Catalysis of Oxygen-18 Exchange between Inorganic Phosphate and Water by the Gastric H,K-ATPase†

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ABSTRACT: The gastric H,K-ATPase is shown to catalyze ^{18}O exchange between P_i and HOH. Mg^{2+} is the only ion required for the reaction. K^+ increases the rate of isotope exchange, which is directly proportional to specific ATPase activity. Ouabain, which potently inhibits the Na,K-ATPase, has no effect on the exchange reaction. Conversely, omeprazole, which is specific for the H,K-ATPase, completely inhibits ^{18}O exchange. Vanadate inhibition of exchange can be explained by competitive binding with P_i. The rate of ^{18}O exchange is faster than the hydrolytic rate and about equal to the dephosphorylation rate. Thus, the ionic requirements for exchange, inhibition of exchange, and the rate of exchange are all compatible with catalysis occurring via the same phosphoenzyme intermediate formed during hydrolysis of ATP. The distribution of ^{18}O-labeled P_i species formed with time indicates that P_i loss is only about twice as fast as covalent bond formation. This kinetic pattern is unaffected by K^+, temperature, or the specific activity of the enzyme preparation. Invariance of the kinetic pattern could mean isotope exchange is always catalyzed by the same form of the enzyme, and K^+ and higher temperature accelerate the reaction by increasing the relative amount of the active conformer. Independence of the kinetic pattern from specific activity implies that the catalytic mechanism of active enzyme molecules is unaffected by inactive proteins in gastric microsomal membranes.

The potassium-stimulated, proton-translocating adenosinetriphosphatase (EC 3.6.1.3) (H,K-ATPase)[1] isolated from gastric mucosae belongs to the class of active transport enzymes that effects catalysis by forming a covalent phosphoenzyme intermediate. The two more extensively studied enzymes in this class, the Na,K-ATPase and the Ca-ATPase, can be phosphorylated by P_i as well as by ATP (Post et al., 1975; Kanazawa, 1975). Both of these enzymes also catalyze ^{18}O exchange between P_i and water (Shaffer et al., 1978; Kanazawa & Boyer, 1973), presumably by reversal of the final steps in the overall hydrolysis reaction

$$E + H\bullet OP\bullet O_3^{2-} \rightleftharpoons E{\cdot}H\bullet OP\bullet O_3^{2-} \xrightarrow{H\bullet OH} E{-}P\bullet O_3^{2-} \xrightarrow{HOH} E{\cdot}HOP\bullet O_3^{2-} \longrightarrow E + HOP\bullet O_3^{2-} \quad (1)$$

where ● represents ^{18}O and the reaction is written as irreversible to the right, because the natural abundance of ^{18}O in the solvent is negligible.

Evidence on whether the gastric H,K-ATPase can be phosphorylated by P_i is inconclusive. Two reports of ^{32}P incorporation from [^{32}P]P_i have appeared (Sachs et al., 1980; Schrijen, 1981), but the phosphorylation levels were less than half those reached with ATP and the fractions chased by K^+, which accelerates dephosphorylation (Wallmark & Mardh, 1979), were not given. ^{31}P NMR measurements are reported demonstrating that the gastric H,K-ATPase catalyzes ^{18}O exchange between P_i and water. The rate of isotope exchange, the ionic requirements for exchange, and inhibition of exchange are all compatible with catalysis occurring by reversal of the terminal steps in the hydrolytic reaction. Some of these results have been communicated in preliminary form (Faller et al., 1983b).

Experimental Procedures

Materials

Gastric Vesicles. The H,K-ATPase was isolated from hog stomachs by zonal centrifugation (Chang et al., 1979). The enzyme is found in vesicles of the microsomal fraction recovered from the 0.25 M sucrose–7% (w/w) Ficoll interface of a step gradient. The vesicles were made freely permeable to alkali metal ions by suspension in deionized water, lyophilization, and storage at −80 °C. Sample protein was determined by the Lowry method using bovine serum albumin standards (Lowry et al., 1951). ATPase activity was assayed colorimetrically by stopping the reaction with perchloric acid, complexing the released P_i with molybdenum, and extracting the complex with butyl acetate (Yoda & Hokin, 1970).

Labeled P_i. The 93.3–99 atom % ^{18}O-enriched P_i was obtained from Miles Laboratories. Contaminating paramagnetic ions were removed by adjusting the pH to 7.4 with concentrated NaOH and passing the labeled P_i through a Chelex 100 column equilibrated with 40 mM Tris-HCl buffer at pH 7.4. The concentration of P_i in an aliquot of the measurement sample was determined colorimetrically as the phosphomolybdenum complex.

Reagents. Omeprazole was a gift from Bjorn Wallmark, AB Hassle Research Laboratories, Molndal, Sweden. [^{48}-

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[1] Abbreviations: H,K-ATPase, magnesium-dependent, hydrogen ion transporting, and potassium-stimulated adenosinetriphosphatase (EC 3.6.1.3); Na,K-ATPase, magnesium-dependent and sodium- and potassium-stimulated adenosinetriphosphatase; Ca-ATPase, calcium- and magnesium-dependent adenosinetriphosphatase; ATP, adenosine 5′-triphosphate; P_i, inorganic phosphate; MES, 4-morpholineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane, pNPPase, *p*-nitrophenylphosphatase; FITC, fluorescein isothiocyanate; NMR, nuclear magnetic resonance; IEF, isoelectric focusing; SA, specific activity; AE, average isotopic enrichment.

