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STUDIES ON (K^+ + H^+)-ATPase

III. BINDING OF ADENYLYL IMIDODIPHOSPHATE

F.J.M.VAN DE VEN, J.J. SCHRIJEN, J.J.H.H.M. DE PONT and S.L. BONTING *

Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)

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Summary

1. Adenylyl imidodiphosphate (AMPPNP) binds to (K^+ + H^+)-ATPase from pig gastric mucosa with a dissociation constant (K_d) of 50 μM for the AMPPNP-enzyme complex.

2. Monovalent cations reduce the amount of AMPPNP bound in the following order of effectiveness $Tl^+ > K^+ > Rb^+ > Cs^+ > Na^+$, Li^+, choline$^+$.

3. AMPPNP binding to the enzyme has a pH optimum at pH 7.0—7.5 in the absence of added ions, which is shifted to pH 8 upon addition of $MgCl_2$.

4. Cyclodiaminotetraacetic acid (CDTA, Tris salt) inhibits binding of AMPPNP. This inhibition is not due to chelation of Mg^{2+}. It may be due to direct binding of CDTA to the enzyme or to removal of stabilizing cations other than Mg^{2+}.

5. Binding curves determined in the presence of various concentrations of Mg^{2+} show that at low Mg^{2+} concentrations (less than 0.5 mM), the apparent number of binding sites is reduced, while at higher Mg^{2+} concentrations (greater than or equal to 0.5 mM), the binding of AMPPNP is inhibited in a competitive way.

6. From these observations it is concluded that the enzyme has two binding sites for AMPPNP and only one for Mg-AMPPNP (or two with strong anticooperativity), and that Mg^{2+} inhibits binding of Mg-AMPPNP. This finding is interpreted in terms of a model involving a dimeric form of the enzyme.

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* To whom correspondence should be addressed.

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid; AMPPNP, adenylyl imidodiphosphate.

Introduction

The tubulovesicular and apical membranes of the gastric parietal cells contain a Mg^{2+}-dependent, K^+-stimulated ATPase, $(K^+ + H^+)$-ATPase, which is believed to be involved in the gastric acid secretion. In the presence of Mg^{2+} and ATP, the enzyme forms a phosphorylated intermediate [1] which is hydrolysed under the influence of K^+ [1,2]. In the binding of ATP, which precedes phosphorylation, a specific arginine group appears to play a crucial role [3,4]. Arginine-modification studies with butanedione suggest that K^+ reduces ATP binding. On the other hand, Mg^{2+} seems to induce an enzyme conformation in which the essential arginine group in the ATP binding site appears to be more exposed; consequently, it was thought that Mg^{2+} may favor substrate binding [4].

Sulfhydryl group modification with 5,5'-dithiobis(2-nitrobenzoic acid) presents further evidence for specific conformations of the $(K^+ + H^+)$-ATPase, induced by activating monovalent cations and Mg^{2+} [5]. An essential sulfhydryl group, which is reactive in the absence of Mg^{2+} and which is most probably not located in the ATP binding site, seems to play a role in the dephosphorylation process. Of the two sulfhydryl groups, which are discernible in the presence of Mg^{2+}, the slowly reacting group appears to be involved in a step leading to enzyme phosphorylation, viz. substrate binding or phosphorylation.

These indirect measurements have led us to study substrate binding under the influence of Mg^{2+} and monovalent cations. In order to study the binding step per se, we have used a non-phosphorylating ATP analogue, adenylyl imidodiphosphate (AMPPNP). The effects of Mg^{2+}, CDTA, various monovalent cations and pH on binding of AMPPNP have been investigated and the findings are interpreted in the form of a dimeric model of the enzyme.

Materials and Methods

Materials. [2,8-^{3}H]AMPPNP (sodium salt) was purchased from ICN Pharmaceuticals, Cleveland, OH; nonradioactive AMPPNP (lithium salt) from Boehringer Mannheim, F.R.G.; Dowex 50W-X4 from Fluka, Buchs, Switzerland; Pico-Fluor 15 from Packard, Brussels, Belgium, and all other reagents in highest available purity from Merck, Darmstadt, F.R.G.

Isolation and assay. A $(K^+ + H^+)$-ATPase-containing membrane fraction is isolated from pig gastric mucosa as previously described [4]. The membrane fraction obtained after gradient centrifugation is washed once with 10 mM cyclohexanediaminotetraacetic acid (CDTA) adjusted with Tris to pH 7.4, and once with Tris-HCl buffer (25 mM, pH 7.4). The pellet is then resuspended in 0.25 M sucrose to a protein concentration of 6 mg/ml and stored at −20°C.

The ATPase assay is carried out as previously described [4], whilst protein is determined by the method of Lowry et al. [6] with bovine serum albumin as standard. The enzyme preparations routinely have a specific K^+-stimulated activity of 80—120 μmol P_i liberated/h per mg protein, which is more than 90% of the total ouabain-insensitive ATPase activity. The $(Na^+ + K^+)$-ATPase activity of these preparations amounts to about 30% of the value found for

the $(K^+ + H^+)$-ATPase activity [4]. The $(K^+ + H^+)$-ATPase activity of freeze-thawed preparations is not stimulated by the ionophores valinomycin and gramicidin, suggesting a high permeability for K^+ or an open vesicle structure.

