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Eosin, a fluorescent marker for the high-affinity ATP site of $(K^+ + H^+)$-ATPase

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Eosin has been used as a fluorescent probe for studying conformational states in $(K^+ + H^+)$-ATPase. The eosin fluorescence level is increased by Mg^{2+} ($K_{0.5} = 0.2$ mM). This increase is counteracted by K^+ ($I_{0.5} = 1.3$ mM) and choline ($I_{0.5} = 17.2$ mM) and by ATP. Binding studies with eosin indicate that the increase and decrease in fluorescence is due to changes in binding of eosin to the enzyme. The Mg^{2+}-induced specific binding has a K_d of 0.7 μM and a maximal capacity of 3.5 nmol per mg enzyme, which is equivalent to 2.5 site per phosphorylation site. These experiments and the fact that eosin competitively inhibits $(K^+ + H^+)$-ATPase towards ATP, suggest that eosin binds to ATP binding sites.

Introduction

In the mammalian stomach a K^+-activated ATPase is located in the acid secreting regions of the mucosa and appears to be involved in gastric acid secretion [1]. It transports protons in a neutral exchange for K^+, hence the abbreviation $(K^+ + H^+)$-ATPase is used.

Mg^{2+} appears to play an important role in the catalytic cycle. It is an essential factor in phosphorylation of the enzyme [2]. There are several indications for the existence of a particular Mg^{2+} induced conformation of the $(K^+ + H^+)$-ATPase, such as higher reactivity of an essential arginine group [3], an increased number of reactive sulfhydryl groups [4] and a smaller radiation sensitive volume [5]. Mg^{2+} and K^+ seem to play antagonizing roles in the catalytic cycle: the lowering of the steady-state phosphorylation level by K^+ appears to be antagonized by Mg^{2+} [6]. A K^+-Mg^{2+} antagonism is also noticed in their effect on the ATP-ADP exchange reaction [7].

In the light of this K^+-Mg^{2+} antagonism we have looked for a fluorescent marker with which a K^+ and a Mg^{2+} form of the enzyme can be distinguished, since the use of such markers has been successful in studies on $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase [8–14]. In studies on the $(Na^+ + K^+)$-ATPase by Skou and Esmann [15] eosin is used as a probe for the different conformational states of the enzyme. This probe binds to $(Na^+ + K^+)$-ATPase in a non-covalent way so that the ATPase activity is reversibly inhibited. Binding of eosin is at the ATP site and addition of Mg^{2+}, Na^+ or K^+ to the enzyme in the presence of eosin gives distinct levels of fluorescence. In analogy with these studies we have used eosin with $(K^+ + H^+)$-ATPase in order to study the transition from the Mg^{2+}-form of the enzyme to the K^+-form and vice versa.

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Materials and Methods

Enzyme preparation. A $(K^+ + H^+)$-ATPase containing membrane fraction is isolated from porcine gastric mucosa according to Schrijen et al. [3]. The isolated preparation is stored at −20°C in 0.25 M sucrose. The specific activity of the enzyme preparations ranges from 90 to 120 μmol ATP hydrolyzed per hour per mg protein.

Fluorescence measurements. Changes in fluorescence of eosin Y in the presence of enzyme are measured in 20 mM histidine-HCl (pH 7.5) and 1 μM eosin in the presence of 0.15 mg/ml $(K^+ + H^+)$-ATPase. Fluorescence is measured on a Shimadzu RF 510 spectrofluorimeter with excitation at 530 nm and emission at 560 nm (slit width 10 nm). The fluorescent probe eosin Y (C.I.45380) is purchased from Gurr and will be referred to as eosin.

Binding experiments. Binding of eosin to the enzyme is measured by incubating a certain amount of enzyme (0.2–0.5 mg) in 1 ml of 20 mM histidine-HCl (pH 7.5), with eosin in various concentrations and with various ligands.

After 10 min incubation at room temperature, centrifugation takes place at 140 000 × *g* for 1 h at 4°C to separate enzyme and supernatant. From the fluorescence of the supernatant and a calibration curve, the concentration of free eosin and the amount of eosin bound to the enzyme are calculated.

