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STUDIES ON THE PHOSPHORYLATED INTERMEDIATES OF A K^+-STIMULATED ATPase FROM RABBIT GASTRIC MUCOSA

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SUMMARY

A density gradient-purified microsomal membrane preparation from rabbit fundic gastric mucosa was used for a detailed study of the K^+-stimulated ATPase and associated intermediate reactions. Membranes incubated with γ-[^{32}P]ATP show the rapid incorporation of ^{32}P into phosphoprotein. Phosphoprotein levels were markedly reduced (1) when ATP hydrolysis went to completion or (2) upon addition of unlabeled ATP, thus suggesting the participation of a rapid turnover phosphorylated intermediate in the gastric microsomal ATPase. Addition of K^+, Rb^+ or Tl^+ greatly reduced the level of the intermediate while stimulating ATPase activity; the observed affinities of these cations were similar for the effects on both ATPase and intermediate levels, with $Tl^+ > K^+ > Rb^+$. Neither ATPase nor intermediate were stimulated by Na^+, and ouabain was without effect on the reactions, thus differentiating this system from the $(Na^+ + K^+)$-ATPase. Addition of various inhibitors showed differential effects on the partial reactions of the gastric ATPase system. *N*-ethylmaleimide and Zn^{2+} showed characteristics of completely abolishing the K^+-stimulated component of ATPase as well as the effects of K^+ in reducing the level of intermediate, thus suggesting that these agents exert their inhibitory effect on a phosphoprotein phosphatase partial reaction. F^- abolished the K^+-stimulated ATPase, but its more complex effects on the intermediate suggested an additional reaction step within the domain of the phosphorylated intermediate. Results are consistent with a model system for the gastric microsomal ATPase involving a Mg^{2+}-dependent protein kinase, a phosphorylated intermediate(s), and a K^+-stimulated phosphoprotein phosphatase.

INTRODUCTION

Previous reports from our laboratory have demonstrated the presence of a K^+-stimulated ATPase and K^+-stimulated *P*-nitrophenylphosphatase in the oxyntic region of gastric mucosa from various species, including frogs, rats, rabbits and

Abbreviation: MalNEt, *N*-ethylmaleimide.

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pigs [1–3]. The enzymes are associated with a very light microsomal membrane fraction, and in addition to stimulation by K^+, they require Mg^{2+} for their activity. The K^+-stimulated enzyme systems are quite unique for the oxyntic glands [3, 4], and unlike the $(Na^+ + K^+)$-ATPase, are insensitive to Na^+ and ouabain. We have also previously reported that these K^+-ATPase rich membrane fractions give a phosphoprotein when incubated with Mg^{2+} and γ-[^{32}P]ATP [5]. Preliminary characterization of the phosphoprotein suggested that it might be the phosphorylated intermediate of the K^+-stimulated ATPase. Since the K^+-stimulated ATPase has been implicated to be involved in some aspect of the proton transport process of oxyntic cells [4, 6, 7], it was important to study the detailed molecular mechanisms and intermediate compounds associated with this interesting enzyme.

The present paper extends our earlier work using a purified microsomal preparation of rabbit gastric microsomes to study the ATPase reaction and associated phosphorylated intermediates. We have established that most of the ^{32}P-labeled membrane bound phosphoprotein has characteristics of being the intermediate of the K^+-stimulated ATPase. Furthermore, using inhibitors of K^+-stimulated ATPase, like F^-, Zn^{2+} and *N*-ethylmaleimide (MalNEt), we have been able to resolve the total ATPase reaction into several distinct steps.

EXPERIMENTAL PROCEDURE

Methods

The light membrane fractions used for these studies were isolated from rabbit gastric mucosa by the procedure described previously [1]. Briefly, the oxyntic glandular region of the gastric mucosa was scraped and homogenized for cell fractionation in 0.25 M sucrose containing 5 mM Tris · HCl (pH 7.5) and 0.2 mM ethylenediaminetetra acetic acid (EDTA). All operations were carried out in the cold (0–4 °C). A crude fraction of microsomes was isolated by differential centrifugation as the pellet which sedimented between 12 000 × *g* for 15 min and 100 000 × *g* for 90 min. Purification of the microsomes was carried out by centrifugation overnight on a linear sucrose density gradient (20–55 % sucrose, w/v). A band of membranes appears in the density region of 1.09–1.15 gm/ml (measured at room temperature). This band of membranes will be designated as the purified light microsomes and has been used for all the studies. The membranes were stored at 0–4 °C and were usually used from 2–3 days after the membranes were harvested (aged membranes).

Labeling of the membranes

The labeling reaction was generally carried out at room temperature (20–23 °C). Unless it is stated otherwise, the incubation mixture contained in a total volume of 0.13 ml, 50 mM Tris/acetate (pH 7.0), 6–20 μM γ-[^{32}P]ATP (about $8 \cdot 10^6$ cpm), about 5 μg membrane protein, 0.05 mM Mg^{2+} and other test substances. The reactions were stopped after 15–30 s with 200 μl of 35 % ice cold perchloric acid. An aliquot of 20 μl was transferred to 1.0 ml 5 % ice cold perchloric acid for assay of P_i release. The rest was used for assay of phosphorylated intermediate. A blank without enzyme was run in parallel with each assay.

Assay of ATPase (release of $^{32}P_i$)

An aliquot from the tube designated for P_i assay was counted before and after Norite A charcoal treatment according to the method of Crane [8]. The activity of the ATPase is expressed as μmol P_i liberated/mg protein/hour at the specified temperature. The term basal ATPase is used to designate enzyme activity when Mg^{2+} was the only metal ion activator and is differentiated from the K^+-stimulated ATPase which refers to activity with K^+ plus Mg^{2+}.