Magnesium determination. Samples containing approx. 5 nmol Mg^{2+} are dried at 100°C and digested for 30 min at 150°C with 200 µl $H_2SO_4/HClO_4/HNO_3$ (1 : 3 : 12, v/v/v) in quartz tubes. After cooling to room temperature, 1.5 ml 0.5% (w/v) $LaCl_3$ in 0.1 M HCl is added. After mixing, magnesium is determined in an atomic absorption spectrometer (Pye Unicam SP 1950), using suitable standards of $MgSO_4$.

The enzyme preparations routinely contain 20—100 µmol Mg^{2+}/g protein. When washing with CDTA during isolation is omitted, the Mg^{2+} content is double or more. Attempts at removing Mg^{2+} by multiple washings with CDTA, gel filtration and dialysis against a Dowex 50W-X4 suspension failed to reduce the Mg^{2+} content below 10 µmol/g protein, and caused nearly complete loss of enzyme activity. Hence, preparations washed once with CDTA have been used in all further experiments.

The rather firmly bound 20—100 µmol Mg^{2+}/g protein corresponds to 3—7 mol Mg^{2+}/mol enzyme (70% M_r 100 000 enzyme protein according to SDS-gel electrophoresis). This high molar ratio suggests that part of the Mg^{2+} is bound to phospholipids and membrane proteins other than $(K^+ + H^+)$-ATPase.

AMPPNP binding assay. Radioactive [2,8-^{3}H]AMPPNP, mixed with non-radioactive AMPPNP to a specific activity of 75 Ci/mol, is converted to the Tris-salt by passage over a Dowex 50W-X4 cation-exchange resin (200—400 mesh) in the Tris form.

Equal volumes of enzyme preparations (1 mg/ml) in 50 mM imidazole hydrochloride buffer (pH 7) and of [2,8-^{3}H]AMPPNP in the same buffer are mixed. Ligands (Mg^{2+}, K^+, CDTA) are added to the AMPPNP buffer solution. After 10 min incubation at room temperature (22 ± 1°C) a 50 µl aliquot of the mixture is layered over 50 µl of a mixture of corn oil/dibutyl phthalate (1 : 3, v/v; d = 1.01 g/ml at 22°C). After 5 min centrifugation at 160 000 × g in an air-driven Beckman ultracentrifuge, the supernatant is removed by suction. The bottom of the centrifuge tube with the pellet is cut off and is placed in a tube containing 0.4 ml 10% (w/v) sodium dodecyl sulfate solution. After vigorous mixing, the tube is allowed to stand for 1 h at room temperature. The solubilized pellet is mixed with 4.0 ml of Pico-fluor and counted for ^{3}H radioactivity. Blanks are prepared by including an additional 5 mM non-radioactive AMPPNP in the incubation medium.

Results

AMPPNP binding without ligands

The amount of AMPPNP bound to $(K^+ + H^+)$-ATPase protein has been determined as a function of the AMPPNP concentration at pH 7.0 and at 22°C. The dissociation constant (K_d) of the enzyme-AMPPNP complex and the maximal binding capacity (y_{max}), derived from a double reciprocal plot as shown in Fig. 1, are found to vary from 40 to 60 µM and from 2 to 3.5 µmol/g protein, respectively, for the various preparations used in this study. For a single preparation, the results are reproducible to within 10%.

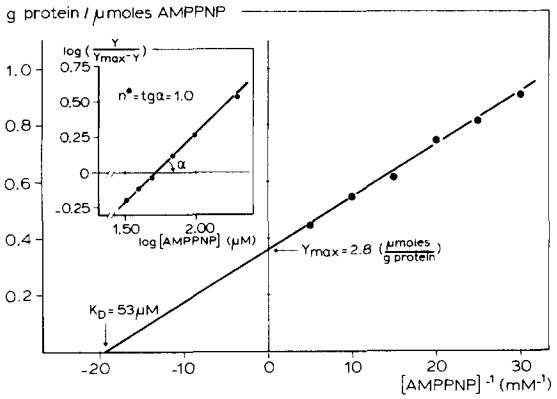


Fig. 1. Binding of AMPPNP to $(K^+ + H^+)$-ATPase from pig gastric mucosa. Double-reciprocal plot for a typical experiment out of six. Assay conditions are: 0.5 mg/ml enzyme protein, 50 mM imidazole hydrochloride buffer (pH 7), AMPPNP in the indicated concentrations. Incubation for 10 min at 22°C. No ligands added (endogenous Mg^{2+} 25 μM). Inset: Hill plot derived from these data.

Since at higher AMPPNP concentrations high blank values are obtained, it is impossible to determine whether any binding of AMPPNP to 'low-affinity sites' occurs. Up to 500 μM AMPPNP, no deviation from linearity of the Lineweaver-Burk plots is observed. This suggests the absence of (anti-) cooperativity, which can also be concluded from the slope of the Hill-plot: $n^* = 1.0$ (Fig. 1, inset).

