(^{30}\) and molecular dynamics simulations\(^{55}\) indicated a conformational change in different Na\(^+\)-PPases induced by substrate or its analog binding. To the best of our knowledge, our study provided the first demonstration of two conformations of the same enzyme species (presumably, the enzyme–substrate complex). Finally, although our data refer to Na\(^+\)-PPase, a similar conformational change may occur in H\(^+\)-PPase.

### 4 MATERIALS AND METHODS

#### 4.1 Reagents

Tetramethylammonium (TMA) PP\(_i\) was prepared by passing a solution of tetrasodium PP\(_i\) through a Dowex 50 W X8 (Serva) column of charged with TMA\(^+\). PP\(_i\)
concentration in the eluate was determined by measuring the amount of $P_i$ after boiling with 1 M hydrochloric acid to hydrolyze $PP_i$. $3^{2}P_i$ was obtained from Perkin Elmer, $D_2O$ from Cambridge Isotope Laboratories, buffer components and most other chemicals from Sigma–Aldrich.

4.2 | Protein preparation

$Na^+$-transporting mPPases from $T. maritima$, $M. mazel$, $D. acetoxidans$, $Bacteroides vulgatus$, and $H^+$-transporting mPPase from $De sulfotobacterium hafniense$ were produced in $Escherichia coli C41(DE3)$ cells. IMVs were prepared by a French press method and isolated using a three-step ultracentrifugation procedure. $3,29$ The membrane pellet was suspended to $15 – 25$ mg ml$^{-1}$ in storage buffer (10 mM MOPS-TMA hydroxide, pH 7.2, 150 mM sucrose, 1 mM MgCl$_2$, and 40 mM EGTA), frozen in liquid-N$_2$, and stored at $-70^\circ$C. The Tm-mPPase used in the pH studies was partially purified by heating the IMV suspension to $70^\circ$C and sedimenting the precipitate formed in a microcentrifuge. This additional treatment yielded $30 – 50\%$ pure Tm-mPPase with a complete recovery of activity. $25$ IMV and partially purified Tm-mPPase were quantified according to their protein content, which was estimated using the Bradford assay. $54$

4.3 | Activity measurements

mPPase activity was assayed by following $P_i$ production from PP$_i$ using an automatic $P_i$ analyzer$^{35}$ at a sensitivity of $2 – 4$ μM $P_i$ per recorder scale. The reaction mixture typically contained 100 mM MOPS-KOH or MOPS-NaOH buffer, pH 7.2. The concentrations of MgCl$_2$, KCl, and NaCl are found in the descriptions of the particular experiments. The reaction was started by the addition of PP$_i$ (tetrasodium salt), and $P_i$ liberation was continuously recorded for 3–4 min. When significant deviations from linearity were observed, initial velocities were estimated from analyzer recordings as described previously. $35$ The concentrations of Mg$_2$PP$_i$ (the actual substrate) and free Mg$^{2+}$ ions in the assay medium were maintained as described in the same publication. The rates of hydrolysis (mPPase activities) are given below in terms of the amount of total PP$_i$ hydrolyzed per 1 min.

4.4 | Active site titration

Tm-mPPase-containing IMVs (0.5 mg total protein) were preincubated for 1 min with 0–50 nM tightly binding PP$_i$ analog, AMDP, in 25 ml of 50 mM MOPS-TMA hydroxide, pH 7.2, 5 mM MgCl$_2$, and 100 mM NaCl at 25°C. PP$_i$ (8 μM) was added, and the enzymatic reaction was continuously monitored with the automatic $P_i$ analyzer. The active-site concentration was determined from the dependence of activity on AMDP concentration.

4.5 | Quenched-flow measurements

Equal volumes (14 μl each) of IMV suspension containing 12–20 mg ml$^{-1}$ total protein (6–10 μM Tm-mPPase) and 158 μM tetrasodium PP$_i$ solution (including $\sim$0.03 μCi $3^{2}P_i$) were mixed in an RQF-3 Quench-Flow Instrument (KinTek) at 40°C. The medium for both reactants was buffer Q (100 mM MOPS-TMA hydroxide, pH 7.2, 5.2 mM MgCl$_2$, 50 μM EGTA) supplemented with either 100 mM NaCl or 10 mM NaCl and 50 mM KCl. The final concentration of PP$_i$ after mixing, 79 μM, corresponded to 50 μM Mg$_2$PP$_i$ complex (assumed true substrate). $35$ After 5–1,000 ms, the reaction was terminated with 85 μl of 1 M hydrochloric acid. The product mixture was collected into a microcentrifuge tube containing 530 μl of 1.8 mM KH$_2$PO$_4$ and stored on ice until the whole kinetic curve samples were acquired. After removing the precipitated protein by centrifugation (5 min, 14,000g), the supernatants were transferred to new tubes containing 25 mg of glass beads (150–212 μm, Sigma), added to facilitate the subsequent washing steps. $3^{2}P_i$ was precipitated from the product mix as a $P_i$:molybdate: triethylamine complex $^{55}$ by adding 200 μl of 20 mM ammonium molybdate and 50 μl of 100 mM triethylamine-HCl, pH 5.0. After 5 min, the yellow $P_i$ precipitate was isolated by centrifugation (8,000g, 5 min). Residual $3^{2}P_i$ was removed from the pellet by resuspension in 800 μl of washing solution (prepared by combining 1 volume of 100 mM triethylamine-HCl, pH 5.0, 1.6 volumes of 1 M hydrochloric acid, 4 volumes of 20 mM ammonium molybdate, and 10.8 volumes of water), followed by centrifugation; the resuspension/centrifugation cycle was repeated three times. The final $P_i$ sediment was dissolved in 100 μl of 1 M aqueous ammonia, 1 ml of Ultima Gold cocktail (Perkin Elmer) was added, and $3^{2}P_i$ was counted by liquid scintillation. Control experiments indicated $\sim$95% recovery of $3^{2}P_i$, while the amount of copurified $3^{3}P_i$ was negligible. Appropriate corrections were made for contaminating $3^{2}P_i$ in $3^{2}P_i$ stocks (2–4%, depending on the batch).