Determination of phosphorylated intermediate

The phosphorylated intermediate was isolated by the method described previously [9]. The perchloric acid-stopped reaction medium was brought to 10 mM with respect to carrier ATP and P_i by adding a solution of unlabeled ATP and P_i. The precipitated membranes were then collected by filtration on paper discs (Whatman No. 3) using a suction device and followed by repeated washings with cold 5 % perchloric acid containing 10 mM P_i and 2 mM carrier ATP. The membrane precipitate was then washed twice with ethanol to remove most of the lipids, dried and then counted in 10 ml Aquasol (New England Nuclear).

Treatment with hydroxylamine

The phosphorylated membrane proteins were subject to treatment with hydroxylamine according to the method of Lipmann and Tuttle [10]. Thoroughly washed and dried filter paper discs containing the phosphorylated membranes were transferred into 4.0 ml of ice-cold, freshly prepared, 28 % hydroxylamine (pH adjusted to 6.5 by NaOH). The paper discs were left for 30 min at 0 °C. After treatment, the filter paper discs and aliquots of the supernatants were counted.

Treatment with different pH values

The washed and dried filter paper discs containing the phosphorylated intermediate were transferred into 4.0 ml buffers of different pH values and incubated for 60 min at room temperature. At the end of the incubation period, an aliquot of the medium and the paper discs were counted.

Materials

γ-[^{32}P]ATP was purchased from New England Nuclear in the form of the triethylammonium salt. Perchloric acid was purchased from Mallinckrodt. Tris/ATP, ADP and MalNEt were from Sigma. [^{14}C]ATP was obtained from Calbiochem.

RESULTS

Preliminary characterization of phosphorylation reaction

Table I shows the following observations. The membranes, when incubated with γ-[^{32}P]ATP and Mg^{2+} at pH 7.0 produced the phosphorylated intermediate. Unlike the (Na^++K^+)-ATPase [11], Na^+ did not increase the level of intermediate formation. K^+ reduced the level of intermediate. No intermediate was formed when the K^+-stimulated ATPase was destroyed by heating the membranes at 100 °C for 3 min. Presence of excess EDTA in the incubation medium prevented the formation

TABLE I

ENZYMATIC NATURE OF LABELING OF THE RABBIT GASTRIC MICROSOMAL MEMBRANES

EDTA was added as the sodium salt.

System	pmol of intermediate formed/mg protein	
	without K^+	with 1 mM K^+
Control	630	160
With 1 mM Na^+	600	150
With 2 mM EDTA (without Mg^{2+})	25	0
With 2 mM EDTA and 2 mM Mg^{2+}	600	160
After heat inactivation at 100 °C for 3 min	8	7
[^{14}C]ATP in place of AT^{32}P	15	12

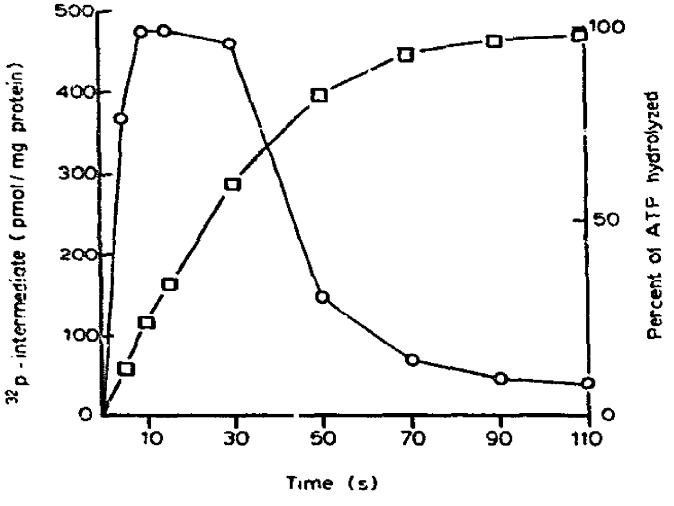

Fig. 1. Time course of incorporation of ^{32}P from γ-[^{32}P]ATP into the microsomal membranes and release of ^{32}P into the medium. 6.85 μg of protein, 7.0 μM γ-[^{32}P]ATP and 50 μM Mg^{2+} were present in the incubation medium containing 20 mM Tris, pH 7.0. Each tube was incubated for the desired length of time at room temperature and processed as described in Methods. (○), ^{32}P incorporation into membranes; (□), percent of total ATP hydrolyzed.

of the intermediate which could be restored by addition of Mg^{2+}. When incubated with [^{14}C]ATP, no significant labeling of the membranes was detected.

That the membrane phosphoprotein is an intermediate in an ATPase reaction is suggested by the time course of labeling and hydrolysis of ATP shown in Fig. 1. The labeling reaction was complete within 10 s. The intermediate remained at the same level as long as sufficient ATP remained. When the ATP was hydrolyzed beyond 60 %, the level of intermediate fell down sharply. After the hydrolysis of ATP was > 90 % complete, the level of the intermediate reached a very low value. Separate experiments showed that the amount of the phosphorylated intermediate increased linearly with the amount of membrane protein.

TABLE II

RELEASE OF COUNTS FROM THE ^{32}P-LABELED INTERMEDIATE AFTER TREATMENT AT DIFFERENT pH VALUES

Treatment for 60 min at	Percent of counts released from the phosphorylated intermediate
pH 3.0	1
pH 4.0	8
pH 5.0	10
pH 6.0	14
pH 7.0	21
pH 8.5	65

As shown in Table II, the intermediate was quite stable at acidic pH, moderately stable at neutral pH and very unstable at alkaline pH. Treatment with NH_2OH at acid pH released more than 70 % of the membrane-bound phosphate as a soluble form. These chemical properties suggest that the gastric microsomal intermediate occurs in the form of an acyl phosphate linkage and are similar to what has been found for other transport ATPases [12].

