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ATP/ADP EXCHANGE ACTIVITY OF GASTRIC ($H^+ + K^+$)-ATPase

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The ATP/ADP exchange is shown to be a partial reaction of the ($H^+ + K^+$)-ATPase by the absence of measurable nucleoside diphosphokinase activity and the insensitivity of the reaction to P^1, P^5-di(adenosine-5') pentaphosphate, a myokinase inhibitor. The exchange demonstrates an absolute requirement for Mg^{2+} and is optimal at an ADP/ATP ratio of 2. The high ATP concentration ($K_{0.5} = 116\ \mu M$) required for maximal exchange is interpreted as evidence for the involvement of a low affinity form of nucleotide site. The ATP/ADP exchange is regarded as evidence for an ADP-sensitive form of the phosphoenzyme. In native enzyme, pre-steady state kinetics show that the formation of the phosphoenzyme is partially sensitive to ADP while modification of the enzyme by pretreatment with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the absence of Mg^{2+} results in a steady-state phosphoenzyme population, a component of which is ADP sensitive. The ATP/ADP exchange reaction can be either stimulated or inhibited by the presence of K^+ as a function of pH and Mg^{2+}.

Introduction

Several lines of evidence suggest that the gastric ($H^+ + K^+$)-ATPase is responsible for acid secretion by the parietal cell [1,2]. The ability of this enzyme to generate proton gradients appears to involve an H^+ for K^+ exchange [3] that is electroneutral. In contrast to the proton pump of mitochondria and chloroplasts [4], a phosphoprotein intermediate is formed during incubation with MgATP, and is discharged upon the addition of K^+ [5,6]. Analysis of the enzyme mechanism by transient kinetics has suggested a reaction scheme whereby the initial step is formation of an enzyme-ATP complex followed by an Mg^{2+}-catalysed transphosphorylation. K^+ bound to the cytosolic face of the enzyme competes with H^+ and ATP, thus inhibiting formation of the phosphoenzyme EP. EP breaks down to enzyme and P_i at an increasing rate as pH increases, or if K^+ is present at the luminal face of the enzyme [7]. As such, therefore, there is considerable similarity between this mechanism and the ($Na^+ + K^+$)- and Ca^{2+}-ATPase reaction pathway [8–10]. Based on the schemes elaborated, it was postulated that two forms of EP existed, an ADP-sensitive, K^+-insensitive form and an ADP-insensitive, K^+-sensitive form [5]. The most direct evidence for this idea, namely discharge of EP by ADP addition, was difficult to obtain either with flow quench or manual techniques. Moreover, non-linear Eadie-Hofstee plots of basal Mg^{2+}-dependent ATPase activity [5] and more recent data using HVO_4^{2-} inhibition of ATP hydrolysis [11] are in-

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Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); Pipes, 1,4-piperazine-diethanesulfonic acid.

consistent with a single functional ATP site. Thus the single site, single state, scheme previously presented did not adequately describe some of the enzyme properties. In this study, some of the properties of the ATP/ADP exchange activity of the ATPase are described, whereby the existence of an ADP-sensitive EP form of the enzyme can be established, and the behavior of this EP in the catalytic cycle explored.

Experimental procedures

Materials

The gastric ($K^+ + H^+$)-ATPase was prepared from yearling hog stomachs by previously published procedures [1]. In this method the enzyme was purified by differential and zonal density gradient centrifugation followed by free-flow electrophoresis. This fraction is enriched in one group of peptides ($M_r = 105000$, as defined by sodium dodecyl sulfate polyacrylamide gel electrophoresis) and was lyophilized and stored at −80°C until use.

[γ-^{32}P]ATP was obtained from Amersham Corporation (spec. act. 3000 Ci/mmol). [^{14}C]ADP was obtained from New England Nuclear, (spec. act. 40 Ci/mol). Tris-ADP and Tris-ATP were obtained from Sigma and immediately stored as a 20.0 mM stock solution at −80°C. PEI impregnated cellulose plates were obtained from Brinkmann and refrigerated until use. All other chemicals were of the highest purity available.

Methods

Steady-state kinetics. ATP/ADP exchange was measured by the incorporation of [^{14}C]ADP into ATP. This was accomplished typically in a 50.0 μl reaction volume containing 100.0 mM Tris-HCl, pH 7.4, 5.0 μg enzyme, 0.5 mM ATP, 1.0 mM [^{14}C]ADP (spec. act. 3500 cpm/nmol) and 50.0 μM $MgCl_2$. The 2.0 to 5.0 min room temperature incubation was stopped by the addition of ice-cold CDTA 15–150-fold in excess of Mg^{2+}. A 4.0 μl aliquot of the reaction solution was spotted on a plastic coated PEI plate and an ATP standard was added to aid in visualization. The chromatogram was developed in 1.2 M LiCl for 75 min. Following drying, the ATP spots were revealed by ultraviolet light, scraped and then counted in 10.0 ml of ACS counting fluid. Variation of experimental conditions are indicated in individual experiments.

