Gastric H/K-ATPase Liberates Two Moles of P_i from One Mole of Phosphoenzyme Formed from a High-Affinity ATP Binding Site and One Mole of Enzyme-Bound ATP at the Low-Affinity Site during Cross-Talk between Catalytic Subunits†

Kazuhiro Abe, Shunji Kaya, Toshiaki Imagawa, and Kazuya Taniguchi*

Biological Chemistry, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

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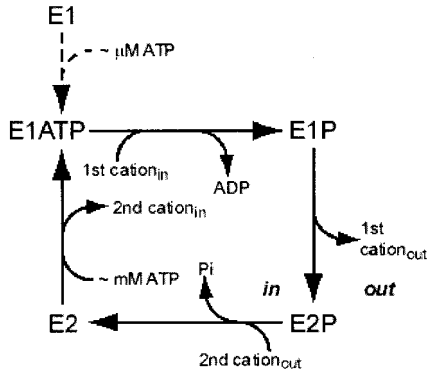
ABSTRACT: The maximum amount of acid-stable phosphoenzyme ($E^{32}P$)/mol of α chain of pig gastric H/K-ATPase from [γ-^{32}P]ATP ($K_{1/2}$ = 0.5 μM) was found to be ∼0.5, which was half of that formed from $^{32}P_i$ ($K_{1/2}$ = 0.22 mM). The maximum ^{32}P binding for the enzyme during turnover in the presence of [γ-^{32}P]ATP or [α-^{32}P]ATP was due to 0.5 mol of $E^{32}P$ + 0.5 mol of an acid-labile enzyme-bound [γ-^{32}P]ATP (EATP) or 0.5 mol of an acid-labile enzyme-bound [α-^{32}P]ATP, respectively. The $K_{1/2}$ for EATP formation in both cases was 0.12 ∼ 0.14 mM. The turnover number of the enzyme (i.e., the H^+-ATPase activity/(EP + EATP)) was very close to the apparent rate constants for EP breakdown and P_i liberation, both of which decreased with increasing concentrations of ATP. The ratio of the amount of P_i liberated to that of EP that disappeared increased from 1 to ∼2 with increasing concentrations of ATP (i.e., equal amounts of EP and EATP exist, both of which release phosphate in the presence of high concentrations of ATP). This represents the first direct evidence, for the case of a P-type ATPase, in which 2 mol of P_i liberation occurs simultaneously from 1 mol of EP for half of the enzyme molecules and 1 mol of EATP for the other half during ATP hydrolysis. Each catalytic α chain is involved in cross-talk, thus maintaining half-site phosphorylation and half-site ATP binding which are induced by high- and low-affinity ATP binding, respectively, in the presence of Mg^{2+}.

The catalytic subunit of a P-type ATPase, such as Na/K-ATPase, sarcoplasmic reticulum Ca/H-ATPase, and gastric H/K-ATPase, forms an acid-stable phosphoenzyme (EP)[1] during ATP hydrolysis (*1*−*6*). These three enzymes have homologous primary structures (*7*−*9*). They are believed to be *ping-pong* enzymes, which hydrolyze ATP in the presence of Mg^{2+}. The transfer of the γ-phosphate group of ATP to a β-aspartyl group of the enzyme is dependent on the first cation (Na^+, Ca^{2+}, or H^+, respectively) to form the ADP-sensitive phosphoenzyme (E1P), prior to the formation of E2P. The subsequent K^+, H^+, or K^+ (the second cation) induces E2P breakdown (dephosphorylation), as shown in a simplified form (Scheme 1) (*1*−*6*). In this scheme, E1 and E2 represent enzyme forms which are reversibly phosphorylated by ATP and P_i, respectively, and E2 binds ATP with low affinity. E1 accepts the first cation with high affinity, accompanied by phosphorylation from ATP. E2P accepts the second cation with high affinity, accompanied by dephosphorylation. "In" designates the sidedness of the enzyme for the binding of ATP, Mg^{2+} (not shown for simplicity), and the first cation and for the release of the second cation. "Out" designates the sidedness for the release of the first cation after E1P formation and the binding of the second cation accompanying dephosphorylation. Thus, the active transport of the first cation in one direction occurs during the phosphorylation of E1ATP to form E2P via the first cation-occluded enzyme form, E1P. The second cation transport in the opposite direction occurs during the dephosphorylation of E2P to form E1ATP via the second cation bound or the occluded enzyme form, E2. The transition from E2 to E1 is accelerated by the low-affinity binding of ATP. Thus, active transport occurs in a sequential manner (Scheme 1).

Scheme 1: Sequential Model for P-Type ATPases



It is generally accepted that two different ATP binding sites are present in a P-type ATPase, namely, the E1 and E2

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* Corresponding author. Phone: 81-11-706-2698. Fax: 81-11-736-2074. E-mail: ktan@ccms1.hucc.hokudai.ac.jp.

[1] Abbreviations: EGTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethly)-1,3-propanediol; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; LDS, lithium dodecyl sulfate; TCA, trichloroacetic acid; EP, phosphoenzyme; EATP, trichloroacetic acid-labile ATP−H/K-ATPase complex.

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sites. The E1 form has a high-affinity ATP binding site ($K_{1/2}$ = sub μM) and is precursor to ADP sensitive phosphoenzyme (E1P) formation. The E2 has a low-affinity ATP binding site ($K_{1/2}$ = sub mM), causing the high V_{max} and playing a regulatory role in accelerating the partial reactions (*3*−*6*). The issue of whether the functional unit of the P-type ATPase is a protomer in the case of Ca-ATPase and an $\alpha\beta$ heteroprotomer in the case of Na/K- and H/K-ATPase or higher oligomers remains controversial. If it is a protomer, the catalytic subunit would be expected to have 1 mol of ATP binding site where the E1 or E2 site appears, alternatively (the Scheme 1), or 2 mol of ATP binding sites are present simultaneously, a phosphorylation site for E1 and an acceleration site for ATP binding to E2 on the nucleotide domain (*10*). If it is a diprotomer or higher oligomer, the subunit would be expected to contain only 1 ATP binding site (i.e., the E1 and E2 site should be present in different subunits, with each showing half-site reactivity via cross-talking (*3*−*6*, *11*−*15*)).

