Evidence Calcium Pump Binds Magnesium before Inorganic Phosphate

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SUMMARY

Calcium pump-catalyzed $^{18}$O exchange between inorganic phosphate and water was studied to test the hypothesis that all P-type pumps bind Mg$^{2+}$ before P$_i$ and validate utilization of the rate equation for ordered binding to interpret differences between site-directed mutants and wild-type enzyme. The results were remarkably similar to those obtained earlier with sodium pump [Kasho et al. (1997) Biochemistry 36, 8045-8052]. The equation for ordered binding of Mg$^{2+}$ before P$_i$ fit the data best with only a slight chance (0.6%) of P$_i$ binding to apoenzyme. Therefore, P$_i$ is the substrate, and Mg$^{2+}$ is an obligatory cofactor. The intrinsic Mg$^{2+}$ dissociation constant from metalloenzyme ($K_M = 3.5 \pm 0.3$ mM) was experimentally indistinguishable from the sodium pump value. However, the half-maximum concentration for P$_i$ binding to metalloenzyme ($K'_P = 6.3 \pm 0.6$ mM) was significantly higher (~6-fold), and the probability of calcium pump forming phosphoenzyme from bound P$_i$ ($P_c = 0.04 \pm 0.03$) was significantly lower (~6-fold) than for sodium pump. From estimates of the rate constants for phosphorylation and dephosphorylation, calcium pump appears to catalyze phosphoryl group transfer less efficiently than sodium pump. Ordered binding of Mg$^{2+}$ before P$_i$ implies that both calcium pump and sodium pump form a ternary enzyme-metal-phosphate complex, consistent with molecular structures of other haloacid dehalogenase superfamily members that were crystallized with Mg$^{2+}$ and phosphate, or a phosphate analogue, bound.
INTRODUCTION

The calcium and sodium pumps are classified as P-type because the energy required for generating ion gradients across cell membranes is derived from ATP by catalyzing hydrolysis in two, Mg-dependent steps (1). The γ-phosphoryl group is initially transferred to an aspartyl side chain of the protein in one conformation (E₁) forming a covalent phosphoenzyme intermediate. Hydrolysis is completed after a conformational change by transferring the phosphoryl group to water. The second step can be reversed by reacting the second conformation (E₂) with P₁ and can be followed either by radioactive ³²P incorporation into the enzyme, or by stable oxygen isotope (¹⁸O) exchange between P₁ and H₂O. Phosphorylation by P₁ is a partial reaction that can be uncoupled from other reactions in the pump cycle experimentally, so that the mechanism is simple enough for derivation of the rate equation. Therefore, measurements combining either ³²P incorporation or ¹⁸O exchange with site-directed mutagenesis are potentially a powerful way of identifying amino acids essential for catalyzing phosphoryl group transfer and of learning their function from estimates of intrinsic constants for interaction of reactants with the enzyme.

The problem considered in this paper is the mechanism of Mg-dependent phosphorylation by P₁. The solution is important because the interpretation of observed differences between mutants and wild-type enzyme depends upon the way in which intrinsic properties of the protein enter into the expressions for the half-maximum substrate concentration and either the maximum amount of ³²P bound, or the maximum rate of ³²P incorporation or ¹⁸O exchange. Currently, different mechanisms are assumed in interpreting studies of the calcium and sodium pumps. A recent review article states categorically that Mg and P₁ bind randomly to Ca-ATPase (2). Most published studies of purified enzyme in aqueous solution support this interpretation (3-7). However, a study in an organic solvent-water mixture concluded that the true substrate is Mg·P₁ (8), and recently the equation for the half-maximum Mg·P₁ concentration
was used to interpret an increased half-maximum $P_i$ concentration when the upstream threonine in a signature amino acid sequence for P-type pumps (DKTGT) was changed to serine (9). The interpretation given to most studies of Na,K-ATPase is also that Mg$^{2+}$ and $P_i$ bind randomly (10-12). However, we concluded from fits of rate equations for alternative mechanisms to the $^{18}$O-exchange rate as a function of reactant concentrations that Mg$^{2+}$ binds to purified Na,K-ATPase before $P_i$ (13). We estimated the intrinsic affinity of apoenzyme for Mg$^{2+}$ with the rate equation for ordered binding of Mg$^{2+}$ before $P_i$ and interpreted mutations of amino acids in two other signature sequences for P-type pumps DPPR (14) and DGVND (15) by comparing mutant Mg$^{2+}$ dissociation constants with the wild-type Na,K-ATPase value.

Phosphorylation of the calcium and sodium pumps by different mechanisms seems unlikely because the amino acids implicated in phosphoryl group transfer are in conserved sequences and the phosphorylation domain containing most of them has the characteristic fold of HAD superfamily members (16). Therefore, we tested the hypothesis that calcium pump also binds Mg$^{2+}$ before $P_i$ by studying purified Ca-ATPase-catalyzed exchange of $^{18}$O between $P_i$ and water as a function of an array of Mg$^{2+}$ and $P_i$ concentrations. The results resembled those obtained earlier with Na,K-ATPase (13). Neither the equation for $P_i$ binding before Mg$^{2+}$, nor the equation for reaction with Mg$\cdot$P$_i$, fit the data well. The equations for ordered binding of Mg$^{2+}$ before $P_i$ and for random binding fit the data comparably. However, the fit of random binding was achieved by estimating more than two orders of magnitude higher affinity of apoenzyme for Mg$^{2+}$ than for $P_i$. Therefore, the dominant pathway for calcium pump, as well as sodium pump, is ordered binding of Mg$^{2+}$ before $P_i$. Catalysis of $^{18}$O-exchange by the same mechanism strengthens the justification for quantitatively interpreting data for site-directed mutants of P-type pumps with the rate equation for ordered binding of Mg$^{2+}$ before $P_i$. The
implication of ordered binding to the E₂ conformation for the molecular mechanism is that P-type pumps form a ternary enzyme-metal-phosphate complex (E₂·Mg·Pᵢ).

EXPERIMENTAL PROCEDURES

**Enzyme Preparation and Purification**—Ca-ATPase was prepared from washed SR membranes as described by MacLennan (17). The specific activity (mean ± sd) at 37 °C determined by a NADH-coupled assay (18) was 1.8 ± 0.2 µmol min⁻¹ mg⁻¹ (n = 4) of protein estimated by the Lowry (19) method. The amount of phosphoenzyme formed using a published protocol (20) from 12.5 mM ³²P-labeled Pᵢ and 22 mM MgCl₂ was 5.9 ± 0.5 nmol mg⁻¹ (n = 3) in 30% (v/v) DMSO/buffer at pH 6 and 2.6 ± 0.2 nmol mg⁻¹ (n = 3) under the conditions used to measure ¹⁸O exchange, viz. 50 mM KCl in aqueous buffer at pH 7. The theoretical phosphorylation level for a molecular mass of 109,485 daltons is 9.13 nmol mg⁻¹. Therefore, 65 ± 6% of the Lowry protein in the membranes was functional enzyme, assuming complete phosphorylation in the organic solvent-water mixture at the lower pH (8).

**Synthesis of ¹⁸O-enriched Inorganic Phosphate**—We synthesized stable isotope-enriched Pᵢ by reacting ultrapure PCl₅ (Alpha Products) with ¹⁸O-enriched water (Icon) in a dry box (21). The average enrichment (∑jP₁⁸O_j¹⁶O₄⁻⁴j/4∑₁⁸O_j¹⁶O₄⁻⁴j where j = 0, 1, 2, 3, or 4) of the preparation used in the reported experiments was 96.1%. The filled bars in Fig. 1 show the starting distribution of phosphate isotopomers (P₁⁸O_j¹⁶O₄⁻⁴j). A 78.5 mM stock solution of the enriched Pᵢ was titrated to pH 7 with Tris base and stored frozen at –20 °C.