In view of the presence of considerable $(Na^+ + K^+)$-ATPase activity in the membrane fraction, a control experiment is carried out in which $1 \cdot 10^{-4}$ M ouabain and 5 mM Mg^{2+} are included in the binding assay medium, which should greatly inhibit substrate binding to $(Na^+ + K^+)$-ATPase [7]. Since the binding of AMPPNP to the gastric microsomal membranes is not detectably decreased, only a very small amount of the total AMPPNP binding can be due to binding to the $(Na^+ + K^+)$-ATPase. Hence, we have omitted ouabain in subsequent experiments.

In another control experiment (not shown), the incubation time has been

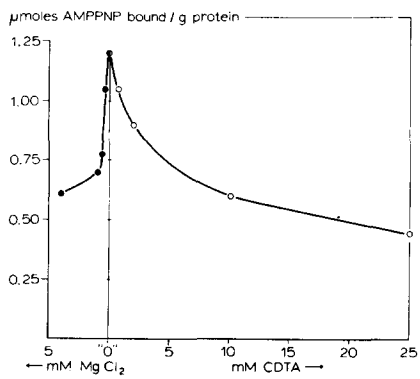


Fig. 2. Effect of $MgCl_2$ and CDTA on binding of AMPPNP to $(K^+ + H^+)$-ATPase. Assay conditions are: 0.5 mg/ml enzyme protein, 100 μM AMPPNP, 50 mM imidazole hydrochloride (pH 7.0), ligands added as indicated (endogenous Mg^{2+} 25 μM). Incubation for 10 min at 22°C. Typical experiment out of two.

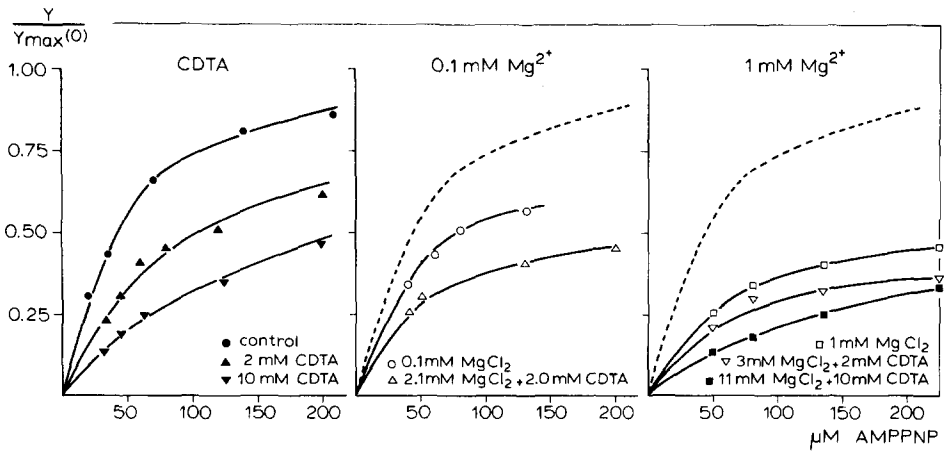


Fig. 3. Effects of $MgCl_2$ and CDTA on binding of AMPPNP to ($K^+ + H^+$)-ATPase. Assay conditions are: 0.5 mg/ml protein, 50 mM imidazole hydrochloride (pH 7.0); AMPPNP, $MgCl_2$ and CDTA as indicated in the figures. y = μmol AMPPNP bound/g protein; $y^{(0)}_{max}$ = maximal value for y (obtained from a double-reciprocal plot) in the absence of added ligands.

varied from 5 min to 2 h, and the imidazole-buffer concentration from 25 to 200 mM. No deviations from the values obtained under standard conditions (10 min incubation, 50 mM imidazole) are observed, which suggests that true equilibrium values are determined and that changes in ionic strength have little effect on the binding properties of the enzyme. In view of the latter finding, we have not tried to maintain constant ionic strength in experiments where various amounts of ions (Mg^{2+}, K^+) are added to the assay medium.

In a third control experiment, we have preincubated (1 h at 22°C) either the membrane suspension or the AMPPNP solution or both with ligands (Mg^{2+}, K^+) added in concentrations so as to produce the same final ionconcentrations after mixing the enzyme and AMPPNP. Identical binding levels are obtained in all three cases, which indicates that equilibrium is reached.

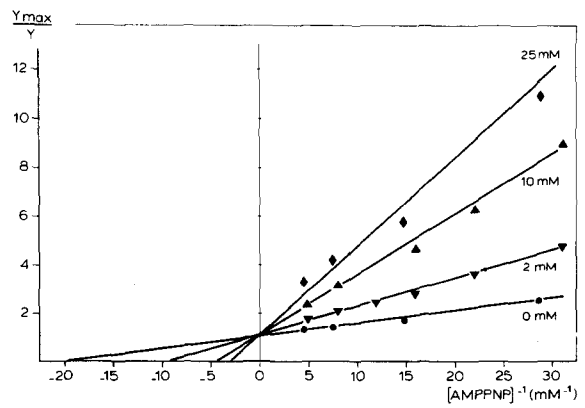


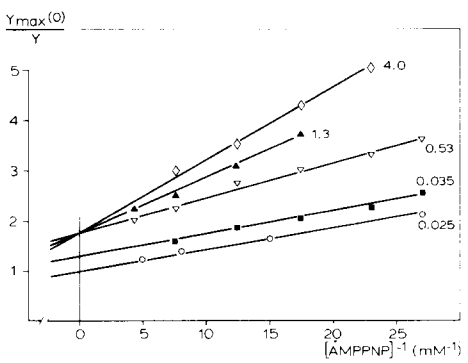
Fig. 4. Inhibition by CDTA of binding of AMPPNP to ($K^+ + H^+$)-ATPase. Assay as described in Materials and Methods with CDTA (Tris-salt) added to the medium in final concentrations: 0 mM (●); 2 mM (▼); 10 mM (▲) and 25 mM (◆, as sodium salt). Typical experiment out of two.