All-sites phosphorylation has been proposed for H/K-ATPase from studies using P_i or acetyl phosphate (*16*), Na/K-ATPase from P_i using ouabain (*17*), and Ca/H-ATPase using ATP or P_i (*18*). Half-site phosphorylation has also been reported in studies of H/K-ATPase using ATP (*16*), Na/K-ATPase using ATP (*14*), and Ca/H-ATPase using P_i (*19*). Studies involving phosphorylation kinetics (*20*−*22*), cross-linking (*23*, *24*), the stoichiometry of ligand binding (*11*, *12*, *14*−*16*, *19*, *25*, *26*), chemical modification (*19*, *27*, *28*), fluorescence energy transfer (*29*, *30*), electron microscopy (*14*), low-angle laser light scattering photometry (*31*), and others (*11*−*15*, *32*) strongly point to the oligomeric nature of the P-type ATPases (*15*). These data point to some necessity for modifying Scheme 1, where the oligomeric properties are not included. The atomic structure of Ca/H-ATPase (*10*) shows strong evidence for the presence of an ATP binding pocket and a phosphorylation domain. These are present in the same cytosolic loop and are separated by up to 25 Å, but the former should come into proximity with the latter when a γ-phosphoryl group of ATP is transferred to form the acid-stable EP, which then contains an aspartyl phosphate bond at Asp351. This structure also shows the presence of a typical Rossmann fold in the phosphorylation domain, where the site of phosphorylation (Asp351) is situated in the C-terminal region of the central β strand and where critical residues for ATP hydrolysis are clustered. These data are consistent with an ATP binding site/catalytic site (*10*).

ATP binding at a low-affinity site accelerates the transition of the K^+-occluded Na/K-ATPase (E2) form to the Na^+-bound (E1) form (*3*, *4*, *13*). Recently, the simultaneous presence of 0.5 mol of an acid-labile ATP binding to Na/K-ATPase at a low-affinity site with 0.5 mol of an acid-stable EP (EATP:EP) or with the Rb^+ (K^+ congener)-occluded enzyme (EATP:RbE) has been reported (*14*). However, the fate of the bound ATP at a low-affinity site is not clear, nor is the issue of whether the bound ATP is capable of accelerating some other reactions as the result of simple binding without hydrolysis or by hydrolysis, either without or with the phosphorylation of the enzyme.

The maximum amount of the acid-stable phosphoenzyme (EP) from ATP in H/K-ATPase was found to be nearly half that from P_i or acetyl phosphate (*16*). The extent of increase of tryptophan fluorescence divided by the amount of EP accompanying phosphorylation with ATP was approximately 4-fold larger than that for the case of acetyl phosphate (*16*). These data suggest the possibility of the simultaneous presence of enzyme-bound ATP and EP in H/K-ATPase during turnover rather than the alternative appearance of E1ATP and E2ATP sites.

In this paper, we show, for the first time for a P-type ATPase, that 2 mol of P_i are liberated, one from each mol of EP formed as the result of high-affinity ATP binding in half of the enzyme and the other from EATP formed as a result of low-affinity ATP binding in another half of a cross-talking oligomeric H/K-ATPase.

MATERIALS AND METHODS

Methods have been described for the preparation of vesicles (G1 fraction) which contain pig gastric H/K-ATPase (*16*) and their further purification by SDS (*33*). Enzyme preparations were stored in 250 mM sucrose containing 0.5 mM EGTA/Tris (pH 7.4) at −80 °C. Specific activities of H/K-ATPase preparations assayed in the presence of 2 mM $MgCl_2$, 25 mM sucrose, 0.1 mM EGTA/Tris, 40 mM HEPES/Tris (pH 7.0), 10 mM KCl, and 1 mM ATP/Tris were greater than 400 μmol of P_i/mg of protein/h at 37 °C. Protein concentration was measured by the method of Bradford (*34*) using bovine serum albumin as standard (*16*). Methods for the determination of the amount of EP from [γ-^{32}P]ATP and that of the liberated P_i have been described previously (*14*). All phosphorylation, dephosphorylation, and binding experiments were done at pH 7.0 and at 0 °C, unless otherwise stated. To estimate the amount of bound ^{32}P from [γ- or α-^{32}P]ATP, the enzyme (0.2 ∼ 4.0 mg of protein/mL) was incubated at 0 °C with 25 μL of a solution containing 40 mM HEPES/Tris (pH 7.0), 2 mM $MgCl_2$ and various concentrations of [γ- or α-^{32}P]ATP, and 50 mM [^{3}H]-glucose to estimate water space. Approximately 20 μL of the mixture, with or without the enzyme, was applied to a set of two membrane filters (upper: a Bio-Rad nitrocellulose filter with a pore size of 0.45 μm for trapping the enzyme; lower: a type AA Millipore filter with a pore size of 0.8 μm for trapping the filtrate). About 3 μL of the reaction mixture containing little enzyme protein was then trapped in the Millipore filter by aspirating for 5 s at 3 °C (*35*). The Millipore filters were incubated with 1 mL of a solution containing unlabeled 5 mM ATP/Tris and 50 mM glucose for 30 min. After the removal of the filter, a scintillation cocktail was added and counted. The ratio of radioactivity ^{32}P/^{3}H in the filtrate could be measured accurately when the radioactivity (cpm) of [^{3}H]glucose was around 2∼3 times higher than that of ^{32}P, and the concentrations of ^{32}P in the filtrate were calculated from the ratio. The amount of ^{32}P bound to the enzyme was calculated from the difference between the ratio of radioactivity ^{32}P/^{3}H of the filtrate in the absence and presence of the enzyme in the reaction mixture. The amount of [γ-^{32}P]ATP bound to the enzyme in the presence of CDTA by the filtration method (*14*, *35*) was compared with that by a centrifugation method (*27*). The data obtained were the same within experimental error, which indicates that the recovery of the enzyme-ligand complex is quantitative by either method. The [α- or γ-^{32}P]-ATP and [^{3}H]glucose were purchased from Amersham Phamacia Biotech. The $H_3{}^{32}PO_4$ was purchased from NEN

