Kinetic Studies of the Gastric H,K-ATPase
EVIDENCE FOR SIMULTANEOUS BINDING OF ATP AND INORGANIC PHOSPHATE*

William W. Reenstra, Jeffrey D. Bettencourt, and John G. Forte

From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720

The steady state rate of ATP hydrolysis (v) by the gastric H,K-ATPase and the steady state level of phosphoenzyme (E-P) have been measured at 0 and 10 mM KCl; both v and E-P have a nonhyperbolic dependence on the ATP concentration that is consistent with negative cooperativity. The ratio of the rate of hydrolysis to phosphoenzyme (v/E-P) was found to vary with the concentration of ATP. Thus, for the rate law v = [E-P] - k, k must be a function of the ATP concentration. This requires that ATP be able to bind to E-P or to an enzyme form that occurs after E-P but prior to an irreversible step, such as the loss of inorganic phosphate (P). At low ATP concentrations, product inhibition by P_i gives concave downward plots of 1/v against P_i concentration. P_i increases the apparent K_m and decreases the apparent V_max. At saturating ATP concentrations, P_i is a noncompetitive inhibitor. These data show that ATP and P_i can bind to the H,K-ATPase simultaneously. They are inconsistent with mechanisms where the binding of ATP and P_i is mutually exclusive.

The gastric H,K-ATPase originally isolated by Ganser and Forte (1), has been shown to be the primary pump for acid secretion in the stomach. During acid secretion the H,K-ATPase is located in the apical membrane of the oxyntic cell where it catalyzes a one-for-one exchange of cytoplasmic potassium for luminal potassium (2-4). Based on numerous kinetic and structural similarities with the Na,K-ATPase, the H,K-ATPase has been classified as an E_I:E_E ATPase. These similarities include 1) a high degree of homology in primary amino acid sequence (5, 6), 2) the formation of a K⁺-sensitive acyl-phosphate during ATP hydrolysis (7, 8), 3) identical amino acid sequences around the aspartic acid to which phosphate is bound (9), 4) K⁺ stimulated hydrolysis of para-nitrophenylphosphate (10, 11), and 5) inhibition by vanadate (12, 13). Several studies have shown that for both enzymes the rate of ATP hydrolysis is not a hyperbolic function of the ATP concentration and that both enzymes exhibit negative cooperativity (14, 15). For the Na,K-ATPase numerous studies have sought to explain this effect, and current models propose that ATP binds with a low affinity to a form of the enzyme that occurs between the phosphoenzyme, E-P, and the free enzyme (16). Despite the similarities in the kinetics of ATP hydrolysis for the two enzymes, most models for the H,K-ATPase have failed to incorporate a second mode of ATP binding and are therefore unable to account for the dependence of ATP hydrolysis on the ATP concentration (17).

In the present study we have examined the H,K-ATPase-catalyzed rate of ATP hydrolysis and the steady state concentration of E-P as a function of the ATP concentration. We have also measured inhibition by inorganic phosphate, P_i. The results of these experiments require that the mechanisms of ATP hydrolysis includes steps where P_i and ATP simultaneously bind to the enzyme. A mechanism that is consistent with these findings is shown in Fig. 1. The essential feature of this mechanism is that ATP can bind only to free enzyme, E, but also to the phosphate containing forms E-P or E-P_i. For reasons of clarity the interaction of K⁺ with the H,K-ATPase has not been included in the scheme but in the presence of KCl the hydrolysis of ATP is postulated to follow the same mechanism.

EXPERIMENTAL PROCEDURES

Materials
Gastric microsomes containing the H,K-ATPase were isolated from hfg fundus as previously described (18, 19). Briefly, crude microsomes were harvested as the membrane fraction that sedimented between 14,600 x g for 15 min and 143,000 x g for 45 min. These membranes were further purified by centrifugation in a sucrose gradient for 4 h at 131,000 x g. Microsomes were collected at the interface between 20 and 27% sucrose. Intact microsomes were broken by suspension in lyophilization buffer, 5 mM Pipes-Tris, 0.2 mM EDTA, pH 7.0, sedimented at 143,000 x g for 45 min, resuspended in buffer, and lyophilized. Lyophilized membranes, which do not reform tight vesicles, were resuspended in lyophilization buffer at 3 mg/ml and stored in aliquots at -20°C until use. For the experiments described five preparations of microsomes were used and from these 11 batches of lyophilized vesicles were made.

All chemicals were purchased from commercial sources and used without further purification. [γ-32P]ATP was obtained from Du Pont-New England Nuclear. Stock solutions of inorganic phosphate were made by titration of H_3PO_4 with Tris.