Effects of Mg^{2+} and CDTA

The membrane suspension is incubated in a medium containing 0.1 mM AMPPNP to which either $MgCl_2$ (0—4 mM) or CDTA (0—25 mM) is added, and AMPPNP binding is determined. Fig. 2 shows that addition of Mg^{2+} sharply reduces the amount of AMPPNP bound to the membrane protein. Since addition of CDTA also reduces AMPPNP binding, it appears that either there exists an optimal Mg^{2+} concentration which happens to be close to the amount of Mg^{2+} already present in the enzyme preparations, or that Mg^{2+} and CDTA inhibit binding by different mechanisms.

In order to discriminate between these two possibilities, binding curves have been determined in the presence of mixtures of $MgCl_2$ and CDTA (Fig. 3). It is obvious that the concentration of free Mg^{2+} is not the only parameter determining the reduction of binding and that CDTA itself also acts as an inhibitor of AMPPNP binding to the enzyme. Binding curves at various CDTA concentrations, shown as double-reciprocal plots in Fig. 4, indicate that CDTA only affects K_d, the apparent dissociation constant of the enzyme-AMPPNP complex, and not y_{max}, which is proportional to the apparent number of sites.

It is rather difficult to determine the effects of low concentrations of Mg^{2+} on the binding process, because the enzyme preparations contain appreciable amounts of firmly bound Mg^{2+}, while CDTA, to be used to buffer the free Mg^{2+} concentration, inhibits the binding of AMPPNP independently. However, the effects of Mg^{2+} at concentrations above 25 μM can be determined, as already indicated in Fig. 2 (left side). This has been studied more systematically by determining AMPPNP binding curves at Mg^{2+} concentrations between 25 μM (endogenous Mg^{2+} only) and 4 mM (Fig. 5). At low Mg^{2+} concentrations



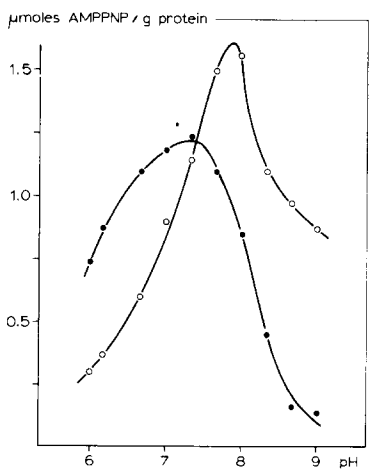


Fig. 5. Effect of Mg^{2+} on binding of AMPPNP to $(K^+ + H^+)$-ATPase. The assay procedure is described in Materials and Methods. Total magnesium concentrations (endogenous Mg^{2+} + added $MgCl_2$) are 25 μM (●), 35 μM (■); 0.53 mM (▼); 1.3 mM (▲); 4.0 mM (◆). Typical experiment out of three ($y^{(o)}_{max} = y_{max}$ for 25 μM Mg^{2+}).

Fig. 6. Effect of pH on binding of AMPPNP to $(K^+ + H^+)$-ATPase. Assay conditions are: 0.5 mg/ml protein, 100 μM AMPPNP, 50 mM imidazole (adjusted with 4 M HCl to produce the indicated pH values), with (○) and without (●) 5 mM $MgCl_2$. Typical experiment out of two.

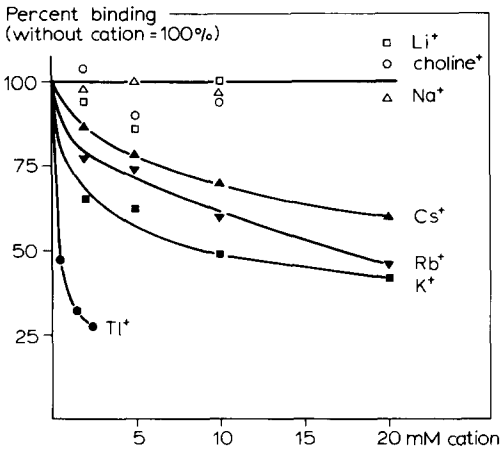


Fig. 7. Effects of monovalent cations on binding of AMPPNP to $(K^+ + H^+)$-ATPase. AMPPNP (^3H) is mixed with the enzyme to produce final concentrations: 0.5 mg/ml protein, 100 μM AMPPNP, 50 mM imidazole hydrochloride (pH 7.0) and salts as indicated in the figure. Salts are: Choline chloride (○); LiCl (□); NaCl (△); CsCl (▲); RbCl (▼); KCl (■) and TlCl (●). Typical experiment out of two.