Preliminary experiments indicated exchange increased linearly from 1.0 to 15.0 min. Typical incubations were 5.0 min with reduction to 2.0 min when nucleotide concentrations were below 100.0 μM in order to keep the sum of exchange activity and hydrolysis below 10% of the nucleotide initially present. All results are the average of assays performed in duplicate. As a test of myokinase activity the enzyme was incubated for 15.0 min at room temperature with 0.75 mM P^1, P^5-di(adenosine-5′) pentaphosphate before measurement of ATP/ADP exchange activity [12]. At this concentration, the exchange activity was insensitive to the addition of inhibitor. Nucleoside diphosphokinase was measured essentially as described by Mourad and Parks [13] with reduction of ATP to 1.0 mM and phospho*enol*pyruvate to 1.5 mM. In this assay no evidence was seen for activity other than that which could be ascribed to the ATPase activity.

Conditions for ATP hydrolysis were identical to that of the exchange assay except that [γ-^{32}P]ATP was used instead of [^{14}C]ADP. ATP hydrolysis was assayed as the release of $^{32}P_i$ from [γ-^{32}P]ATP [16]. After the reaction was stopped by the addition of excess CDTA the reaction volume was diluted by the addition of 1.0 ml of water and 1.5 ml of 5% ammonium molybdate in 2 M H_2SO_4. The [^{32}P]phosphomolybdate was then extracted into 2.5 ml of isobutanol/benzene (1 : 1 v/v). ATP hydrolysis was calculated from the $^{32}P_i$ contained in an aliquot of this layer.

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) treatment. Three 200.0 μl aliquots were taken as controls from a 1500.0 μl sample containing 1.8 mg · ml^{-1} enzyme suspended in 25.0 mM imidazole buffer, pH 7.3. At zero time 81.0 μl of 5.0 mM DTNB was added to the 900-μl aliquot remaining. Following a 30.0 min room temperature incubation 200-μl aliquots were layered upon 9.5 cm Econo columns packed with G-25 fine Sephadex. After adsorption of the protein suspension, successive aliquots of 220 μl and 1500 μl of 25 mM imidazole, pH 7.3, were layered onto the columns. The 220-μl eluate collected during the first addition of buffer was discarded. The protein was

collected in the next 700 μl of eluate. The untreated control suspension was applied and collected from the columns in a similar manner. The eluate was placed on ice. The phosphoenzyme sensitivity of this treated sample was immediately measured. This removal of DTNB by gel filtration is a modification of a procedure detailed by Bonting et al. [14]. Protein was measured by the method of Lowry et al. [15].

Pre-steady state kinetics; These experiments were carried out in a flow quench apparatus using methods which have been previously described [5,16]. By the use of one or two mixers in series either the formation or breakdown of the phosphorylated intermediate could be studied.

Following a reaction time of 5.0 to 120 ms the reaction was quenched by expelling the mixture into 5.0 ml of 10% $HClO_4$ (v/v) containing 5 mM ATP and 40 mM inorganic phosphate. The precipitated protein was collected on a 3.0 μm Millipore filter which was subsequently washed with 70.0 ml of 5% $HClO_4$ containing 10 mM P_i.

The radioactivity trapped on the filter was assayed by measuring its Čerenkov radiation in a Wallace LKB 81000 liquid scintillation counter. Details of the solutions are provided in the individual figure legends.

Results

Mg^{2+}. In the absence of added Mg^{2+}, no nucleotide exchange was detected. Thus, as shown in Table I, where exchange activity is presented as a function of total added Mg^{2+} concentration, it can be seen that approx. 60% of maximal exchange activity was obtained at 15 μM Mg^{2+}. A broad maximum occurred up to a concentration of 0.5 mM, but at higher Mg^{2+} levels inhibition of exchange was found. In this experiment ADP was 1 mM and ATP 0.5 mM.

TABLE I

Mg^{2+} REQUIREMENT FOR ATP/ADP EXCHANGE

5.0 μg of enzyme was incubated in a reaction solution consisting of 45.0 mM Pipes/Tris, pH 7.4 and $MgCl_2$ ranging from 15.0 μM to 3.0 mM. At zero time the reaction was started by the addition of 0.5 mM ATP and 1.0 mM [^{14}C]ADP.

Mg^{2+} (mM)	Exchange activity ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)
0.015	6.0
0.050	7.75
0.500	9.35
1.000	8.15
1.500	6.15
3.000	3.75

Nucleotide ratio. The initial steps at the high-affinity ATP site leading to formation of EP are considered to take place as follows [5]:

$$E + ATP \rightleftharpoons E \cdot ATP \quad (1)$$

$$E \cdot ATP + Mg^{2+} \rightleftharpoons Mg^{2+} \cdot EP \cdot ADP \quad (2)$$

$$Mg^{2+} \cdot EP \cdot ADP \rightleftharpoons Mg^{2+} \cdot EP + ADP \quad (3)$$

Given a single site/single function mechanism, maximal exchange would be anticipated following saturation of the substrate sites by 5 μM ATP. However, when the ADP/ATP ratio was fixed at 1 and the concentration of both nucleotides varied (Mg^{2+} = 50 μM), the apparent $K_{0.5}$ with respect to ATP was found to be 116 μM (data not shown). In this case the maximal rate of exchange was 8.7 $\mu mol \cdot mg^{-1} \cdot h^{-1}$.