Methods

ATP Hydrolysis—Reactions, performed at room temperature (21-23°C), were started by adding [γ-32P]ATP to 1.0 ml of assay buffer containing lyophilized vesicles (at 0.01-0.08 mg protein/ml), 10 mM Pipes-Tris, 1 mM MgSO_4, and 0 or 10 mM KCl at pH 7.0. Aliquots (200 μl) were removed at four time points between 5 and 90 s and quenched by vortexing in 1.8 ml of ice-cold 5.0% trichloroacetic acid containing 3.0 mM phosphoric acid and 0.3 g of acid-washed charcoal.

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†To whom correspondence should be addressed: Dept. of Physiology-Anatomy, University of California, Berkeley, CA 94720.

The abbreviations used are: E-P, H,K-ATPase with covalently bound phosphate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); E-P_i, H,K-ATPase with noncovalently bound inorganic phosphate.
in order to adsorb ATP (20). Tubes were centrifuged and aliquots of the supernatants containing released inorganic phosphate were counted by liquid scintillation spectrometry. Rates of ATP hydrolysis were calculated from the linear least square slopes of cpm recovered against time, the specific activity of the [γ-32P]ATP, and the protein concentration. Least squares lines had correlation coefficients greater than 0.99 in all cases, and the standard error was always less than 10% of the calculated rate and did not vary with the concentration of ATP. In all cases less than 15% of the total ATP added was hydrolyzed during the time of the reaction. In the presence of KCl, the observed rate of ATP hydrolysis, not the increase due to K+, is reported.

Phosphoenzyme Formation—The steady state level of phosphoenzyme (E-P) was obtained with a filtration assay. Reaction mixtures at pH 7.0 contained lyophilized vesicles (0.01-0.06 mg protein) 10 mM Pipes-Tris, 1 mM MgSO4, 0 or 10 mM KCl, and [γ-32P]ATP in 200 μl. Reactions, at room temperature, were started with the addition of ATP and quenched after 10 s by vortexing with 1.0 ml of ice-cold 7% trichloroacetic acid containing 4 mM phosphoric acid. Samples were diluted with 10 ml of ice-cold wash (5% trichloroacetic acid, 5 mM trichloroacetic acid, 5 mM H3PO4 and 1 mM ATP) and poured over cellulose filters. Gelman GN6. The reaction tube and filter were rinsed with a second 10 ml of the wash, and the filter was then washed with 10 ml of ice-cold ethanol. Filters were dissolved in scintillation mixture and counted by liquid scintillation spectrometry. Blanks were obtained in the same manner except that a 100-fold excess of unlabeled ATP was added 15 s after the reaction was started, and the reaction was quenched after an additional 10 min of incubation. Phosphoenzyme was determined from the difference between the radioactivity of the experimental and the control samples, the specific activity of the ATP, and the concentration of protein in the reaction mixture. All points were determined in duplicate. The maximum difference in the calculated level of E-P, determined with duplicate samples, was less than 7% of the calculated level of E-P and did not vary with the ATP concentration. The radioactivity in the control samples was proportional to the ATP concentration. Reaction times and conditions were chosen so that no more than 15% of the ATP was consumed during the incubation.

Rate of Hydrolysis of Acid Precipitated E-P—The rate of hydrolysis of total acid stable phosphoprotein was determined using phosphoprotein that was obtained in the same manner as described for the measurement of E-P. After quenching with 7% trichloroacetic acid containing 4 mM phosphoric acid, samples were twice pelleted at 8000 x g for 1 min, and resuspended in 1.0 ml of ice-cold wash (5% trichloroacetic acid, 5 mM phosphoric acid, and 1 mM ATP). After the second wash samples were applied to filters, Gelman GN6, and washed as described above. The filters were placed in 5 ml of 500 mM potassium phosphate buffer, pH 6.5, with or without 50 mM NaN3, or 500 mM glucose/NaOH buffer, pH 9.0. Aliquots of 500 μl were removed at various times, 200 μl of 50% trichloroacetic acid was added, and the sample centrifuged at 8000 x g for 1 min. Radioactivity in a 500-μl sample of the supernatant was determined by liquid scintillation spectrometry. The total acid soluble 32P, in the incubation buffer was calculated at each time point, and the data were fit by a nonlinear least squares routine to the form of equation (1) where f is the total soluble 32P, at a given time, fmax is the recovered 32P, and k is the observed rate constant, and t is time.

The rate of 32P-phosphoenzyme loss from native H,K-ATPase following the addition of a 100-fold excess of unlabeled ATP was measured as follows. Lyophilized membranes were reacted with [γ-32P]ATP as described for the measurement of E-P. Unlabeled ATP was added 10 s after the reaction was started, and separate 200-μl reaction mixtures were quenched with 1.0 ml of 7% trichloroacetic acid, 4 mM phosphoric acid at various times between 1 and 15 s after the addition of unlabeled ATP. Samples were washed two times and then filtered as described above. Filters were counted by liquid scintillation spectrometry.