**Measurement and Analysis of ¹⁸O Exchange**—Most measurements were made at pH 7.0 in 50 mM HEPES buffer containing 50 mM KCl and 0.5 mM EGTA. Each experiment consisted of a control titration including 1 mM CaCl₂, which converts the enzyme to the E₁ conformation, and duplicate titrations of the E₂ conformation without CaCl₂ at eight concentrations of MgCl₂ or ¹⁸O-enriched Pᵢ with the concentration of the other reactant fixed. Figure 1 shows that ignoring
the time dependence of the background exchange did not introduce significant error because Ca\(^{2+}\) inhibited 99% of the observed exchange in a representative experiment. The total \([\text{Mg}^{2+}]_0\) and \([\text{Pi}]_0\) concentrations needed to give free concentrations of titrant equaling 1, 2, 3, 4, 5, 10, 15, and 20 mM at fixed concentrations of 0.2, 0.5, 1, 2, 5, 7.5 mM Mg\(^{2+}\) or 2, 3, 3.5, 5, 6, 10 mM Pi were approximated with the quadratic equation for formation of a 1:1 complex between Mg\(^{2+}\) and Pi (9, Equation 5) with dissociation constant \(K_d = 8.5\) mM (22). The actual free concentrations were estimated from \([\text{Mg}^{2+}]_0\) and \([\text{Pi}]_0\) with the chelator program BAD (23). BAD iteratively solves nonlinear equations like the quadratic equation for Mg·Pi for all the equilibria between metals and chelators in our experiments, including Mg\(^{2+}\) binding to EGTA and Pi coordination by K\(^+\), using consensus dissociation constants that take into account the ionization state of the chelator, as well as the effects of ionic strength and temperature on proton and metal-ion equilibria. The concentration of free Pi was found by summing the concentrations of Pi in different protonation states. The concentration of free Mg·Pi was found by summing the concentrations of the protonated and unprotonated forms of Pi with Mg\(^{2+}\) bound. The reaction was started by adding 0.07 mg mL\(^{-1}\) of protein in a final volume of 200 µL and run for 60 min at 24 °C in a thermomixer. As a control for the effect of K\(^+\) on the phosphorylation mechanism, 50 mM ChoCl was substituted for KCl in duplicate titrations with 2 mM fixed Mg\(^{2+}\) or Pi. In this control, the reaction time was also lengthened (260 min) to compensate for a lower protein concentration (0.02 mg mL\(^{-1}\)) and an anticipated slower exchange rate. The ionic strength (µ) was increased from 102 mM (including 50 mM KCl) to 163 mM with ChoCl at fixed total concentrations of Mg\(^{2+}\) and Pi (both 5 mM) as a control for variation of the rate with µ. The methods used to quench the reaction, isolate Pi, synthesize volatile triethyl phosphate, and analyze the product with a Hewlett-Packard 5972A GCMS, which was upgraded during the study with an Agilent Technologies G1896A autosampler and HP G1701BA software and
operated in the SIM mode, have all been described previously (13). Bio-synthesis Inc.,
Lewisville, TX synthesized ENTS, the precursor for the diazoethane used to derivatize Pi.

Theory of $^{18}$O Exchange—Oxygen exchange between Pi and water requires covalent bond
cleavage. The non-enzymatic reaction is extremely slow with a half time at 100 °C of one and
one-half weeks (24). The E$_2$ conformation of P-type pumps catalyzes the reaction by initially
forming a noncovalent “Michaelis” complex (E$_2$·Pi) that is converted into a covalent
phosphoenzyme (E$_2$–P) by nucleophilic attack of an aspartyl oxygen atom (25).

$$
\begin{align*}
\text{E}_2 + \text{Pi} & \rightarrow \text{E}_2\cdot\text{Pi} \\
\text{E}_2\cdot\text{Pi} & \rightarrow \text{E}_2\text{--P} + \text{H}_2\text{O}
\end{align*}
$$

The reaction can be followed either by synthesizing $^{18}$O-enriched Pi, or by running the reaction
in [$^{18}$O]H$_2$O. Oxygen exchange is a pseudo first-order process with hydrolysis rate constant
$k'_{+\text{H}_2\text{O}}$ because the concentration of unenriched H$_2$O (55.6 M) does not change (Equation 2).

$$
\nu_{\text{ex}} = k'_{+\text{H}_2\text{O}}[\text{E}_2 \cdot \text{P}] = k'_{+\text{H}_2\text{O}}E_0\chi_{E_2\cdot\text{P}} = V_{\text{max}}\chi_{E_2\cdot\text{P}}
$$

The measured isotope exchange rate is slower than the oxygen exchange rate ($\nu_{\text{ex}}$) because $^{18}$O
exchange depends upon the enrichment of the labeled reactant and upon the number of times the
covalent bond with the enzyme is formed before Pi dissociates. The partition coefficient ($P_c$) is
the probability of bound Pi forming phosphoenzyme, which depends upon the rate constants for
Pi ($k_{-\text{Pi}}$) and water ($k_{-\text{H}_2\text{O}}$) loss from the Michaelis complex (Equation 3).

$$
P_c = \frac{k_{-\text{H}_2\text{O}}}{k_{-\text{Pi}} + k_{-\text{H}_2\text{O}}}
$$

Hackney (26) derived equations for estimating $P_c$ and a first-order rate constant ($k$) from the
observed isotopomer distribution as a function of time and the starting isotopomer distribution of
$^{18}$O-enriched Pi by assuming equivalence of the oxygen atoms in the transition state. We wrote a
nonlinear least squares program (available upon request) for estimating $k$ and $P_c$ from a single time point and calculated $v_{ex}$ in $\mu$atom min$^{-1}$ mg$^{-1}$ from the estimated exchange parameters and the total concentrations of protein in mg mL$^{-1}$ ($P_o$) and $P_i$ in molarity ($[P_i]_o$) with Equation 4 (27).

$$v_{ex} = k \frac{P_c}{(1 - P_c)} \frac{[P_i]_o}{P_o} \quad (4)$$

The rate equation relating $v_{ex}$ to reactant concentrations depends upon the mechanism of Mg$^{2+}$-dependent phosphorylation. The theoretical maximum rate ($V_{max}$) is $k'_{+H_2O}E_o$ (Equation 2), where $E_o$ is the concentration of functional enzyme in mg mL$^{-1}$. The observed rate depends upon the fraction of the enzyme with $P_i$ covalently attached ($\chi_{E_2-P} = [E_2-P]/E_o$) calculated by assuming all of the binding equilibria are rapid and applying the stationary-state assumption to $E_2-P$. The steady-state solutions ($\chi_{E_2-P_{ss}}$) for the mechanisms proposed for P-type pumps (I, III-IV) in the cited references (3-8, 10-13), for ordered binding of $P_i$ before Mg$^{2+}$ (II), and for ordered binding of Mg$^{2+}$ before $P_i$ with noncompetitive inhibition by Mg-$P_i$ were substituted into Equation 2 to obtain the rate equations in Table I. $K_{MP}$ is the dissociation constant of bound Mg-$P_i$ from apoenzyme ($E_2$); $K_M$ is the dissociation constant of Mg$^{2+}$ from metalloenzyme (Mg-$E_2$); $K_P$ is the dissociation constant of bound $P_i$ from apoenzyme; $K'_P$ is the dissociation constant of bound $P_i$ from metalloenzyme; $K'_M$ is the dissociation constant of bound Mg$^{2+}$ from apoenzyme with $P_i$ noncovalently bound ($E_2-P_i$); $\alpha$ equals either $K'_P/K_P$ or $K'_M/K_M$; $K_h$ is the equilibrium constant between noncovalently and covalently bound $P_i (k_{-H_2O}/k'_{+H_2O})$, and the reactant concentrations are the free concentrations estimated from $[Mg^{2+}]_o$ and $[P_i]_o$ with BAD. We investigated the phosphorylation mechanism and estimated the parameters for Mechanisms I-V by fitting the rate equations in Table I to plots of $v_{ex}$ as a function of the free Mg$^{2+}$ and $P_i$ concentrations with the SigmaPlot 4 nonlinear-least-squares algorithm.
RESULTS