Inhibition experiments. ATPase activity of the enzyme is measured in 30 mM imidazole-HCl (pH 7.0), 5 mM Mg^{2+}, 0.1 mM ouabain, 20 mM KCl or choline chloride and ATP in concentrations ranging from 20 to 50 μM. After 10–20 min incubation at 37°C, the reaction is stopped by adding a 2 M HCl-molybdate solution and P_i is determined with malachite-green according to Carter and Karl [16]. The $(K^+ + H^+)$-ATPase activity is obtained from the difference in activity with KCl and with choline chloride. Protein is determined by the Lowry method, using bovine serum albumin as a standard.

Results

Ligand-induced conformational changes

Fig. 1 shows the fluorescence excitation and emission spectra of 1 μM eosin in 30 mM histidine-HCl (pH 7.5) in the absence and presence of $(K^+ + H^+)$-ATPase. In the absence of enzyme the excitation maximum of eosin in 518 nm and the emission maximum is 538 nm (Fig. 1, curve d). Addition of enzyme (0.2 mg/ml) to a K^+ containing eosin solution has only a slight effect on the excitation and emission spectra (curve c). When the solution contains Mg^{2+} instead of K^+ there is a pronounced effect (curve a): the fluorescence is increased, the excitation maximum (emission at 560 nm) is shifted from 518 to 523 nm, the emission maximum (excitation at 500 nm) is shifted from 538 to 541 nm and a shoulder appears on the excitation curve around 490 nm. When no extra ions such as K^+ and Mg^{2+} are added (curve b), the spectra are intermediate between the Mg^{2+} and the K^+ spectra. This is probably due to the endogenous Mg^{2+} present in the $(K^+ + H^+)$-ATPase preparation (20–100 nmol per mg protein). With heat-inactivated enzyme (15 min, 95°C), there is no difference in fluorescence with Mg^{2+} or K^+ and the fluorescence intensity is decreased to the level of eosin in buffer. So the observed effects of Mg^{2+} and K^+ need a fully active enzyme to occur.

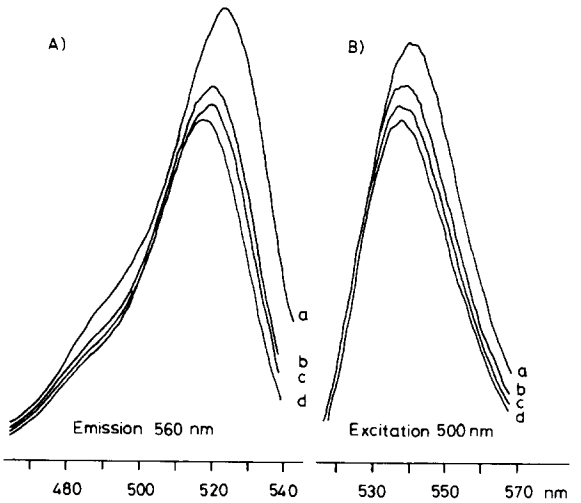

Fig. 1. Fluorescence excitation (A) and emission spectra (B) of 1 μM eosin in 30 mM histidine-HCl (pH 7.5) without (d) or with (a, b, c) 0.2 mg $(K^+ + H^+)$-ATPase per ml in the presence of 4 mM Mg^{2+} (a), 10 mM K^+ (c), or without the addition of extra ions (b), at 20°C. A slit width of 10 nm is used for both excitation and emission.

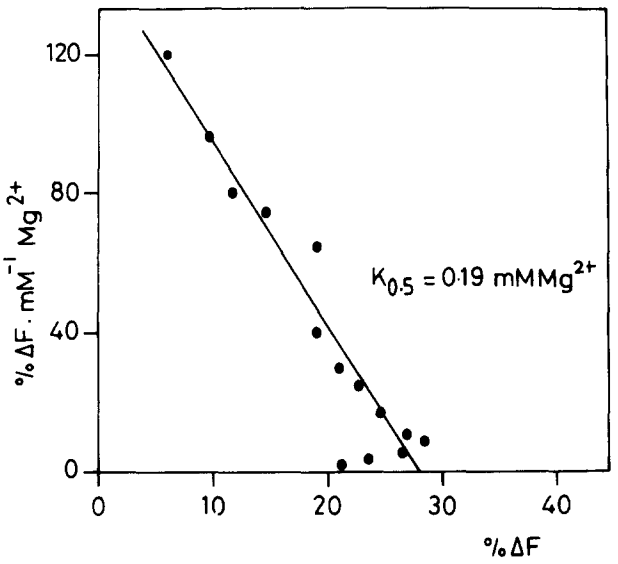

Fig. 2. Percentage stimulation of eosin fluorescence as a function of the Mg^{2+} concentration, presented in an Eadie-Scatchard plot. Fluorescence is measured of 1 μM eosin, 0.15 mg enzyme per ml in 20 mM histidine-HCl (pH 7.5) at 20°C. Excitation 530 nm, emission at 560 nm, slit width 10 nm.