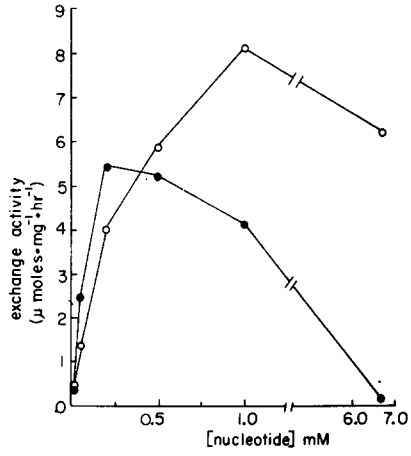

Fig. 1. Influence of the ATP/ADP ratio on the exchange reaction. ATP/ADP exchange activity was measured following a 2.0 min incubation at room temperature. 5.0 μg of enzyme was incubated in a reaction solution containing 45.0 mM Pipes/Tris, pH 7.4 and 50.0 μM $MgCl_2$. ADP was maintained at 0.5 mM while ATP was varied from 15.0 μM to 6.7 mM (●—●). ATP was maintained at 0.5 mM while ADP was varied from 15.0 μM to 6.7 mM (○——○).

Fig. 1 shows the data from two types of experiments, one in which ADP concentration was fixed at 0.5 mM and ATP varied and another in which ATP concentration was fixed at 0.5 mM and ADP varied. In both experiments the maximal rate of exchange was obtained at an ADP/ATP ratio of 2.0. The exchange reaction is slightly sensitive to variations in the ADP concentration at ADP/ATP ratios above 1 since a ratio of 13 is approx. 75% of maximal rate. A similar increase in ratio with varying ATP eliminates the exchange reaction.

Cation sensitivity of the ATP/ADP exchange. The exchange was measured in the presence of K^+. In Fig. 2 a biphasic effect of K^+ is noted at pH 7.4. Exchange is progressively stimulated by K^+ as the cation concentration is increased from 5.0 μM to 1.0 mM. The K^+-stimulated component of exchange is progressively inhibited as the concentration is increased from 20.0 to 100.0 mM. At 100.0 mM K^+ the exchange activity is equal to the basal Mg^{2+}-dependent exchange. In Table II the order of effectiveness for stimulation of exchange by monovalent cations is listed as $K^+ \equiv Rb^+ > NH_4^+ \equiv Tl^+ > Na^+ \equiv Cs^+ \equiv Li^+$.

pH dependence of ATP/ADP exchange. The primary function of the $(H^+ + K^+)$-ATPase is to couple the K^+-stimulated hydrolysis of ATP to the transmembrane movement of H^+ and K^+. To investigate the effect of proton concentration on the exchange reaction both the rate of basal Mg^{2+}- and K^+-stimulated exchange activity was measured over a pH range of 5.5 to 8.5. In Fig. 3 it is

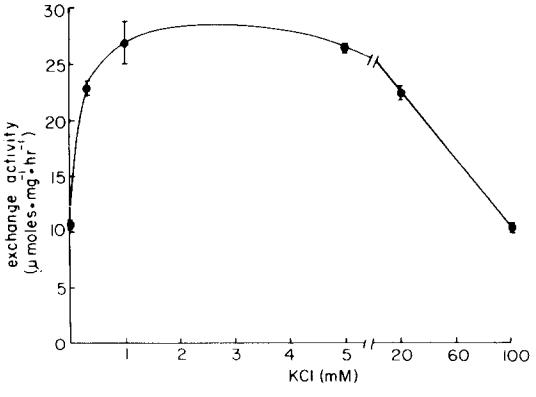

Fig. 2. Biphasic effect on ATP/ADP exchange of KCl. ATP/ADP exchange activity was measured following a 5.0 min incubation at room temperature. The 50.0 μl reaction solution contained 45 mM Pipes/Tris, pH 7.4, 1.0 mM ADP, 0.5 mM ATP, 1.0 mM $MgCl_2$ and 5.0 μg enzyme. The KCl concentration was varied from 0.1 mM to 100.0 mM. $MgCl_2$ dependent exchange was measured in the absence of added KCl. The reaction was stopped after 5.0 min by addition of excess ice-cold CDTA and exchange assayed as described in Methods.