The estimates of $v_{ex}$ and $P_c$ as a function of total Mg$^{2+}$ and Pi concentrations are reported in Table II. In compiling the data, two criteria were used to identify outliers. First, isotopomer distributions giving $P_c$ estimates greater than two standard deviations from the mean or indistinguishable from zero (rounded value < $10^{-6}$) were discarded, even though retaining the points improved the fits. Second, points outside the 90% confidence interval for the two most likely mechanisms (III, IV) and an additional mechanism (V), which was considered to explain inhibition of exchange at high concentrations of both Mg$^{2+}$ and Pi, were rejected. Rounded mean values ± sd for each ([Pi]$_o$, [Mg$^{2+}$]$_o$) pair are tabulated to save space. All the averaged $v_{ex}$ estimates in Table II from titrations with Mg$^{2+}$ (triangles) or Pi (circles) are plotted against the free Mg$^{2+}$ and Pi concentrations in Fig. 2. The sd values in the table for $n = 2$ indicate the precision of duplicate measurements. The reproducibility of $v_{ex}$ estimated from different titrations is illustrated by the Mg$^{2+}$ titration at approximately 2 mM free Pi (inverted triangles), which was repeated ($n = 4$) as a control for upgrading and reprogramming the GCMS. The estimated percentage sd in $P_c = 0.04 ± 0.03$ (sem = 0.002) is large (75%) because three of the twelve titrations gave consistently higher estimates of $P_c$. However, there was no discernible effect on the estimates of $v_{ex}$. Figure 3 shows that points for the same choice of ([Pi]$_o$, [Mg$^{2+}$]$_o$) from different titrations superimpose regardless of the titrant or $P_c$ estimate. In Fig. 3, estimates of $v_{ex}$ from Mg$^{2+}$ titrations (triangles) are plotted on the Pi titrations (circles) without discernible Mg·Pi inhibition ($0.16 \leq [\text{Mg}^{2+}]_\text{free} \leq 6.05$ mM). The mean data points plotted in Fig. 3 are indicated by bold type in Table I. The ionic strength increased from 69 to 207 mM with added Mg$^{2+}$ and Pi. However, no trend or change in exchange rate was observed ($v_{ex} = 1.32 ± 0.06$ μatom min$^{-1}$ mg$^{-1}$) when $\mu$ was increased from 102 to 163 mM with ChoCl in a control experiment ($n = 3$). Substituting Cho$^+$ for K$^+$ reduced the estimates of $v_{ex}$ at corresponding free
Mg\textsuperscript{2+} and P\textsubscript{i} values roughly one-half to one-third, but the shape of the Mg\textsuperscript{2+} or P\textsubscript{i} titration curves (not shown) did not change significantly, and the conclusion drawn about the mechanism from multivariate nonlinear regression analysis was the same, i.e. Mg\textsuperscript{2+} binds before P\textsubscript{i}. The regression lines in Figs. 2 and 3 are spline curves drawn through the origin and points calculated with the parameters estimated by globally fitting all the data points in Table II (n = 184) with Mechanism V, or the data points in bold type (n = 107) with Mechanism I, II, III, or IV.

Table III summarizes the parameter estimates for Mechanisms I-V. Values obtained by fitting the data in Fig. 2 or 3 with the equations in Table I (regression coefficients) are shown in bold type. Values in normal type were calculated either from two regression coefficients (Mechanism IV), or from the regression coefficient containing the intrinsic substrate (S) dissociation constant (K\textsubscript{S}) and an independent estimate of the equilibrium constant between covalently and noncovalently bound P\textsubscript{i} (Mechanisms I, II, III, and V). In the ordered-binding mechanisms (II, III, and V), K\textsubscript{S}/(1 + K\textsubscript{h}) is the half-maximum substrate concentration when the cofactor sites are filled (e.g. [Mg\textsuperscript{2+}] > K\textsubscript{M} in III and V). The standard error of the parameter estimate is reported when P < 0.0001 and omitted when P > 0.8 (see > 100%). Column 11 contains one measure of “goodness of fit”, viz. the multiple correlation coefficient (28) adjusted for the number of degrees of freedom (R\textsubscript{adj}). Two other criteria for choosing between alternative mechanisms are shown in Fig. 4. The residuals (circles) between the measured and calculated (solid black line) values of the dependent variable (v\textsubscript{ex}) are plotted, along with the standard error of the estimate (gray dashed lines) for each mechanism. The standard error of the estimate is the square root of the “average” (division by n minus the number of parameters, i.e. the number of degrees of freedom) squared deviation of the data about the regression surface. Two-thirds of the measurements fall within the envelope formed by the dashed lines. The data were also analyzed with Mechanism III assuming that the true substrate is the monoprotonated form of P\textsubscript{i}. 
(HPO_4^{2-})$, the diprotonated form of P_i (H_2PO_4^-), or the sum of the monoprotonated and diprotonated forms of P_i instead of the sum of all the protonation states. The free concentrations of the fully protonated (H_3PO_4) and completely unprotonated (PO_4^{3-}) forms of P_i were both negligible. The differences in the multiple correlation coefficients for the three substrates that fit the data best (P_i, H_2PO_4^-1, or HPO_4^{-2}) were in the fourth place after the decimal point, and the differences in the standard errors of the estimate of the surface were in the thousandth (H_2PO_4^{-1}) or in the ten thousandth (P_i, HPO_4^{-2}) place.

**DISCUSSION**

The rate equations for Mechanisms I-V predicts hyperbolic dependence of $v_{ex}$ on concentration (Table I). The choice of independent concentration variable (X) is arbitrary. However, the expressions for the observed maximum rate ($V_{X_{max}}$) and half-maximum titrant concentration ($K_{X_{0.5}}$) differ and can be used to choose between alternative mechanisms. We concluded from an earlier study that Mg$^{2+}$ binds to sodium pump before P_i (13). Mechanism I was rejected because $K_{X_{0.5}}$ depended upon [Mg$^{2+}$] when $v_{ex}$ was plotted against [Mg·P_i] (*ibid.*, Fig. 4c). Mechanism II was rejected because $V_{X_{max}}$ depended upon [P_i] when $v_{ex}$ was plotted against [Mg$^{2+}$] (*op cit.*, Fig. 4b). Neither experimental observation is predicted by the equations for Mechanism I or Mechanism II in Table I. Mechanisms III and IV fit the data for Na,K-ATPase comparably. However, the estimates of $K_M$ and $K_P$ obtained with the equation for Mechanism IV indicated that >98% of the reaction would go through the pathway in which Mg$^{2+}$ binds before P_i, if the concentrations of free Mg$^{2+}$ and free P_i were equal. Therefore, we concluded that P_i is the substrate. Mg$^{2+}$ is a cofactor that binds to sodium pump and is required for P_i binding and phosphoryl group transfer to the enzyme.
Evidence Mg\(^{2+}\) Binds to Calcium Pump Before Pi—The results obtained with Ca-ATPase are remarkably similar to those just summarized for Na,K-ATPase with one complication. Reduced activity is apparent (especially in two-dimensional plots) of the Mg\(^{2+}\) titrations at the two highest fixed Pi concentrations and perceptible in the plot of \(v_{ex}\) versus [Pi] at the highest fixed Mg\(^{2+}\) concentration (Fig. 2). Bell-shaped titration curves were observed previously for calcium pump when the amount (6,8,29) or rate (7) of E–P formation from Pi measured by counting \(^{32}\)P was plotted against [Mg\(^{2+}\)]. The decrease in E–P was attributed to a low-affinity inhibitory Mg\(^{2+}\) site (8) or Mg\(^{2+}\) binding to Ca\(^{2+}\) sites causing a shift in the conformational equilibrium toward unreactive E\(_1\) (6). However, the dip in the surface formed by the data points in Fig. 2 correlates with high free Mg·Pi concentrations (>12 mM). Tabulated (30) values for the solubility of MgHPO\(_4\)·7H\(_2\)O (17-25 mM) suggest that precipitation could be a complication in experiments at high concentrations of both Mg\(^{2+}\) and Pi. However, a low-affinity inhibitory Mg·Pi site cannot be ruled out because of a similar dip in the data-point surface for the Na,K-ATPase mutant D717N (15) at lower free Mg·Pi concentrations (>3 mM). The solid lines in Fig. 2 show that the data can be fit with ordered binding of Mg\(^{2+}\) before Pi by assuming that Mg·Pi is a noncompetitive inhibitor (Mechanism V) with \(K_{MP} = 17 \pm 3\) mM (Table III). Another possibility is a parallel pathway in which Mg·Pi binds to the active site of Ca-ATPase and turns over more slowly than Pi bound to the metalloenzyme because Mg\(^{2+}\) forms a bidentate complex with Pi in solution, whereas Pi (or an analogue) occupies a single coordination site of Mg\(^{2+}\) in the crystal structures of HAD superfamily members PN (31) and PSP (32). The exact explanation for loss of exchange activity at high free Mg·Pi concentrations was not pursued because \(^{18}\)O measurements at higher total Mg\(^{2+}\) and Pi concentrations than those reported are not feasible.