Protein concentrations were determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

Derivation of the Rate Laws—Steady state rate laws were generated for all models tested with the aid of a program written by Runyan and Guttm (22). For the mechanisms shown in Fig. 1, where ATP can bind to both the free enzyme and to either E-P, or to E-P, steady state rate laws in the presence of Pi have the form shown in Equation 1.

\[
\frac{u}{E_i} = \frac{A \cdot [ATP]^a \cdot B \cdot [ATP] + E \cdot [ATP] \cdot [Pi]}{1 + C \cdot [ATP]^a + D \cdot [ATP] + F \cdot [Pi] + G \cdot [ATP]^a \cdot [Pi] + H \cdot [ATP] \cdot [Pi] + J \cdot [Pi]^a + K \cdot [Pi]^b \cdot [ATP]}
\]

where the coefficients are functions of the rate constants shown in Fig. 1 and E is the total enzyme concentration. For the two limiting cases, shown in Fig. 1, expressions for the coefficients in terms of the mechanisms in Fig. 1 are

\[\text{Table I}
\]

\[\text{Definition of the coefficients in Equations 1 and 2 in terms of the mechanisms in Fig. 1.}
\]

Mechanism A

\[\begin{align*}
A & k_1 k_2 k_3 k_4 k_5 k_6 \\
B & k_1 k_2 k_3 (k_4 + k_5) + k_8 \\
C & k_1 k_2 [k_3 + k_4 + k_5] + k_8 \\
D & k_1 (k_3 + k_4 + k_5) + (k_4 + k_5) \cdot [k_8 + k_9] + k_8 k_9 k_{10} \\
E & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
F & k_1 (k_3 + k_4 + k_5 + k_9) + (k_3 + k_9) \cdot [k_1 + k_2 + k_3 + k_4 + k_9 + k_10] + k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
G & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
H & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
J & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
K & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
L & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
M & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
N & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
O & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
P & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
Q & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
R & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
S & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
T & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
U & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
V & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
W & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
X & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
Y & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
Z & k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9 k_{10} \\
\end{align*}\]

\[a^*\] For Mechanism A all coefficients have a denominator of \( k_b (k_1 + k_2 + k_3 + k_4) \) and for Mechanism B all coefficients have a denominator of \( k_b (k_1 + k_2 + k_3 + k_4) \).
rate constants are given in Table I. In the absence of P, Equation 1 simplifies to

$$v/E_t = \frac{A \cdot [ATP]^2 + B \cdot [ATP]}{1 + C \cdot [ATP]^2 + D \cdot [ATP]} \tag{2}$$

Under the same conditions the fraction of total enzyme that has covalently bound phosphate is given by Equation 3,

$$E-P/E_t = \frac{A' \cdot [ATP]^2 + B' \cdot [ATP]}{1 + C' \cdot [ATP]^2 + D' \cdot [ATP]} \tag{3}$$

C and D have the same meaning as in Equation 1, and $A'$ and $B'$ are functions of the rate constants in Fig. 1, for both mechanisms in Fig. 1, expressions for $A'$ and $B'$ are given in Table I. The requirement that the coefficients $C$ and $D$ be equal in both Equations 2 and 3 follows from the fact that both $v/E_t$ and $E-P/E_t$ can be expressed as ratios where the denominators are given by the sum of the concentrations of the enzyme forms present at the steady state, thus the denominator in Equation 2 must be the same as the denominator in Equation 3; the same is also true for any intermediate in a rate law that follows a hyperbolic rate law.

Both in the presence and the absence of $K^+$ the data for $v$ and $E-P$, in Figs. 2 and 3, are fit simultaneously so that the coefficients $A, A', A^*,$ and $B'$ were obtained with one fitting. The data were also fit by the same procedure to a hyperbolic model, which generated values for $V_m, K_m,$ and the maximal steady state level of $E-P$. Since the errors in $v$ and $E-P$ were a constant fraction of $v$ or $E-P$ all data were assigned a weight proportional to $1/v^2$ or $1/E-P^2$ (23). The numerical values for the data were scaled by a constant factor so that the data for $v$ and for $E-P$ were of the same magnitude, and the average weights of data for $v$ and $E-P$ were equal. Statistical comparisons of the residuals were done with an F test as described by Mannervik (23). The nonlinear least squares fits were generated with a modified basic program originally described by Duggleby (24).