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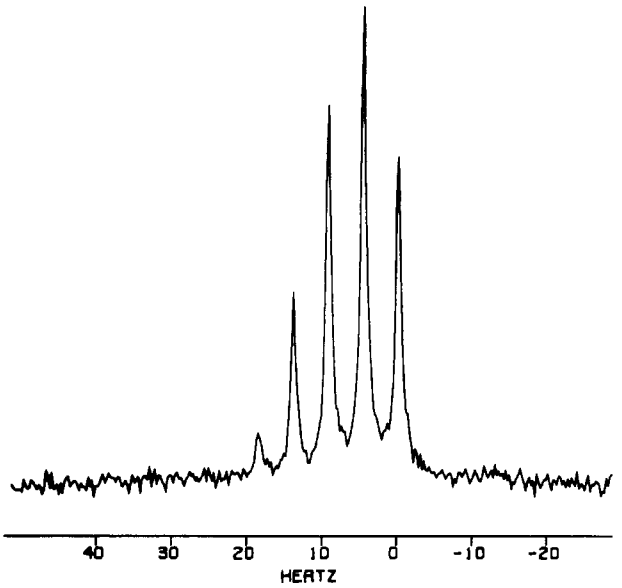

FIGURE 1: ^{31}P NMR spectrum of ^{18}O-enriched P_i. 48 scans in a 500-MHz (protons) spectrometer were averaged, beginning 70 min after the addition of gastric microsomal vesicles containing H,K-ATPase with SA = 120 μmol mg^{-1} h^{-1}. The protein concentration was 50 μg mL^{-1}, and the P_i concentration was 20 mM. 85% of the P_i was initally present as the $P^{18}O_4$ species (peak centered at 0 Hz). Other conditions: 1 mM EDTA, 3 mM $MgCl_2$, 7 mM KCl, and 50% D_2O in 40 mM Tris-HCl buffer at pH 7.4 and 37 °C. The volume was 2 mL, and the sample tube diameter was 10 mm.

V]$VOCl_3$ was purchased from Amersham. Vanadium-free ATP from Sigma and sodium orthovanadate from Fisher Scientific Company were used. All other reagents were the highest grade available.

Methods

NMR. The distribution of $P^{18}O_j{}^{16}O_{4-j}$ species, where $O \leq j \leq 4$, was determined by ^{31}P NMR (Cohn & Hu, 1978). The spectra were obtained with 9.4 or 11.75 T Bruker instruments at a frequency of 162 or 202.5 MHz for phosphorus. Forty-eight scans were averaged and then Fourier transformed. At the higher frequency the peaks were completely resolved, and the fractional contribution of each $[^{18}O]P_i$ species to the spectrum could be evaluated directly from the peak heights. Figure 1 is a typical spectrum obtained at the National Science Foundation's Southern California Regional NMR Facility at Caltech. Details of the reaction mixture are given in the figure legend. The mean deviation in the contribution of the jth species to this spectrum calculated from the peak heights, compared with integrating the area under the curve, was less than 3%. Partially resolved spectra were simulated by a linear combination of individual Lorentzian peaks with the appropriate half-width and separation.

Omeprazole Inhibition. Omeprazole (74 μM) was reacted with gastric microsomes at pH 5.5 in 100 mM MES buffer for 30 min. Measurements of the isotope exchange rate were made immediately after diluting to a final omeprazole concentration of 10 μM in pH 7.4, Tris-HCl buffer.

Vanadate Binding. Vanadate binding was measured as previously described (Faller et al., 1983a). [^{48}V]Vanadate was incubated with gastric microsomal vesicles for 5 min and then the vesicles were rapidly sedimented by centrifuging at 95 000 rpm in a Beckman airfuge (Howlett et al., 1978). The concentration of free vanadate remaining in the supernatant and the amount of bound vanadate in the pellet were determined by scintillation counting. Control experiments in which EDTA was added were used to correct for trapped counts and the small amount of nonspecific binding below 10 μM vanadate.

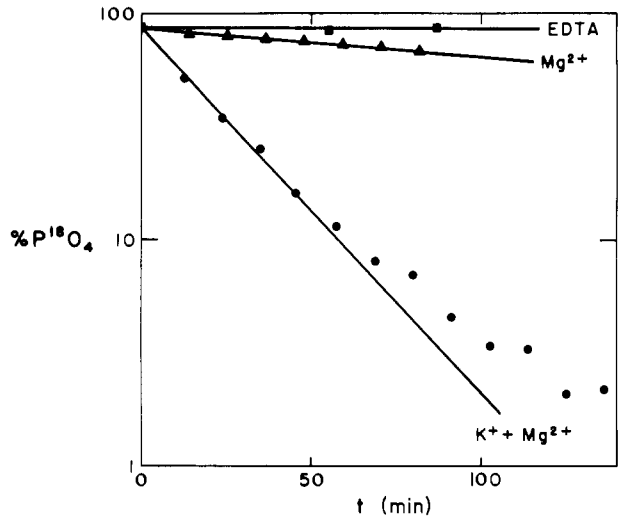

FIGURE 2: Ion requirements for catalysis of ^{18}O exchange by the gastric H,K-ATPase. Semilogarithmic plots of the percentage of P_i remaining in the $P^{18}O_4$ form as a function of time: (■) 1 mM EDTA, (▲) 1 mM EDTA plus 3 mM $MgCl_2$, (●) 1 mM EDTA plus 3 mM $MgCl_2$ plus 7 mM KCl. Other experimental conditions are given in the legend to Figure 1. The pseudo-first-order rate constants calculated from the slopes of the asymptotes shown drawn to the curves and the specific activity of the preparation are given in Table I.