The Mg^{2+}-enhanced fluorescence of eosin in the presence of $(K^+ + H^+)$-ATPase is shown in Fig. 2 as a function of the Mg^{2+} concentration in an Eadie-Scatchard plot. A half-maximal concentration ($K_{0.5}$) of 0.2 mM is calculated from the slope. From the same plot it can be observed that at concentrations above 4 mM Mg^{2+}, the fluorescence decreases, which may be an effect of ionic strength. Ca^{2+} increases the fluorescence to the same extent but is less efficient than Mg^{2+} ($K_{0.5}$ = 1.0 mM). Mg^{2+}-like effects of Ca^{2+} on phosphorylation and fluorescence of fluorescein-isothiocyanate (FITC) have been reported before by Jackson et al. [17]. Other divalent cations such as Mn^{2+} and Co^{2+} also increase fluorescence but the maximal increase is lower than with Mg^{2+} and higher $K_{0.5}$ values are observed (not shown).

Choline, which we have used to study the effect of ionic strength, also increases the fluorescence at concentrations below 20 mM (Table I). At higher concentrations the fluorescence decreases, which effect is also observed with very high Mg^{2+} concentrations. Choline is not so effective as Mg^{2+} in stimulating fluorescence as the maximal effect is less and more choline is needed ($K_{0.5}$ = 7.5 mM).

TABLE I

FLUORESCENCE LEVELS OF EOSIN IN THE PRESENCE OF $(K^+ + H^+)$-ATPASE AND VARIOUS IONS

Fluorescence level of 2 μM eosin and 0.15 mg/ml $(K^+ + H^+)$-ATPase in 20 mM histidine- HCl (pH 7.5) in the presence of various ions. The presented data are from one typical experiment out of three. The asterisked values are measured with 6 μM eosin and 0.5 mg enzyme per ml.

No enzyme, no additions	49
No additions	56
1 mm ATP	51
4 mM Mg^{2+}	98
4 mM Mg^{2+}, 1 mM ATP	53
10 mM choline	74
10 mM choline, 1 mM ATP	52
20 mM choline	81
40 mM choline	79
100 mM choline	72
10 mM K^+	55
5 mM Ca^{2+}	95
4 mM Mg^{2+}, 10 mM K^+	56
4 mM Mg^{2+}, 10 mM choline	74
No additions *	80 *
10 mM K^+ *	76 *
20 mM K^+ *	82 *

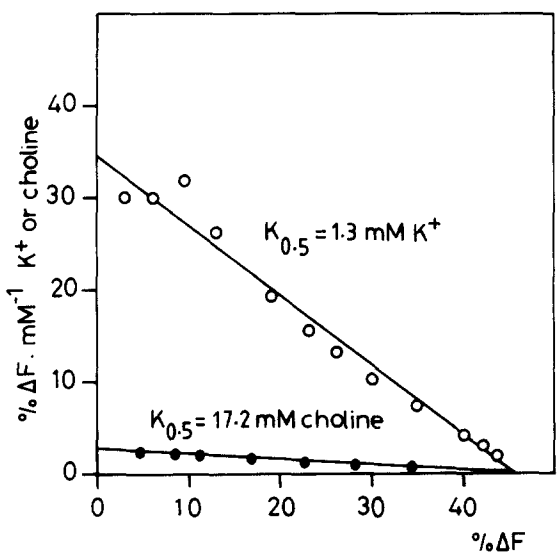

Fig. 3. Percentage decrease of Mg^{2+}-induced fluorescence of eosin as a function of the K^+ (○) or choline (●) concentration, plotted as an Eadie-Scatchard plot. Fluorescence of 1 μM eosin, 0.15 mg enzyme per ml in 4 mM Mg^{2+}, 20 mM histidine-HCl (pH 7.5) at 20°C. Excitation at 530 nm, emission at 560 nm, slit width 10 nm.