**RESULTS**

**Kinetics of ATP Hydrolysis**—In Fig. 2 the observed rates of ATP hydrolysis at 0 and 10 mM KCl are plotted as $v$ against $v/[ATP]$. In agreement with previous studies, the rate of ATP hydrolysis is not a hyperbolic function of the concentration of ATP (14) and Equation 2 can be fit to the data at both 0 and 10 mM KCl. Best fit parameters for the coefficients $A - D,$ obtained by the nonlinear least squares regression procedure described under "Experimental Procedures" are given in the figure legend. Calculated values for the rate at saturating ATP, $V_m,$ are 39.3 ± 1.0 and 652 ± 15 nmol/min/mg protein at 0 and 10 mM KCl, respectively. At limiting ATP, where the rate of ATP hydrolysis is a linear function of the ATP concentration, $V_m/K_m$ is 136 ± 21 and 28.0 ± 1.4 nmol/min/mg protein/mM for 0 and 10 mM KCl, respectively. The data were also fit to a hyperbolic model by the procedure described under "Experimental Procedures." Also at both 0 and 10 mM KCl, the sum of the squares of the rate data was significantly greater for the hyperbolic model than for the fit to Equation 2 ($p < 0.05$). Also at both 0 and 10 mM KCl, the distribution of positive and negative residuals was random for the fit to Equation 2 but not for the hyperbolic fit ($p < 0.05$) (23). The values for $V_m$ and $V_m/K_m$ at 0 KCl are similar to values reported by Wallmark et al. (14).

In parallel experiments the steady state levels of acid stable phosphoenzyme formed from ATP was measured at the same time and under the same conditions as ATP hydrolysis. The results, at 0 and 10 mM KCl, are shown in Fig. 3 as plots of $E-P$ against $E-P/[ATP].$ As with the data for ATP hydrolysis, the concentration of $E-P$ is not a hyperbolic function of the concentration of ATP, and Equation 3 can be fit to the data at both 0 and 10 mM KCl. Best fit parameters for the coefficients $A', B', C$, and $D$ were obtained by nonlinear least squares regression as described under "Experimental Procedures" and are given in the figure legend. At both 0 and 10 mM KCl, the sum of the squared residuals for $E-P$ formation was shown to be significantly less for the fit to Equation 3 than for the fit to the hyperbolic model ($p < 0.05$). Also at both 0 and 10 mM KCl, the distribution of positive and negative residuals was shown to be random for the fit to Equation 3 but not for the hyperbolic fit ($p < 0.05$) (23). The values for $V_m$ and $V_m/K_m$ at 0 KCl are similar to values reported by Wallmark et al. (14).
> ATP; the \( K_m \) values reported were similar to the low ATP \( K_m \) in the absence of KCl seen in this study. Two studies have measured E-P with ATP concentrations up to 100 \( \mu M \) (12, 14); these data showed a 30% increase in the steady state level of E-P between 1 and 100 \( \mu M \), which is somewhat smaller than the increase in E-P seen in the present study.

The dependence of \( v \) and E-P on the ATP concentration is shown in Fig. 4, where the data at 0 KCl and ATP concentrations less than 20 mM in Figs. 2 and 3 are replotted against the ATP concentration. A similar difference in the apparent \( K_m \) for \( v \) and E-P formation is also seen with the data at 10 mM KCl (data not shown). Since for the hyperbolic model the concentration of ATP giving half-saturation of the rate of hydrolysis must be equal to the concentration of ATP that gives half-saturation of the steady state intermediate E-P, these data and the analysis of the data plotted in Figs. 2 and 3 demonstrate that the data cannot be fit to a hyperbolic dependence of \( v \) or E-P on the ATP concentration.

Characterization of the Isolated Phosphoprotein—Before a detailed analysis of the dependence of the isolated phosphoprotein was attempted, it was necessary to show that under all conditions tested the isolated phosphoprotein is the acid stable phosphoenzyme intermediate E-P. Several effects other than phosphorylation of the H,K-ATPase at the catalytic site of the H,K-ATPase at the catalytic site could give rise to the nonhyperbolic dependence of E-P on the concentration of ATP. These include phosphorylation of the H,K-ATPase at a regulatory site or the phosphorylation of other proteins in the gastric mucosae. This latter possibility is a cause for concern as several groups have reported alkaline stable phosphoproteins in oxyntic cell microsomal membranes (26, 27). We have used three tests to show that phosphoprotein isolated at high ATP concentrations and in the presence of KCl is chemically identical to the phosphoprotein formed at low ATP concentrations which has been previously characterized (28).

Studies with the Na,K-ATPase (29), and the H,K-ATPase (28) have shown that the phosphoenzyme intermediate is formed via an anhydride linkage between phosphate and the carboxyl group of an aspartic acid residue on the enzyme. Anhydride linkages are more subject to base hydrolysis than phosphate esters on serine or threonine and are attacked by hydroxylamine, NH\(_2\)OH (30). Data for the hydrolysis at pH 6.5 of acid-denatured phosphoprotein in the presence and the