Table I: Ion Requirements for ^{18}O Exchange

additions	k' (h^{-1})[a]	k'_{KM}/k'_M	SA[b]	SA_{KM}/SA_M
1 mM EDTA	<4E − 4			
3 mM Mg^{2+}[c]	0.058		15	
		10		9
3 mM Mg^{2+} + 7 mM K^+	0.6		138	
3 mM Mg^{2+}	0.16		17	
		13		14
3 mM Mg^{2+} + 7 mM K^+	2.0		235	
2 mM Ca^{2+}[d]	0.0078			
		3		
2 mM Ca^{2+} + 7 mM K^+	0.025			

[a] Pseudo-first-order constant. The subscripts K and M indicate the presence of 7 mM K^+ and 2 mM free Mg^{2+}, respectively. Values quoted for 50 μg mL^{-1} Lowry protein. [b] Specific activity in μmol mg^{-1} h^{-1}. [c] 20 ± 1 mM P_i and 50% (v/v) D_2O in the NMR measurements, 40 mM Tris-HCl, pH 7.4, 37 °C. [d] 10 mM P_i, pH 7.2.

Results

Ionic Requirements for Exchange. Semilogarithmic plots of the percentage of P_i completely labeled with ^{18}O (j = 4) against time are linear for at least the first 80% of the reaction (Figure 2). The pseudo-first-order rate constant for decay of this peak can therefore be used to compare the effectiveness of different cofactors in the exchange reaction. No measurable isotope exchange occurred in the absence of divalent cations, that is, in the presence of 1 mM EDTA. K^+ accelerated the exchange rate observed with Mg^{2+} 10–13-fold, which is in satisfactory quantitative agreement with the effect of K^+ on the overall hydrolysis rate (Table I). Thus, the ionic requirements for ^{18}O exchange parallel those for catalysis of ATP hydrolysis by the gastric enzyme, suggesting a common mechanism.

Ca^{2+} Can Substitute for Mg^{2+}. Substitution of Ca^{2+} for Mg^{2+} has been reported to result in a different conformation of the H,K-ATPase, because neither dephosphorylation nor hydrolysis was accelerated by K^+ when Ca^{2+} was the activating divalent cation, even though phosphorylation and hydrolysis were both catalyzed (Mendlein & Sachs, 1982). It was therefore of interest to investigate the effect of substituting Ca^{2+} for Mg^{2+} on the ^{18}O exchange reaction. The results are summarized in Table I. Ca^{2+} can substitute for Mg^{2+}, but

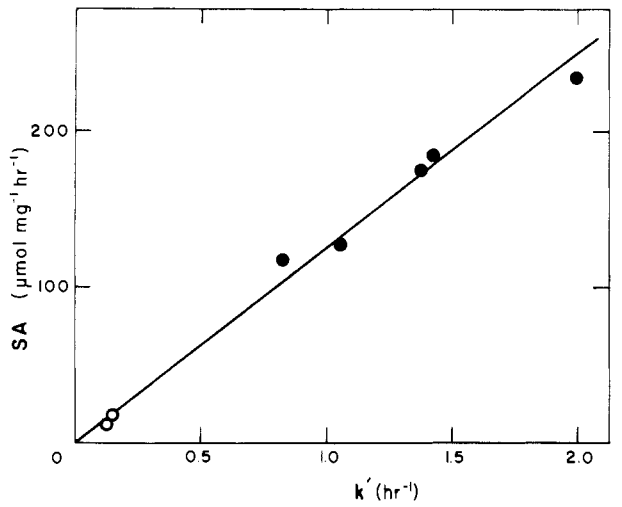

FIGURE 3: Dependence of ^{18}O exchange rate on active enzyme concentration. Specific activity is plotted against the pseudo-first-order rate constant ($k' = k[E]_0$) for decay of the $P^{18}O_4$ species: (●) different vesicle preparations; (○) heat-inactivated enzyme. Other conditions were the same as in the legend to Figure 1.

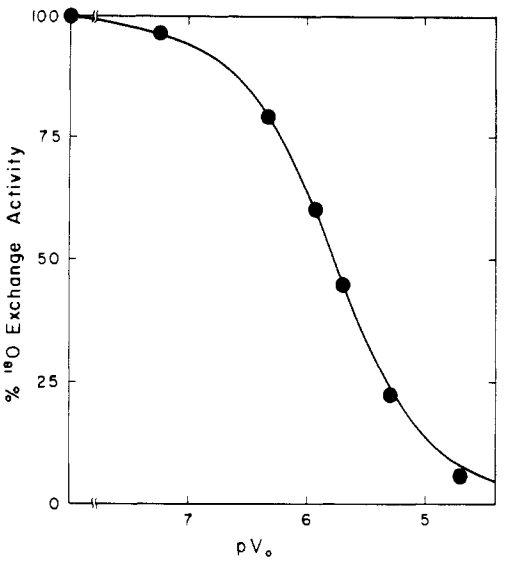

FIGURE 4: Vanadate inhibition of ^{18}O exchange activity. The percentage of ^{18}O exchange activity remaining is plotted against minus the logarithm of the total vanadate concentration ($[V]_0$). The solid line was calculated for an apparent vanadate inhibition constant of 1.6 μM. The experimental conditions were those given in the legend to Figure 1.