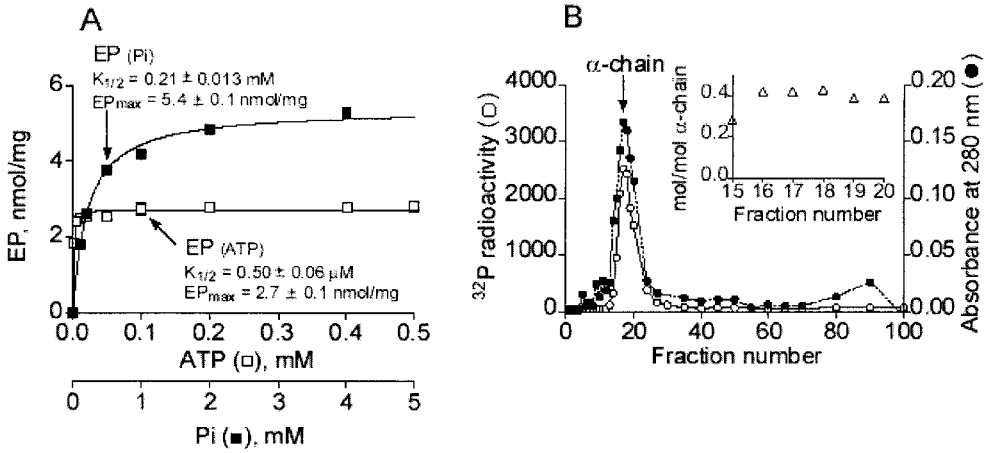

FIGURE 1: Determination of the maximum amount of phosphorylation/α chain using a Superdex 200 gel filtration column. (A) Phosphorylation was initiated by adding 0.05 mL of 0.002, 0.01, 0.02, 0.1, 0.2, 0.4, 0.8, and 1.0 mM of [γ-^{32}P]ATP/Tris or 0.2, 0.4, 1.0, 2.0, 4.0, and 8 mM $^{32}P_i$/Tris to 0.05 mL of a reaction mixture containing 4 mM $MgCl_2$, 80 mM HEPES/Tris (pH 7.0), and 0.01−0.1 mg of protein of SDS-treated H/K-ATPase preparation. The reaction was allowed to continue for 10 s at 0 °C or for 5 min at 37 °C for the phosphorylation from ATP and P_i, respectively. The reaction was terminated by the addition of 0.3 mL of an ice-cold 10% trichloroacetic acid solution containing 10 mM H_3PO_4 and 1 mM ATP (TCA solution). The denatured enzyme was centrifuged at 15 000 rpm (24 000*g*) at 4 °C for 10 min using a Hitachi Himac CF15D RT15A3 rotor. The precipitates were suspended and counted as EP from ATP (□) or P_i (■). (B) For the measurement of the amount of EP/α chain, an SDS-treated H/K-ATPase preparation (1 mg of protein) was phosphorylated in a 0.5 mL solution containing 2 mM $MgCl_2$, 40 mM HEPES/Tris (pH 7.0), and 0.5 mM [γ-^{32}P]ATP/Tris for 10 s at 0 °C. The reaction was terminated by the addition of 0.5 mL of the TCA solution, and the denatured enzyme (1 mg) was precipitated by centrifugation at 70 000 rpm (265 000*g*) for 5 min at 4 °C (Optima, TLA 100.3; Beckman). The pellet was suspended in ice-cold distilled water and centrifuged a second time. The addition of 4% lithium dodecyl sulfate (LDS) (*23*) containing 50 mM CH_3COOLi (pH 4.2) and 100 mM Li_2SO_4 solubilized up to 80% of the radioactivity. The supernatant was applied to a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech; 1.0 × 30 cm) equilibrated with a 50 mM CH_3COOLi (pH 4.2) elution buffer containing 100 mM Li_2SO_4, 1 mM H_3PO_4, and 1% LDS. Each 0.2 mL fraction was collected at a flow rate of 15 mL/h at 4 °C. The radioactivity (○) and absorbance (●) at 280 nm were measured. The inset shows the content of phosphorylated α chain (△) which was calculated from the ratio of the amount of phosphorylated α chain to the total amount of α chain. The former and the latter were estimated from the specific activity of the ^{32}P bound to the α chain and the absorbance at 280 nm using an extinction coefficient of 105 500 M^{-1} cm^{-1}. Data shown are for the second column chromatographic separation of the peak fractions obtained from the first column.

Research Products. Other chemicals were of the highest grade commercially available.

RESULTS AND DISCUSSION

Maximum Amount of Phosphoenzyme from ATP or P_i per α Chain. Determination of the phosphorylation capacity and the amount of bound ATP/α chain during turnover is essential for the understanding of the ATP-induced molecular events in H/K-ATPase. Figure 1A shows the amount of acid-stable phosphoenzyme, (E^{32}P)/mg of protein, of the SDS-purified H/K-ATPase under steady-state conditions versus the concentration of [γ-^{32}P]ATP or $^{32}P_i$. The maximum amount of EP obtained from ATP is exactly half the amount obtained from P_i. Similar observations were made before in H/K-ATPase membrane preparations without SDS treatment (*16*).