![Fig. 4. Comparison of the steady state level of phosphoenzyme (E-P) and the rate of ATP hydrolysis (v) at low ATP concentrations. Both E-P (A) and \( v \) (O) were determined as described under "Experimental Procedures" with lyophilized vesicles in 10 mM Pipes-Tris, 1 mM MgSO\(_4\), and 0 mM KCl at pH 7.0 with \([\text{ATP}] = [\text{ATP}]^{0.2}\).]
Simultaneous Binding of ATP and Pi to the H,K-ATPase

\[ \frac{v}{[E-P]} = \frac{B/A' + A/A' \cdot [ATP]}{B'/A' + [ATP]} \]  

(4)

can be fit to the data; \( A \) and \( B \) are the coefficients in the numerator for \( v/[E] \), and \( A' \) and \( B' \) are analogous coefficients for \( E-P/E \). Best fit values at 0 and 10 mM KCl are given in the figure legend. As a hyperbolic dependence of \( v \) on ATP concentration requires that \( v/[E-P] \) be independent of the ATP concentration, the data in Fig. 6, A and B, further emphasizes the point that the hydrolysis of ATP cannot be fit by a hyperbolic dependence on the ATP concentration.

Inhibition by Inorganic Phosphate—In order to characterize further the mechanism of ATP hydrolysis, inhibition of ATP hydrolysis by inorganic phosphate has also been investigated. In the absence of KCl and at ATP concentrations less than 0.5 \( \mu M \), where the squared terms in Equation 4 represent less than 15% of either the denominator or the numerator, Dixon plots (Fig. 7A) of \( 1/v \) against [Pi] in the range 0 to 10 \( \mu M \) Pi, are nonlinear. At each concentration of ATP, 10 mM Pi, appears to give a limiting nonzero rate of ATP hydrolysis. Inhibition of ATP hydrolysis by Pi, in the presence of 10 mM KCl was also biphasic (data not shown). At each concentration of ATP, a nonlinear fit of Equation 5 to the data was made.

\[ 1/v = \frac{d + h \cdot [Pi]}{1 + e \cdot [Pi]} \]  

(5)

The best fit parameters were used to generate the solid lines in Fig. 7A. Provided the second order terms in Pi, are not significant the model in Fig. 1 predicts that \( e = E/B \) where \( B \) and \( E \) are defined in Table I. (Squared terms in \( P_i \) must contribute to the numerator of Equation 5 but as shown in Fig. 8 they are not significant at concentrations of Pi less than 10 mM so that the data can be fit by Equation 5.) In accord with the model a replot of \( 1/v \) against \( [Pi] \) gives a half-maximal increase in \( 1/v \), against the ATP concentration shows that \( e \) is independent of the ATP concentration.

FIG. 6. Dependence of \( v/[E-P] \) on the concentration of ATP. E-P and \( v \) were determined with lyophilized vesicles in 10 mM Pipes-Tris, 1 mM MgSO\(_4\), and 0 (panel A) or 10 mM KCl (panel B) at pH 7.0 with [\(^{33}\)P]ATP. Concentrations of ATP ranged from 0.1 to 200 \( \mu M \). Solid lines are for best fit parameters to Equation 5. Best fit values for KCl = 0 mM are \( B/A' = 58 \pm 33 \mu M/min \), \( A/A' = 36.8 \pm 2.6 \) min\(^{-1} \), and \( B'/A' = 4.2 \pm 2.1 \mu M \); for KCl = 10 mM \( B/A' = 4.19 \times 10^2 \pm 787 \mu M/min \), \( A/A' = 813 \pm 21 \) min\(^{-1} \), and \( B'/A' = 13.1 \pm 2.1 \mu M \).

FIG. 7. Inhibition by inorganic phosphate (Pi) at low concentrations of ATP. Panel A, rates were determined with lyophilized vesicles in 10 mM Pipes-Tris, 1 mM MgSO\(_4\), at 0 mM KCl and ATP concentrations, in \( \mu M \), of 0.05 (C), 0.1 (\( \Delta \)), 0.2 (C), and 0.5 (V). Data from three separate experiments are shown. Solid lines are calculated from best fit parameters to Equation 6. Panel B, replots of the best fit values for \( 1/v \), the concentration of Pi, that gives a half-maximal increase in \( 1/v \), against the concentration of ATP; indicated errors are S.E. Solid line is for a least squares linear fit to the data. Panel C, replots of \( 1/v \) against \( 1/[ATP] \) at 0 (\( \Delta \)) and at 10 mM Pi, (O). Calculated values of \( 1/v \) for a high \( K_a \) and a low \( K_a \) enzyme (see text) are indicated by the lines.
parameters to drolysis by inorganic phosphate. Rates were determined with lyophilized vesicles in 10 mM Na,K-ATPase
solutions. Pipes-Tris, 1 mM MgSO₄, at 0 mM KCl and Pi concentrations of 0 (○), 50 (△), 100 (□), and 200 (V) mM. Solid lines are for best fit parameters to $v = \frac{V_m \cdot [ATP]}{K_m + [ATP]}$.