TABLE II

STIMULATION OF EXCHANGE BY MONOVALENT CATIONS

ATP/ADP exchange was measured following a 5.0 min incubation at room temperature. The assay medium consisted of 45.0 mM Pipes/Tris, pH 7.4, 1.0 mM [^{14}C]ADP, 0.5 mM ATP, 1.0 mM $MgCl_2$, 5.0 μg enzyme and 20.0 mM monovalent cation.

Cation addition	Relative stimulation (cation/K^+)
Mg^{2+} only	-
KCl	1
RbCl	0.89
NH_4Cl	0.63
TlCl	0.56
NaCl	0.06
CsCl	0.03
LiCl	0.02

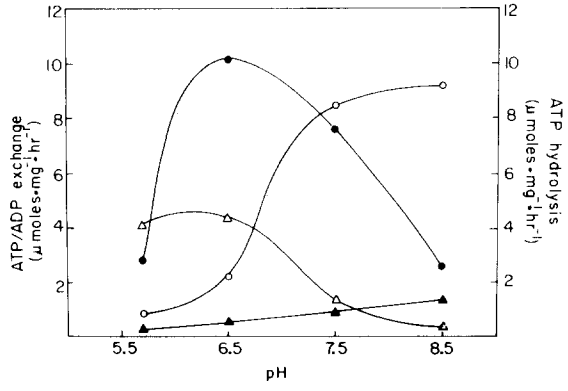

Fig. 3. Comparison of the pH dependence of ATP/ADP exchange and ATP hydrolysis. Both ATP/ADP exchange activity and ATP hydrolysis were measured following a 5.0 min incubation at room temperature. In these assays 5.0 μg of enzyme was added to a 50.0 μl volume of reaction solution containing a 45.0 mM buffer ranging in pH from pH 5.5 to pH 8.5 and 50.0 μM $MgCl_2$. In the ATP/ADP exchange assay the reaction was started by the addition of 0.5 mM ATP plus 1.0 mM [^{14}C]ADP to the reaction medium in the presence (●——●) or absence (○——○) of 20 mM KCl. In the ATP hydrolysis assay the reaction was started by the addition of 1.0 mM ADP plus 0.5 mM [^{32}P]ATP to the reaction medium in the presence (△——△) or absence (▲——▲) of 20.0 mM KCl. Each assay was stopped by the addition of excess ice-cold CDTA.

shown that Mg^{2+}-dependent exchange is progressively stimulated by an increasingly alkaline medium. The exchange measured at pH 8.5 is almost 10-fold that measured at pH 5.5. The curve of pH dependence of the K^+-stimulated component of exchange is bell shaped. K^+ stimulation of exchange increases to pH 6.5. Above this pH the exchange activity decreases. At pH 7.5 and above there is K^+-dependent inhibition of exchange which produces rates of exchange which are clearly below those observed in the presence of Mg^{2+} alone. The rate of hydrolysis measured under identical conditions is also shown in Fig. 3. In the absence of K^+, the basal Mg^{2+}-ATPase activity increases as the medium pH increases. Little stimulation of hydrolysis is seen at pH 7.5 while above this pH, 20.0 mM K^+ inhibits hydrolysis. It was noted that from pH 5.7 to 6.5 hydrolysis increases very little while the stimulation of exchange occurs.

Fig. 4 shows that the inhibition of exchange at pH 8.5 is dependent on K^+ concentration. 100.0 μM K^+ at this pH produces an apparent slight stimulation of exchange though this difference is within assay error. Progressive K^+ dependent inhibition is seen from 1.0 to 20.0 mM K^+.

In most of the experiments measuring exchange the Mg^{2+} concentration was limited to 50.0 μM. Since the relationship between Mg^{2+}, ATP concentration, pH and the K^+ concentration necessary to stimulate hydrolysis at the pH is complex, the pH activity profiles for exchange and hydrolysis were investigated following substitution of 1.0 mM Mg^{2+} for the 50.0 μM Mg^{2+} used in the experiments reported in Figs. 3 and 4. In Fig. 5 it is seen that stimulation of the Mg^{2+}-dependent exchange is dependent on the alkalinity of the medium as was observed at low Mg^{2+} concentrations. The apparent pH optimum of the K^+-dependent component of exchange, however, has been shifted from pH 6.5 to pH 7.5 by the increase in medium Mg^{2+}. The pH optimum for K^+-stimulated hydrolysis remains at pH 6.5 and rapidly declines at pH 7.5 and above. The K^+-dependent inhibition is less pronounced at 1 mM Mg^{2+} (Fig. 5) than at 50 μM Mg^{2+} (Fig. 3).