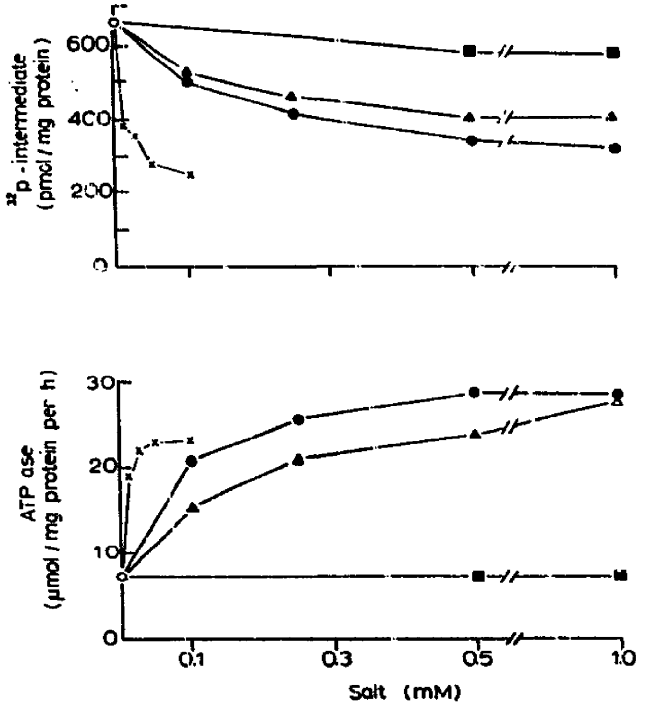

Fig. 2. Effects of different monovalent cations on the gastric ATPase and the phosphorylated intermediate in the presence and absence of K^+. 7.5 μg membrane protein and 20 μM γ-[^{32}P]ATP were used. Incubation time was 20 s at 37 °C. Na^+(■), K^+ (●), Rb^+ (▲) and Tl^+ (×).

Relationship between the K^+-stimulated ATPase and the ^{32}P-intermediate

To assess whether the ^{32}P-intermediate might be a functional part of the K^+-stimulated ATPase, the effects of various agents and co-factors in altering the enzyme rate and the level of the intermediate were tested. Fig. 2 shows the effects of different monovalent cations on the gastric ATPase and the phosphorylated intermediate. K^+, Rb^+ and Tl^+ reduce the level of the intermediate while stimulating the rate of hydrolysis of ATP. The degree of stimulation of ATPase by these cations at different concentrations bears an inverse relationship to the decrease in the level of the intermediate. It is of interest that Tl^+, which has an ionic radius close to K^+ and which has frequently been shown to be an excellent substitute for K^+ [13], has a very high affinity for the enzyme in the activation process. Although Na^+ has no appreciable effect on the ATPase, it has a small but reproducible effect in reducing the level of the intermediate.

An interesting feature of the gastric K^+-stimulated ATPase is that in the fresh membrane preparation, the enzyme activity is greatly stimulated by ionophores [14], such as gramicidin, in presence of K^+. Table III shows that gramicidin, which increases the rate of K^+-stimulated hydrolysis of ATP, also decreases the level of intermediate further than that produced by K^+ alone. Various interpretations for this ionophoric effect have been discussed before [2]. In freshly prepared membrane vesicles, K^+ activation of the ATPase is restricted, possibly due to some kind of permeability barrier for K^+, which is eliminated by gramicidin. Gramicidin effects are lost after storage for several days at 0–4 °C. In order to maximize the K^+ effect, we used the "aged" membranes for most of the studies reported here.

TABLE III

EFFECT OF GRAMICIDIN ON THE LEVEL OF PHOSPHORYLATED INTERMEDIATE AND HYDROLYSIS OF ATP

Each tube contained 3.3 μg of freshly prepared membranes and 7 μM γ-[^{32}P]ATP. Reaction was carried out for 30 s at room temperature.

	^{32}P intermediate (pmol/mg protein)	ATPase (μmol/mg protein per h)
Basal state (0 K^+)	109.0	3.3
2 mM K^+	89.4	4.6
2 mM K^+ plus 10^{-5} M gramicidin A	63.2	6.1

Fig. 3 shows results of an experiment in which the concentration of free Mg^{2+} was varied using EDTA in the incubation medium. Both the basal, as well as the K^+-stimulated ATPase were gradually reduced with an increasing EDTA concentration; the K^+-stimulated activity appears more dependent on Mg^{2+} than the basal ATPase. The phosphorylated intermediate, both in the presence and absence of K^+, also decreased with the reduction in ATPase activity. Under the condition when the ratio of Mg^{2+} to EDTA was 1 : 1 (Fig. 3 and Table I), the formation of the intermediate and hydrolysis of ATP were not appreciably affected. This indicates that the

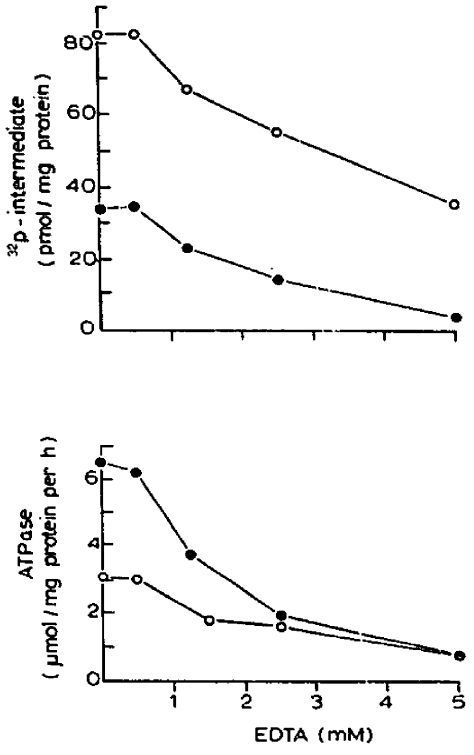

Fig. 3. Effects of different concentrations of EDTA on the level of ^{32}P-intermediate and hydrolysis of ATP in the presence (●) and absence (○) of 2 mM K^+. The Mg^{2+} concentration was 0.5 mM. Reactions were carried out for 30 s at room temperature. 3.8 µg of membrane protein was used for each experiment.