For $3^{2}P_i$ pulse-chase experiments, the Quench-Flow Instrument was supplemented with a syringe charged with 600 μM nonlabeled tetrasodium PP$_i$ and 25 mM NaCl. Equal volumes (14 μl each) of 1.6–2 μM Tm-mPPase and 5 μM $3^{2}P_i$ solutions in buffer Q with or without 50 mM KCl were mixed and allowed to react for
5–150 ms before mixing with 85 μl of 600 μM nonlabeled PPi. The mixture was further incubated for 1 s to hydrolyze enzyme-bound 32PPi, quenched with hydrochloric acid, and processed as described above.

4.6 Measurement and analysis of solvent isotope effects

The buffers used to measure kinetic SIEs were prepared in H2O or D2O and contained 100 mM MOPS, 20 mM MgCl2, 0.1 mM EGTA, and varying concentrations of NaCl and KCl. The buffers were adjusted to pHread values of 6.2 (Tm-mPPase), 7.2 (Mm-mPPase), or 6.7 (Dh-mPPase and Bv-mPPase) with concentrated KOH or NaOH solutions in H2O or D2O. TAPS replaced MOPS in the pH 8.5 buffer (Da-mPPase). The components of the D2O buffers were dissolved in D2O and lyophilized prior to use. Proton inventories were constructed by varying the atom fraction of D2O (n) from 0 to 0.95. The IMVs were incubated for 2 min in the respective buffer at 40°C (Tm-mPPase) or 25°C (other mPPases), and the addition of 114 μM PPI started the hydrolysis reaction. The reaction progress curves were linear, signifying no kinetically significant H/D exchange during the measurement. Two-fold changes in Na+, K+, or PPi concentration had negligible effects on the observed rate, signifying that D2O did not affect the saturation of the metal and PPi binding sites. Replacing H2O with D2O did not affect PPi analyzer sensitivity.

4.7 Isothermal titration calorimetry

Heat production accompanying Mg2+ binding with PPi was measured at 25 and 40°C in a VP-iTC calorimeter (MicroCal). Tetrasodium PPi and MgCl2 solutions were prepared in 0.1 M MOPS/KOH buffer (pH 7.2) containing 10 mM NaCl. Titrations were performed by successive 10- or 20-μl injections of 30 mM MgCl2 solution into 1.4 ml of 0.5 mM PPi at 25°C or 0.3 mM PPi at 40°C; the interval between injections was 5 min. Measured heat values were corrected for ligand dilution effects.

5 CONCLUSIONS

Dual Na+/?H+ transport specificity is not uncommon among cation transporters, but all such transporters use indirect coupling mechanisms with easy switching between the cations, both of which come from the solution and cross the membrane independently. mPPase uses a direct-coupling mechanism to pump proton, the product of the hydrolysis reaction, but an indirect-coupling mechanism to pump Na+ ion, which comes from the cytoplasm. This unique combination of two alternative mechanisms has never been observed for any cation transporter. The direct-coupling H+ transport mechanism emerged straightforwardly from the transporter structure, making H+-Pase the first example of a non-oxidoreductase proton transporter with such a mechanism. The question is how this mechanism coexists with indirectly coupled Na+ transport. The principal outcome of the current study consists of providing the first experimental evidence of a unique solution for Na+ transport—the “billiard-type” mechanism. Specifically, two types of data—pre-steady-state kinetics and kinetic isotope effects—demonstrate that the proton-generating step is rate-limiting in Na+-Pase catalysis. Pulse-chase measurements of PPi-binding indicated that Na+ binds independently of the substrate. These findings are entirely consistent with the “billiard-type” mechanism, although they do not prove it unequivocally.

Future studies will test and extend the proposed Na+ transport mechanism. One task is to determine how and in which form the Na+ ion passes the ion-conducting channel. Are there intermediate Na+/-binding sites forming a “Na+ wire,” similar to the proton wire, between the cytoplasm and the gate? Significant functional and structural asymmetry of the mPase dimer suggests that its subunits may work alternately, which raises the possibility of coordinated Na+ and H+ transport by different subunits. Concurrent transportation of both Na+ and H+ in one catalytic cycle is also conceivable.