(25—35 μM) the slopes of the double-reciprocal plots appear to be unaffected, whereas the apparent number of AMPPNP binding sites (y_{max}) is decreased with increasing Mg^{2+} level. At high Mg^{2+} concentrations (0.5—4.0) y_{max} is not changed further but the K_d values are increased.

Effect of pH and monovalent cations

Since mutual binding of Mg^{2+}, AMPPNP and enzyme would be expected to be pH-dependent, the effect of pH on the binding of AMPPNP has been studied with and without added Mg^{2+}. At a constant AMPPNP concentration of 100 μM, binding has been assayed at pH values ranging from 6 to 9 (Fig. 6). In the absence of added Mg^{2+}, maximal binding occurs at pH 7.0—7.5, whereas in the presence of 5 mM $MgCl_2$ the pH optimum is shifted to pH 8 and the optimum binding level is increased by 35% in the presence of 100 μM AMPPNP.

Butanedione experiments indicate indirectly that certain monovalent cations antagonize ATP binding [4]. Hence, we have determined AMPPNP binding in the presence of various concentrations of TlCl, KCl, RbCl,CsCl, NaCl, LiCl and choline chloride (0—20 mM, Tl^+; 0—2.5 mM) in the assay medium (Fig. 7). The ions inhibit binding in the following order of effectiveness: $Tl >> K^+ > Rb^+ > Cs^+ > Na^+$, Li^+, choline, the latter three having no effect at concentrations up to 20 mM. This order of effectiveness is the same as that found for their activation of the overall enzyme activity and for their protection against butanedione inactivation in the absence of ATP [4].

Discussion

Introductory remarks

The purpose of our study was to investigate the effect of various ligands (monovalent cations, Mg^{2+}, CDTA) on substrate binding to $(K^+ + H^+)$-ATPase

of gastric mucosa. The non-phosphorylating ATP analogue AMPPNP has been used, since ATP in the presence of Mg^{2+} leads to phosphorylation. Experiments with purified $(Na^+ + K^+)$-ATPase from kidney outer medulla have shown that at pH 7.0, AMPPNP reaches the same binding level as ATP, although the K_d value for the analogue (2 μM) is 6-times as high as for ATP (Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., de Pont, J.J.H.H.M. and Bonting, S.L., unpublished observations).

Our gastric microsomal membranes have a limited purity with regard to $(K^+ + H^+)$-ATPase: the M_r 100 000 band represents only 70% of the protein after sodium dodecyl sulfate gel electrophoresis, while also other ATPase activities, viz. Mg^{2+}, HCO_3^- and $(Na^+ + K^+)$-stimulated ATPase are present [4]. The last enzyme activity is present in considerable amount but a control experiment with $1 \cdot 10^{-4}$ M ouabain and 5 mM $MgCl_2$ present in the binding assay medium reveals that it can bind only a very minor part of the total amount of AMPPNP bound by the membranes. This can be explained by considering that the $(Na^+ + K^+)$-ATPase has a much higher turnover number than the $(K^+ + H^+)$-ATPase and consequently is present in a much lower molar concentration than the latter enzyme. Moreover, because of the much higher nucleotide affinity of the $(Na^+ + K^+)$-ATPase, its binding sites will be completely occupied by AMPPNP at the concentrations used in our experiments. Hence, the observed effects at these relatively high AMPPNP concentrations cannot be significantly influenced by the presence of this enzyme.

The maximal binding level of 3000 μmol AMPPNP/mg protein, observed in these experiments, would represent 0.3 ml AMPPNP/mol M_r 100 000 subunit at 100% protein purity. In fact, the M_r 100 000 band represents only 70% of total protein. Moreover, this band appears to be composed of three proteins, as has recently been concluded from tryptic inactivation studies [8]. Furthermore, during the centrifugation step in the binding assay some of the bound AMPPNP may dissociate from the enzyme. Hence, the true molar-binding value will be considerably higher than the calculated value. An estimation of the molar-binding ratio per mol $(K^+ + H^+)$-ATPase cannot be given because the molecular weight and subunit composition of the enzyme are still uncertain.

Effects of pH and monovalent cations

The effects of pH on binding (Fig. 6) can readily be explained by assuming electrostatic interaction between AMPPNP and basic amino acid residues of $(K^+ + H^+)$-ATPase. Optimal binding is expected to occur at a pH value which is high enough to ensure dissociation of protons of AMPPNP but low enough for association of protons to basic amino acid residues in the AMPPNP binding centre of the enzyme. In the presence of 5 mM $MgCl_2$, when Mg-AMPPNP is the substrate, these amino acid residues should be less protonated (less positive) for optimal binding to occur and, hence, the pH optimum is shifted to the alkaline region.

Monovalent cations like Tl^+, K^+, Rb^+ and Cs^+ stimulate the $(K^+ + H^+)$-ATPase activity by enhancing its dephosphorylation. They also decrease the binding of AMPPNP in the same order of effectiveness as is found for overall enzyme activity [4] and dephosphorylation [1]. This parallelism confirms the earlier

conclusion from butanedione modification studies [4] that the binding of these monovalent ions induces a conformational change in the enzyme, which reduces substrate binding and increases dephosphorylation.