A second series of experiments was performed in the absence of KCl and at concentrations of ATP greater than 75 μM. Under these conditions, inhibition of ATP hydrolysis was noncompetitive with ATP, as shown in Fig. 8, where $1/v$ is plotted against $1/[ATP]$. At saturating ATP concentrations, Pi inhibited ATP hydrolysis with a $K_i$ of 165 mM.

**DISCUSSION**

The results of this study demonstrate three features of the mechanism of ATP hydrolysis by the H,K-ATPase that have not been previously reported. 1) Like the rate of ATP hydrolysis the steady state concentration of the acid stable phosphoenzyme, E-P, is not a hyperbolic function of the ATP concentration, but shows negative cooperativity. 2) The rate of Pi, loss from E-P is catalyzed by ATP. In regard to this point we must emphasize that we have not measured the rate of E-P bond cleavage, but the effective rate constant for the overall reaction from E-P to enzyme plus free Pi. 3) Inhibition of ATP hydrolysis by Pi is complex. At low ATP concentrations Pi gives partial inhibition; at 10 mM Pi the rate of ATP hydrolysis appears to be insensitive to the concentration of Pi. At high ATP concentrations inhibition by Pi is noncompetitive with respect to ATP.

Previous work with the enzyme has shown that ATP binds to the enzyme with two distinctly different binding constants, (14, 32) yet most models (14, 17) like the mechanism in Scheme 1 can only account for ATP binding to the

$$E + ATP \xrightarrow{k_1} E \cdot ATP \xrightarrow{k_2} E - P \xrightarrow{k_3} E \cdot P \xrightarrow{k_4} E + Pi$$

**SCHEME 1**

free the enzyme and therefore fail to provide an explanation for the nonhyperbolic dependence of $v$ and E-P on ATP concentration. This model is also inconsistent with kinetics of inhibition by Pi, seen at either low or high ATP concentrations.

**Mechanisms Consistent with the Data—** As described for the Na,K-ATPase (34), the nonhyperbolic dependence of the rate of ATP hydrolysis with the concentration of ATP can be explained by several kinetically indistinguishable mechanisms. These mechanisms include 1) two enzymes following hyperbolic kinetics but with appropriate differences in their $V_m$ values and $K_m$ values for ATP; 2) mechanisms where ATP alters the kinetic parameters by chemically modifying the enzyme (e.g. by phosphorylation); 3) mechanisms where at high ATP the order of substrate binding and product release is altered causing a change in the rate of ATP hydrolysis; and 4) mechanisms with a multimeric enzyme where binding of substrate to a second active site on an adjacent subunit causes the rate of hydrolysis at the first site to be increased. In the absence of the product, $P_i$, all of these mechanisms will give rise to rate laws of the form of Equation 2 where the physical meaning of the constants $A-D$ is dependent upon the underlying mechanism. Although these mechanisms are all consistent with the nonhyperbolic dependence of $v$ on the ATP concentration, and all can be made consistent with the nonhyperbolic dependence of E-P on the concentration of ATP, several fail to provide reasonable explanations for the Pi inhibition data obtained in this study.

The data are inconsistent with the presence of two enzymes each with a hyperbolic dependence of $v$ on the ATP concentration, Equation 6,

$$V = \frac{V_m \cdot [ATP]}{K_m + [ATP]}$$

For two separate enzymes each following a mechanism like the one shown in Scheme 1, inhibition by Pi should be competitive with ATP, since ATP and Pi, both bind to the same form of the enzyme. As shown in Fig. 8 at high ATP concentrations inhibitions by Pi is not competitive with ATP. At low ATP concentrations and 10 mM Pi, where the rate of ATP hydrolysis appears to be insensitive to the concentration of Pi, the observed rate is not consistent with kinetic constants determined for the high $K_a$ enzyme. The data in Fig. 8 suggest that 10 mM Pi does not cause a measurable change in the kinetic properties of ATP hydrolysis at high ATP concentrations. Therefore, if two enzymes are present, the rate in the presence of 10 mM Pi should be consistent with the kinetic constants for the high $K_a$ enzyme. The kinetic constants in Fig. 2 can be used to generate kinetic constants for the mechanism in Equation 6 (34). In the absence of KCl $V_m$, and $V_m$ are 7.5 and 32.4 nmol/min/mg protein, respectively, and $K_m$ and $K_m$ are 0.05 and 10.9 mM, respectively. The curves in Fig. 7C are drawn for enzymes having these kinetic constants.

The observed rates of ATP hydrolysis in Fig. 7A at 0 and 10 mM Pi are replotted in Fig. 7C. The data in the absence of Pi is superimposable with the corresponding data from Fig. 2, but the data at 10 mM Pi fall below the calculated values for the high $K_a$ enzyme. Therefore at 10 mM Pi, the observed rate of hydrolysis is greater than can be accounted for by the high $K_a$ enzyme. Thus, while the dependence of $v$ and E-P on ATP concentration does not permit the exclusion of two enzymes the kinetics of inhibition by Pi is not consistent with the presence of two enzymes and allows these two alternatives to be distinguished. It should also be noted that the chemical similarities of the phosphoproteins formed a low and high ATP concentrations also strongly suggest that the observed kinetics of ATP hydrolysis is not due to the presence of two distinct ATPases.