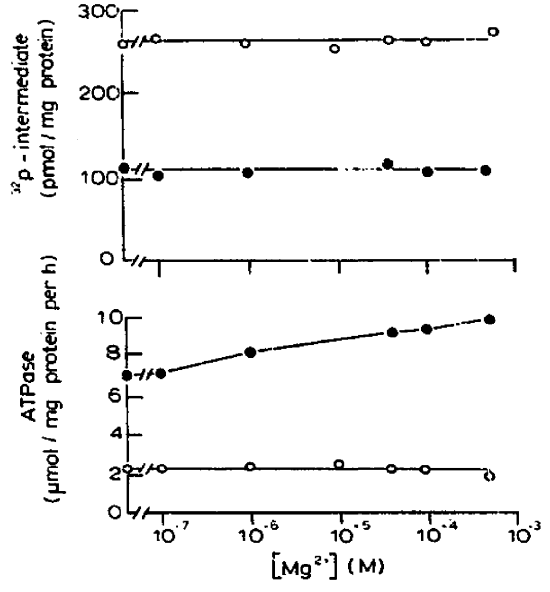

Fig. 4. Levels of phosphorylated intermediates and hydrolysis of ATP in the presence of different concentrations of added Mg^{2+}. Incubation time was 30 s at room temperature. 2.75 µg of membrane protein and 7 µM ATP were used for each study. (○), without K^+; (●), with K^+.

Mg^{2+} requirement for this ATPase is very low at 7 μM ATP concentration. In the absence of EDTA, and at low ATP concentration, the membranes can maintain the production of intermediate and hydrolysis of ATP at a normal rate, even in the absence of added Mg^{2+} (Fig. 4). Increasing the Mg^{2+} concentration from 10 μM to 500 μM to the incubation medium did not have any effect on the intermediate and only a small effect on the rate of hydrolysis (Fig. 4). Since EDTA in the absence of any added Mg^{2+} completely blocked the formation of the intermediate, which could be restored by the addition of Mg^{2+} (Table I), we concluded that there is some residual Mg^{2+} firmly bound to these membranes which can maintain intermediate formation and hydrolysis at a low substrate concentration. Similar kinds of observations and conclusions have been made in the case of $(Na^+ + K^+)$-ATPase of kidney membranes [15].

We tested the relationship between the phosphorylated intermediate and hydrolysis of ATP as a function of ATP concentrations (Fig. 5). The intermediate concentration reached a saturated level even at 5 μM ATP, the lowest substrate concentration tested in this experiment, while for maximum hydrolysis a far greater substrate concentration was needed. To discover the saturation kinetics for the formation of the intermediate, an experiment was conducted at 0–4 °C using very low concentrations of ATP (Fig. 6). Although it was not possible to measure the rate of ATP hydrolysis at the low temperature conditions of the experiments shown in Fig. 5, a comparison of the results of Figs. 5 and 6 show that the ATP saturation kinetics are clearly different for the intermediate and the enzymic hydrolysis. The apparent K_m

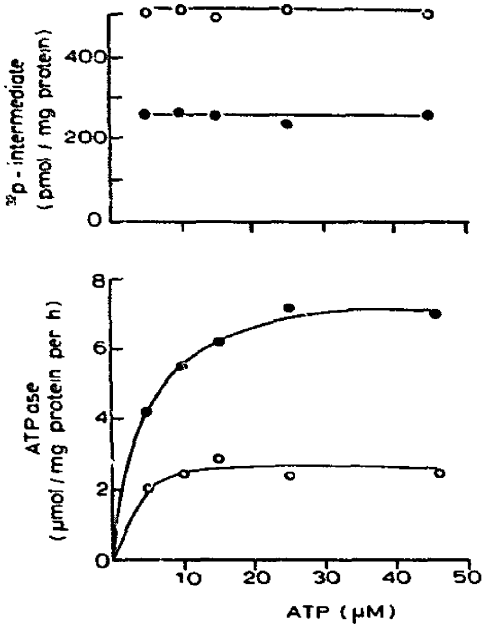

Fig. 5. Effects of different concentrations of γ-[^{32}P]ATP on the level of the phosphorylated intermediate and hydrolysis of ATP. Incubations were carried out for 20 s at room temperature using 6.9 μg membrane protein (○), without K^+; (●), with K^+.

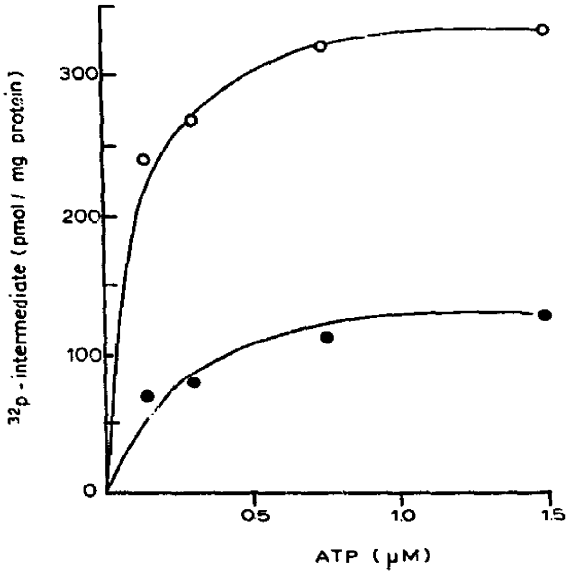

Fig. 6. Levels of ^{32}P-intermediates at different concentrations of ATP in the presence (●) and absence (○) of K^+. Incubations were performed at 0–4 °C for 12 s.

for the intermediate was in the order of 0.1 μM, while that for the ATPase was about 5 μM.