exchange is almost an order of magnitude slower when Ca^{2+} is the activating divalent cation. The Ca^{2+} and Mg^{2+} data are not exactly comparable, because the limited solubility of calcium phosphate necessitated a slightly lower pH and lower divalent cation and phosphate concentrations in the measurements of Ca^{2+}-activated exchange. K^+ accelerated the exchange rate, which appears to contradict either the cited report that K^+ does not accelerate dephosphorylation or hydrolysis or the tentative conclusion that isotope exchange is catalyzed by a partial reaction in the hydrolytic pathway. It is possible that the observed exchange resulted from endogenous Mg^{2+}, since EDTA binds Ca^{2+} approximately 100 times tighter than Mg^{2+} (Dawson et al., 1969) and the rate in the presence of both Ca^{2+} and K^+ was still slower than the exchange rate in the presence of Mg^{2+} alone. However, then an order of magnitude or greater acceleration of exchange by K^+ should have been observed, instead of the 3-fold increase in the exchange rate that was measured when K^+ was added with Ca^{2+}. The possibility that K^+ stimulated only a small fraction (3–4%) of the enzyme complexed with endogenous Mg^{2+} cannot be excluded.

Dependence on Enzyme. Additional evidence that medium[2] ^{18}O exchange occurs by a partial reaction in the hydrolytic pathway is presented in Figure 3. The rate of isotope exchange was found to be directly proportional to specific ATPase activity (micromoles of P_i formed from ATP per hour per milligram of Lowry protein), even though the assayed activity of the microsomal enzyme preparations used in this study varied considerably (closed circles). Deliberate inactivation by heating for 30 min at 50 °C also resulted in parallel losses of ^{18}O exchange and ATPase activity (open circles). The direct proportionality between the exchange rate and ATPase activity facilitates comparison of the isotope exchange reaction with other partial reactions of the gastric enzyme, because it means that data obtained with different vesicle preparations can be normalized to the same specific activity.

Inhibition of ^{18}O Exchange. Since the exchange observed could be catalyzed by an enzyme with similar ion requirements that copurifies with the H,K-ATPase, inhibition of ^{18}O exchange by specific inhibitors of the H,K-ATPase and Na,K-ATPase was studied.

(*1*) *Ouabain.* The Na,K-ATPase catalyzes rapid ^{18}O exchange between medium P_i and water (Dahms & Miara, 1983). Therefore, the exchange observed in the present investigation could be an artifact, because some preparations of the H,K-ATPase from hog gastric mucosae have been reported to contain Na,K-ATPase activity (Schrijen, 1981). To exclude this possibility, the effect of the cardiac glycoside ouabain on the exchange reaction has been studied. Ouabain potently inhibits the Na,K-ATPase but does not affect the H,K-enzyme (Schuurmans Stekhoven & Bonting, 1981). Under otherwise identical conditions, the measured pseudo-first-order rate constant for ^{18}O exchange (k') was 0.51 h^{-1} both in the absence and in the presence of 0.1 mM ouabain. Millimolar ouabain was also without effect. Therefore, the exchange measured in this study is not due to contamination with the Na,K-enzyme.

(*2*) *Omeprazole.* The substituted benzimidazoles picoprazole and omeprazole have the opposite specificity of ouabain. H,K-ATPase activity is inhibited by these compounds with apparent constants in the micromolar range, while Na,K-ATPase activity is unaffected (Wallmark et al., 1983). ^{18}O exchange was completely inhibited by 10 μM omeprazole, providing direct evidence that exchange is catalyzed by the H,K-ATPase.

(*3*) *Vanadate.* Vanadium, in the form of the vanadate ion $H_2VO_4^-$, is a potent inhibitor of the H,K-ATPase (Faller et al., 1983a). Figure 4 is a plot of percent ^{18}O exchange activity, calculated from the pseudo-first-order constants for disappearance of the $P^{18}O_4$ peak, against the negative logarithm of the total vanadate concentration. The solid line was calculated for an apparent inhibition constant of 1.6 μM.

Inhibition of the ATPase and pNPPase activities of the gastric enzyme could be explained by competitive binding of the substrates with vanadate ions (Faller et al., 1983a). Figure 5 is a plot of the average number of vanadate ions bound in the presence of 20 mM P_i as a function of free vanadate concentration. The solid line was calculated by assuming 3 nmol of vanadate bind per mg of Lowry protein with an ap-

[2] "Medium" exchange is used to contrast isotope exchange between medium P_i and HOH with "intermediate" exchange, in which the P_i is an intermediate in the hydrolysis of ATP.

