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EOSIN, A FLUORESCENT PROBE OF ATP BINDING TO THE ($Na^+ + K^+$)-ATPase

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(1) Eosin bound to the ($Na^+ + K^+$)-ATPase in the presence of K^+ has practically the same fluorescence as eosin without enzyme while in the presence of Na^+ the fluorescence is higher, the excitation maximum is shifted from 518 to 524 nm, the emission maximum from 538 to 542 nm, and a shoulder appears at about 490 nm on the excitation curve. (2) The amount of eosin bound increases with the K^+ concentration but with a low affinity. With equal concentrations of Na^+ and K^+ more is bound in the presence of Na^+, and the difference between 150 mM Na^+ and 150 mM K^+ shows one high-affinity eosin binding site per ^{32}P-labelling site (K_D 0.45 μM). With lower concentrations of the cations there are between one and two Na^+-dependent high-affinity eosin binding sites per ^{32}P-labelling site. (3) ATP (and ADP) prevents the high-affinity Na^+-dependent eosin binding and there is competition between eosin and ATP for the hydrolysis in the presence of Na^+ ($+Mg^{2+}$). (4) Eosin, like ATP, increases the Na^+ relative to K^+ affinity ($Na^+ + K^+$ = 150 mM) for Na^+ activation of hydrolysis and for Na^+ protection against inactivation by *N*-ethylmaleimide. (5) The results suggest that the high affinity eosin binding site is an ATP binding site and that it is located on the enzyme in an environment with a low polarity, i.e., the conformational change induced by Na^+ opens a high-affinity site for ATP while K^+ closes the site (or decreases the affinity to a low level). The experiments suggest, furthermore, that the ATP which increases the Na^+ relative to K^+ affinity of the internal sites is not the ATP which is hydrolyzed, i.e., in a turnover cycle in the presence of $Na^+ + K^+$ the system reacts with two different ATP molecules.

Introduction

In a previous paper [1] it was shown that eosin maleimide may be a useful fluorescent probe for measuring the effect of Na^+ and K^+ on the conformation of the ($Na^+ + K^+$)-ATPase. After an incubation period the reaction between enzyme and eosin maleimide was stopped by adding mercaptoethanol followed by washing three times by centrifugation to remove the mercaptoethanol-reacted eosin maleimide from the enzyme. By this procedure most but not all mercaptoethanol-eosin maleimide was removed.

In an attempt to find procedures to remove all the non-covalently bound eosin maleimide in order to locate that which is covalently bound, eosin was added to the enzyme and it was observed that when Na^+ and when K^+ was added to the enzyme the non-covalently bound eosin responded with a difference in fluorescence identical to that which had been observed with the eosin maleimide-labelled enzyme. The same thing was observed when eosin maleimide, which had been reacted with mercaptoethanol, was added and this indicated that the previously observed effect of Na^+ and of K^+ on the fluorescence of the eosin maleimide-reacted enzyme was due to a difference in the non-covalent binding of the remaining mercaptoethanol-reacted eosin maleimide to the enzyme. It prompted us to investigate the effect of the ligands on the binding of eosin to the ($Na^+ + K^+$)-ATPase.

Methods

The enzyme was prepared from rectal glands of *Squalus acanthias* as previously described [2]. The

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specific activity was 1 276 ± 32 μmol/mg protein per h and with 2.1 ± 0.08 ^{32}P-labelling sites/mg protein (n = 4).

The fluorescent probes eosin Y (C.I. 45380), erythrosin Y (C.I. 45425) and phloxin B (C.I. 45410) were from Gurr. Eosin B (C.I. 45400) and erythrosin B (C.I. 45430) were from Difko, phloxin (C.I. 45405) from Merck, eosin S (C.I. 45386) from Ridel-de Haén, and eosin maleimide from Molecular Probes.

The fluorescence was measured on a Perkin Elmer spectrofluorimeter MPF 44A [1].