The following facts suggest that the nonhyperbolic dependence of the rate of ATP hydrolysis and the steady state level of E-P cannot be caused by phosphorylation at a regulatory site. 1) At both high and low ATP concentrations the measured phosphoenzyme exhibits a rapid rate of turnover. 2) The chemical reactivity of the E-P formed at all ATP concentrations suggests that E-P is formed via an anhydride bond (28) while regulatory phosphoproteins are usually formed at sites not subject to NH₂OH catalyzed hydrolysis. 3) Finally, at constant ATP concentrations inhibition by Pi, gives nonlinear
Simultaneous Binding of ATP and P, to the H,K-ATPase

Dixon plots. The noncompetitive inhibition by P, at high ATP concentrations is not consistent with a mechanism like the one in Scheme 1 that has the additional feature that ATP increases the rate by binding to a regulatory site. However, more complicated mechanisms that postulate ATP binding to a regulatory site only during specific steps cannot be excluded with this data.

The final two classes of mechanisms both postulate that ATP binds to the H,K-ATPase at the active site thereby changing 1) the kinetic order of substrate binding and product release, and 2) the rate constant for the rate-limiting step. One mechanism that possesses these properties is shown in Fig. 1. Two limiting cases of this mechanism will be considered: mechanism 1A where ATP binding occurs to an active site on the E·P form, and mechanism 1B where ATP binds to the E·P form. In both cases the order of substrate binding and product release is changed by ATP binding, and the rate of conversion of E·P to E·ATP could be increased. The model could describe either a monomeric enzyme where ATP initially binds at a site that is displaced from the phosphorylation site, or a dimeric enzyme with interacting catalytic sites. The kinetic properties of both the dimeric and the monomeric enzyme will show negative cooperativity (35, 36) and no change in the steady state turnover number, v/\[E-P\], at saturating ATP concentrations where the second order terms for ATP are significant.

However, for the monomeric model the simultaneous binding of ATP and P, (see below) probably requires that ATP initially bind to the enzyme at a site that is distinct from the phosphorylation site and that a subsequent conformational change brings the bound ATP to the phosphorylation site. Models incorporating this type of conformational change have been proposed for the Ca"-ATPase (37).

The model in Fig. 1 is not only consistent with the nonlinearity in plots of v against v/[ATP] and v against E·P/[ATP], it also predicts that the ratio v/[E·P] should be a function of the concentration of ATP. Models where ATP binds either after P, has been lost, such as those proposed by Ljungstrom and Marsh (38) and Helmich-De Jong et al. (25), or after an irreversible cleavage of the E·P bond, require that v/[E·P] be independent of the ATP concentration and therefore inconsistent with the data. This follows from the fact that the steady state rate, v, equals the steady state concentration of E·P times k, where k is a function of all the rate constants between E·P and the first irreversible step such as the loss of P. If ATP binds only after P, is lost, then k must be independent of the ATP concentration. The observation in Fig. 6 that v/[E·P] varies with the ATP concentration requires that ATP bind before P, is lost and requires the formation of a ternary complex of enzyme ATP and P,.

The partial inhibition of ATP hydrolysis by P, at low ATP concentrations can also be explained by a mechanism that incorporates the simultaneous binding of P, and ATP. According to the rate law in Equation 1, under conditions where second order terms in [ATP] or [P,] do not contribute, 1/v can be approximated by an equation of the form of Equation 5, where d = (1 + D·[ATP])/B·[ATP], h = (l + D·[ATP])/B·[ATP], and e = E/B and D, E, F, and H are defined in Table I. The concentration of P, that gives a half-maximal increase in 1/v, B/E, will be independent of the concentration of ATP, as shown in Fig. 7B. At saturating concentrations of ATP, where the second order terms for ATP are significant, both cases in Fig. 1 predict that P, should be a noncompetitive inhibitor of ATP hydrolysis as it can bind to E·ATP. Thus, the models in Fig. 1 are able to account for all the data obtained in this study. In contrast, a model where ATP binds only after P, loss will not allow for partial inhibition of ATP hydrolysis at low ATP concentrations because the enzyme forms that accumulate in the presence of P, E·P, and E·P, cannot bind ATP. Such a model also predicts that P, will be a competitive inhibitor at all ATP concentrations because P, and ATP always bind to the same form of the enzyme.