Discharge of EP. The 116 μM $K_{0.5}$ for the exchange reaction may indicate a lower affinity for

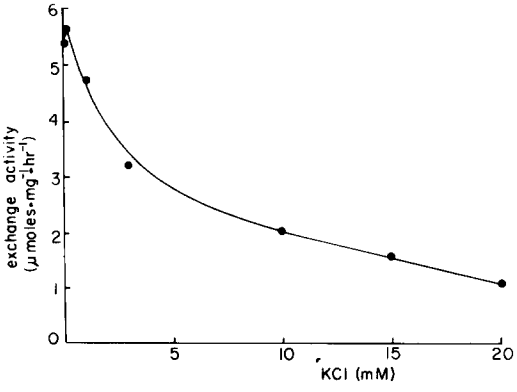

Fig. 4. Inhibition of ATP/ADP exchange by KCl at high pH. ATP/ADP exchange was measured following a 5.0 min incubation at room temperature. In these assays 5.0 μg of enzyme was incubated in a 50.0 μl volume of reaction solution containing 45.0 mM Tris-HCl, pH 8.5, 50.0 μM $MgCl_2$, 0.5 mM ATP, 1.0 mM [^{14}C]ADP and KCl from 0.1 mM to 20.0 mM. $MgCl_2$-dependent exchange activity was measured in the absence of KCl. The activity was determined as described in Methods following a stop by the addition of excess ice-cold CDTA.

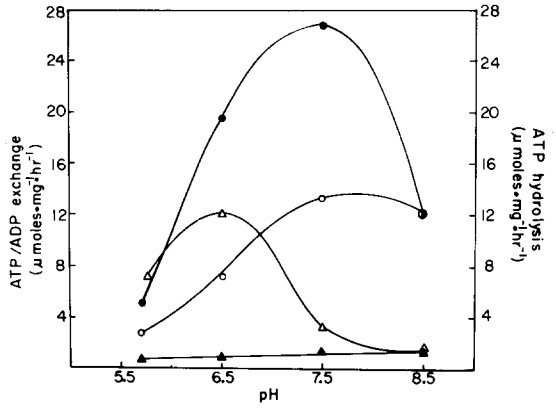

Fig. 5. Comparison of the pH dependence of ATP/ADP exchange and ATP hydrolysis at high Mg^{2+}. Both ATP/ADP exchange activity and ATP hydrolysis were measured following a 5.0 min incubation at room temperature. In these assays 5.0 μg of enzyme was added to a 50.0 μl volume of reaction solution containing 45.0 mM buffer from pH 5.5 to pH 8.5 and 1.0 mM $MgCl_2$. In the ATP/ADP exchange assay the reaction was started by the addition of 0.5 mM ATP plus 1.0 mM [^{14}C]ADP to the reaction medium in the presence (●———●), or absence (○———○), of 20 mM KCl. In the ATP hydrolysis assay the reaction was started by the addition of 1.0 mM ADP plus 0.5 mM [^{32}]ATP to the reaction medium in the presence (△———△), or absence (▲———▲) of 20 mM KCl. The activity was determined as described in Methods following a stop by the addition of excess ice-cold CDTA.

the nucleotide binding site following formation of the EP. Discharge of label from the phosphoenzyme found by either 5.0 μM or 200.0 μM ATP was measured in order to approach apparent substrate requirements for either the phosphorylation or the ATP/ADP exchange reaction. Using a flow quench apparatus where EP was formed in the presence of 5 μM or 200 μM ATP and 2 mM Mg^{2+} for approx. 130 ms , ADP was added in a second mix at either 2.0 or 0.8 mM and the rate of loss of EP followed up to 180 ms. 180 ms after the addition of ADP not more than 15% loss of EP was found, and this was identical to the loss observed with a chase with unlabelled ATP. Hence this slow loss of EP is not selectively induced by ADP and may correspond to the rate of turnover of EP (Fig. 6) [17].

In a further attempt to demonstrate ADP sensitivity of EP, it was considered that the formation of ADP-insensitive EP was extremely rapid, and kinetic evidence for an ADP-sensitive form of EP could only be obtained if ADP was present during phosphorylation. If enzyme is premixed with [^{32}P]ATP prior to the addition of Mg^{2+}, the rate of formation of EP is faster than if Mg^{2+} and

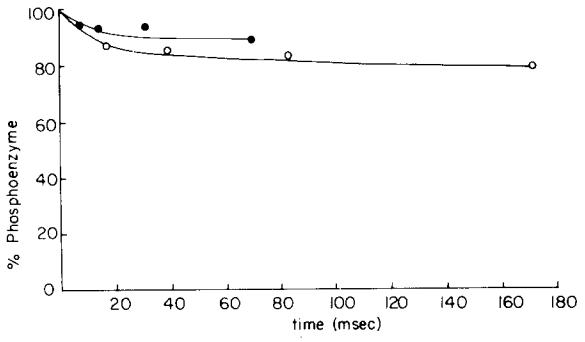

Fig. 6. Dephosphorylation of phosphoenzyme by ADP. The phosphoenzyme was formed in a flow quench apparatus at 21–22°C. All solutions contained 40 mM Tris-HCl, pH 7.4 and 2.0 mM $MgCl_2$. The reaction was started by the addition of 5.0 μM [^{32}P]ATP (●——●) or 200.0 μM [^{32}P]ATP (○——○) and was allowed to proceed for 120 to 130 ms, whereafter a second nucleotide addition is taken as zero time in the figure. At zero time 10.0 mM CDTA plus either 2.0 mM ADP (●——●) or 0.8 mM ADP (○——○) was added. At the indicated times the reaction was stopped and the amount of phosphoenzyme analyzed as described in Methods. The amount of phosphoenzyme at 130 ms was taken as the 100% value as this incubation time was sufficient to produce the maximal phosphoenzyme level [5].