Characterization of the different steps involved in the phosphorylation and dephosphorylation of the enzyme

Once the relationship between the phosphorylated intermediate and the hydrolysis of ATP was established, it was of interest to characterize the steps which might be involved in the total ATPase reaction. The simplest mechanism would involve a kinase step where ^{32}P from γ-[^{32}P]ATP is incorporated into the protein giving rise to the phosphorylated intermediate and a phosphatase step where the phosphorylated intermediate is broken down into inorganic phosphate and the enzyme. We have shown before that Mg^{2+} is absolutely necessary for the kinase step because in the presence of EDTA the formation of intermediate was abolished (Table I). In order to determine whether the phosphatase step requires Mg^{2+}, we first labeled the membranes in the presence of Mg^{2+}, then chelated all Mg^{2+} by adding excess of EDTA and followed the fate of the intermediate under different conditions. The results presented in Fig. 7 show that within 15 s after chelation of Mg^{2+} by EDTA the intermediate achieved a new low level, which is not significantly altered in the next 15 s, nor is it significantly affected by the addition of K^+ or unlabeled ATP. Thus, within the 15 s time of resolution tested here, either Mg^{2+} was not required for the dephosphorylation of the intermediate or the intermediate was unstable in the absence of the divalent cation. Addition of unlabeled ATP, instead of EDTA, brings the level of the intermediate down to the same level as did EDTA indicating that the residual intermediate represents a part of a slow turnover component probably not associated with the intermediate of the K^+-ATPase.

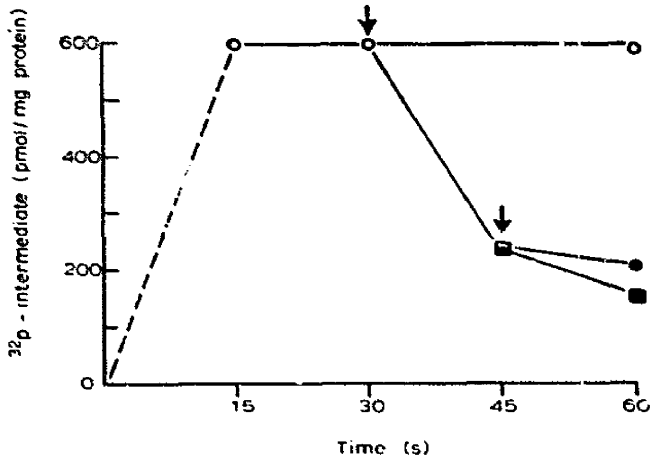

Fig. 7. Effects of EDTA, unlabeled ATP and K^+ on the steady-state levels of the phosphorylated intermediates. Membranes (2.7 μg protein) were first labeled with 7 μM [γ-^{32}P]ATP. Different agents were then added at the times indicated by arrows. At 30 s the additions were buffer (○), 10 mM EDTA (□) or 0.12 mM ATP (■). At 45 s tubes previously treated with 10 mM EDTA then received additions of 2 mM K^+ (●) or 0.12 mM ATP (■).

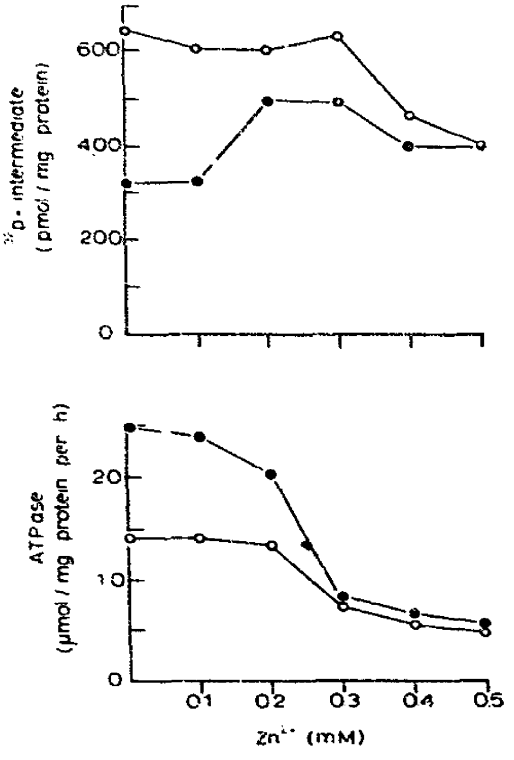

Fig. 8. Effects of different concentrations of Zn^{2+} on the level of the phosphorylated intermediate and hydrolysis of ATP. Incubation time was 20 s at room temperature. Reactions were initiated by adding 5.4 μg of membrane proteins. Mg^{2+} and γ-[^{32}P]ATP concentrations were 500 μM and 10.7 μM, respectively. (○), without K^+; (●), with K^+.

Zn^{2+}, MalNEt and F^- have been found to be good inhibitors of K^+-stimulated ATPase and K^+-stimulated *p*-nitrophenylphosphatase [1, 3]. We, therefore, attempted to determine the site of action of these inhibitors in the total ATPase reaction sequence. The effects of different concentrations of Zn^{2+} on the level of phosphorylated intermediate and hydrolysis of ATP in the presence and absence of K^+ are shown in Fig. 8. Incorporation of ^{32}P into membrane protein in the

TABLE IV

EFFECTS OF *N*-ETHYLMALEIMIDE ON THE LEVEL OF ^{32}P-INTERMEDIATE AND HYDROLYSIS OF ATP

Reactions were started by adding 3.3 μg membrane protein to each tube also containing: 3 mM β-mercaptoethanol and 5 μM γ-[^{32}P]ATP, then incubating for 30 s at room temperature.