Instead of adding a poorly understood inhibition term to every mechanism, fits to the smaller array of consistent data from Mg\(^{2+}\) and Pi titrations with [Mg·Pi]\(_{free}\) < 8.5 mM in Fig. 3
(bold-type points in Table II) were compared (Table III). All of the models “fit” the data in the sense that the null hypothesis (no correlation between the dependent and independent variables) could be rejected \( (P < 0.0001) \), but not all of the regression coefficients were statistically different from zero for ordered binding of \( P_i \) before \( \text{Mg}^{2+} \) (Mechanism II) or random binding (Mechanism IV). The standard error of the estimate is tabulated (bold type) for statistically significant parameter estimates \( (P < 0.0001) \). The reason all of the regression equations describe the data reasonably well is that the independent variables are correlated (9, Equation 5). Therefore, the concentrations of all of the substrates considered in Table I increase along with the exchange rate in the same total \( \text{Mg}^{2+} \) and \( P_i \) concentration range. “Multicollinearity” (28) is also the reason some of the regression coefficients in some of the mechanisms cannot be estimated precisely. \( R_{adj} \) values closer to 1.0000 (perfect fit) in Table III mean that Mechanisms III and IV fit the data better than Mechanisms I and II. The lines in Fig. 3 illustrate the poorer fit. Dash-dot-dot (— · ·) lines are used to represent the superposition of calculated curves for two mechanisms because the regression lines for Mechanisms I and II (gray lines) and for Mechanisms III and IV (black lines) were visually indistinguishable. Figure 4 shows the trend in the residuals for the mechanisms that gave poorer fits. The differences between the observed and calculated values of \( v_{ex} \) for Mechanisms I and II scatter about lines (not shown) that start below and end above the theoretical (solid) line, whereas the residuals for Mechanisms III and IV scatter about zero. The same ordinate scale is used in all of the residual plots to illustrate that the standard error of the estimate for the data array was smallest \( (±0.0992 \ \mu \text{atom min}^{-1} \text{mg}^{-1}) \) for ordered binding of \( \text{Mg}^{2+} \) before \( P_i \) (Mechanism III). Random binding (Mechanism IV) fit the data almost as well \( (see = ±0.1007 \ \mu \text{atom min}^{-1} \text{mg}^{-1}) \), but only because the lower pathway in the reaction scheme (Equation 5), in which \( P_i \) binds before \( \text{Mg}^{2+} \), was essentially ignored.
This conclusion can be reached either by using the values of $K_M$ and $K_P$ for Mechanism IV in Table III to estimate that only 0.6\% $[E_2\cdot P_i/(Mg\cdot E_2 + E_2\cdot P_i) = K_P/(K_P + K_M)]$ of the reaction goes through the lower pathway of the scheme in Equation 5 when the concentrations of Mg$^{2+}$ and Pi are equal, or by interpreting the high probability that $K_P$ is zero ($P = 0.8369$) to mean that Pi binding to apoenzyme does not affect the exchange rate significantly. Statistically indistinguishable fits were obtained when the monoprotonated or diprotonated form of Pi, instead of the sum of all the protonation states, was assumed to be the substrate (Results). Therefore, our results do not support a claim that only H$_2$PO$_4^{-1}$ forms phosphoenzyme (33). The conclusion that Mg$^{2+}$ binds before Pi was not affected by fitting the larger array in Fig. 2, but Table III shows that the affinities for Mg$^{2+}$ and Pi could be 15-33\% lower (Mechanism V) than estimated by ignoring Mg–Pi inhibition (Mechanism III). The inference from ordered binding that P-type pumps form a ternary E$_2$·Mg·Pi complex is supported by the crystal structures of HAD superfamily members PSP (32) and PN (31), in which Mg$^{2+}$ coordinated to both an aspartyl group of the enzyme and Pi(PSP), or a Pi analogue (PN), was observed.

In addition to an obligatory role in Pi binding, Mg$^{2+}$ presumably participates directly in the catalytic mechanism. In one mechanism for phosphoryl group transfer, a pentacoordinate intermediate is formed by nucleophilic attack upon phosphorus (34). Metal ions lower the potential-energy barrier by neutralizing excess negative charge on the pentacoordinate transition state, and the stereochemistry of the product is inverted. Evidence P-type pumps catalyze
phosphoryl group transfer by this mechanism includes the following: (a) Mg$^{2+}$ is required (35); the nucleophile in transfer to the enzyme is an aspartyl oxygen atom (25); pentacoordinate orthovanadate is a transition-state analogue (36); and hydrolysis of ATP, which involves two transfers, occurs with retention of the stereochemistry of the $\gamma$–phosphoryl group (37).

**Comparison with Published Studies**—Since establishing the mechanism of Na,K-ATPase-catalyzed $^{18}$O exchange between P$_i$ and H$_2$O by purified enzyme as ordered binding of Mg$^{2+}$ before P$_i$, we have analyzed four preparations of wild-type enzyme (one sheep and three human) expressed in yeast membranes and thirty-two site-directed mutants. Thirteen of the mutants could be studied as a function of both Mg$^{2+}$ and P$_i$ concentration, and in every case the rate equation for ordered binding of Mg$^{2+}$ before P$_i$ fit the data best, thereby adding to the evidence for Mechanism III. Table IV summarizes the wild-type Na,K-ATPase results, including a totally independent estimate of $K_M$ from stopped-flow studies of the E$_1$ $\rightleftharpoons$ E$_2$ conformational change in purified enzyme (38), and compares them to the parameters estimated for Ca-ATPase from the data in Fig. 3. The two statistically significant differences are the estimates of $K_P'(1 + K_h)$ and $P_c$, which differ nearly 6-fold from the average Na,K-ATPase values in opposite directions. An increase in the P$_i$-off rate could explain both observations because $k_{P_i}$ is in the numerator of $K_P'$ ($k_{P_i}/k_{-P_i} = [\text{Mg}\cdot\text{E}_2][\text{P}_i]/[\text{Mg}\cdot\text{E}_2\cdot\text{P}_i]$) and in the denominator of $P_c$ (Equation 3).

To test the hypothesis that the differences between the calcium and sodium pumps are attributable to a single reaction step, the rate constants for $^{18}$O exchange catalyzed by calcium pump were estimated. There are four unknown rate constants in Equation 1, but only three equations relating them to parameters estimable from $^{18}$O-exchange data, viz. Equation 3 for $P_c$ and the expressions for $V_{p_{\text{max}}}$ and $K_{P_{0.5}}$ in Table I for Mechanism III. Therefore, $k'_{-\text{H}_2\text{O}}$ (12.0 ± 1.6 sec$^{-1}$) was estimated with Equation 2 from the value of $\nu_{ex}$ (1.9 ± 0.1 µmol min$^{-1}$ mg$^{-1}$).
calculated with the exchange parameters in Table III (bold type) for the total P<sub>i</sub> (12.5 mM) and Mg<sup>2+</sup> (22 mM) concentrations used to measure the amount of [E–P] formed from [³²P]P<sub>i</sub> (2.6 ± 0.2 nmol mg<sup>-1</sup>) in the exchange buffer (Materials). The error in v<sub>ex</sub> is the standard error of the estimate (Fig. 4) for the fit of Mechanism III to the array of data in Fig. 3. The errors in v<sub>ex</sub> and [E–P] were propagated with the total differential of k′<sub>′+H₂O</sub>. There was a fifth unknown in our experiments because the cofactor sites were not always saturated (e.g. in measurement of [E–P]), but K<sub>M</sub> could be estimated with confidence (P < 0.0001) and was experimentally the same for the calcium and sodium pumps (Table IV). V<sub>max</sub> (4.3 ± 0.9 µmol min<sup>-1</sup> mg<sup>-1</sup>) calculated with k′<sub>′+H₂O</sub> and the ³²P estimate (Materials) of E<sub>o</sub> (5.9 ± 0.5 nmol mg<sup>-1</sup>) was equal to the estimate of V<sub>Pmax</sub> (4.4 ± 0.2 µmol min<sup>-1</sup> mg<sup>-1</sup>). Therefore, K<sub>h</sub> could not be evaluated, but k<sub>H₂O/k′+H₂O</sub> must be >1. Assuming a difference greater than the aggregate uncertainties in V<sub>max</sub> and V<sub>Pmax</sub> (1.1 µmol min<sup>-1</sup> mg<sup>-1</sup>) could be detected experimentally, K<sub>h</sub> > 3 and k<sub>H₂O</sub> > 36 sec<sup>-1</sup>. The lower limit on k<sub>H₂O</sub> and the estimate of P<sub>c</sub> were substituted into Equation 3 to calculate a minimum value of k<sub>Pi</sub>. Finally, a lower limit for K′<sub>P</sub> was calculated from the estimate of K′<sub>P</sub>/ (1 + K<sub>h</sub>) and used to estimate k<sub>′+Pi</sub>.