As shown in Table I, K^+ and choline are also able to decrease the stimulation of fluorescence by Mg^{2+}. A more detailed presentation is given in Fig. 3, which shows in an Eadie-Scatchard plot the inhibitory effect of K^+ and choline on the Mg^{2+}-enhanced fluorescence, as a function of the concentration. $K_{0.5}$ values of 1.3 and 17.2 mM for K^+ and choline, respectively, are calculated. ATP in millimolar concentrations lowers the fluorescence level to about the level of eosin in the absence of enzyme.

As pointed out by Skou and Esmann [15] changes in the excitation and emission spectra of eosin, as are observed in the presence of Mg^{2+}, may be caused by a shift towards lower polarity of the environment of eosin. As a consequence the question arises: what causes the increase of eosin fluorescence in the presence of Mg^{2+}? One possible explanation is that in the presence of Mg^{2+} more eosin is bound to the $(K^+ + H^+)$-ATPase than in the presence of K^+. Another explanation is that under both circumstances the same amount of eosin is bound, while a change in the environment of the bound eosin causes the fluorescence change.

A titration with enzyme at a fixed eosin concentration (1 μM, Fig. 4) shows that the difference in fluorescence with Mg^{2+} and K^+ approaches saturation. The maximal level of fluorescence in this experiment is reached at a protein concentration of 0.2 mg/ml. This concentration depends on the eosin concentration used and increases with increasing eosin concentration. Conversely titration with eosin at a fixed protein concentration (0.15 mg/ml, Fig. 5) shows that the difference in fluorescence with Mg^{2+} and K^+ also approaches saturation. This saturation behaviour suggests that the increase in fluorescence of eosin in the presence of Mg^{2+} is caused by an increase in binding of eosin to the enzyme. In order to verify this we have directly measured the binding of eosin to the enzyme.

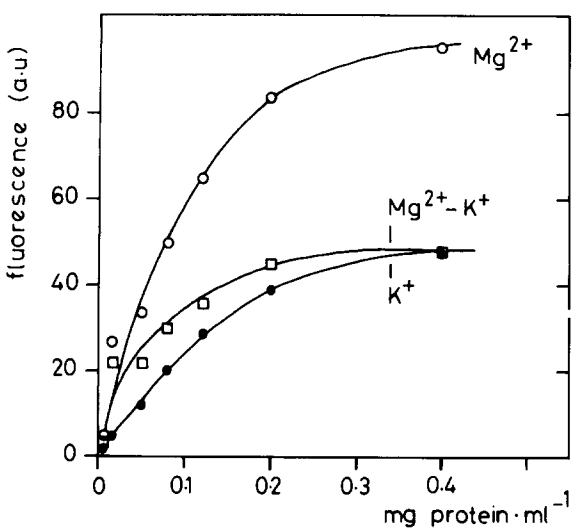

Fig. 4. Fluorescence of 1 μM eosin in the presence of 4 mM Mg^{2+} (○) or 10 mM K^+ (●), respectively, as a function of the protein concentration in 20 mM histidine-HCl (pH 7.5) at 20°C. The difference in fluorescence with Mg^{2+} and K^+ is plotted (□). Excitation 530 nm and emission 560 nm, slit width 10 nm.

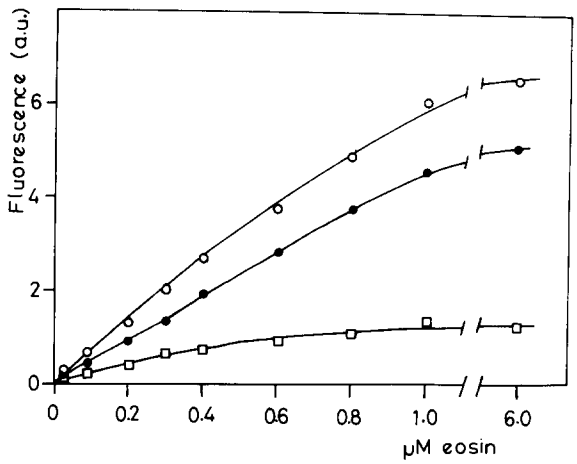

Fig. 5. Fluorescence of eosin in the presence of 0.15 mg $(K^+ + H^+)$-ATPase per ml and 4 mM Mg^{2+} (○) or 10 mM K^+ (●) as a function of the eosin concentration. The difference of the two curves is given (□). Other conditions are as described in the legend to Fig. 4.