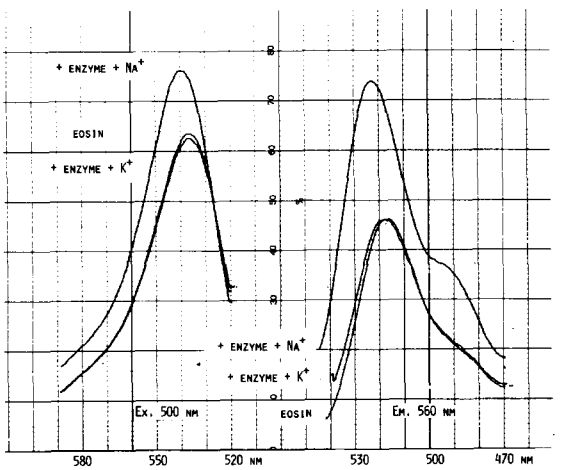
Binding of eosin Y to the enzyme was measured by incubating a certain amount of enzyme (0.7–0.9 mg protein) in 2 ml of a 30 mM histidine buffer, pH 7.2, with the ligands and eosin Y for 5 min at room tmperature in the dark followed by a centrifugation at 60 000 rev./min for 1 h to separate enzyme and supernatant. From the fluorescence of the supernatant and a control without enzyme the concentration of free eosin and the amount of eosin bound to the enzyme was calculated.

^{32}P labelling was measured as previously described [3].

Results

The excitation maximum of eosin Y in a 30 mM histidine buffer, pH 7.2, is 518 nm and the emission maximum 538 nm (Fig. 1). Addition of enzyme in a K^+-containing eosin solution has only a very slight effect on the excitation and the emission spectra (see Fig. 1). If the solution contains Na^+ instead of K^+ there is a pronounced effect: the fluorescence is increased, the excitation maximum (emission at 560 nm) is shifted from 518 to 524 nm, the emission maximum (excitation at 500 nm) from 538 to 542 nm and a shoulder appears on the excitation curve around 490 nm. The effect of enzyme in the presence of Na^+ on the eosin Y fluorescence can be mimicked by dissolving eosin (no enzyme or cations) in ethyl alcohol, (compare Figs. 1 and 2), indicating that eosin Y in the presence of Na^+ is bound to the enzyme in an environment with a low polarity.

With heat inactivated enzyme (5 min, 65°C) there is no difference in fluorescence with Na^+ and with K^+ and the fluorescence intensity, the excitation and the emission spectra are identical to those seen with eosin in buffer.



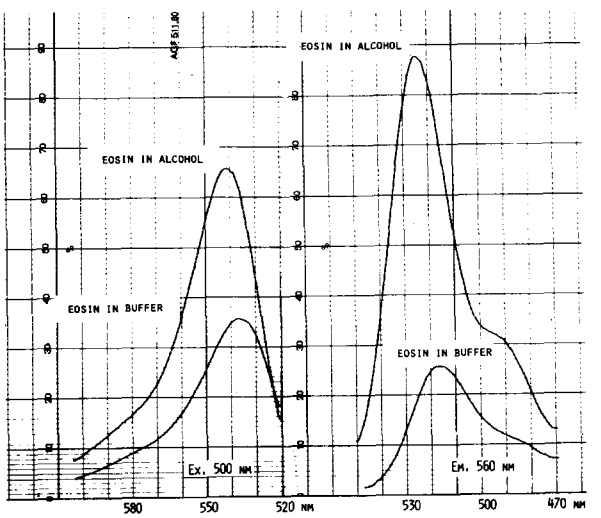


Fig. 1. Fluorescence excitation and emission spectra of 30 nM eosin in a 30 mM histidine buffer, pH 7.2, without and with 0.1 mg ($Na^+ + K^+$)-ATPase/ml with 12 mM K^+ and with 20 mM Na^+, respectively, at 22°C. The specific activity of the ($Na^+ + K^+$)-ATPase was 1 270 μmol P_i/mg protein per h, and with 2.1 nmol ^{32}P labelling sites per mg protein. 10 nm slit for both excitation and emission.

Fig. 2. Excitation and emission spectra of 30 nM eosin in 30 mM histidine, pH 7.2 and in ethyl alcohol, respectively.