To determine the absolute amount of EP/α chain, SDS-purified enzyme was fully phosphorylated by [γ-^{32}P]ATP and solubilized, as described in the legend to Figure 1B. The resulting supernatant was subjected to column separation in order to isolate the α chains. A major single peak of radioactivity, which also absorbed UV, appeared with a shoulder of absorption, indicating that the former and latter were mainly α (catalytic subunit) and β (glyco-peptide) chains, respectively. Little significant radioactivity was detected in the fractions containing unlabeled P_i (not shown). Thus, an acid-stable EP can be isolated quantitatively by a column chromatographic separation at 4 °C, as has already been reported in the case of Ca/H-ATPase (*18*, *19*) and Na/K-ATPase (*28*). However, SDS gel electrophoresis of the main radioactive peak fractions showed evidence of contamination by the β chain. Thus, the main peak fraction was subjected to a second column chromatographic separation, as shown in Figure 1B. The ratio of the amount of phosphorylated α chain to that of total α chain was calculated from each fraction (Figure 1B, inset). The ratio reached a maximum constant value of 0.43 ± 0.01/mol of α chain. Differences in absorption between the two different pairs from fraction numbers 15−20 were obtained in order to minimize the effect of light scattering on the absorption so that the amount of α chain could be correctly estimated. When these values were used, the amount of phosphorylated α chain was found to be 0.55 ± 0.08 mol/mol of α chain (n = 11) except for four pairs, which had very small differences in absorption. These data show that phosphorylation from ATP occurs in only half of the α chains and suggest that ATP binds to the other half during turnover, as in the case of Na/K-ATPase (*14*, *28*).

Dependence of the Amount of Acid-Stable E^{32}P and Acid-Labile Enzyme-Bound ^{32}P on the Concentration of AT^{32}P. The results of the experiments depicted in Figure 2A (closed squares) showed that the amount of acid-stable E^{32}P formed in the presence of 2 mM Mg^{2+} depended on the concentration of [γ-^{32}P]ATP ($K_{1/2}$ = 1.1 μM) and that the maximal level of 0.5 mol of E^{32}P/mol of α chain was obtained. When the enzyme was similarly exposed to varying concentrations of [γ-^{32}P]ATP and the amount of bound ^{32}P to the enzyme that was not acid-denatured was measured (Figure 2A, closed circles), a biphasic curve was obtained ($K_{1/2}$ values: 1.1 μM and 0.15 mM), and the maximal level of enzyme-bound ^{32}P

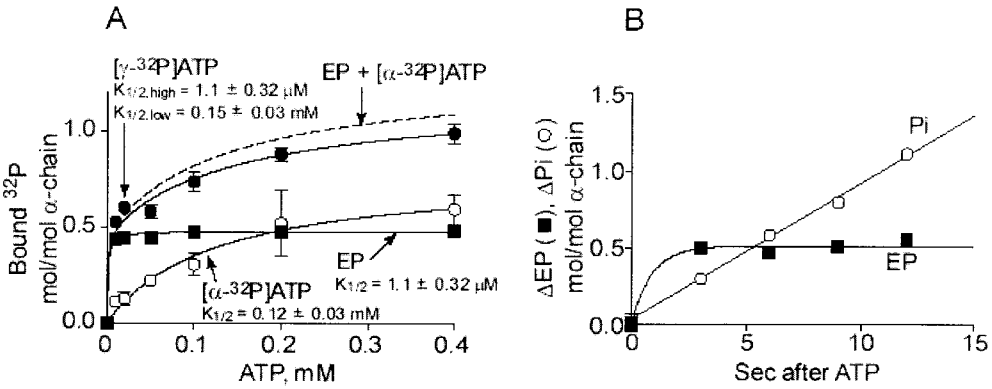

FIGURE 2: Effects of varying concentrations of [α- or γ-^{32}P]ATP on the amount of ^{32}P bound to the enzyme during turnover and on the amount of acid stable phosphoenzyme (EP) from [γ-^{32}P]ATP. (A) ^{32}P bound to the SDS-treated H/K-ATPase preparations in the presence of [γ-^{32}P]ATP/Tris (●) and [α-^{32}P]ATP/Tris (○) were estimated from the difference between the counts of the reaction mixture and the counts of the filtrate in the Millipore filter (*31*). The amounts of ^{32}P bound/mg of protein were corrected as mol/mol of α chain from the maximum amount of TCA-stable E^{32}P (closed squares) to be 0.5 mol/mol of α chain. The data obtained are plotted against the initial concentrations of ATP added; 0.01, 0.02, 0.05, 0.1, 0.2, and 0.4 mM. Data shown in this paper are the means ± SD for three or four independent experiments. (B) Time course of E^{32}P formation (■) and $^{32}P_i$ liberation (○) was initiated by the addition of 0.05 mL of 0.2 mM [γ-^{32}P]ATP/Tris to 0.05 mL of a reaction mixture containing 2 mM $MgCl_2$, 40 mM HEPES/Tris, and 0.1 mg protein of H/K-ATPase membrane preparation (G1 fraction). The reaction was stopped at the indicated time in the figure by the addition of 0.3 mL of the TCA solution. The denatured enzyme was centrifuged as described in Figure 1A. The precipitates were counted as EP, and the resulting supernatant was treated with charcoal to estimate the amount of P_i liberated (*14*).

was ~1 mol/mol of α chain. The results of similar binding experiments with varying concentrations of [α-^{32}P]ATP showed a monophasic curve ($K_{1/2}$ = 0.12 mM) and a maximal level of ~0.5 mol/mol of α chain (Figure 2A, open circles). These data suggest that 1 mol of the enzyme-bound ^{32}P that is obtained in the presence of [γ-^{32}P]ATP is due to the accumulation of 0.5 mol of an acid-stable E^{32}P and 0.5 mol of an acid-labile enzyme-bound [γ-^{32}P]ATP (EATP) or $^{32}P_i$ (EP_i). In experiments of Figure 2B, the time course for $^{32}P_i$ liberation, assayed after stopping the reaction with trichloroacetic acid, was determined. Little initial burst of $^{32}P_i$ production was noted. This excludes the presence of an acid-labile EP_i complex. On the other hand, the data obtained in the presence of [α-^{32}P]ATP (Figure 2A) suggest the possibility of the presence of 0.5 mol of an acid-labile [α-^{32}P]-ATP (EATP) or [α-^{32}P]ADP (EADP) bound to the enzyme. These experimental data can best be explained by assuming that H/K-ATPase accumulates 0.5 mol of an acid-stable EP ($K_{1/2}$ = 1.1 μM) plus 0.5 mol of an acid-labile EATP ($K_{1/2}$ = 0.12 mM) per α chain in the presence of Mg^{2+} and high concentrations of ATP (i.e., [1 mol of EP + 1 mol of EATP]/ 2 α chains). The maximum amount of EP/mol of α chain from P_i was shown to be 1 (Figure 1A). In fact, the amount of ^{32}P binding/α chain in the presence of [γ-^{32}P]ATP (Figure 2A, closed circles), as detected by double membrane filtration during turnover (*14*, *35*), was very close to the sum (dotted line) of the directly detected acid-stable E^{32}P formed in the presence of [γ-^{32}P]ATP (closed squares) and acid-labile EAT^{32}P (open circles) formed in the presence of [α-^{32}P]ATP (i.e., about 1 mol/mol of α chain (Figure 2A)).