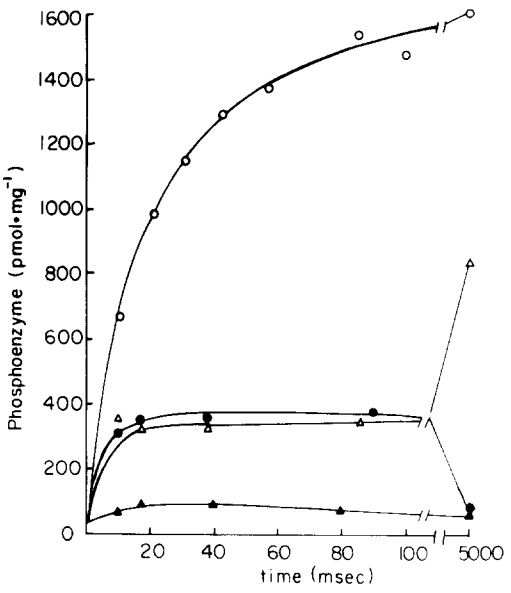

Fig. 7. Formation of phosphoenzyme from $MgCl_2$ and ATP. The phosphoenzyme was formed in a rapid mixing apparatus at 21–22°C. The phosphorylation was carried out in 40.0 mM Tris-HCl buffer, pH 7.4. The membranes were suspended in a solution containing 0.3 mM CDTA and were allowed to incubate with 5.0 μM [^{32}P]ATP for about 120–130 ms before the reaction was started with 2 mM $MgCl_2$ and ATP, ADP or ATP plus ADP. The time of this second addition is taken as zero time in the figure. The reagents added in the second mix with 2 mM $MgCl_2$ are: 0.3 mM ATP (●——●), no other addition (○——○), 0.3 mM ADP (△——△) and 0.3 mM ATP plus 0.3 mM ADP (▲——▲). At the indicated times the reaction was stopped and the phosphoenzyme was assayed as described in Methods.

[^{32}P]ATP are added simultaneously. This has been interpreted as evidence for the existence of an E · ATP conformation of the enzyme [5]. It is seen in Fig. 7 that if unlabelled ATP was added along with Mg^{2+}, the level of radioactive EP declined. This is considered to be due to rapid reversal of the reaction.

$$E + ATP \rightleftharpoons E \cdot ATP$$

ADP also inhibited the phosphorylation level as shown in Fig. 7. This could also be due to the substitution of ADP at the nucleotide binding site or due to the discharge of phosphoenzyme during its transition from ADP-sensitive to ADP-insensitive form. If the effect of ADP were identical to

that of ATP, and saturating levels of ATP were present, an additional effect of ADP superimposed upon that of ATP would be unexpected. Thus in Fig. 7 approx. 80% of EP formation was suppressed by the addition of 0.3 or 0.6 mM ATP (latter concentration not shown) or 0.3 mM ADP when these nucleotides were added, along with Mg^{2+} to the enzyme premixed with [^{32}P]ATP. However, a mixture of 0.3 mM ATP and 0.3 mM ADP reduced detectable EP to less than 10% of control. This suggests that ADP has an action in addition to the loss resulting from the displacement of labelled triphosphate.

Inhibition of the EP interconversion. In an effort to modify the extent of the conversion of the phosphoenzyme to its ADP-insensitive form, the ATPase was partially reacted with the sulfhydryl group reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Treatment of the enzyme with 200 nmol DTNB per mg of protein, at room temperature, produced a time-dependent inhibition of K^+-stimulated hydrolysis up to 50 min (not shown). After 30 min (the reaction time chosen in the following experiments) approx. 40% inhibition of K^+-stimulated hydrolysis was observed.