Effect of CDTA

In an attempt to study the binding of AMPPNP in the absence of Mg^{2+}, we have added CDTA as a complexant for Mg^{2+}. From the evidence shown in Figs. 2 and 3, it is clear that CDTA has an inhibitory effect of its own, which cannot be explained by complexation of Mg^{2+}. Similar direct effects of chelating agents have been reported for $(Na^+ + K^+)$-ATPase [9].

Fig. 4 shows that CDTA inhibits the binding of AMPPNP to $(K^+ + H^+)$-ATPase in a competitive-like fashion. This can be interpreted in two different ways: (1) CDTA reduces the affinity of the enzyme for AMPPNP, (2) CDTA complexes a cation which stabilizes the enzyme. The main difference between the two models is that for the second one we must expect that addition of CDTA and a certain amount of ion X would produce the same binding curve as is determined in the absence of CDTA. Discrimination between these two possibilities is not possible, since the mathematical derivations for the two models reveal the same dependence of the apparent dissociation constant K_d on the CDTA concentration.

Effect of Mg^{2+}

From Fig. 2 we have concluded that added Mg^{2+} (in addition to the endogenously present 20—100 μmol Mg^{2+}/g protein) greatly inhibits the binding of AMPPNP. From the results in Fig. 5, we conclude that addition of upto 0.5 mM Mg^{2+} lowers the apparent number of AMPPNP binding sites (y_{max}) by about half. Further addition of Mg^{2+} (upto 4 mM) does not reduce the number of binding sites anymore but it increases the dissociation constant.

In interpreting these finding we must bear in mind the following points. First, AMPPNP binds Mg^{2+} with a K_c for the Mg-AMPPNP complex of approx. 0.1 mM at pH 7 [10,11] and this dissociation constant is pH-dependent. Secondly, the concentration of free Mg^{2+} and the (Mg-AMPPNP)/(AMPPNP) ratio are not constant in any of the curves of Fig. 5. Thirdly, the fractions of Mg^{2+} and AMPPNP bound to the enzyme are negligibly small at the relatively high concentrations used.

The concentrations of free Mg^{2+} and AMPPNP can be calculated with the following equations:

$$\text{AMPPNP: } [A] = \frac{K_t \cdot [A]_{tot}}{K_c + [M]} \tag{1}$$

$$\text{Mg-AMPPNP: } [MA] = [A]_{tot} - [\text{AMPPNP}] = \frac{[A]_{tot} \cdot [M]}{K_c + [M]} \tag{2}$$

$$\text{Mg}^{2+}\text{: } [M] = \tfrac{1}{2}([M]_{tot} - [A]_{tot} - K_c) + \tfrac{1}{2}\{([M]_{tot} - [A]_{tot} - K_c)^2 + 4\,K_c \cdot [M]_{tot}\}^{1/2} \tag{3}$$

where $[A]$ and $[A]_{tot}$ are the concentrations of free and total AMPPNP, respectively, and $[M]$ and $[M]_{tot}$ those of free and total Mg^{2+}, respectively (see Appendix, last part).

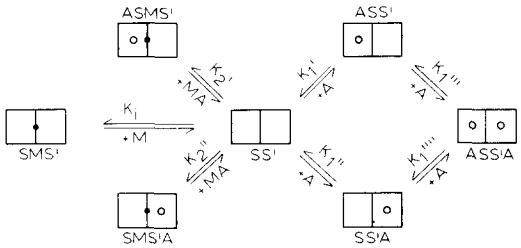


Fig. 8. Schematic presentation of the dimeric model of $(K^+ + H^+)$-ATPase. Symbols: □□, dimeric $(K^+ + H^+)$-ATPase molecule; ○, AMPPNP; ●, Mg^{2+}; A, AMPPNP; M, Mg^{2+}; S and S', the two catalytic subunits of a $(K^+ + H^+)$-ATPase molecule. The same symbols are used in the Appendix.

The lowering of the apparent number of AMPPNP binding sites (y_{max}) at increasing Mg^{2+} concentration cannot be explained by means of these equations, when only one AMPPNP binding site per enzyme molecule is assumed. The simplest model that can account for our observations involves a dimeric model of the enzyme.

Dimeric model of $(K^+ + H^+)$-ATPase

We assume that there are two independent, identical AMPPNP sites per enzyme molecule and that after binding of Mg^{2+} both sites have a strong anti-cooperative interaction, making them mutually exclusive with respect to binding of AMPPNP. This is schematically represented in Fig. 8.

In the Appendix, we derive an equation describing AMPPNP binding to $(K^+ + H^+)$-ATPase according to this model:

$$\frac{y}{y_{max}} = \frac{\frac{[A]}{K_1}\left(1 + \frac{[A]}{K_1}\right) + \frac{[MA]}{K_2}}{\left(1 + \frac{[A]}{K_1}\right)^2 + 2\frac{[MA]}{K_2} + \frac{[M]}{K_i}} \qquad (4)$$

where [A], [MA] and [M] are the concentrations of AMPPNP, Mg-AMPPNP and Mg^{2+}, respectively, not bound to the enzyme. The values of these concentrations can be calculated from the total concentrations by means of Eqns. 1, 2 and 3.