Taken together, the data of Figure 2 appear to negate possibilities such as 2 mol of ATP binding sites/α chain or 1 mol of EP + 1 mol of EATP/α chain. The simultaneous formation of EP and EATP would not occur in the presence of the low concentrations of ATP that are sufficient to form EP ($K_{1/2}$ = 1.1 μM) but too low to form EATP ($K_{1/2}$ = 0.12 mM). We conclude that H/K-ATPase, in the presence of high concentrations of ATP, accumulates 1 mol of EP/mol of α chain in half of the enzyme molecules and 1 mol of EATP/ mol of α chain in another half of the enzyme molecules rather 1 mol of each EP and EATP in the same α chain.

Time Course of EP and EATP Breakdown and P_i Liberation. To investigate the fate of EATP which is simultaneously present with EP in the presence of high concentrations of ATP, experiments were conducted in which the enzyme was first incubated with a selected fixed concentration [γ-^{32}P]-ATP under turnover conditions, as in Figure 2. After 10 s, an excess of nonradioactive ATP was added, and the time course of the breakdown of E^{32}P and the liberation of $^{32}P_i$ was determined. For each selected fixed initial concentration of [γ-^{32}P]ATP used, the amounts of E^{32}P and EAT^{32}P present prior to the chase were calculated based on the Michaelis−Menten equation and the constants ($K_{1/2}$ and maximal binding values) derived in experiments of Figure 2A. These calculated initial amounts of E^{32}P and EAT^{32}P, and the experimentally determined values of E^{32}P and $^{32}P_i$, were then used to express the results of the time course experiments (Figure 3).

When 0.01 mM [γ-^{32}P]ATP was used, and the E^{32}P and EAT^{32}P accumulated prior to the chase were 0.45 and 0.04 mol/mol of α chain, respectively, the addition of nonradioactive ATP induced the breakdown of ~0.45 mol of E^{32}P accompanied by the same amount of liberation of $^{32}P_i$ with similar rate constants (Figure 3A). The ratio (crosses) between the amount of $^{32}P_i$ liberated (ΔP_i) and that of E^{32}P which had disappeared (ΔEP) induced by the addition of nonradioactive ATP was ~1 (dotted line). When 0.2 mM [γ-^{32}P]ATP was used, which produced 0.5 mol E^{32}P + ~0. 3 mol EAT^{32}P/mol of α chain (Figure 2), the addition of nonradioactive ATP was found to induce the breakdown of E^{32}P accompanied by the liberation of ~1.5-fold of $^{32}P_i$ with the same rate constants (Figure 3B). When both E^{32}P and EAT^{32}P were accumulated in the presence of 0.4 mM [γ-^{32}P]-ATP, which produced 0.5 mol E^{32}P + ~0. 4 mol EAT^{32}P/ mol of α chain, the addition of nonradioactive ATP induced the breakdown of E^{32}P accompanied by the liberation of ~1.8-fold of $^{32}P_i$. These data clearly show that the breakdown of EAT^{32}P is accompanied by $^{32}P_i$ production. The time