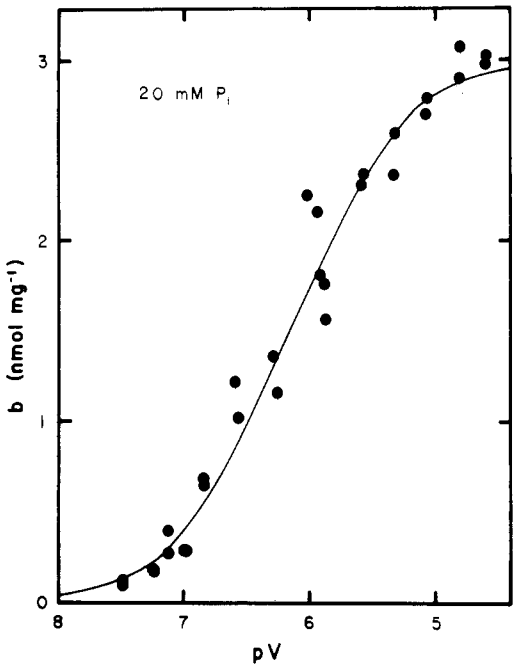

FIGURE 5: Vanadate binding to gastric microsomal vesicles in the presence of 20 mM P_i. Bound vanadate (b) is plotted against minus the logarithm of the free vanadate concentration ([V]). The solid line was calculated for specific binding of 3 nmol mg^{-1} vanadate with an apparent dissociation constant of 0.69 μM. Experimental conditions: 20 mM P_i, 2 mM $MgCl_2$, 7 mM KCl, 250 μg mL^{-1} protein, and 40 mM Tris-HCl buffer at pH 7.4 and room temperature.

parent dissociation constant of 0.69 μM. When Figures 4 and 5 are compared, inhibition of ^{18}O exchange by vanadate is qualitatively compatible with displacement of P_i from the enzyme. The data are considered quantitatively under Discussion.

Time-Dependent Distribution of [^{18}O]P_i Species. The power of ^{18}O exchange as a mechanistic probe of enzymes that catalyze hydrolysis of oxygen–phosphorus bonds has been greatly increased by technical innovations (Eargle et al., 1977; Cohn & Hu, 1978) that make it possible to measure the distribution of [^{18}O]P_i species. In terms of the minimal mechanism that can account for medium exchange

$$E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{-}P + HOH \qquad (2)$$

the average number of oxygens exchanged per encounter ordinarily depends on the relative rates of water ($\propto k_2$) and phosphate ($\propto k_{-1}$) loss.

In the simplest limiting case ($k_{-1} \gg k_2$), there is an obligatory exchange of one oxygen atom per turnover. Several tests have been developed for assessing whether a single oxygen is exchanged per encounter. In one test, the measured distribution at several times is compared with the distribution calculated from the average isotopic enrichment

$$AE = \frac{\sum_{j=0}^{4} jP^{18}O_j{}^{16}O_{4-j}}{4\sum_{j=0}^{4} P^{18}O_j{}^{16}O_{4-j}} \qquad (3)$$

and the binomial terms

$$(1 - AE)^4,\ 4(1 - AE)^3AE,\ 6(1 - AE)^2AE^2,\ 4(1 - AE)AE^3,\ AE^4 \qquad (4)$$

since the predicted distribution remains random (Bock & Cohn, 1978). In Table II random distributions are compared with experimental distributions found for catalysis of exchange by the gastric H,K-ATPase. Only the starting distribution of [^{18}O]P_i species is random. In another test, the pseudo-first-order rate constants for disappearace of the fully enriched phosphate species and decay of the average enrichment are compared. This ratio

$$R_4 = \frac{k_{P^{18}O_4}{}'}{k_{AE}{}'} \qquad (5)$$

is 4 if only one oxygen exchanges per turnover, because there are four oxygens per P_i molecule (Hackney & Boyer, 1978). Semilogarithmic plots of the experimental points are compared with the curve expected for a single exchange in Figure 6. R_4 is significantly smaller than predicted for the simplest exchange mechanism.

If P_i does not diffuse away much faster than HOH is eliminated, multiple reversals of the second step in eq 2 may result in exchange of more than one ^{18}O atom during a single encounter between P_i and enzyme. If it is assumed that P_i can rotate freely in the E·P_i complex so that all four oxygens are equivalent, the partition coefficient

$$P_c = k_2/(k_{-1} + k_2) \qquad (6)$$

and the ratio of the rate constants for water and P_i loss

$$R = k_2/k_{-1} = P_c/(1 - P_c) \qquad (7)$$

can be calculated from the observed R_4

$$P_c = (4 - R_4)/3 \qquad (8)$$

The values of the distribution parameters for catalysis of medium ^{18}O exchange by the gastric H,K-ATPase are compiled in Table III.

Table II: Comparison of Observed with Random Distribution[a]

	initial		24 min		70 min	
%	obsd	calcd	obsd	calcd	obsd	calcd
AE	96.7		73.0		45.2	
$P●_4$	86.8	87.4	35.4	28.3	8.1	4.2
$P●_3O$	13.2	11.9	33.9	42.0	22.0	20.2
$P●_2O_2$		0.6	20.2	23.4	27.4	36.8
$P●O_3$			8.0	5.8	27.4	29.8
PO_4			2.6	0.5	15.1	9.1

[a] Calculated as described in the text.

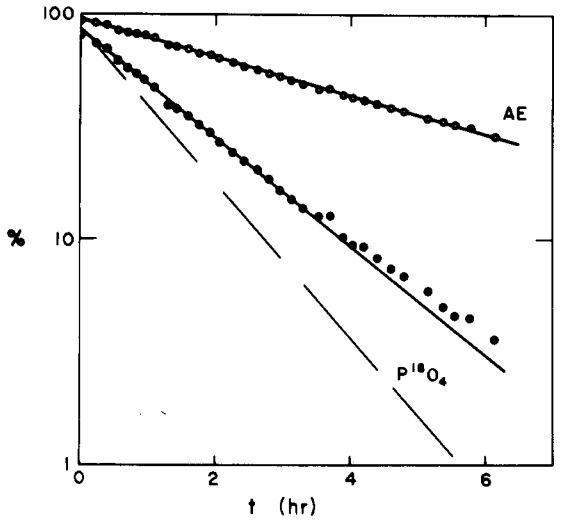

FIGURE 6: Relative rates of $P^{18}O_4$ and AE loss. Semilogarithmic plots of the percentage of P_i remaining as $P^{18}O_4$ and of the percentage of ^{18}O remaining in P_i vs. time. The data are for room temperature. Other experimental conditions are given in the legend to Figure 1. The dashed line is the expected rate of loss of $P^{18}O_4$ for the observed rate of loss of AE, when it is assumed that a single oxygen atom is exchange per turnover.