	^{32}P intermediate (pmol/mg protein)	ATPase (μmol/mg protein per h)
Control membranes		
0 K^+	106.0	3.4
2 mM K^+	66.9	6.3
Membranes pretreated with 10 mM *N*-ethylmaleimide for 30 min		
0 K^+	104.9	3.5
2 mM K^+	103.1	3.7

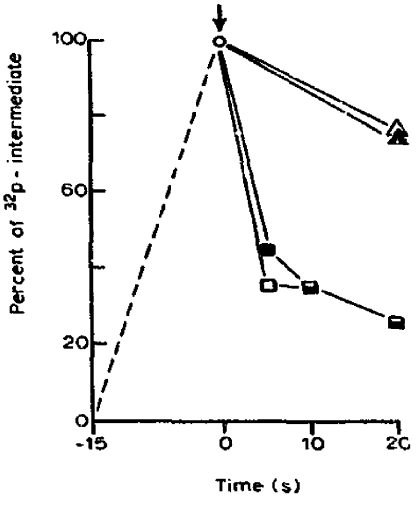

Fig. 9. Effects of unlabeled ATP and ADP on the steady-state level of the phosphorylated intermediate formed by control and MalNEt-treated membranes. Membranes were first preincubated with and without 10 mM MalNEt for 30 min at room temperature. 2.7 μg membrane protein and 14 μM γ-[^{32}P]ATP were used for each experiment. After 15 s of incubation at room temperature, the following additions were made (indicated by arrow): 150 μM unlabeled ATP, 300 μM ADP or 5 μl of buffer. The reactions were stopped at designated times and assayed for the ^{32}P-intermediate. ATP (□, control membranes; ■, MalNEt membranes); ADP(△, control membranes; ▲, MalNEt membranes).

absence of K^+ showed only an inhibitory effect at about the same concentration of Zn^{2+} as the diminution of the basal ATPase. In the presence of K^+, there was an interesting elevation in the intermediate in the range of Zn^{2+} where the K^+-stimulated ATPase was most severely depressed. The K^+-stimulated component of the ATPase is especially sensitive to lower levels of Zn^{2+}. Thus, elevation of the intermediate at Zn^{2+} concentrations between 0.1–0.3 mM in presence of K^+ may be explained on the basis of inhibition of the dephosphorylation step by the heavy metal.

The effects of MalNEt on the hydrolysis of ATP and level of the intermediate is shown in Table IV. In MalNEt pretreated membranes the K^+ sensitivity of the intermediate, as well as the ATPase, was lost. However, the level of the intermediate for the MalNEt-treated membranes remains almost the same as that of the control membranes. The results strongly suggest that K^+ stimulates the hydrolysis of ATP by enhancing the dephosphorylation of the intermediate, while MalNEt specifically blocks or interferes with the K^+-coupled step of the enzyme. We tested the turnover of the intermediate for control and MalNEt-treated membranes in order to get more information about the mechanism of action of the ATPase. Fig. 9 shows that the control membranes reached a new steady state within a few seconds after addition of unlabeled ATP, whereas MalNEt-treated membranes took about 10 s to reach the same steady-state level. Although the difference between control and MalNEt-treated

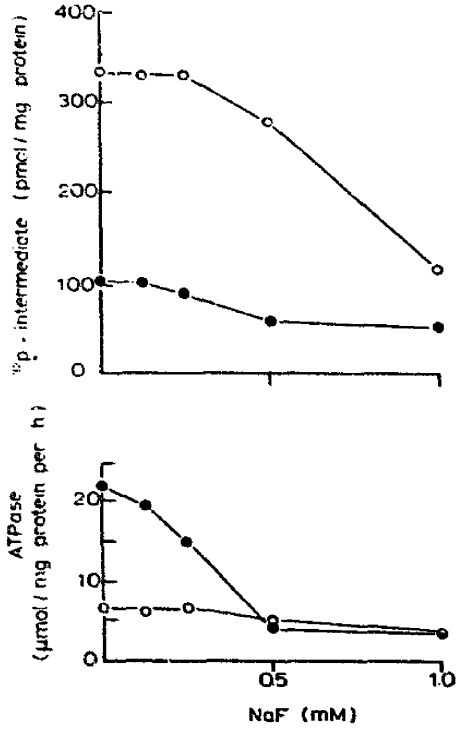

Fig. 10. Effects of different concentrations of NaF on the level of phosphorylated intermediate and hydrolysis of ATP. 2.7 μg of membrane protein was preincubated with different concentrations of NaF for 15 min at room temperature. The reactions were initiated by adding 14 μM γ-[^{32}P]ATP and incubated for 30 s at room temperature. (○), without K^+; (●), with K^+.

membranes appear small, it was very reproducible giving similar kinetic differences in four separate experiments. Since the ATP-hydrolytic activity in the absence of K^+ is the same for both control and MalNEt-treated membranes, the apparently slower turnover rate of the MalNEt-intermediate indicates some additional site of action of *N*-ethylmaleimide in the ATPase reaction.

Our results testing the effects of different concentrations of NaF on the formation of intermediate and hydrolysis of ATP are shown in Fig. 10. In the presence of K^+, the typical decrease in the level of the intermediate occurred with no F^- present; as F^- concentration was increased, a small, but consistent, further drop in the level of the intermediate was observed. In the absence of K^+, the level of the intermediate was not affected below 0.25 mM F^-, whereas the level systematically decreased with higher concentrations of F^-. On the other hand, the K^+-stimulated ATPase continuously decreased with an increase in F^- concentration and reached the basal value at 0.5 mM. The fact that K^+ can reduce the level of the intermediate at all concentrations of F^- tested suggests that F^- inhibits the K^+-stimulated ATPase by acting at a step other than the dephosphorylation step.