Stoichiometry of eosin binding to the $(K^+ + H^+)$-ATPase

Binding of eosin to the $(K^+ + H^+)$-ATPase is measured by incubating a certain amount of enzyme with varying eosin concentrations. The eosin-enzyme complex and free eosin are separated by centrifugation after which the concentration of bound eosin is determined from the decrease in the concentration of free eosin.

Table II presents data on the binding of eosin to the $(K^+ + H^+)$-ATPase in the presence of various ions, all at about the same ionic strength. In

TABLE II

BINDING LEVEL OF EOSIN TO THE $(K^+ + H^+)$-ATPase IN THE PRESENCE OF VARIOUS IONS

Amount of eosin bound to the $(K^+ + H^+)$-ATPase enzyme. The enzyme (0.2 mg/ml) is incubated in 2 μM eosin in 20 mM histidine-HCl (pH 7.5) in the presence of various ions. The amount of eosin bound to the enzyme is determined as described in Materials and Methods. In the table values are given in nmol/mg with S.E. and number of experiments

	Eosin bound (nmol/mg)
4 mM Mg^{2+}	2.1 ±0.10 (4)
10 mM K^+	0.51 ± 0.07 (4)
10 mM choline	0.62 ± 0.05 (4)
4 mM Mg^{2+}, 1 mM ATP	0.03 ± 0.02 (4)
10 mM choline, 1 mM ATP	0.05 ± 0.03 (4)
10 mM K^+, 1 mM ATP	0 (4)
1 mM ATP	0 (4)

the presence of 4 mM Mg^{2+} more eosin is bound than in the presence of 10 mM K^+. In the presence of 10 mM choline slightly more eosin is bound to the $(K^+ + H^+)$-ATPase than in the presence of 10 mM K^+. Under all circumstances the amount of eosin bound to the enzyme is decreased to zero by the addition of ATP.

Fig. 6 shows the amount of eosin bound to the $(K^+ + H^+)$-ATPase as a function of the eosin concentration in the presence of either 4 mM Mg^{2+} or 10 mM K^+. With K^+ there is a low-affinity binding of eosin to the enzyme, which increases with the eosin concentration. Due to the low affinity for eosin in the presence of K^+ it is not possible to determine the affinity for eosin or the number of binding sites under this condition. In the presence of Mg^{2+} the eosin bound to the enzyme also increases with the eosin concentration but more eosin is bound than in the presence of K^+.

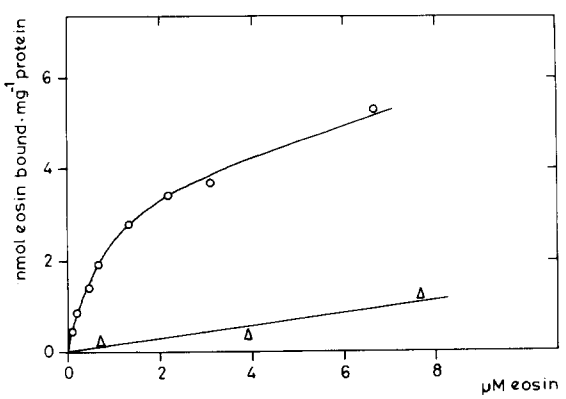

Fig. 6. The amount of eosin bound to the $(K^+ + H^+)$-ATPase as a function of the free eosin concentration in the presence of either 4 mM Mg^{2+} (○) or 10 mM K^+ (△), respectively. Binding is measured as described in Materials and Methods. Typical experiment out of four.