At very low concentrations of Mg^{2+} ($[M] << K_i$, $[MA] << K_2$ and thus $[A] \simeq [A]_{tot}$) Eqn. 4 simplifies to

$$\frac{y_{max}}{y} = 1 + \frac{K_1}{[A]_{tot}}$$

At high concentrations of Mg^{2+} ($[A] << K_1$ and thus $[MA] \simeq [A]_{tot}$) Eqn. 4 reduces to

$$\frac{y_{max}}{y} = 2 + \left(1 + \frac{[M]}{K_i}\right) \cdot \frac{K_2}{[A]_{tot}}$$

These equations do indeed describe the observed double-reciprocal plots at low and high Mg^{2+} concentrations (Fig. 5), including the shift in y_{max} and the competitive-like behaviour of Mg^{2+}. We have calculated double-reciprocal plots

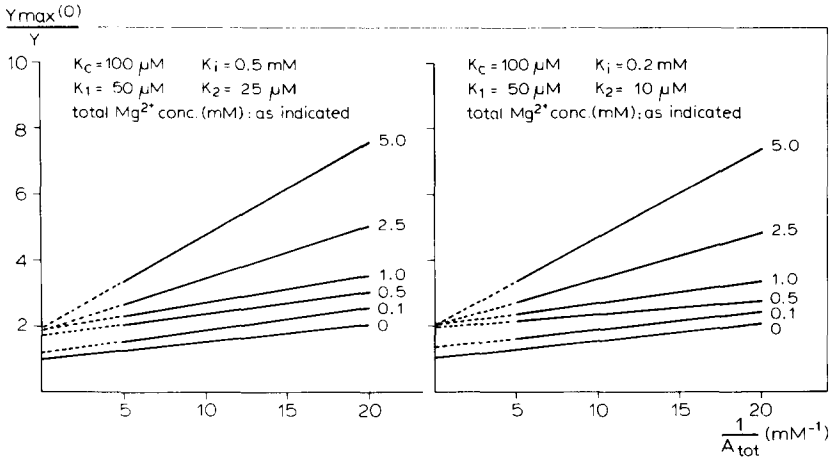


Fig. 9. Theoretical double-reciprocal plots for the effect of Mg^{2+} on binding of AMPPNP to $(K^+ + H^+)$-ATPase. Plots are calculated from Eqns. 3, 4, 5 and 6, described in the text. At values $1/[A]_{tot} << 5\ mM^{-1}$ the curves deviate from linearity and intersect the ordinate at $y^{(o)}_{max}/y = 1$ (blank values are too high to obtain reliable experimental data in this region).

for various values of all parameters (K_c, K_i, K_1, K_2, $[M]_{tot}$ and $[A]_{tot}$), two examples of which are shown in Fig. 9. Comparing these curves with the experimental curves in Fig. 5, we conclude that the dimeric model can qualitatively explain our observations.

We have not attempted to 'fit' the theoretical parameters to the experimental results because the parameters are strongly interdependent, especially K_i and K_2 (as can be seen from Fig. 8), and because the experimental values for different preparations vary too much to allow unambiguous determination of the theoretical parameters. The variations in the experimental values are correlated with differences in the amounts of endogenous magnesium, although the same qualitative results are obtained.

Since Ray and Forte [1] found that the phosphorylation is Mg^{2+}-dependent, while we find that the actual nucleotide binding shows no Mg^{2+} requirement, we may conclude that Mg^{2+} is involved in the mechanism of transfer of the γ-phosphate from ATP to the enzyme. It is remarkable that the amount of endogenous Mg^{2+} suffices to yield maximal phosphorylation [1] but not to saturate either AMPPNP or $(K^+ + H^+)$-ATPase with Mg^{2+} in our experiments. A more detailed comparison of AMPPNP binding and phosphorylation must await further experimental data.

Our theoretical curves (Fig. 9) show optimal agreement with the experimental curves when we choose $K_2 < K_1$ (Fig. 8), which means that Mg-AMPPNP binds stronger than AMPPNP at pH 7.0. This may seen contradictory to the observations that addition of Mg^{2+} leads to decreased binding, but this is due to a reduction of the number of binding sites and to competitive inhibition by Mg^{2+} at higher Mg^{2+} concentrations. We interpret this as a result of a Mg^{2+}-induced conformational change, more particularly a Mg^{2+}-regulated interaction of the subunits. Chemical-modification studies of $(K^+ + H^+)$-ATPase with the arginine modifying reagent butanedione [4] and with the sulfhydryl

reagent 5,5'-dithiobis(2-nitrobenzoic acid) [5] also indicate that Mg^{2+} affects the conformational state of the enzyme.

In the proposed model, we assume a dimeric form of the $(K^+ + H^+)$-ATPase enzyme system, which agrees with conclusions of other authors [12] based on radiation-inactivation experiments [13] and on analysis of reconstituted membranes [14]. The exact form of the model is in our opinion less important than its characteristic properties: two sites for Mg-AMPPNP and inhibition of the Mg-AMPPNP binding by Mg^{2+}.