Table III: Summary of Distribution Parameters[a]

additions	T (°C)	k'_{P_i} (h^{-1})	k'_{AE} (h^{-1})	R_4	P_c	$R = k_2/k_{-1}$
3 mM Mg^{2+}	37	0.067	0.022	2.97	0.39	0.52
+7 mM K^+	37	1.99	0.65	3.06[b]	0.31[b]	0.45[b]
		0.86	0.28	3.09	0.34	0.43
	31	0.35	0.13	2.67	0.44	0.79
	25	0.28	0.098	2.83	0.39	0.64

[a] 20 mM P_i, 1 mM EDTA, 50% D_2O, 50 μg mL^{-1} protein, and 40 mM Tris-HCl at pH 7.4. [b] $R_4 = 3.0 \pm 0.2$, $P_c = 0.33 \pm 0.07$, and $R = 0.51 \pm 0.16$ ($n = 9$).

Discussion

ATP is a necessary and sufficient energy source for gastric acid secretion (Malinowska et al., 1981). The ATPase isolated from gastric epithelia exchanges protons electroneutrally for K^+ ions and is therefore thought to be the proton pump. Recently support for this idea has come from the demonstration that substituted benzimidazoles, which are effective in blocking gastric acid secretion clinically, inhibit the H,K-ATPase (Fellenius et al., 1981). Exactly how ATP hydrolysis is coupled to ion transport remains largely a matter for speculation (Tanford, 1983).

The discovery that an acid-stable phosphoenzyme is formed from ATP (Ray & Forte, 1976) has permitted some dissection of the catalytic mechanism. The phosphoenzyme was shown to be competent to function as an intermediate in the hydrolytic reaction by measuring the phosphorylation and dephosphorylation rates and demonstrating that both are greater than the turnover number (Wallmark & Mardh, 1979). Catalysis of ^{14}C isotope exchange between ADP and ATP is consistent with the existence of a high-energy phosphoenzyme form (Rabon et al., 1982). Recently phosphorylation has been shown to occur on the β-carboxyl group of an aspartic acid residue, forming a carboxylic–phosphoric anhydride intermediate (Walderhaug et al., 1983).

The present study demonstrates that the gastric ATPase can also be phosphorylated by P_i. Three arguments in support of the conclusion that ^{18}O exchange is catalyzed via the terminal step in the hydrolytic pathway were presented under Results. First, the cofactors for ^{18}O exchange and ATP hydrolysis are the same. The only requirement for catalysis of medium isotope exchange is a divalent cation (Figure 2). Ca^{2+} can substitute for the physiological metal Mg^{2+} but is much less effective (Table I). K^+ accelerates Mg^{2+}-activated ^{18}O exchange and ATP hydrolysis to the same extent (Table I). Second, the ^{18}O exchange rate correlates directly with the specific ATPase activity of different vesicle preparations (Figure 3). Third, inhibition of medium ^{18}O exchange parallels inhibition of H,K-ATPase activity. Ouabain, which is specific for the Na,K-ATPase, does not inhibit exchange. Conversely, omeprazole, which is specific for the H,K-ATPase, completely inhibits ^{18}O exchange. Vanadate ions, which inhibit hydrolysis by binding competitively with ATP, inhibit ^{18}O exchange (Figure 4) by displacing P_i (Figure 5).

It remains to be asked whether medium ^{18}O exchange is fast enough to be catalyzed by a step in the hydrolytic reaction. A sufficient criterion is that any partial reaction in the catalytic pathway must be at least as fast as the rate-determining step. In Table IV the measured specific ATPase activity of a gastric vesicle preparation is compared with the specific activity calculated from the rate of disappearance of the $P^{18}O_4$ species. Since there is a direct proportionality between the rate of $P^{18}O_4$ disappearance and ATPase activity (Figure 3), the result is independent of the vesicle preparation chosen. Medium ^{18}O exchange is catalyzed 3–4 times faster than hydrolysis of ATP.

Table IV: Comparison of ^{18}O Exchange and ATPase Activities[a]

T (°C)	ATPase	^{18}O exchange	k_{dephos} fast[b]	k_{dephos} slow[c]
37	117	346[d]		
25	26	111[e]	369	72

[a] μmol mg^{-1} h^{-1}. [b] 4100 min^{-1} (Wallmark & Mardh, 1979). [c] 800 min^{-1} (Wallmark & Mardh, 1979). [d] $k' = 0.86$ h^{-1}; 50 μg mL^{-1} protein. [e] $k' = 0.55$ h^{-1}; 100 μg mL^{-1} protein.