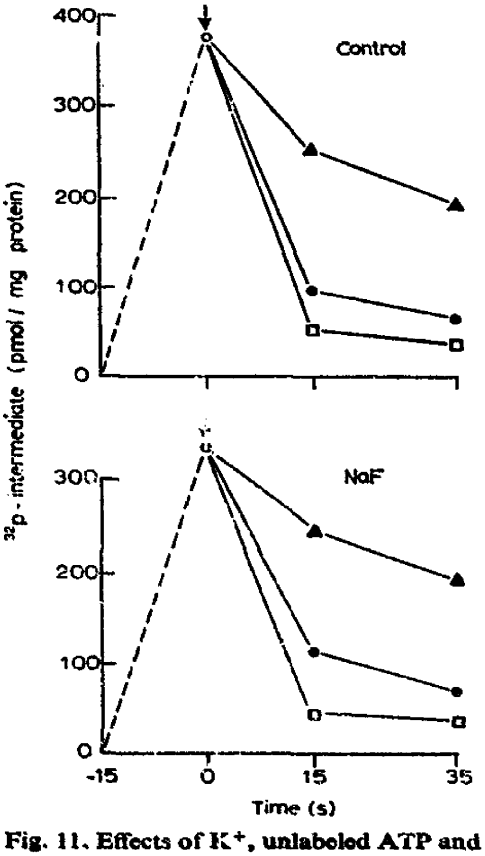

Fig. 11. Effects of K^+, unlabeled ATP and ADP on the steady-state level of the intermediate formed by the control and F^--treated membranes. Membranes were first preincubated with and without 0.5 mM NaF for 15 min at room temperature. The reaction medium contained 2.7 μg protein and 7 μM γ-[^{32}P]ATP. The point of addition of unlabeled 0.12 mM ATP (□), 0.24 mM ADP (▲), or 2 mM K^+ (●) is shown by arrow.

In order to get further insight as to the mechanism of F^- inhibition, we studied the turnover of the F^--treated and control membranes. Fig. 11 shows that ATP with K^+ lowers the level of the intermediate to about the same extent in both F^--treated and control membranes.

DISCUSSION

Some ATPases, such as the $(Na^+ + K^+)$-ATPase from a variety of tissues [16, 17] and the ATPase of muscle sarcoplasmic reticulum [18] give phosphorylated intermediates which have been characterized as part of the ATPase reaction. In this paper, we have described the properties of the phosphorylated intermediate of an ATPase which is associated with a light membrane fraction isolated from rabbit gastric mucosa. This K^+-stimulated ATPase is found in oxyntic glands and, unlike $(Na^+ + K^+)$-ATPase, is insensitive to Na^+ and ouabain. Mg^{2+} is absolutely necessary for the formation of the gastric membrane intermediate. Na^+, which stimulates the formation of the intermediate of $(Na^+ + K^+)$-ATPase, does not do so in the case of the gastric ATPase; however, K^+ and its analogs significantly stimulate hydrolysis of ATP and reduce the level of the intermediate for both of these ATPases. The cationic affinities for reducing the intermediate and stimulating gastric ATPase activity are about the same, with the order of effectiveness being $Tl^+ > K^+ > Rb^+$ (Fig. 2). Although Na^+ does not stimulate the hydrolysis of ATP for the gastric system, it does reduce the level of intermediate to a small extent. At present, this latter effect of Na^+ is not clear, but it is very different from what is found in the case of $(Na^+ + K^+)$-ATPase.

The relationships found between the intermediate and ATPase give clues to features of a possible reaction sequence. Mg^{2+} is necessary for the formation of the intermediate, and K^+ catalyzes the dephosphorylation step, which breaks down the intermediate. A simple model for the sequence of ATPase reaction which would be consistent with data so far discussed can be represented as follows:

$$E + ATP \xrightarrow{Mg^{2+}} E{-}P \xrightarrow{K^+} E + P_i$$

In the first Mg^{2+}-requiring step, the terminal phosphate group of ATP is transferred to the enzyme with a release of ADP. In a subsequent step, hydrolysis of the enzyme-phosphate bond is accelerated by K^+. The differential rate constants of the combined steps make up a K^+-stimulated ATPase activity. Since the level of intermediate is reduced by K^+ in the presence of partially inhibitory concentrations of EDTA (Fig. 3), it is likely that the dephosphorylation step is independent of Mg^{2+}.

The apparent K_m for the hydrolysis of ATP is about 50-times higher than the K_m for formation of the intermediate (Figs. 6 and 7). This might suggest the possibility of two different enzymes, one of high K_m value and another of low K_m. Since the level of the intermediate does not increase with increasing concentration of ATP, the low K_m enzyme would have to produce almost all of the phosphorylated intermediate and show low turnover. The high K_m enzyme producing virtually no intermediate would unlikely be sensitive to K^+. Under these conditions, it would be difficult to explain the enhanced rate of hydrolysis by K^+ at higher ATP concentration using the two enzyme hypothesis. An alternative possibility for the apparent differences in K_m value is that ATP may have a secondary effect at higher concentration, perhaps

at some point beyond the initial enzyme-substrate complex, which increases the turnover of the enzyme. Such an explanation would satisfy the intermediate and hydrolysis data at different concentrations of ATP, and it also implies the involvement of multiple steps and delicate control mechanisms for this ATPase reaction. It is of interest to mention that in the case of $(Na^+ + K^+)$-ATPase the apparent K_m of ATP for phosphorylation catalyzed by Na^+ has been reported to be 300-fold lower than the K_m of ATP for hydrolysis in the presence of both Na^+ and K^+ [19], and the K_m for hydrolysis varies directly with concentration of K^+ [20]. The data of Post et al. [19] strongly suggest that higher concentrations of ATP (0.1 mM) may act as an activator of the ATPase in a fashion functionally distinct from its role as a phosphate donor.