Appendix

Derivation of Eqn. 4

$SS' = (K^+ + H^+)$-ATPase, composed of subunits S and S'.

A = AMPPNP M = Mg^{2+} MA = Mg-AMPPNP

The interaction of the enzyme, composed of subunits S and S', with AMPPNP (A), Mg^{2+} (M) and Mg-AMPPNP (MA) is shown in Fig. 8 where:

$$K_1' = \frac{[SS'][A]}{[ASS']} \quad K_1'' = \frac{[SS'][A]}{[SS'A]}$$

$$K_1''' = \frac{[ASS'][A]}{[ASS'A]} \quad K_1'''' = \frac{[SS'A][A]}{[ASS'A]} \tag{1}$$

$$K_2' = \frac{[SS'][MA]}{[ASMS']} \quad K_2'' = \frac{[SS'][MA]}{[SMS'A]} \quad K_i = \frac{[SS'][M]}{[SMS']}$$

The binding equation is:

$$\frac{y}{y_{max}} = \frac{[ASS'] + [SS'A] + 2[ASS'A] + [ASMS'] + [S'MSA]}{2\{[SS'] + [SMS'] + [ASS'] + [SS'A] + [ASS'A] + [ASMS'] + [S'MSA]\}} \tag{2}$$

When independent and identical sites are assumed, we have:

$$K_1' = K_1'' = K_1''' = K_1'''' = K_1 \text{ and } K_2' = K_2'' = K_2 \tag{3}$$

Substitution of Eqn. 1 in Eqn. 2, with the use of Eqn. 3, yields:

$$\frac{y}{y_{max}} = \frac{2\frac{[A]}{K_1} + 2\left(\frac{[A]}{K_1}\right)^2 + 2\frac{[MA]}{K_2}}{2 + 2\frac{[M]}{K_i} + 4\frac{[A]}{K_1} + 2\left(\frac{[A]}{K_1}\right)^2 + 4\frac{[MA]}{K_2}} \tag{4}$$

This can be rearranged to:

$$\frac{y}{y_{max}} = \frac{\frac{[A]}{K_1}\left(1 + \frac{[A]}{K_1}\right) + \frac{[MA]}{K_2}}{\left(1 + \frac{[A]}{K_1}\right)^2 + \frac{[M]}{K_i} + 2\frac{[MA]}{K_2}} \quad \text{(Eqn. 4 in the text.)} \tag{5}$$

[A], [M] and [MA] are calculated as follows:

$$[A] + [M] \underset{K_c}{\rightleftharpoons} [MA]$$

$$K_c = \frac{[A][M]}{[MA]} \tag{6}$$

$[A]_{tot} \approx [A] + [MA]$ (neglecting terms ASS', etc.)

$[M]_{tot} \approx [M] + [MA]$ (neglecting terms SMS', etc.)

From these equations it follows:

$$[A]_{tot} = [A]\left(1 + \frac{[M]}{K_c}\right) \tag{7}$$

$$[M]_{tot} = [M]\left(1 + \frac{[A]}{K_c}\right) \tag{8}$$

Elimination of [A] from Eqn. 7 and Eqn. 8 yields Eqn. 3 in the text:

$$[M] = \tfrac{1}{2}([M]_{tot} - [A]_{tot} - K_c) + \tfrac{1}{2}\sqrt{([M]_{tot} - [A]_{tot} - K_c)^2 + 4\,K_c[M]_{tot}}$$

Substitution of Eqn. 8 and Eqn. 6 yields [A]; while [MA] is calculated from $[MA] = [A]_{tot} - [A]$.

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References

1 Ray, T.K. and Forte, J.G. (1976) Biochim. Biophys. Acta 443, 451—467
2 Wallmark, B. and Mårdh, S. (1979) J. Biol. Chem. 254, 11899—11902
3 Bonting, S.L., de Pont, J.J.H.H.M., van Amelsvoort, J.M.M. and Schrijen, J.J. (1980) Ann. N.Y. Acad. Sci. 341, 335—356
4 Schrijen, J.J., Luyben, W.A.H.M., de Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 597, 331—344
5 Schrijen, J.J., van Groningen-Luyben, W.A.H.M., de Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 640, 473—486
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Hansen, O., Jensen, J. and Nørdby, J.G. (1971) Nat. New Biol. 234, 122—124
8 Saccomani, G., Dailey, D.W. and Sachs, G. (1979) J. Biol. Chem. 254, 2821—2827
9 Klodos, I. and Skou, J.C. (1975) Biochim. Biophys. Acta 391, 474—485
10 Yount, B.C., Babcock, D., Ballantyne, W. and Ojala, D. (1971) Biochemistry 10, 2484—2489
11 Yount, B.C. (1975) Adv. Enzymol. 43, 1—56
12 Sachs, G., Spenney, J.G. and Lewin, M. (1978) Physiol. Rev. 58, 106—173
13 Chang, H.H., Saccomani, G., Rabon, E., Schackmann, R. and Sachs, G. (1977) Biochim. Biophys. Acta 464, 313—327
14 Goodall, M.C. and Sachs, G. (1977) J. Membrane Biol. 35, 285—301