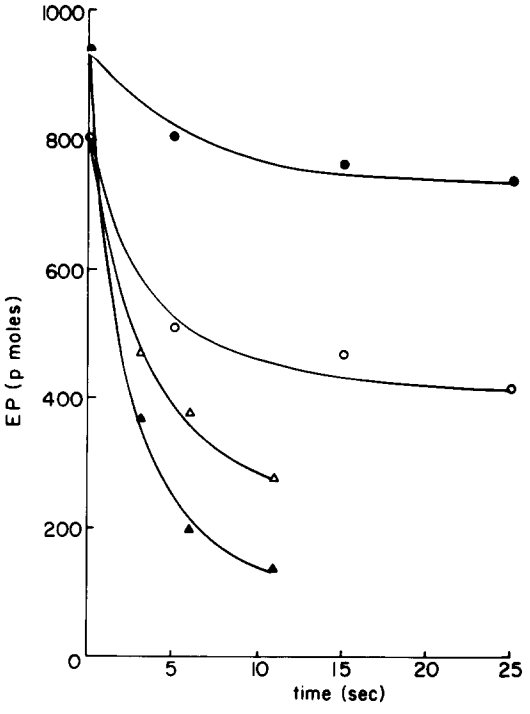

Fig. 8. DTNB modification of ADP and K^+ sensitivity of the phosphoenzyme (EP). Steady-state levels of EP were attained following a 15 s incubation of 22.5 μg of gastric enzyme in a 0.5 ml suspension of 150.0 mM choline chloride, 2.0 mM $MgCl_2$, 5.0 μM [γ^{32}P]ATP and 21 mM imidazole, pH 7.4 at 4°C. At this time (zero time) either a 1.0 mM ADP or a 4.0 mM K^+ chase was introduced and the EP levels determined at the listed intervals. ●———●, ADP chase of untreated material; ○———○, ADP chase of DTNB-treated material; ▲———▲, K^+ chase of untreated material; △———△, K^+ chase of DTNB-treated material.

TABLE 3

ATP/ADP EXCHANGE AFTER DTNB TREATMENT

Exchange activity measured for 5.0 min at room temperature following reaction of 200 nmol DTNB per mg protein for 30.0 min at room temperature. Values are expressed as $\mu mol \cdot mg^{-1} \cdot h^{-1}$. Conditions for exchange assay are: 30 mM Pipes/Tris, pH 7.4, 150 mM choline chloride, 2.0 mM $MgCl_2 \pm 2.0$ mM KCl, 1.0 mM ADP, 0.5 mM ATP and 5.0 μg protein per assay.

	+DTNB	−DTNB	%of control
Mg^{2+}	13.8	8.5	162
K^+	4.8	11.5	42

In Fig. 8 the loss of phosphoenzyme following the addition of ADP or KCl is shown before and after enzyme treatment with DTNB. In the untreated sample the quantity of phosphoenzyme sensitive to the presence of ADP is small while K^+ addition stimulates the rapid turnover of the phosphoenzyme label. Following DTNB treatment, the addition of ADP stimulates a rapid loss of approx. 35% of the phosphoenzyme label within the initial 5 s of ADP addition. The treated enzyme maintains a partial sensitivity to K^+ though the percentage of phosphoenzyme sensitive to this cation is clearly reduced.

The effect of this DTNB treatment on the exchange reaction is shown in Table III. The Mg^{2+}-dependent component of exchange is stimulated 62% by treatment with DTNB. Though the K^+-stimulated component is still apparent following the DTNB treatment, it is reduced approx. 60% in comparison to the untreated enzyme.

Discussion

As for the $(Na^+ + K^+)$- or Ca^{2+}-ATPases, transient kinetic studies have shown that the kinet-

ics of phosphoenzyme formation and decay are compatible with the participation of a phosphoprotein intermediate in the catalytic cycle of the ATPase reaction [16–19]. In analogy to the above transport enzymes, it was reasoned that the phosphoenzyme existed in at least two forms, a form destabilised by luminal K^+, K^+-sensitive EP, and a preceding form, an ADP sensitive EP [8,20,21]. However, under either steady-state or pre-steady state conditions, it was not possible to show a loss of EP beyond that of spontaneous turnover upon ADP addition to phosphorylated enzyme [17].

The presence of an ATP/ADP exchange activity associated with the $(H^+ + K^+)$-ATPase is evidence for the postulated initial form of EP. The absence of measurable nucleoside diphosphokinase activity and insensitivity of the reaction to diadenosine pentaphosphate, a myokinase inhibitor, show that the exchange is not due to those contaminating enzymes. Moreover, pretreatment of the enzyme with DTNB in the absence of added Mg^{2+} was shown to reduce the K^+ sensitivity of the EP [14]. With this treatment it was possible to shown that a population of the EP formed after DTNB pretreatment was ADP sensitive and that this pretreatment stimulated the ATP/ADP exchange. These data indicate that an ADP-sensitive form of EP is involved in ATP hydrolysis by the gastric ATPase, but, in contrast to some preparations of the $(Na^+ + K^+)$-ATPase, the formation of the K^+-sensitive form of EP is favored making detection of the ADP-sensitive form of EP in the unmodified enzyme difficult.

Using flow quench conditions, it has been shown that premixing of enzyme with [^{32}P]ATP prior to Mg^{2+} addition results in a rapid formation of EP [5,17]. Addition of unlabeled ATP at the time of Mg^{2+} addition reduced EP levels due, presumably to dissociation of the E-[γ-^{32}P]ATP complex. The observed loss of EP with ADP addition is either due to displacement of bound ATP or due to reversal of EP formation. That the latter may also be occurring was shown by the larger effect of a mixture of ADP and ATP as opposed to either nucleotide alone.