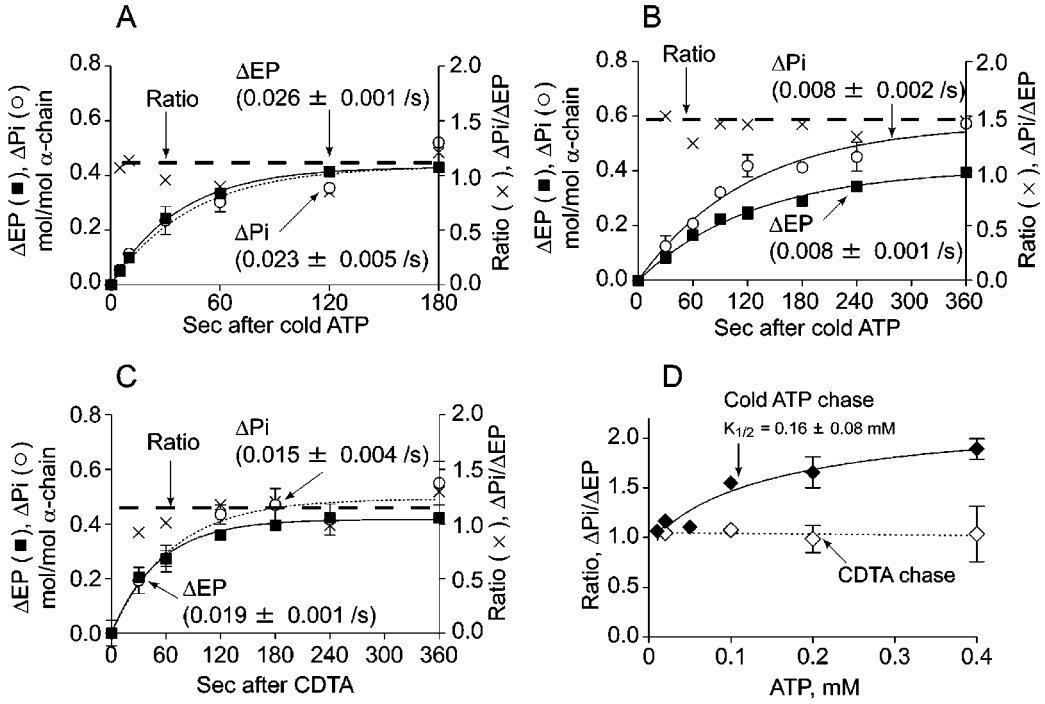

FIGURE 3: Time course for EP breakdown and P_i liberation in the absence and presence of EATP. Phosphorylation was initiated by adding 0.05 mL of a solution containing 0.02, 0.04, 0.1, 0.2, 0.4, and 0.8 mM of [γ-^{32}P]ATP/Tris to 0.05 mL of a reaction mixture containing 4 mM $MgCl_2$, 80 mM HEPES/Tris, and 0.05 to 0.2 mg protein of SDS-treated H/K-ATPase preparation. The reaction was chased after 10 s with 0.5 mL of a solution containing 40 mM HEPES/Tris and 5 mM $MgCl_2$ with 0.2, 0.4, 1, 2, 4, and 8 mM nonradioactive ATP/Tris or 40 mM HEPES/Tris with 20 mM CDTA/Tris. It was terminated at various times by the addition of 0.3 mL of ice-cold 20% TCA containing 10 mM H_3PO_4. The denatured enzyme was centrifuged as described in Figure 1A. The precipitates were suspended and counted as EP (■). The supernatants were treated with charcoal and the amounts of P_i liberated (○) were measured (*14*). Blank experiments were done by the addition of solution containing radioactive + nonradioactive ATP to the enzyme to measure background levels of $E^{32}P$ and also by measuring $^{32}P_i$ liberation. The data showed negligible increase over the time range studied. The amount of $^{32}P_i$ liberated (ΔP_i, ○) and that of $E^{32}P$ which had disappeared (ΔEP, ■) and the ratio ($\Delta P_i/\Delta EP$, ×, ◊, ♦) were plotted. The apparent first-order rate constants of EP breakdown and P_i liberation were estimated by PRISM 2.01. (A) One-tenth milliliter of the phosphorylation solution contained 0.01 mM [γ-^{32}P]ATP/Tris, and 0.5 mL of the chase solution contained 0.2 mM of nonradioactive ATP/Tris. (B) One-tenth milliliter of phosphorylation solution contained 0.2 mM [γ-^{32}P]ATP/Tris, and 0.5 mL of the chase solution contained 4 mM of nonradioactive ATP/Tris. (C) One-tenth milliliter of phosphorylation solution contained 0.2 mM [γ-^{32}P]ATP/Tris, and 0.5 mL of the chase solution contained 20 mM CDTA/Tris. (D) One-tenth milliliter of phosphorylation solution contained 0.01, 0.02, 0.05, 0.1, 0.2, and 0.4 mM [γ-^{32}P]ATP/Tris and 0.5 mL of the chase solution contained 0.2, 0.4, 1, 2, 4, and 8 mM nonradioactive ATP/Tris, or 0.1 mL of phosphorylation solution contained 0.02, 0.1, 0.2, and 0.4 mM [γ-^{32}P]ATP/Tris and 0.5 mL of the chase solution contained 20 mM CDTA/Tris. To obtain better signal-to-noise ratio, the ratios of the amount of $^{32}P_i$ liberation to the decrease of the amount of $E^{32}P$ at 240 s after a chase were taken. They were plotted with various initial concentrations of [γ-^{32}P]ATP with Mg^{2+} (♦) or with CDTA (◊).

course for the breakdown of $E^{32}P$ was nearly the same as that for the $^{32}P_i$ liberation, within experimental error. When the ratios between ΔP_i and ΔEP were plotted against ATP concentrations (Figure 3D, closed diamonds), the ratio increased from 1 to ~2 with increasing concentrations of ATP ($K_{1/2}$ = 0.16 mM).

If EP and EATP break down with the same rate constant, the time course for the decrease in $E^{32}P$ and total enzyme-bound ^{32}P should be the same. The time courses for the disappearance of enzyme-bound ^{32}P (0.5 mol of $E^{32}P$ + ~0.3 mol of $EAT^{32}P$) and 0.5 mol of acid-stable $E^{32}P$ in the presence of 0.2 mM [γ-^{32}P]ATP after the addition of a 100 fold excess of nonradioactive ATP were nearly the same within experimental error (0.015 ± 0.007/s and 0.020 ± 0.002/s) with the ratio of $\Delta^{32}P_i/\Delta E^{32}P$ = ~1.5. These data suggest that both EP and EATP are reaction intermediates for the H^+-ATPase in liberating stoichiometric amounts of P_i from EP and EATP.

State of Bound Mg^{2+} for Liberation of P_i from EP and EATP. When both $E^{32}P$ and $EAT^{32}P$ were accumulated in the presence of 0.2 mM [γ-^{32}P]ATP, and the reaction was chased with CDTA to chelate Mg^{2+}, 0.5 mol of $E^{32}P$ breakdown was accompanied by ~0.5 mol of $^{32}P_i$ liberation (Figure 3C), although ~0.3 mol of $EAT^{32}P$ had been present (Figure 2A). The data suggest that Mg^{2+} is required for P_i liberation from EATP. The presence of CDTA had little effect on the stoichiometry of $^{32}P_i$ liberation from $E^{32}P$ (Figure 3C,D). These data suggest that $MgAT^{32}P$ at a low-affinity site in half of the enzyme molecules binds tightly to liberate a stoichiometric amount of $^{32}P_i$, despite the presence of a large excess of nonradioactive MgATP. However the Mg^{2+} could be chelated by CDTA rather easily to inhibit $^{32}P_i$ liberation. On the other hand, the Mg^{2+} required to liberate $^{32}P_i$ from $E^{32}P$ in the other half of the enzyme is embedded deeply such that it is not chelated with CDTA (Figure 3C), as in the case of Na/K-ATPase (*36*).