It has previously been shown that phosphorylation from ATP and dephosphorylation of the H,K-ATPase are faster than ATP turnover. Comparison of the ^{18}O exchange and dephosphorylation rates is complicated by the fact that biphasic dephosphorylation kinetics were reported, and the amount of phosphoenzyme decaying in the fast and slow phases depended on the K^+ concentration (Wallmark & Mardh, 1979; Wallmark et al., 1980). The specific activities calculated from both the fast and slow dephosphorylation rate constants by using the maximum phosphoenzyme level formed from ATP (1.5 nmol mg^{-1}) are recorded in Table IV. The rate of ^{18}O exchange and the slower phase of dephosphorylation are in reasonable quantitative agreement; therefore, the rate of isotope exchange is compatible with catalysis of the reaction by the same phosphoenzyme intermediate formed during ATP hydrolysis.

A second common feature of the active transport enzymes that catalyze ATP hydrolysis by forming a covalent phosphoenzyme intermediate is a complex dependence of the rate on substrate concentration. Explanations premised either on the existence of two nucleotide sites or on different conformational states of a single site have been proposed (Norby, 1983). Studies of vanadate binding to the H,K-ATPase and inhibition of ATPase activity were interpreted as evidence for two sites, because vanadate binding was heterogeneous and vanadate inhibition of ATPase activity was biphasic. The possibility of phosphoenzyme formation at only one site was raised to explain vanadate binding and inhibition with twice the stoichiometry of acid-stable phosphoenzyme formation from ATP (Faller et al., 1983a). The data shown in Figures 4 and 5 for vanadate inhibition of medium ^{18}O exchange and for vanadate binding in the presence of P_i are compatible with isotope exchange occurring via phosphorylation of either one or two sites.

Considering first phosphorylation of a single site, in the absence of P_i 3 nmol of vanadate bind per mg of protein, half tightly with dissociation constant K_{V1} = 5–20 nM and the rest weakly with K_{V2} = 0.5–0.6 μM. Catalysis of ^{18}O exchange only at the 1.5 nmol mg^{-1} of sites phosphorylatable by ATP and inhibitable by high-affinity vanadate (Faller et al., 1983a) would imply a P_i dissociation constant (K_P) in the 60–250 μM range. K_P has been estimated by substituting the apparent inhibition constant of 1.6 μM found experimentally for ^{18}O exchange in 20 mM P_i (Figure 4) into the expression for competitive binding of P_i at the high-affinity vanadate site

$$K_P = K_{V1}[P_i]_0/(K_{I(app)} - K_{V1}) \quad (9)$$

K_P has not been measured for the H,K-ATPase, but its magnitude is given approximately by the concentration of P_i required for half-maximal phosphorylation ($K_{0.5}$) in the case of related enzymes like the Ca-ATPase (Pickart & Jencks, 1984). $K_{0.5}$ in the range 100–200 μM was found in one of the previously cited studies reporting phosphorylation of the H,K-ATPase by P_i (E. Rabon, personal communication). While the present study was in progress, a third report of phosphorylation of the H,K-ATPase by P_i appeared that gives $K_{0.5}$ =

60 μM (Jackson & Saccomani, 1984). Therefore, the measured $K_{I(app)}$ is consistent with competitive binding of P_i at the high-affinity vanadate site. The total vanadate binding stoichiometry of 3 nmol mg^{-1} observed experimentally could be explained in the one-phosphorylation-site interpretation by noncompetitive filling of the low-affinity vanadate site, since K_{V2} is approximately equal to the apparent vanadate binding constant of 0.69 μM in the presence of 20 mM P_i (Figure 5). Thus, the results presented in Figures 4 and 5 are compatible with inhibition of ^{18}O exchange by competitive binding with P_i at the high-affinity vanadate site and noncompetitive vanadate binding to the low-affinity site.

Considering next the possibility that both sites are phosphorylated and catalyze ^{18}O exchange, apparently monophasic inhibition of exchange (Figure 4) and apparently monophasic vanadate binding in the presence of P_i (Figure 5) would have to be explained by a fortuitous combination of vanadate and P_i dissociation constants. Evidence for tight P_i binding of about the right magnitude was discussed in the preceding paragraph. Substituting K_{V2} for K_{V1} in eq 9 give $K_{P2} \simeq 10$ mM for P_i binding at the low-affinity vanadate site. The phosphoenzyme levels reported recently (2.5–2.7 nmol mg^{-1}), which approach twice those reached with ATP, were obtained by reacting the gastric enzyme with 5 mM P_i (R. J. Jackson, personal communication). P_i dissociation constants from sarcoplasmic reticulum Ca-ATPase in the 8–37 mM range have been reported by a number of laboratories (Epstein et al., 1980; McIntosh & Boyer, 1983; Pickart & Jencks, 1984). Thus, there is both precedent and some evidence for weak P_i binding, so that the two-phosphorylation-site interpretation of the data in Figures 4 and 5 cannot be excluded. Preliminary evidence that FITC, which completely inhibits ATPase activity without affecting pNPPase activity (Jackson et al., 1983), halves the ^{18}O exchange rate (Faller & Mendlein, 1984) is easier to explain by a two-site model.