Table V compares the rate constants estimated from the oxygen exchange data for Ca-ATPase in Fig. 3 and the ³²P data characterizing the preparation in Materials with published values for the calcium (39,40), proton (27), and sodium pumps (13). To facilitate comparing the P-type pumps, K′<sub>P</sub>, K<sub>h</sub>, and P<sub>c</sub> are also tabulated. Readers are referred to the original papers for how the parameters were estimated and how the estimates were combined to calculate other constants. Only error estimates smaller than the parameter estimate are reported. The uncertainty in the remaining parameters is greater than 100% either because of fewer unknowns than equations or error propagation. The previously published numbers in Table V generally confirm our observations for Ca-ATPase, but they do not support the hypothesis that the
observable differences between the calcium and sodium pumps are attributable to a single reaction step. The estimates of $P_c$ for calcium pump from different laboratories (rows 1-3) are in good agreement, and the significantly lower probability of phosphorylating Ca-ATPase than either of the other P-type pumps cannot be explained by the differences in experimental pH (footnotes) because $P_c$ is inversely related to pH for all three pumps (27,39,41). The two more recent studies of calcium pump are in agreement that $P_i$ binds weaker ($K'_{P}$ larger) to calcium pump than to the proton or sodium pump. This can be seen empirically by comparing the $P_i$-concentration range of titrations of the proton and sodium pumps (0-10 mM) in References 27 (Fig. 6) and 13 (Fig. 4a) with Fig. 3 and with Fig. 5 in Reference 40 ($0 \leq [P_i] \leq 20-30$ mM). However, the phosphoryl group transfer steps in Equation 1 appear to be as responsible as $P_i$ binding for the empirical differences between calcium pump and the two other P-type ATPases. Both dephosphorylation and phosphorylation of Ca-ATPase seem to be less efficient, with the caveat that the estimate of the rate constant for the latter ($k_{H2O}$) depends upon the equilibrium between noncovalently and covalently bound $P_i$, and different assumptions were made in estimating the $K_h$ values for different pumps in Table V.

The most striking difference between this study and earlier studies of calcium pump is the conclusion that $P_i$ binds only to the metalloenzyme. The same conclusion was reached for sodium pump (13), in disagreement with previous studies that all claimed Mg$^{2+}$ and $P_i$ bind randomly (10-12). However, in the case of sodium pump, the disagreement was only apparent because the free reactant concentrations, which appear in the equations, were not calculated and/or experimental conditions, which could affect the mechanism, were different (13). Published data from another laboratory (12) for a different technique (deocclusion) actually supported our conclusion from oxygen exchange experiments that Na,K-ATPase binds Mg$^{2+}$ before $P_i$ when data for phosphorylation-stimulated Rb$^+$ release was analyzed as a function of the
free, instead of total, Mg\(^{2+}\) and P\(_i\) concentrations (13). Different conclusions from \(^{32}\)P and \(^{18}\)O studies of calcium pump cannot be rationalized in the same way. All the cited mechanistic studies of Mg\(^{2+}\)-dependent phosphorylation of Ca-ATPase ignored inhibition, if observed (6-8), and omitted K\(^+\). We included K\(^+\) in our experiments, so that we could compare our \(^{18}\)O exchange results with published experiments (39). However, we confirmed that the mechanism does not depend upon the monovalent cation by control experiments in which Cho\(^+\) was substituted for K\(^+\) (Results). All but one (5) of the studies of Ca-ATPase took coordination of P\(_i\) by Mg\(^{2+}\) into account and used the free concentrations to analyze \(^{32}\)P rate or binding data.

Another difference between earlier studies of calcium pump and this study was that nonlinear-least-squares software was unavailable, so the data were linearized by plotting reciprocals and analyzed graphically. However, when the data from the two studies with the most data points were reanalyzed with SigmaPlot, only slight differences in the parameter estimates were obtained that could be attributed to errors in reading the data from published figures and/or different values of the Mg·P\(_i\) dissociation constant. Mechanism I fit the data at pH 6 in 15% DMSO (8) best, and Mechanism IV fit the data at pH 7 in aqueous solution (3) better than any of the other mechanisms. Organic solvent must be more important than pH in the change to Mechanism I (8) because the pH varied from 6 to 7 in the studies of calcium pump in water that concluded Mg\(^{2+}\) and P\(_i\) bind randomly (3-7). However, the shift in half maximum with [Mg\(^{2+}\)]\(_o\) predicted by assuming Mg·P\(_i\) is the substrate in organic solvent (Table I, Mechanism I, Row 2) was not observed when phosphorylation data for expressed wild-type Ca-ATPase at pH 6 in 30% DMSO was plotted against [P\(_i\)]\(_o\) in a more recent study (9). The published evidence for random binding in aqueous solution (3-7) is compelling with the caution, first pointed out by Inesi et al. (41), that \(K_h\) can only be calculated if the number of functional enzyme molecules (\(E_o\)) is known because there are fewer equations than unknowns. When 5
instead of 4 parameters were estimated from the Punzengruber-Kolassa data (3,4), only $K_M$ and $K_P$ were statistically significant ($P < 0.0001$). The estimate of $K_h$ was more than an order of magnitude smaller than the value that Inesi et al. (41) obtained from direct $^{32}$P-measurements of the phosphorylation and hydrolysis rates ($K_h = 16$), which is consistent with two of the three $^{18}$O exchange estimates (Table V). However, the estimates of $K_M (9 \pm 2$ mM) and $K_P (3.0 \pm 0.6$ mM) make a strong case for random binding. The observable difference between random and ordered binding is that in the former, the probabilities of going through the upper and lower pathways in Equation 5 are roughly the same, whereas in the latter, the observed maximum rate in titrations with the cofactor (P$_i$ in Mechanism II or Mg$^{2+}$ in Mechanism III, Table I) is limited by the fixed concentration of substrate. Titrations with Mg$^{2+}$ and P$_i$ approached approximately the same E–P$_{\text{max}}$ (3-7), or maximum rate (7) in all of the $^{32}$P studies of Ca-ATPase, whereas the three-dimensional plot of the $^{18}$O exchange data is asymmetrical (Fig. 2). The titrations with P$_i$ approach the same maximum exchange rate, whereas the observed maximum rate in titrations with Mg$^{2+}$ increases with the fixed P$_i$ concentration. The increase in the observed maximum $^{18}$O-exchange rate of Mg$^{2+}$ titrations with $[P_i]_0$ is easier to see in Fig. 4b of Reference 13 for sodium pump because inhibition by Mg·P$_i$ did not complicate the picture.

The simplest explanation for different conclusions about the mechanism depending upon the technique used to observe Mg$^{2+}$-dependent phosphorylation of Ca-ATPase is that $^{32}$P and $^{18}$O detect different reactions. However, the mechanisms in the literature for E–P formation (3-7) and $^{18}$O exchange (39,40) are the same, and the equations in the literature for the amount or rate of E–P formation by different mechanisms can be rearranged to give the equations in Table I. An alternative possibility is that an inherent limitation of one of the methods introduces a systematic error that distorts the data. In both methods, the reaction must be quenched for observation$^2$, and the time required is significant compared to the relaxation time for the
unimolecular step in Equation 1 \((\tau = k_{-H_2O} + k'_{+H_2O})\) estimated with the values of the rate constants for calcium pump in Table V \((8 \leq \tau \leq 21 \text{ msec})\). However, the two techniques differ fundamentally in what is measured. The amount of covalent phosphoenzyme \((E-P_{\text{max}} \sim 3 \text{ nmol, assuming a 1 mL reaction volume in Reference 3})\) is measured in \(^{32}\text{P}\)-incorporation experiments, whereas the isotopomer distribution of solvent \(P_i\) \((0.2-8 \text{ mmol of } P_i \text{ in our experiments})\) is measured in \(^{18}\text{O}\)-exchange experiments. Therefore, any shift in the equilibrium between covalently and noncovalently bound \(P_i\) during the observation would distort \(^{32}\text{P}\) measurements more than \(^{18}\text{O}\) measurements, although how a larger percentage systematic error in one technique than the other would lead to different mechanistic conclusions is unclear.

The importance of the reaction mechanism for the interpretation of data comparing site-directed mutants with wild-type enzyme is clear from preliminary \(^{18}\text{O}\)-exchange studies of the serine mutant of the upstream threonine in the signature DKTGT sequence of sodium pump. Mechanism I did not fit oxygen exchange data. Moreover, the intrinsic \(\text{Mg}^{2+}\) dissociation constant estimated with Mechanism III was significantly larger (5-fold) than the wild-type value (unpublished results). Neither result was expected because a \(^{32}\text{P}\) study of the corresponding mutant in calcium pump \((9)\) assumed the substrate is \(\text{Mg} \cdot P_i\) (Mechanism I) and concluded that \(\text{Mg}^{2+}\) binding is unaffected.