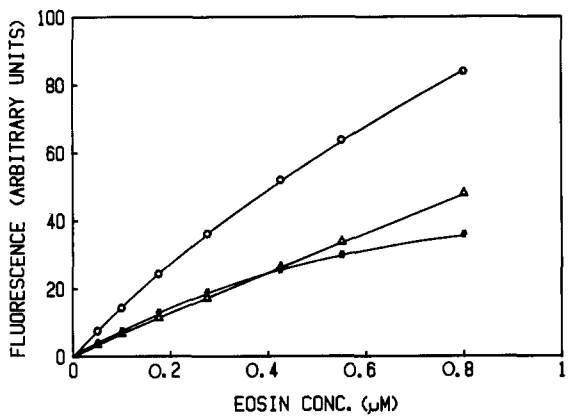


Fig. 3. Eosin fluorescence in the presence of $(Na^+ + K^+)$-ATPase with 20 mM Na^+ (o———o) and with 20 mM K^+ (Δ———Δ), respectively, as a function of the eosin concentration. $(Na^+ + K^+)$-ATPase represented 0.1 mg protein per ml in a 30 mM histidine buffer, pH 7.2, 22°C. The difference in binding with Na^+ and with K^+ is shown by *———*. Excitation 530 nm, emission 560 nm.

The difference in fluorescence (excitation at 530 nm, emission at 560 nm) with Na^+ and with K^+ approaches a saturation when the eosin Y concentration is increased (Fig. 3), and the fractional increase in fluorescence with Na^+ decreases.

A titration with enzyme in the presence of 30 nM eosin Y with Na^+ and with K^+ is shown in Fig. 4. The maximum difference in fluorescence is obtained with about 240 μg protein/ml (0.50 μM enzyme).

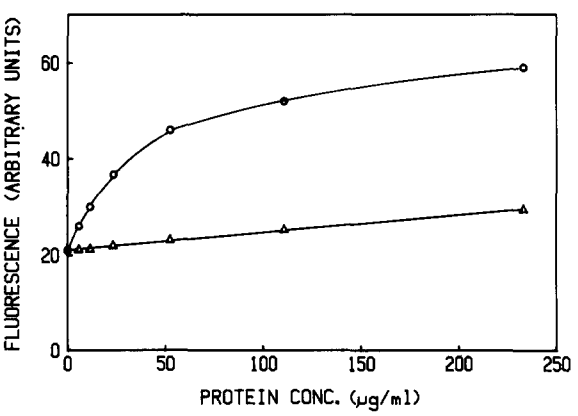


Fig. 4. Eosin fluorescence in the presence of $(Na^+ + K^+)$-ATPase with 20 mM Na^+ (o———o) and 20 mM K^+) (Δ———Δ), respectively, as a function of the enzyme concentration in 30 mM histidine buffer (pH 7.2), 30 nM eosin, 22°C. Excitation 530 nm, emission 560 nm.

The chemical composition of eosin Y is shown in Fig. 5. An ethyl group on the carboxyl group (eosin S) has no effect on the difference in fluorescence with Na^+ or with K^+ in the presence of enzyme; neither has the replacement of the ethyl group with a maleimide group (eosin 5′-maleimide) when the maleimide group is blocked by mercaptoethanol, dithiothreitol or cysteine. Nor has removal of two of the bromides in the molecule (eosin B) any effect. However, when bromide is replaced by iodide as in erythrosin B or Y (see Fig. 5), the effect of Na^+ in the presence of enzyme on the fluorescence of erythrosin is as with eosin Y, while K^+ gives a change in fluores-

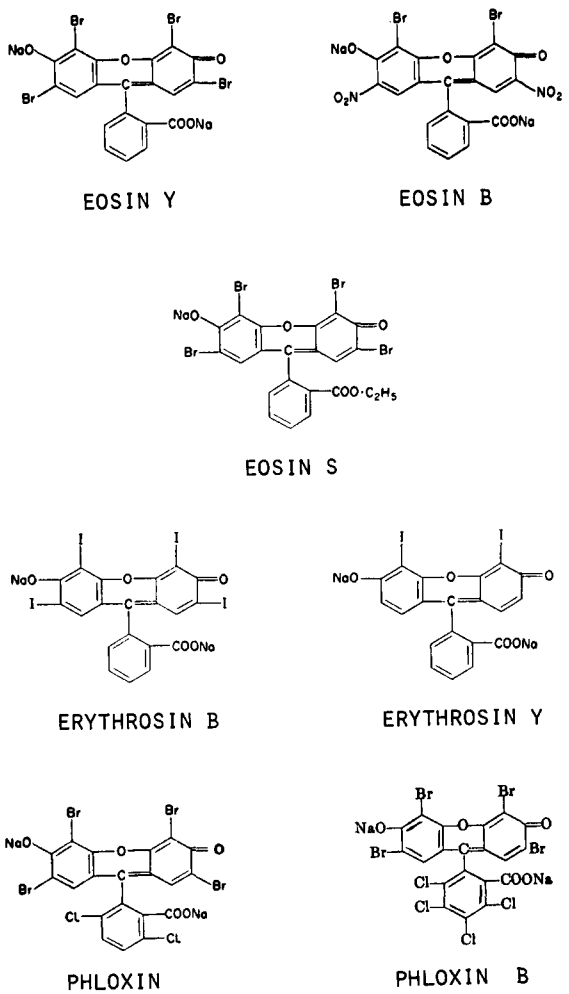


Fig. 5. Chemical structures of the fluorescent compounds tested.