The present study demonstrates that the gastric H,K-ATPase shares with the Na,K- and Ca-ATPases a remarkable capacity for phosphorylation from P_i, with Mg^{2+} the only requirement for the reaction (Jencks, 1980). A possible difference is the mean lifetime of the E·P_i complex compared to the rate of covalent bond formation. For the Na,K-ATPase $R \ll 1$ at neutral pH (Dahms & Miara, 1983) and for the sarcoplasmic reticulum Ca-ATPase $R = 0.08$ (Boyer et al., 1977). The distribution of [^{18}O]P_i species formed during catalysis of medium exchange by the H,K-ATPase deviates significantly from random (Table II), and the $P^{18}O_4$ species decays slower, compared to the average enrichment, than expected for exchange of a single oxygen atom per turnover (Figure 6). Hackney (1980) has derived expressions for calculating the theoretical distribution of [^{18}O]P_i species with time when reversals of the phosphorylation step occur, assuming the oxygens are exchanged statistically. Figure 7 shows the fit obtained for one set of data with $P_c = 0.31$, $K' = 1.99$ h^{-1}, and the distribution of [^{18}O]P_i species measured initially. Comparable fits, without any pattern in the deviations from theoretical, were obtained for other data sets by using the distribution parameters recorded in Table III. These results demonstrate that release of P_i from the gastric enzyme is only about twice as fast as covalent bond formation and probably much slower than diffusion controlled. Recently an increase in R to 0.4 was reported for the sarcoplasmic reticulum Ca-ATPase at high ATP concentrations and attributed to modulation of the properties of enzyme-bound P_i by ATP binding (McIntosh & Boyer, 1983).

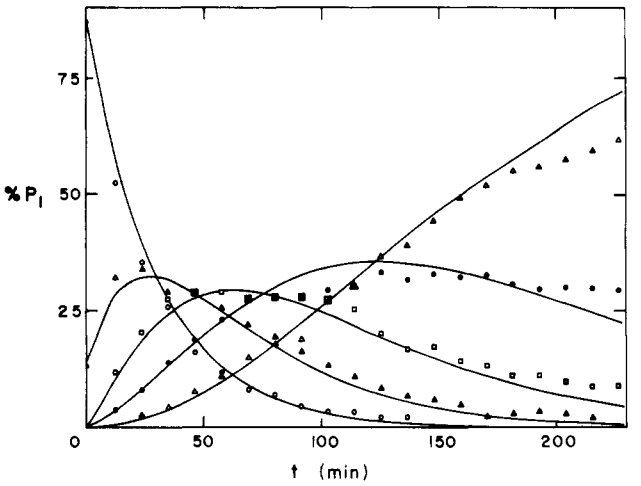

FIGURE 7: Fit of experimental [^{18}O]P_i distribution to theory. The experimentally measured percentage of P_i containing (○) four, (▲), three, (□) two, (●) one, and (△) zero ^{18}O atoms is plotted together with theoretical curves calculated for $P_c = 0.31$, $k' = 1.99$ h^{-1}, and initial % $P^{18}O_4 = 85$ as described in the text. This data set was obtained with 50 μg mL^{-1} protein and SA = 235 μmol mg^{-1} h^{-1}. Other experimental conditions are given in the legend to figure 1.

Finally, several comparisons between the distribution parameters in Table III are worth emphasizing. First, vesicle preparations differing in specific activity by a factor of more than 2 (rows 2 and 3) showed exactly the same kinetic distribution pattern. This result, together with the strict linearity between ^{18}O exchange rate and active enzyme, demonstrates that the catlytic center of the enzyme is conserved even when other criteria of purity, such as IEF gels, may indicate considerable heterogeneity among vesicle preparations. The remarkable reproducibility of kinetic data obtained with different vesicle preparations, including some purified on a continuous gradient followed by free-flow electrophoresis, was noted in a previous study of vanadate inhibition of the gastric H,K-ATPase (Faller et al., 1983a). Second, the [^{18}O]P_i distribution was unaffected by K^+ (rows 1 and 2), which accelerated exchange more than 10-fold (Table I). Third, no pattern was observed in the variation of R with temperature (rows 4–6). The observed variation in R_4 from 2.7 to 3.1 corresponds to only a 15% change in one or both of the rate constants and is not significant experimentally. The kinetic distribution of [^{18}O]P_i species formed during catalysis of medium exchange by the Na,K-ATPase is not affected by temperature either (Miara & Dahms, 1981). An activation energy of about 15 ± 2 kcal mol^{-1} for catalysis of ^{18}O exchange by the H,K-ATPase can be estimated by assuming an Arrhenius plot of the data in Table III (ln k' vs. reciprocal temperature in kelvin) is linear. Evidently K^+ and higher temperature lower the energy barriers to P_i and HOH loss from the H,K-ATPase by about the same amount. One simple explanation for the invariance of R is that ^{18}O exchange is always catalyzed by the same form of the enzyme, and K^+ and higher temperature increase the relative amount of the active conformer. The best evidence for ligand-mediated conformational changes in the H,K-ATPase is quenching of FITC-labeled enzyme fluorescence (Jackson et al., 1983). However, vanadate also induced fluorescent changes, and neither vanadate binding nor inhibition of ATPase activity is compatible with a dynamic equilibrium between two enzyme conformers (Faller et al., 1983a).

The major conclusion of this study is that the gastric H,K-ATPase catlyzes ^{18}O exchange between P_i and HOH via the terminal steps in the hydrolytic reaction. The rate of the reaction correlates rigorously with the amount of active en-

zyme, so that quantitative studies of intermediate exchange can be undertaken confidently. The partition coefficient for medium isotope exchange is significantly different from zero, engendering optimism that systematic study of the factors that influence the kinetic pattern of $[^{18}O]P_i$ species formed can provide insight into the number of sites and/or conformational states of the enzyme involved in catalysis.

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