*Note Added in Proof*—While this paper was being reviewed, a high-resolution structure of Ca-ATPase was published with the aspartate that is phosphorylated in one coordination site of hexacoordinate \(\text{Mg}^{2+}\) and a \(P_i\) analogue \((\text{MgF}_4^{2-}\)) in another \((43)\). Therefore, the new structure confirms formation of a ternary \(E_2 \cdot \text{Mg} \cdot P_i\) complex by P-type pumps. However, the structure is consistent with any of the mechanisms for \(\text{Mg}^{2+}\) and \(P_i\) reaction with the enzyme in Table 1 and does not permit derivation of the half-maximum titrant concentrations needed to interpret equilibrium and kinetic experiments comparing site-directed mutants with wild-type enzyme.
REFERENCES:


FOOTNOTES

1 Abbreviations: Ca-ATPase (calcium pump), Ca\(^{2+}\) and Mg\(^{2+}\)-dependent ATPase (EC 3.6.1.38); H,K-ATPase (proton pump), Mg\(^{2+}\)-dependent, H\(^+\)-transporting, and K\(^+\)-stimulated ATPase (EC 3.6.1.36); Na,K-ATPase (sodium pump), Mg\(^{2+}\)-dependent and Na\(^+\) and K\(^+\)-stimulated ATPase (EC 3.6.1.37); PN, phosphonatase; PSP, phosphoserine phosphatase; HAD, haloacid dehalogenase; P-type, phosphoenzyme forming pump; ATP, adenosine 5\(^{′}\)-triphosphate; P\(_i\), inorganic phosphate; NADH, nicotinamide adenine dinucleotide; DMSO, dimethyl sulfoxide; ChoCl, choline chloride; HEPES, N-(2-hydroxyethyl)piperazine-N\(^{″}\)-2-ethanesulfonic acid; EGTA, ethylene glycol bis(\(β\)-aminoethyl ether)-N,N,N\(^{″}\),N\(^{″}\)-tetraacetic acid; SR, sarcoplasmic reticulum; ENTS, N-ethyl-N-nitroso-p-toluenedisulfonamide; AE, average (isotope) enrichment; BAD, bound and determined (www.stanford.edu/~cpatton/other.html); GCMS, gas chromatograph-mass spectrometer; NMR, nuclear magnetic resonance; SIM, selected ion monitoring; SF, stopped flow; \(P\), probability null hypothesis is correct; \(R_{adj}\), multiple correlation coefficient adjusted for number of degrees of freedom; sd, standard deviation of the mean; see, standard error of the estimate; sem, standard error of the mean.

2 Oxygen exchange can be followed in real time by \(^{31}\)P-NMR spectroscopy (44). However, more material is required, and the method is less sensitive. Therefore, measurements as a function of an array of reactant concentrations are difficult with purified P-type pumps and are currently impossible with mutants of sodium pump expressed in yeast.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Inhibition of $^{18}$O exchange by Ca$^{2+}$. Isotopomer distributions measured in the presence (unfilled bars) and absence (crosshatched bars) of Ca$^{2+}$ are compared with unreacted $^{18}$O-enriched P$_i$ (filled bars). In the experiment shown, Ca-ATPase (0.07 mg mL$^{-1}$) catalyzed $^{18}$O-exchange between 4.35 mM P$_i$ and 55.6 M H$_2$O for 1 hr at pH 7 and 24 °C in 12.35 mM MgCl$_2$. The buffer was 50 mM HEPES containing 50 mM KCl and 0.5 mM EGTA plus or minus 1 mM CaCl$_2$. The average isotope enrichment of P$_i$ decreased from 96.1% to 95.9% when Ca$^{2+}$ was present and to 76.8% when Ca$^{2+}$ was omitted. The figure shows that only the Ca$^{2+}$-free (E$_2$) conformation of Ca-ATPase catalyzes $^{18}$O exchange between P$_i$ and H$_2$O.

Fig. 2. Mg$^{2+}$ and P$_i$ dependence of exchange rate. Averaged estimates of $v_{ex}$ are plotted against the free Mg$^{2+}$ and P$_i$ concentrations calculated with the chelator program bound and determined (23). Triangles indicate titrations with Mg$^{2+}$ at approximately constant P$_i$ concentrations and circles indicate titrations with P$_i$ at roughly constant Mg$^{2+}$ concentrations. The Mg$^{2+}$ titration at the lowest fixed P$_i$ concentration (inverted triangles) was repeated. The buffer and other experimental conditions are given in the legend of Fig. 1. The solid lines are spline curves drawn through the origin and the points calculated with the parameters (Table III) estimated by globally fitting Mechanism V for ordered binding of Mg$^{2+}$ before P$_i$ with noncompetitive inhibition by Mg·P$_i$ to all of the data ($n = 184$). The figure shows $v_{ex}$ decreases at high concentrations of both Mg$^{2+}$ and P$_i$, consistent with noncompetitive inhibition by Mg·Pi.

Fig. 3. Analysis of exchange ignoring inhibition. Mean estimates of $v_{ex}$ ± sd from Mg$^{2+}$ titrations (triangles) with approximate free Mg$^{2+}$ concentrations of 1 (0.9 ≤ [Mg$^{2+}$]$_{free}$ ≤ 1.1), 2 (1.8 ≤ [Mg$^{2+}$]$_{free}$ ≤ 2.2), or 5 (4.3 ≤ [Mg$^{2+}$]$_{free}$ ≤ 4.9) mM are plotted on the P$_i$ titrations (circles) that did not show inhibition (0.16 ≤ [Mg$^{2+}$]$_{free}$ ≤ 6.05). Points plotted in the figure are indicated by bold type in Table II. The lines are spline curves through the values of $v_{ex}$ calculated with the
regression coefficients (Table III) obtained by fitting Mechanism I or II (gray) or Mechanism III or IV (black) to the data ($n = 107$). Dash-dot-dot lines are used to represent the superposition of two regression lines because the calculated lines for Mechanisms I and II and for Mechanisms III and IV were visually indistinguishable. The figure shows that ordered binding of Mg$^{2+}$ before P$_i$ (Mechanism III) and random binding (Mechanism IV) fit the smaller array of data comparably.

**Fig. 4. Residual plots for the mechanisms tested.** The differences (symbols) between the observed and calculated (solid regression line) exchange rates for Mechanisms I-IV ($n = 107$) and Mechanism V ($n = 184$) are plotted against the free P$_i$ concentration. The dashed gray lines are the standard error of the estimate for the fit of the data points to the regression surface. The upward trend of the points in the top two panels show that Mechanism I (substrate Mg-P$_i$) and II (ordered binding of P$_i$ before Mg$^{2+}$) do not fit in the data. Random distribution of the points about the theoretical (solid) line and the smallest see (area enclosed by dashed gray lines) show that Mechanism III (ordered binding of Mg$^{2+}$ before P$_i$) fits the smaller data array best.
Table I. Phosphorylation Mechanisms Tested

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Mechanism</th>
<th>( v_{ex} = \frac{v_{\max}}{v_{\max} + K_{X_{0.5}}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>( V_{\max} )</td>
<td>( K_{X_{0.5}} )</td>
</tr>
<tr>
<td>I. Substrate Mg·Pi</td>
<td>Mg·Pi</td>
<td>( \frac{V_{\max}K_h}{1 + K_h} )</td>
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<tr>
<td></td>
<td>P_i</td>
<td>( \frac{V_{\max}K_h}{1 + K_h} )</td>
</tr>
<tr>
<td>II. Ordered Binding of P_i before Mg^{2+}</td>
<td>Mg^{2+}</td>
<td>( \frac{V_{\max}[Mg^{2+}]}{[Mg^{2+}] + \frac{K_M'\hat{P}_p}{1 + K_h}} )</td>
</tr>
<tr>
<td></td>
<td>P_i</td>
<td>( \frac{V_{\max}[P_i]}{[P_i] + \frac{K_M'\hat{P}_p}{1 + K_h}} )</td>
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<tr>
<td>III. Ordered Binding of Mg^{2+} before P_i</td>
<td>Mg^{2+}</td>
<td>( \frac{V_{\max}K_h}{1 + K_h + \frac{\alpha K_M}{[Mg^{2+}]} \hat{P}_p} )</td>
</tr>
<tr>
<td>IV. Random binding</td>
<td>P_i</td>
<td>( \frac{V_{\max}K_h}{1 + K_h + \frac{\alpha K_M}{[Mg^{2+}]} \hat{P}_p} )</td>
</tr>
<tr>
<td></td>
<td>Mg^{2+}</td>
<td>( \frac{V_{\max}K_h}{1 + K_h + \frac{\alpha K_P}{[P_i]} \hat{P}_p} )</td>
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<tr>
<td>V. Mg^{2+} before P_i with Mg·Pi inhibition</td>
<td>P_i</td>
<td>( \frac{V_{\max}K_h}{1 + K_h \left( \frac{K_{MP}}{[Mg·P_i] + K_{MP}} \right)} )</td>
</tr>
</tbody>
</table>