Dependence of the Turnover Number and the Rate Constant of EP Breakdown on Concentration of ATP. The time course of $E^{32}P$ breakdown after the addition of nonradioactive ATP with or without CDTA were measured in the presence of various concentrations of ATP to compare the turnover number of the enzyme. Figure 4 shows the turnover number (v/EP) and the rate constant for EP breakdown as a function of ATP. The amount of EP from

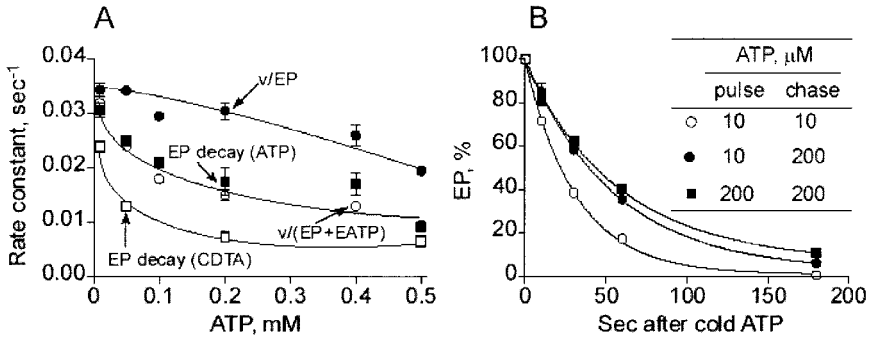

FIGURE 4: Dependence on ATP concentration of the turnover number of the enzyme and the rate constant of breakdown of EP with Mg^{2+} or CDTA. (A) Turnover numbers, v/EP (●) and v/[EP + EATP] (○), were estimated from $^{32}P_i$ liberation due to the H^+-ATPase activity (v) and the amounts of $E^{32}P$ from [γ-^{32}P]ATP/Tris and $EAT^{32}P$ from [α-^{32}P]ATP/Tris, shown in Figure 2A. The H^+-ATPase reaction was initiated by the addition of 0.72 mL of substrate solution containing 0.011, 0.055, 0.111, 0.222, 0.444, and 0.556 mM [γ-^{32}P]ATP/Tris, 2 mM $MgCl_2$, and 40 mM HEPES/Tris to 0.08 mL of enzyme solution containing 40 mM HEPES/Tris, 2 mM $MgCl_2$, and 0.05 mg of protein of SDS-treated H/K-ATPase preparation. The reaction was terminated at 10−60 min before the occurrence of up to 10% [γ-^{32}P]ATP/Tris breakdown by the addition of 0.4 mL of 20% TCA containing 10 mM H_3PO_4. To estimate apparent rate constants for the breakdown of EP, the phosphorylation was initiated by adding 0.04 mL of substrate solution containing 0.02, 0.1, 0.2, 0.4, 0.8, and 1 mM [γ-^{32}P]ATP/Tris to 0.04 mL of an enzyme solution containing 80 mM HEPES/Tris, 4 mM $MgCl_2$, and 0.05 mg of protein of SDS-treated H/K-ATPase preparation. The reaction was chased at 10 s with 0.72 mL of solution containing 40 mM HEPES/Tris and 0.01, 0.05, 0.1, 0.2, 0.4, or 0.5 mM nonradioactive ATP/Tris (■) with 2 mM $MgCl_2$ or with 10 mM CDTA/Tris (○). It was stopped at various times as described in Figure 3. (B) One-twentieth milligram of SDS-treated H/K-ATPase preparation was phosphorylated by 0.01 (○, ●) or 0.2 mM [γ-^{32}P]-ATP/Tris (■) for 10 s in a final volume 0.08 mL. They were chased with 0.72 mL of a solution containing 0.01 mM (○) or 0.2 mM nonradioactive ATP/Tris (●, ■).

ATP under these conditions was constant and almost saturated (Figures 1A and 2A). These rate constants were decreased by the addition of ATP with CDTA more strongly than without CDTA. The turnover number of the intermediates, as estimated from v/[EP + EATP] with increasing concentrations of ATP (open circles) but not v/EP (closed circles), were very close to the apparent rate constants for the break down of $E^{32}P$ (closed squares) induced by the addition of nonradioactive ATP without CDTA. These data support the hypothesis that both EP and EATP are reaction intermediates.

CDTA completely inhibited $^{32}P_i$ liberation from $EAT^{32}P$ in the presence of 0.01−0.4 mM [γ-^{32}P]ATP (Figure 3C,D). These data suggest that bound ATP in the presence of CDTA directly reduced the rate of breakdown of $E^{32}P$ with little effect on the stoichiometry of $^{32}P_i$ liberation from $E^{32}P$ (Figure 3C). High concentrations of ATP may affect oligomeric interactions, because it has been reported that 2 mM ATP inhibited the dimerization of α chains by Cu^{2+}-phenanthroline, independent of Mg^{2+} (*24*).

The question arises as to whether the decrease in the rate of $E^{32}P$ breakdown with increasing [γ-^{32}P]ATP concentrations without CDTA (Figure 4A, closed squares) is due to formation of $E^{32}P$ from $EAT^{32}P$. To investigate this issue, pulse−chase experiments for $E^{32}P$ were performed. As control experiments, 0.45 mol of $E^{32}P$ + ∼0.04 mol of $EAT^{32}P$ (Figure 2) was formed in the presence of 0.01 mM [γ-^{32}P]ATP, and the chase was done with nonradioactive 0.01 and 0.2 mM ATP, respectively. The rate constant for $E^{32}P$ breakdown was 0.032 ± 0.001/s and 0.018 ± 0.001/s, respectively (Figure 4B, open circles and closed circles), showing that breakdown was inhibited to nearly half by 0.2 mM ATP. When 0.5 mol of $E^{32}P$ and ∼0.3 mol of $EAT^{32}P$ were formed in the presence of 0.2 mM [γ-^{32}P]ATP, a chase experiment using 0.2 mM nonradioactive ATP (Figure 4B, closed squares) gave a similar low value (0.017 ± 0.001/s) to that obtained in the presence of 0.01 mM [γ-^{32}P]ATP followed by a 0.2 mM ATP chase.