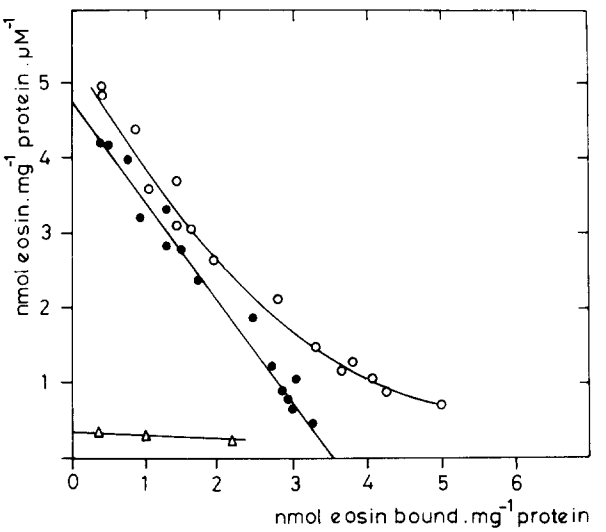

Fig. 7. Relationship of bound and free eosin (Eadie-Scatchard plot) in the presence of $(K^+ + H^+)$-ATPase and either 4 mM Mg^{2+} (○) or 10 mM K^+ (△). The difference of the binding with 4 mM Mg^{2+} and with 10 mM K^+ present is given (●). Values of three experiments are given.

When the amount of eosin bound to the enzyme in the presence of Mg^{2+} is given in a Scatchard plot a curved line is observed (Fig. 7). The difference of binding with Mg^{2+} and with K^+ gives a straight line. This suggests that in the presence of Mg^{2+} two types of binding sites are present, one with a high affinity, which is specific for Mg^{2+} and one with a low affinity which is also observed in the presence of K^+ ions. From Fig. 7 we calculate for the Mg^{2+}-specific binding a K_d of 0.7 μM and a maximal binding capacity of 3.5 nmol per mg enzyme. The enzyme preparation used has a phosphorylation capacity for ATP of 1.4 nmol per mg protein which implies a ratio of 2.5 high-affinity binding sites per ^{32}P labelling site.

Correlation of binding and fluorescence of eosin

Fig. 8 shows the relation between eosin fluores-

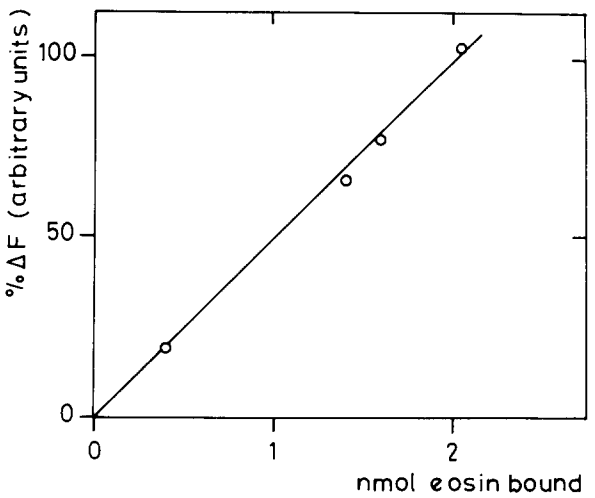

Fig. 8. Relationship of the change in fluorescence and the amount of eosin bound. An amount of 0.5 mg enzyme is incubated in 2 ml 20 mM histidine-HCl (pH 7.5) in the presence of 2 μM eosin and 0, 0.5, 1.0 or 2.0 mM Mg^{2+}. Of the sample half is used to determine binding as described in Materials and Methods. The remaining 1 ml is used to measure fluorescence directly. A sample of 2 μM eosin in buffer is used as a reference.

cence and binding. The various values are obtained by incubating a fixed amount of enzyme with a fixed concentration of eosin but with an increasing Mg^{2+} concentration. From the same sample both fluorescence and the level of eosin binding is measured. As a result of the increasing Mg^{2+} concentration both fluorescence and binding increase in a straight linear relation. We therefore conclude that an increase of fluorescence in the presence of $(K^+ + H^+)$-ATPase is the result of an increase in binding of eosin to the enzyme.

Interaction of eosin and ATP

As shown in Table I, ATP has a pronounced effect on the fluorescence of eosin in the presence of $(K^+ + H^+)$-ATPase as it reduces the Mg^{2+}-enhanced fluorescence to almost the fluorescence of eosine alone. The same is observed in the presence of other ions. It is not possible to reduce the fluorescence completely to the value of eosin alone. This is probably due to the presence of protein in the solution, causing scattering.