\( ^*X \) is the independent variable.  
\( K_{MP} = [E_2][Mg·Pi]/[E_2·MgPi], K_M = [Mg^{2+}][E_2]/[Mg·E_2], K_M' = [Mg^{2+}][E_2·Pi]/[Mg·E_2·Pi], K_P = [E_2][P_i]/[E_2·P_i], K_P' = [Mg·E_2][P_i]/[Mg·E_2·P_i], \alpha = K_M'/K_M = K_P'/K_P, \) and \( K_h = E_2-P/E_2·P_i. \)
Table II. Estimated Oxygen Exchange Parameters

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<th>[F]₀</th>
<th>[Mg²⁺]₀</th>
<th>vₑ ± sd</th>
<th>Pₑ ± sd</th>
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<td>(µatom min⁻¹ mg⁻¹)</td>
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<td>0.019 ± 0.004</td>
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<td>17</td>
<td>2.20 ± 0.05</td>
<td>0.032 ± 0.001</td>
<td>2</td>
</tr>
<tr>
<td>3.8</td>
<td>9.3</td>
<td>0.624 ± 0.009</td>
<td>0.030 ± 0.002</td>
<td>2</td>
<td>14</td>
<td>6.5</td>
<td>1.9</td>
<td>0.038</td>
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<tr>
<td>3.9</td>
<td>1.4</td>
<td>0.42 ± 0.02</td>
<td>0.006 ± 0.03</td>
<td>1</td>
<td>15</td>
<td>0.55</td>
<td>0.47 ± 0.03</td>
<td>0.060 ± 0.001</td>
<td>2</td>
</tr>
<tr>
<td>4.1</td>
<td>0.29</td>
<td>0.099 ± 0.004</td>
<td>0.008 ± 0.002</td>
<td>2</td>
<td>16</td>
<td>1.4</td>
<td>0.97</td>
<td>0.024</td>
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<tr>
<td>4.2</td>
<td>0.74</td>
<td>0.20</td>
<td>0.0371 ± 0.0008</td>
<td>2</td>
<td>17</td>
<td>2.8</td>
<td>1.70 ± 0.04</td>
<td>0.030 ± 0.003</td>
<td>2</td>
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<tr>
<td>4.3</td>
<td>2.8</td>
<td>0.72 ± 0.01</td>
<td>0.032 ± 0.003</td>
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<td>3.37 ± 0.07</td>
<td>0.037 ± 0.003</td>
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<td>4.4</td>
<td>5.4</td>
<td>0.986 ± 0.006</td>
<td>0.030 ± 0.002</td>
<td>2</td>
<td>19</td>
<td>5.5</td>
<td>2.2</td>
<td>0.004</td>
<td>1</td>
</tr>
<tr>
<td>4.5</td>
<td>1.5</td>
<td>0.42 ± 0.01</td>
<td>0.0339 ± 0.0005</td>
<td>2</td>
<td>20</td>
<td>6.7</td>
<td>0.62 ± 0.03</td>
<td>0.058 ± 0.002</td>
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<tr>
<td>4.7</td>
<td>4.2</td>
<td>0.89 ± 0.02</td>
<td>0.028 ± 0.002</td>
<td>2</td>
<td>21</td>
<td>1.7</td>
<td>1.30 ± 0.01</td>
<td>0.027 ± 0.003</td>
<td>2</td>
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<tr>
<td>4.8</td>
<td>6.8</td>
<td>1.0 ± 0.1</td>
<td>0.05 ± 0.03</td>
<td>3</td>
<td>22</td>
<td>2.6 ± 0.03</td>
<td>1</td>
<td>2.0 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>4.9</td>
<td>2.9</td>
<td>0.74 ± 0.02</td>
<td>0.017 ± 0.004</td>
<td>2</td>
<td>23</td>
<td>6.7</td>
<td>2.7</td>
<td>0.046</td>
<td>1</td>
</tr>
<tr>
<td>5.1</td>
<td>0.32</td>
<td>0.116 ± 0.005</td>
<td>0.073 ± 0.002</td>
<td>2</td>
<td>24</td>
<td>8.5</td>
<td>2.8</td>
<td>0.068</td>
<td>1</td>
</tr>
<tr>
<td>5.2</td>
<td>5.6</td>
<td>1.2 ± 0.1</td>
<td>0.04 ± 0.03</td>
<td>3</td>
<td>25</td>
<td>17</td>
<td>2.8</td>
<td>0.068</td>
<td>1</td>
</tr>
<tr>
<td>5.3</td>
<td>0.79</td>
<td>0.27</td>
<td>0.033</td>
<td>1</td>
<td>26</td>
<td>25</td>
<td>2.44 ± 0.08</td>
<td>0.0349 ± 0.0002</td>
<td>2</td>
</tr>
<tr>
<td>5.5</td>
<td>19</td>
<td>1.04 ± 0.03</td>
<td>0.029 ± 0.007</td>
<td>4</td>
<td>27</td>
<td>14</td>
<td>2.44 ± 0.08</td>
<td>0.0349 ± 0.0002</td>
<td>2</td>
</tr>
<tr>
<td>5.6</td>
<td>3.6</td>
<td>0.55 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>4</td>
<td>28</td>
<td>21</td>
<td>2.28 ± 0.04</td>
<td>0.032 ± 0.001</td>
<td>2</td>
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<tr>
<td>5.7</td>
<td>10</td>
<td>0.87</td>
<td>0.034</td>
<td>1</td>
<td>29</td>
<td>14</td>
<td>2.6 ± 0.03</td>
<td>0.071 ± 0.001</td>
<td>2</td>
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<tr>
<td>6.2</td>
<td>3.2</td>
<td>0.91 ± 0.03</td>
<td>0.0754 ± 0.0001</td>
<td>2</td>
<td>30</td>
<td>6.7</td>
<td>2.7</td>
<td>0.046</td>
<td>1</td>
</tr>
<tr>
<td>6.4</td>
<td>7.4</td>
<td>1.1 ± 0.2</td>
<td>0.078 ± 0.005</td>
<td>2</td>
<td>31</td>
<td>17</td>
<td>2.8</td>
<td>0.068</td>
<td>1</td>
</tr>
<tr>
<td>6.5</td>
<td>14</td>
<td>1.6</td>
<td>0.034</td>
<td>1</td>
<td>32</td>
<td>17</td>
<td>2.8</td>
<td>0.068</td>
<td>1</td>
</tr>
<tr>
<td>6.7</td>
<td>1.7</td>
<td>0.666 ± 0.008</td>
<td>0.033 ± 0.001</td>
<td>2</td>
<td>33</td>
<td>17</td>
<td>2.8</td>
<td>0.068</td>
<td>1</td>
</tr>
<tr>
<td>6.8</td>
<td>4.8</td>
<td>1.0 ± 0.1</td>
<td>0.078 ± 0.002</td>
<td>2</td>
<td>34</td>
<td>25</td>
<td>2.44 ± 0.08</td>
<td>0.0349 ± 0.0002</td>
<td>2</td>
</tr>
</tbody>
</table>

aData in bold type are plotted in Figure 3 (n = 107).

bAll data, both normal and bold type, are plotted in Figure 2 (n = 184).
### Table III. Summary of Parameter Estimates for Phosphorylation Mechanisms Tested