The aforesaid data show that both $EAT^{32}P$ and a newly formed nonradioactive EATP were equally effective in reducing the rate of breakdown of $E^{32}P$, independent of the presence of Mg^{2+}, which is required for the liberation of $^{32}P_i$ from $EAT^{32}P$. The slow conversion of $EAT^{32}P$ to $E^{32}P$, resulting in an apparent reduction of $E^{32}P$ breakdown, seems to be unlikely. These data suggest that the liberation of $^{32}P_i$ from $E^{32}P$ in one subunit, and that from $EAT^{32}P$ in the other, constitute rate-determining steps for ATP hydrolysis in each subunit in the presence of high concentrations of ATP. The reason for the reduction is due to ATP binding to the low-affinity site. The possibility remains that ATP hydrolysis from EATP occurs via a phosphorylated intermediate that hydrolyzes rapidly and does not accumulate because of interaction between the half-site phosphorylated enzyme and the half-site ATP bound enzyme. The presence of a rapid transient phosphorylation, preceding the steady-state accumulation of EP, has been reported for Na/K-ATPase (*37*).

Dependence of H^+-ATPase and H/K-ATPase on Concentration of ATP at 0 and 37 °C. The H^+-ATPase activity at 0 °C was inhibited by increasing concentrations of ATP, namely, by EATP in the presence of Mg^{2+} (Figure 4A). To investigate the issue of whether this happens in H/K-ATPase activity, this activity was measured by adding 10 mM KCl to the H^+-ATPase reaction mixture, while varying the ATP concentrations from 0.5 μM to 0.488 mM at 0 °C. Little ATP-induced inhibition was observed over the concentration range studied. A direct linear plot of Cornish-Bowden (*38*) of these data was consistent with a single ATP site with a $K_{1/2}$ = ∼3 μM and V_{max} = 20 μmol/mg of protein/h (∼0.9/s). When H/K-ATPase activity was measured at 37 °C, two different activation sites appeared with $K_{1/2}$ = 2 and 80 μM and with V_{max} = 100 (∼4.6/s) and 460 μmol/mg of protein/h (∼21/s), respectively. Different activation sites for ATP have already been shown at 25 °C (*39*). These data show that a low-affinity ATP binding site or an inhibitory site ($K_{1/2}$ = ∼0.1 mM) appeared at 0 °C when K^+ was absent. However,

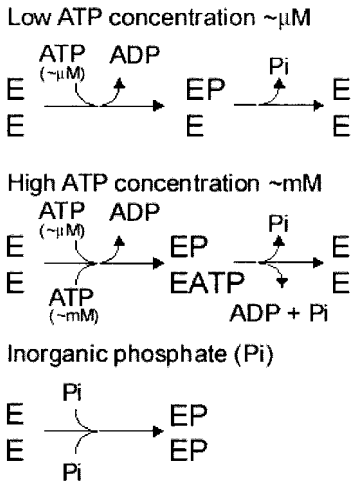

FIGURE 5: H^+-dependent ATP hydrolysis in the presence of low and high concentrations of ATP. Half-site reactivity occurs in the presence of ~μM ATP, resulting in 1 mol of P_i liberation from 1 mol of EP formed in half of the α chains without the formation of EATP in the other half during turnover. All-sites reactivity occurs in the presence of up to sub mM ATP, resulting in 2 mol of P_i liberation from 1 mol of both EP and EATP in each half of the α chains, prohibiting all-sites phosphorylation by ATP because of cross-talking between subunits. All-sites phosphorylation from P_i occurs as shown. Mg^{2+} is not shown for simplicity (see text).

the presence of K^+ accelerated the overall reaction ~20 (0.9/~0.04)-fold. When the temperature was increased, the low-affinity activation site appeared, possibly because of a change in the rate-determining step in the H/K-ATPase reaction.

Half-Site and All-Sites Reactivity. It is possible, but not likely, that these enzyme preparations used were 50% denatured, as has been suggested (*17*), which might lead to the data described here. However, this can be completely ruled out by the following: (1) full-site and half-site phosphorylation, respectively, by P_i and ATP (*16*); (2) 1 mol of ^{32}P binding /mol of α chain in the presence of Mg^{2+} and high concentrations of [γ-^{32}P]ATP (Figure 2); (3) ~2 mol of $^{32}P_i$ liberation from 1 mol of each E^{32}P and EAT^{32}P (Figure 3D); and (4) the reduced rate of P_i liberation in the absence of K^+ by bound ATP or EP (Figure 4A,B). It is well-known that high concentrations of SDS denature this enzyme irreversibly. The question also arises as to whether the lower SDS concentration used to purify the enzyme may have altered some of the properties of the enzyme as compared to the enzyme without detergent treatment. However, this also does not appear to be the case, because the ratio of the amount of EP from ATP to that from P_i to that of [γ-^{32}P]-ATP binding to that of [α-^{32}P]ATP binding were shown to be around 0.5/1/1/0.5 both before and after SDS treatment.

The present data show that H^+-dependent ATP hydrolysis occurs via a single pathway in the presence of ~μM ATP, as has been shown in a widely accepted conventional scheme (Scheme 1) but that it occurs via two parallel pathways with an increase in ATP concentrations, up to sub mM (Figure 5). One mole of P_i liberation occurs from the EP formed as the result of high-affinity ATP binding in one α chain, and another 1 mol from the EATP formed with low affinity ATP binding in another α chain with no detectable amount of corresponding accumulation of EP (Figure 4B). The possibility of the presence of a significant amount of enzyme-bound ADP during ATP hydrolysis can be rejected because ^{32}P binding in the presence of [α-^{32}P]ATP and [γ-^{32}P]ATP was shown, in each case, to be due to ATP binding to the enzyme. Thus, ADP would be liberated from EATP before the next cycle starts.

The present findings provide the first direct evidence for the participation of both EP with tightly bound Mg^{2+} and EATP with loosely bound Mg^{2+} as reaction intermediates for any P-type ATPase under steady-state conditions. At least two catalytic subunits, each of which forms EP and EATP, interact to maintain half-site phosphorylation and half-site ATP binding. Neither ping-pong (*3*) nor flip-flop mechanisms (*40*, *41*) seems to explain the parallel liberation of P_i from both EP and EATP. Further studies will be required to understand the mechanism of ATP hydrolysis through EATP and to determine whether EATP breakdown occurs via EP or not. This would be crucial for a better understanding of the mechanism of the active transport across membranes driven by P-type ATPases.

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