Kinetic Consequences of the Data—The results show that the presence of 10 mM KCl not only increases v, but increases the rate constant for K* binding to the free enzyme, K*, and 6.9 mm at pH 7.2 and 37°C has been obtained from the inhibition of ATP hydrolysis by P3. A value of this magnitude would be consistent with the observed reduction in v/[E·P] seen with 10 mM KCl, since v/[E·P] = v/[E·P] + k*, K*[K+ + K+] + k*.

Although 10 mM KCl reduces the steady state level of E·P under v/[E·P] conditions by over 100-fold, at saturating ATP the reduction is only 24%. In contrast K* stimulates v by 17-fold and reduces v/[E·P] by 4-fold. Since, under all conditions v = [E·P] - k, one consequence is that K* stimulation of k, the rate constant for P, loss from E·P, is independent of the ATP concentration. At v/[E·P] = 10 mM K+ increases k by 23-fold while at v, a 22-fold increase in k is seen.

It is not possible to distinguish between mechanisms where ATP binds to E·P or to E·P, by kinetic means, but values for k/k are calculated provided ATP only binds to E·P, i.e. mechanism 1A is followed. As shown above under v/[E·P] conditions v/[E·P] is equal to k, k/k + k* and at saturating ATP v/[E·P] is equal to k. Accordingly, the data in Fig. 6 give values of 1.7 and 1.6 for k/k at 0 and 10 mM KCl, respectively. By measuring 37O exchange between P and H2O at 37°C Faller et al. (39) have calculated smaller values for k/k = 0.52 and 0.43 at 0 and 7 mM KCl, respectively. Both experiments suggest that the partitioning of E·P, between E·P and E·P + K+, is approximately equal. However a value of 0.5 for k/k would limit the ATP-dependent increase in v/[E·P] to 50% and is therefore not consistent with the data in Fig. 6, A and B. Two explanations can be given. 1) At saturating ATP, ATP binds to E·P; mechanism 1B is followed. 2) Mechanism 1A is followed but rotation of noncovalently bound P, (E·P) is restricted so that k/k calculated from the 37O exchange data, which assumes that all oxygens in the E·P complex are in rapid equilibrium and therefore equivalent (40), gives a reduced value. While these two possibilities cannot be distinguished by steady state kinetics, they could be distinguished by measurement of the effect of ATP, 1) on the rate of E·P bond cleavage or 2) on E·P formation from P, at the present time the issue is unresolved as reports of inhibition by ATP (25) and studies that failed to see an effect of ATP on the rate of E·P bond cleavage (14, 41) have appeared.

Several previous studies have used rapid quench techniques to measure the rate constants for the dephosphorylation of E·P in the presence and the absence of KCl (14, 31–33). In the absence of KCl, Stewart et al. (33) observed a monophasic loss of E·P with a rate constant of 9 min⁻¹, but in the presence of 10 mM KCl the loss of E·P occurred with biphasic kinetics; 50% of the E·P was lost in an initial fast phase with a rate constant of 1500 min⁻¹ after which the apparent rate constant decreased to 400 min⁻¹. Since the dephosphorylation of E·P was measured in the absence of ATP the rate constants should be comparable to the 0 ATP intercepts in Fig. 6, A and B.

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With 0 KCl there is a good agreement with our value of 13.7 min"1. In the presence of 10 mM KCl, the rate constant for E-P loss obtained from Fig. 6B, 314 min"1, is similar to the rate constant for the slow phase of E-P bond cleavage. The biphasic curves for E-P loss upon the addition of KCl could be the result of an initial rapid re-equilibration of E-P and E-P, which is followed by a slower rate of P loss from E-P. In this case the rate constant for the slow phase of E-P loss would be equivalent to the steady state value of v/E-P obtained in this study.

Inhibition of the H,K-ATPase with vanadate (12) has been reported to show two inhibition constants for vanadate, each giving partial inhibition of ATP hydrolysis. Our data show that inhibition by P is also characterized by two inhibition constants and suggest that inhibition by vanadate may also be explained by a mechanism similar to that in Fig. 1.

Comparison of the Kinetic Mechanisms for the H,K-ATPase and the Na,K-ATPase—The data in this study can be compared with the results of similar experiments for the Na,K-ATPase that are suggestive of a ternary complex of enzyme, ATP and P, for the Na,K-ATPase. Of particular relevance to this study is the fact that the Na,K-ATPase shows a nonhyperbolic dependence of Δv on the ATP concentration. However, unlike the H,K-ATPase this effect is seen only when K+ is present (16). As described above a more complex mechanism involving at least two forms of the enzyme, E·ATP and E·Pi, whereas Pi and ATP bind to the same kinetic form with ATP. For the mechanism in Fig. 4, ATPase prior to the loss of Pi. As described above a more precise analysis of v/E-P may resolve this issue. Thus, the mechanisms of the Na,K-ATPase and the H,K-ATPase may show an even greater degree of similarity than previously recognized.

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