We have considered a number of alternative possibilities to interpret our results concerning the basal and K^+-stimulated ATPase and the phosphorylated intermediates associated with these conditions. Our data do not support the hypothesis that either the basal or the K^+-stimulated ATPase alone produce all the intermediates. In the former case K^+ should not reduce the steady-state level of the intermediate, and in the latter unlabeled ATP in the absence of K^+ should not reduce the steady-state level of the intermediate to any significant extent. On the contrary, we observe that in the absence of K^+ the steady-state level of intermediate reaches a new low value within a few seconds after addition of unlabeled ATP. Such results suggest that phosphorylated intermediates are associated with both the basal and K^+-stimulated ATPase, and that they may share some common interrelations in the overall reaction sequence. Studies with inhibitors of K^+-stimulated ATPase, such as Zn^{2+}, MalNEt and NaF, further strengthen this idea. To account for some of the complexities introduced by these various results, we propose the following model for the gastric microsomal ATPase.

$$E + ATP \longrightarrow E \cdot ATP \xrightarrow{Mg^{2+}} E_1 - P \longrightarrow E_1 + P_i$$
$$\updownarrow$$
$$E_2 - P \xrightarrow{K^+} E_2 + P_i$$

In this scheme, an initial enzyme-substrate complex ($E \cdot ATP$) eventually gives rise to two distinct forms of phosphorylated intermediate. The basal ATPase activity is expressed through the turnover of $E_1 - P$, and the K^+-stimulated activity is expressed through the turnover of $E_2 - P$. Since the K^+-stimulated activity appears to be most sensitive to the higher concentrations of ATP without any significant effect on the basal rate, the activating effect of ATP, as discussed previously, may be taking place in the $E_2 - P$ pathway.

Zn^{2+}, MalNEt and F^- have little or no effect on the basal rate of ATPase at such concentrations when the K^+-stimulated rate is abolished. This suggests that the turnover of $E_1 - P$ remains unaffected in presence of the inhibitors.

Elevation in the level of phosphorylated intermediate in the presence of K^+ with a simultaneous reduction of K^+-stimulated ATPase activity at Zn^{2+} concentrations between 0.1 and 0.3 mM (Fig. 10) can be explained by an inhibitory effect of this divalent cation on the K^+-stimulated dephosphorylation step of $E_2 - P$. Similar results for MalNEt pretreated membranes (Table IV) suggest that the K^+-stimulated dephosphorylation of the $E_2 - P$ intermediate is also sensitive to this inhibitor.

MalNEt might also have some effect on another step in the reaction. Since, in the absence of K^+ there appeared to be a decrease in the rate of exchange with unlabeled ATP as compared to the untreated membranes (Fig. 11), but no effect was apparent on the steady-state level of intermediate or on the basal ATPase, an action of MalNEt on the enzyme-substrate formation step is proposed.

Like Zn^{2+} and MalNEt, F^- also appeared to be relatively specific for the K^+-stimulated portion of the ATPase reaction; however, F^- did not abolish the effects of K^+ on reducing the level of the ^{32}P intermediate (Fig. 11). Thus, F^- must have its effect prior to the K^+-dependent dephosphorylation of E_2—P, and since the basal ATPase is virtually unaffected by F^-, the step involving interconversion between E_1—P and E_2—P is suggested. Although it would be expected from such an explanation that after the addition of unlabeled ATP, the E_2—P form of the F^- treated enzyme would disappear at a slower rate than the native enzyme, we could not detect such a distinction in the kinetic experiments (e.g. Fig. 11), possibly due to the fact that the time resolution was not adequate.

It is of interest to compare the effects of MalNEt in our system with those of the $(Na^+ + K^+)$-ATPase reported by Post et al. [21]. In both cases, the intermediates derived from the MalNEt treated membranes lose their sensitivity to K^+. However, the effects of ADP on the MalNEt-poisoned preparation is quite different for the two systems. In the case of $(Na^+ + K^+)$-ATPase, ADP had no significant effect on the steady-state level of intermediate derived from the native enzyme, but had a dramatic effect in reducing the level of the intermediate from NEM-poisoned membranes. In our system, we find that ADP lowers the level of the intermediate from both native and MalNEt treated membranes, and to about the same extent. Also, oligomycin, which has been reported to have effects similar to MalNEt on the $(Na^+ + K^+)$-ATPase [21], shows no appreciable effect on the K^+-stimulated ATPase system of gastric microsomes (unpublished observation). Thus, in spite of certain similarities between these two ATP utilizing systems, there clearly exist some very fundamental differences in specific mechanisms of these enzymes.

In a previous study, it was shown by sodium dodecyl sulfate acrylamide gel electrophoresis that the ^{32}P-intermediate of gastric microsomes migrated as a major band of 90 000–100 000 daltons [3]. The molecular size of this presumed subunit of the gastric ATPase molecule is very similar to that of the $(Na^+ + K^+)$-ATPase or of the ATPase of sarcoplasmic reticulum. For these latter systems, specification of aspartyl phosphate [22], as well as numerous other similarities of amino acid sequence surrounding the active phosphoenzyme site [12], represent a rather intriguing set of comparisons for the two ion transport enzymes. Since the gastric ATPase, which may be involved in some K^+/H^+ transport process in the oxyntic cell, also shares some common features with the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-ATPase, it is tempting to speculate on the principles of commonality which may underlie the function of the various membrane transport proteins. However, before such an analysis could proceed with any real meaning, it will be essential to have a more complete assessment of the kinetic mechanisms and molecular architecture of the transport enzymes.

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