AUTHOR CONTRIBUTIONS

Anssi Malinen: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – review and editing (equal). Viktor Anashkin: Formal analysis (equal); investigation (equal); methodology (equal); validation (equal). Alexander V. Bogachev: Methodology (equal); validation (equal). Reijo Lahti: Methodology (equal); supervision (equal); validation (equal); writing – review and editing (equal). Alexander Baykov: Conceptualization (equal); formal analysis (equal); methodology (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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APPENDIX A

A.1 | STABILITY AND SOLUBILITY OF THE MAGNESIUM–PYROPHOSPHATE COMPLEXES AT ELEVATED TEMPERATURES

Adequate analysis of PPase steady-state kinetics critically depends on maintaining constant free Mg$^{2+}$ concentration, thereby making substrate ($S = Mg_2PP_i$) concentration a single factor affecting catalysis. This is trivially achieved if $[Mg^{2+}] >> [S]$ but requires knowledge of the dissociation constants for the magnesium complexes of PPi if this condition is not satisfied. Such constants are available or can be calculated for a broad pH range at 25°C, the standard temperature for enzyme studies. However, no such data are available for higher temperatures, hampering the studies of the PPases isolated from thermophilic organisms.

We used isothermal titration calorimetry to determine magnesium pyrophosphate stability at 40 and 25°C. The titration curves (Figure A1) were biphasic and indicated that the binding reaction is slightly exothermic at lower and endothermic at higher Mg:PPi ratios. The dissociation constant for MgPPi did not change with temperature, as for HP$O_4^{2-}$, and the dissociation constant for Mg$_2$PPi decreased with increasing temperature (Table A1). The sign and size of the temperature effect on Mg$^{2+}$ binding to MgPPi, which occurs through only one phosphate group of PPi, correlate well with the effect of temperature on H$^+$ and Mg$^{2+}$ binding to phosphate.

The determined MgPPi and Mg$_2$PPi stability constants were used to maintain a constant Mg$^{2+}$ concentration in the experiments measuring the substrate concentration dependence of the hydrolysis rate for Tm-mPPase at 40°C (Figure 4).

The low solubility of Mg$_2$PPi sets an upper limit for permissible substrate concentration, hampering the saturation of the second active site in the dimer. In the presence of 5 mM Mg$^{2+}$, precipitation was visually observed within 5 min at >1 mM Mg$_2$PPi at 25°C and at >0.8 mM Mg$_2$PPi at 40°C (0.1 M MOPS-KOH buffer, pH 7.2). These data were quantitatively confirmed by using centrifugation to separate the precipitated Mg$_2$PPi. To this end, the mixtures of tetrasodium PPi and MgCl$_2$ in the buffer were preincubated for 4–5 min in 1.5-ml Eppendorf tubes at 25, 40, or 60°C and immediately spun off for 1 min in a microcentrifuge. The PPi concentration in the clear supernatant was estimated from the amount of Pi produced upon complete hydrolysis by Streptococcus gordonii soluble PPase.

The results indicated that the highest permissible Mg$_2$PPi concentration, at which <10% PPi precipitated under these conditions, was 1.2 mM at 25°C and 0.8 mM at 40°C. At 60°C, precipitation started at a PPi concentration of 0.2 mM in the presence of 6 mM MgCl$_2$.

| Parameter | Value ± SD |
|-----------|------------|
| $K_1$ (μM) | 56 ± 4 | 56 ± 7 |
| $K_2$ (μM) | 1760 ± 60 | 320 ± 40 |
| $\Delta H_1^0$ (kJ Mol$^{-1}$) | $-4.9 ± 0.5$ | $-9.2 ± 0.8$ |
| $\Delta H_2^0$ (kJ Mol$^{-1}$) | $11.6 ± 0.1$ | $12.8 ± 0.6$ |

FIGURE A1 ITC measurements of magnesium binding to PPi. Top, typical raw data for successive injections of MgCl$_2$ into 0.5 mM PPi solution at 25°C after correction for dilution. Bottom, typical dependencies of integrated heats in PPi titration by MgCl$_2$ at 25 and 40°C. The lines are the best fits of a “sequential binding” model for two sites.
These findings indicated that Mg$_2$P$_i$ is poorly soluble but precipitates slowly from its oversaturated solution on the time scale of the enzyme assay or ITC titration in the concentration ranges used. The following calculations support this conclusion. The literature value for the solubility product of Mg$_2$PP$_i$ at 25°C is 2.5 × 10$^{-13}$ M$^{-3.61}$.

Based on the dissociation constant of 2.5 × 10$^{-9}$ M$^2$ for the Mg$_2$P$_2$O$_7$ complex,$^{17}$ one can calculate that Mg$_2$P$_2$O$_7$ solubility is limited to 10$^{-4}$ M at 25°C. Considering that increasing temperature generally increases solubility, elevated temperatures may affect Mg$_2$PP$_i$ precipitation in oversaturated solutions by increasing its rate.