In addition to demonstrating the presence of a form of EP corresponding to the postulated EP form, the properties of the ATP/ADP exchange activity of the gastric ATPase suggest additional components of the reaction, compared to those described previously. Exchange activity becomes maximal at an ADP/ATP ratio of 2, and this activity is significantly less sensitive to an increase in the ADP/ATP ratio than the reverse when the ratio is decreased by increasing ATP levels at a fixed ADP concentration. The optimal ratio and the high nucleotide requirements suggest the presenc of a functional low affinity nucleotide binding site on EP. A scheme which is compatible with this data is as follows:

$$E + ATP \rightleftharpoons E \cdot ATP \quad (1)$$

$$E \cdot ATP + Mg^{2+} \rightleftharpoons Mg^{2+} \cdot EP \cdot ADP \quad (2)$$

$$Mg^{2+} \cdot EP + ATP \rightleftharpoons Mg^{2+} \cdot EP + ADP \quad (3)$$

$$Mg^{2+} \cdot EP + ATP \rightleftharpoons Mg^{2+} \cdot EP \cdot ATP \quad (4)$$

Reactions 1,2, and 3 indicate that exchange activity involves the direct participation of the phosphoenzyme formed at the high-affinity nucleotide site. Reaction 4 indicates a functional low-affinity nucleotide site which would account for the inhibition of exchange at high ATP/ADP ratios and the high ADP requirement for the ADP/ATP exchange reaction.

The apparent affinity of the nucleotides for the exchange reaction, (i.e. 116 μM) suggests that the exchange reaction involves a lower affinity ATP or ADP site than is required for EP formation. Thus, although posphorylation may initially occur at a high-affinity site, the binding of either ATP or ADP following formation of the EP is of relatively lower affinity. These observations do not necessarily imply two ATP sites and may more simply be accounted for by a single site/dual state function of the ATP binding site [22,23].

The H^+ is a major cationic ligand involved at these sites in the reaction cycle of the gastric ATPase. In the absence of K^+, hydrolysis of the K^+-sensitive EP has been shown to increase with increasing pH [7]. In the experiments reported here both ATP hydrolysis and exchange increase concomitantly with increasing pH, though at a pH of 8.5 exchange activity is about 5-fold that of hydrolysis. This might, in part, be explained by a

pH dependent shift between the ADP-sensitive and K^+ sensitive forms of EP, high pH favoring the former.

Addition of K^+ at low pH (between 5.5 and 6.5) activates exchange and hydrolysis although the degree of exchange stimulation is greater than stimulation of hydrolysis. In addition to the interaction between K^+ and H^+, increasing the Mg^{2+} (or Mg^{2+}-nucleotide) levels exaggerates the discrepancy between K^+ stimulation of exchange and hydrolysis. Thus, while hydrolysis is maximal at pH 6.5, an increase in Mg^{2+} levels shifts the pH optimum for K^+-stimulated exchange from pH 6.5 to pH 7.5.

Inhibition of exchange and hydrolysis by K^+ at high pH values can be explained by the effects of K^+ on the partial reactions of the ATPase. The complex effect of K^+ on the reactions of the $(H^+ + K^+)$-ATPase has indicated that cation binding occurs at least at two functional sites, an activating and an inhibiting site [5,7,24]. Thus K^+ inhibits formation of EP progressively with increasing pH though the rate of K^+-dependent breakdown of EP also rises.

The effect of K^+ in activating the ATP/ADP exchange reaction associated with the $(Na^+ + K^+)$-ATPase has been explained as due to K^+ inhibition of ATP binding to a low-affinity ATP inhibitory site [25]. This explanation is unlikely in the case of the $(H^+ + K^+)$-ATPase. In the absence of K^+, titration of the exchange using either 50 μM or 1.0 mM Mg^{2+} (later data not presented) gives no indication of inhibition by Mg^{2+} nucleotides. Furthermore, the K^+ stimulation of exchange appears due to K^+ binding at the high-affinity K^+ sites located on the luminal face of the enzyme. Inhibition of ATP binding has been attributed only to K^+ binding at a functionally lower affinity site at the external membrane face [5].

Another mechanism to be considered is that K^+ increases turnover of the K^+-sensitive EP. Here, in the absence of K^+, breakdown of EP is rate limiting and the form of EP is the ADP-insensitive form. However, this would predict correlation between K^+ activation of hydrolysis and exchange and, depending on the pH and Mg^{2+} concentration, K^+ may activate exchange while reducing hydrolysis. To account for this latter effect it is necessary to postulate a K^+ dependence in the conversion of the ADP-sensitive to ADP-insensitive EP. The gastric ATPase appears to be a multi-subunit enzyme, based, for example, on target molecular weight [26] and trypsin digestion patterns [27]. The effect of K^+ in stimulating exchange may depend on subunit interactions which have not been detected by transient kinetic methods.

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