Binding of eosin (Table II) is completely reduced to zero by the presence of ATP. It is not possible to determine the concentration dependence of this effect because ATP hydrolyzes during the experiment. We have tested the interaction of eosin and ATP in a hydrolysis experiment by varying the ATP and eosin concentrations. Fig. 9 shows in a Dixon-plot the inhibition by eosin of the K^+-stimulated ATPase activity. In the pres-

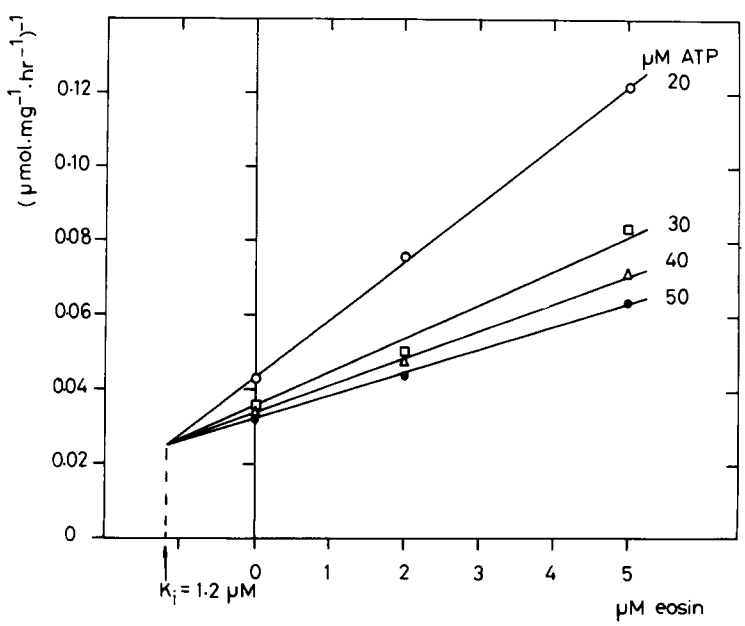

Fig. 9. Reciprocal ATPase activity ($1/v$) as a function of the eosin concentration (Dixon plot). ATP hydrolysis is determined in the presence of 5 mM Mg^{2+}, 20 mM K^+, 30 mM imidazole (pH 7.0) and varying ATP concentrations at 37°C. The P_i production is measured as described in Materials and Methods.

ence of 20 mM K^+ and 5 mM Mg^{2+} eosin inhibits the hydrolysis of ATP, but the effect can be overcome by increasing the ATP concentration. A K_i value for eosin of 1.2 μM is determined, which is of the same order as the K_d value for Mg^{2+} induced eosin binding (0.7 μM), mentioned above. The data indicate competition between eosin and ATP for the same high-affinity binding site.

Discussion and Conclusions

Labelling transport enzymes with a fluorescent marker in order to study conformational changes of the enzyme is a method that has extensively been used for $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase [8–14]. Both enzymes have many characteristics in common with the $(K^+ + H^+)$-ATPase [18,19]. One of the labels used by Skou and Esmann [15] for the $(Na^+ + K^+)$-ATPase, eosin Y, has revealed many characteristics of the $E_2 \rightarrow E_1$ reaction step and its reverse. Eosin binds to the ATP site of the $(Na^+ + K^+)$-ATPase in a non-covalent way and inhibits the overall ATPase reaction in a reversible way.

In the experiments in this paper we observe for $(K^+ + H^+)$-ATPase that eosin inhibits the ATPase activity competitively towards ATP. From this and from fluorescence and binding studies we have concluded that eosin binds at the ATP site of the $(K^+ + H^+)$-ATPase

In addition we correlated the fluorescence level of eosin in the presence of $(K^+ + H^+)$-ATPase to the eosin binding to enzyme. There is a linear relationship between the two which implies that any increase in fluorescence is a direct result of an increased binding of eosin to the enzyme.

From the binding experiments it appears that there are two types of eosin binding to $(K^+ + H^+)$-ATPase. One is the Mg^{2+}-induced high-affinity binding in an environment of low polarity. This is suggested by experiments of Skou and Esmann [15], in which lowering the polarity of the solvent for eosin changed the excitation and emission spectra in the same way as is observed in the presence of Mg^{2+} and the enzyme in our hands. In the presence of Mg^{2+} a K_d of 0.7 μM for the high affinity binding is determined and a maximal binding capacity of 3.5 nmol/mg protein. This K_d for eosin binding at 4°C and 4 mM Mg^{2+} is close to the K_m for ATP in hydrolysis experiments (0.4 μM, 22°C, 2 mM Mg^{2+}) as reported by Wallmark et al. [20]. The maximal binding capacity of eosin is 2.5 times the maximal phosphorylation capacity of the enzyme preparation (1.4 nmol/mg). More often discrepancies have been reported between the maximal phosphorylation and binding capacity of a label that is specific for the ATP site. There are indications for more than one nucleotide site from studies with the fluorescent ATP analogue TNP-ATP [21], from vanadate-binding studies [22], from studies on P_i phosphorylation [23] and from studies in which the enzyme is labeled with 8-azido-ATP, a photoaffinity analogue of ATP [24]. This discrepancy could be explained by assuming the existence of an acid-labile phosphoenzyme which is not detected by the usual way of determining the amount of phosphoenzyme by acid-precipitation [22].