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>(K_M) (mM)</th>
<th>(K'_M) (mM)</th>
<th>(K_S(1 + K_h))</th>
<th>(K_P) (mM)</th>
<th>(K'_P) (mM)</th>
<th>(\alpha)</th>
<th>(K_h)</th>
<th>(K_{MP}) (mM)</th>
<th>(V_{X_{max}}) (µatom min(^{-1}) mg(^{-1}))</th>
<th>(R_{adj})</th>
<th>(n)</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Substrate Mg·P(_i)</td>
<td></td>
<td>2.7 ± 0.2(^c)</td>
<td></td>
<td></td>
<td></td>
<td>&gt;11(^d)</td>
<td></td>
<td>3.2 ± 0.1</td>
<td></td>
<td>0.9649</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>II. P(_i) before Mg(^{2+})</td>
<td>&gt;0.04(^d)</td>
<td>0.01(^e)</td>
<td>2 \times 10(^3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.2 ± 0.2</td>
<td></td>
<td>0.9642</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>III. Mg(^{2+}) before P(_i)</td>
<td>3.5 ± 0.3</td>
<td>6.3 ± 0.6</td>
<td></td>
<td></td>
<td></td>
<td>&gt;25(^d)</td>
<td></td>
<td>4.4 ± 0.2</td>
<td></td>
<td>0.9883</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>IV. Random binding</td>
<td>3.3 ± 0.7</td>
<td>0.12(^e)</td>
<td>4 \times 10(^2)</td>
<td>15(^f)</td>
<td>0.04</td>
<td>1.3</td>
<td></td>
<td>10(^g)</td>
<td></td>
<td>0.9879</td>
<td>107</td>
<td>3</td>
</tr>
<tr>
<td>V. Mg(^{2+}) before P(_i)</td>
<td>5.2 ± 0.2</td>
<td>7.4 ± 0.9</td>
<td></td>
<td></td>
<td></td>
<td>&gt;30(^d)</td>
<td></td>
<td>17 ± 3</td>
<td>6.6 ± 0.8</td>
<td>0.9836</td>
<td>184</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\)Regression coefficients shown in bold type and derived numbers in normal type.

\(^b\)S denotes substrate (Mg\(^{2+}\) in II or P\(_i\) in III and V).

\(^c\)Standard error of parameter estimates tabulated when \(P < 0.0001\).

\(^d\)Calculated from \(K_S/(1 + K_h)\) with \(K_h > 3\) (see Discussion).

\(^e\)Standard error of parameter estimates omitted if \(P > 0.83\).

\(^f\)Calculated with \(\alpha = K'_M/K_M = K'_P/K_P\).

\(^g\)\(V_{X_{max}}K_h\), therefore at high [Mg\(^{2+}\)] \(V_{X_{max}}\) approaches \(V_{X_{max}}K_h/(1 + K_h) = 4.4\) (Table I).
Table IV. Comparison of Parameter Estimates for Ordered Binding of Mg\(^{2+}\) before Pi to Calcium and Sodium Pumps\(^a\)

<table>
<thead>
<tr>
<th>Pump</th>
<th>Preparation</th>
<th>(K_{0.5}(\text{Mg}^{2+}))(^b) (mM)</th>
<th>(K_M) (mM)</th>
<th>(K_P/(1 + K_h)) (mM)</th>
<th>(K_P') (mM)</th>
<th>(K_{0.5}(\text{Pi}))(^c) (mM)</th>
<th>(P_c)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified</td>
<td>2.8 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td>1.5 ± 0.2</td>
<td>0.20 ± 0.02</td>
<td>Stopped flow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.8 ± 0.1</td>
<td>3.8 ± 0.3</td>
<td>0.57 ± 0.03</td>
<td>1.2 ± 0.5(^d)</td>
<td>1.6 ± 0.2</td>
<td>0.26 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>2 ± 1</td>
<td>4.3 ± 3.7(^d)</td>
<td>3 ± 2</td>
<td>0.22 ± 0.04</td>
<td>18O exchange</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.26 ± 0.05</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>2.6 ± 1.2(^d)</td>
<td>1.6 ± 0.2</td>
<td>0.26 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 ± 0.5</td>
<td>4 ± 1</td>
<td>0.7 ± 0.2</td>
<td>1.5 ± 0.8(^d)</td>
<td>2 ± 1</td>
<td>0.18 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Averaged(^e)</td>
<td>0.6 ± 0.3</td>
<td>2.4 ± 1.6</td>
<td>1.1 ± 0.6</td>
<td>2.4 ± 1.4(^d)</td>
<td>1.9 ± 0.6</td>
<td>0.22 ± 0.04</td>
<td>SF or 18O</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>2.6 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>6.3 ± 0.6</td>
<td>&gt; 25(^f)</td>
<td>17 ± 3</td>
<td>0.04 ± 0.03</td>
<td>18O exchange</td>
</tr>
<tr>
<td></td>
<td>Purified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Regression coefficients shown in bold type and derived numbers in normal type.

\(^b\)[Pi]\(_{\text{free}}\) = 2 mM.

\(^c\)[Mg\(^{2+}\)]\(_{\text{free}}\) = 2 mM.

\(^d\)Assuming \(K_h = 1.2 ± 0.4\).

\(^e\)\(n = 4\) or \(5\).

\(^f\)Assuming \(K_h > 3\).
Table V. Comparison of Rate Constants and Exchange Parameters for $^{18}$O Exchange Catalyzed by P-type Pumps

<table>
<thead>
<tr>
<th>Pump</th>
<th>$K'_P$ (mM)</th>
<th>$k_{+P_i}$ (M⁻¹ sec⁻¹)</th>
<th>$k_{-P_i}$ (sec⁻¹)</th>
<th>$k_{H_2O}$ (sec⁻¹)</th>
<th>$k'_{+H_2O}$ (sec⁻¹)</th>
<th>$K_h$</th>
<th>$P_c$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>&gt;25</td>
<td>&gt;3.5 x 10⁴</td>
<td>&gt;8.6 x 10²</td>
<td>&gt;36</td>
<td>12 ± 2</td>
<td>&gt;3</td>
<td>0.04 ± 0.03</td>
<td>This study ²</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>2.8 x 10⁵</td>
<td>3.8 x 10²</td>
<td>32</td>
<td>51 ± &gt;9</td>
<td>0.63</td>
<td>0.08 ± 0.02</td>
<td>39 ³</td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>1.7 x 10⁵</td>
<td>1.1 x 10⁴</td>
<td>120</td>
<td>12</td>
<td>10</td>
<td>0.01</td>
<td>40 ⁴</td>
</tr>
<tr>
<td>Proton</td>
<td>1.7 ± 0.4</td>
<td>5 x 10⁵</td>
<td>1 x 10³</td>
<td>(3 ± 2) x 10²</td>
<td>(5 ± 2) x 10²</td>
<td>0.56 ± 0.12</td>
<td>0.21 ± 0.03</td>
<td>27 ⁵</td>
</tr>
<tr>
<td>Sodium</td>
<td>2.4 ± 1.8</td>
<td>1 x 10⁶</td>
<td>2.4 x 10³</td>
<td>(7 ± 6) x 10²</td>
<td>(6 ± 3) x 10²</td>
<td>1.2 ± 0.4</td>
<td>0.22 ± 0.04</td>
<td>13 ⁶</td>
</tr>
</tbody>
</table>

²At 24 °C and pH 7.0 in 50 mM HEPES containing 0.5 mM EGTA, 50 mM KCl, 0-44 mM MgCl₂, and 0-38 mM $[^{18}$O]Pₐ.
³At 25 °C and pH 6.5 in 20 mM MES containing 0.5 mM EGTA, 50 mM KCl, 20 mM MgCl₂, and 0-50 mM $[^{18}$O]Pₐ.
⁴At 25 °C and pH 7.0 in 50 mM MOPS/TMAH containing 1 mM EGTA, 10 mM MgCl₂, and 2 mM $[^{18}$O]Pₐ.
⁵At 37 °C and pH 7.4 in 40 mM imidazole containing 7 mM KCl, 2 mM MgCl₂, and 0-8 mM $[^{18}$O]Pₐ.
⁶At 25 °C and pH 7.4 in 50 mM Tris containing 10 mM KCl, 0-230 mM ChoCl, 0-46 mM MgCl₂, and 0-22 mM $[^{18}$O]Pₐ.
Figure 1

Isotopomer (%)

Number of $^{18}$O Atoms in P$_i$
Figure 2

\[ v_{ex} (\mu \text{atom min}^{-1} \text{mg}^{-1}) \]

\[ \text{[Mg}^{2+} \text{]}_{\text{free}} \text{ (mM)} \]

\[ \text{[P]}_{\text{free}} \text{ (mM)} \]
Figure 3

\[ \nu_{ex} \text{ (µmol min}^{-1} \text{ mg}^{-1}) \]

\[ [P_i]_{free} \text{ (mM)} \]
Figure 4

Residual (µatom mg⁻¹ min⁻¹) vs [Pᵢ]_free (mM)
Evidence calcium pump binds magnesium before inorganic phosphate
Agnes K. Nagy, David J. Kane, Chinh M. Tran, Robert A. Farley and Larry D. Faller

J. Biol. Chem. published online December 10, 2004

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