For $(Na^+ + K^+)$-ATPase the same phenomenon is observed. Skou and Esmann have shown in studies on this enzyme that in the presence of Na^+ eosin binds to a high-affinity site with a capacity of 1 per phosphorylation site [14]. Mg^{2+} induces an additional site for eosin with the same capacity, yielding a total of 2 binding sites per phosphorylation site [25]. Both sites appear to be ATP binding sites. In measurements of ATP binding one high-affinity ATP binding site is observed per ^{32}P-labelling site [26,27]. However, experiments on binding of Ado*PP*[NH]*P* show in the presence of Mg^{2+} a second site with low affinity, which is in agreement with the eosin experiments [28].

The low-affinity binding of eosin in the presence of K^+ is reflected by an increase of fluorescence, which can only be observed at very high eosin concentrations. Due to experimental difficulties it is not possible to determine the capacity and affinity of this type of binding.

The Mg^{2+}-induced high-affinity binding is calculated by subtracting the binding in the presence of K^+ from the binding in the presence of Mg^{2+}. We assume that the low-affinity binding in the presence of Mg^{2+} and K^+ is the same. As the difference in binding gives a straight line, we assume that this represents an additional binding site specifically induced by Mg^{2+}. Both types of binding in the presence of Mg^{2+} and of K^+ are

specific in the sense that ATP can abolish them.

The nature of eosin binding in the presence of choline is not completely clear. We have used this substance to study the effect of ionic strength on eosin binding. From our experiments it appears that choline can act like K^+ in decreasing Mg^{2+}-stimulated fluorescence. We explain these phenomena in the following way: cations promote binding of eosin to the $(K^+ + H^+)$-ATPase, which is reflected by an increase of fluorescence (Table I). All cations, even K^+, are able to do this (Table I). When ionic strength increases, binding becomes more difficult and fluorescence decreases, which effect is also observed in the presence of high Mg^{2+} concentrations. Mg^{2+} specifically stimulates binding by opening up an extra binding site of high affinity; K^+ specifically counteracts the effect of Mg^{2+} by decreasing binding of eosin and thus lowering its fluorescence. Choline has the same effect but with lower specificity. Antagonistic effects of K^+ versus Mg^{2+} and Ca^{2+} have also been observed with FITC-labeled enzyme [16].

We have considered whether the effect of K^+ is possibly due to lowering of the 'activity' or effective concentration of Mg^{2+} as suggested by Ljungström [29]. This activity is dependent on the presence of other ions including K^+, which would reduce the activity of Mg^{2+} and hence the fluorescence. In our opinion however, this cannot fully explain the K^+-Mg^{2+} antagonism. We conclude from the data of Ljungström that at 20 mM K^+, which gives in our hands a maximal decrease of the Mg^{2+}-fluorescence, the Mg^{2+}-activity is decreased to about half, i.e. to 2 mM. As the K_m for the Mg^{2+}-effect is 0.2 mM, this concentration is still sufficient to give the maximal fluorescence enhancement. We therefore conclude that the effect mentioned by Ljungström cannot explain the K^+-Mg^{2+} antagonism.

The conformation of the enzyme in the presence of Mg^{2+} thus differs from the conformation with K^+ by an additional site for eosin of high affinity and low polarity. High affinity binding of eosin to a site which is opened up by Mg^{2+}, closed by K^+ and eliminated by ATP, combined with the competitive inhibition of ATP hydrolysis by eosin suggest that the high-affinity binding site for eosin in the presence of Mg^{2+} is at an ATP site on the enzyme, required for catalytic activity. The observation that this site is Mg^{2+}-induced explains the requirement of this cofactor as prerequisite for overall enzymatic activity.

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