cence like that seen with Na^+, but a higher concentration of K^+ than of Na^+ is needed to produce the same effect. The same is observed when eosin with two or four chlorides on the lower six-membered ring, phloxin and phloxin B (see Fig. 5), is used.

The change in fluorescence when Na^+ is added shows that eosin Y (hereafter termed eosin) must be bound to the enzyme in the presence of Na^+, while in the presence of K^+ there is no or little change to indicate any binding. However, as will be seen from the following, there is binding of eosin to the enzyme also in the presence of K^+.

Binding of eosin to the enzyme

Effect of K^+ and Na^+. With K^+ there is a low-affinity binding of eosin to the enzyme which increases with eosin as well as with the K^+ concentration; compare the binding in the presence of 20 mM K^+ in Fig. 6 and 150 mM K^+ in Fig. 8.

Because of the low affinity for eosin in the presence of K^+ it is not possible to analyze whether the K^+ effect on binding is due to an effect on the affinity for eosin and/or on the number of binding sites.

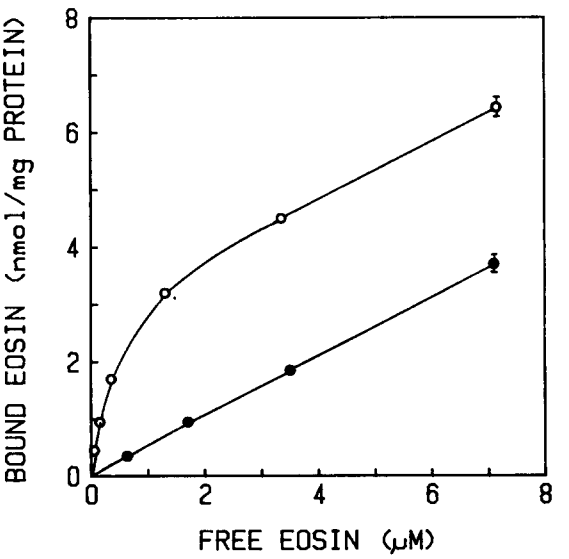


Fig. 6. Amount of eosin bound to the $(Na^+ + K^+)$-ATPase (nmol/mg protein), as a function of the eosin concentration in the presence of 20 mM Na^+ (o———o) (n = 6) and 20 mM K^+ (n = 4) (•———•). In this and the following binding experiment the pH was 7.2 (30 mM histidine) and the temperature 22°C. The S.E. is within the size of the symbols except where shown.

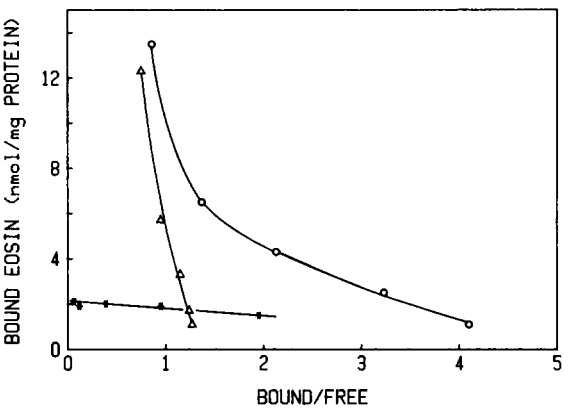


Fig. 7. Scatchard plot of eosin bound to $(Na^+ + K^+)$-ATPase in the presence of 150 mM K^+ (Δ———Δ) and 150 mM Na^+ (o———o), respectively. The difference between the two curves is shown by *———*).

In the presence of Na^+ the eosin bound to the enzyme also increases with the eosin as well as with the Na^+ concentration. However, with the same concentrations of Na^+ and of K^+ more is eosin bound in the presence of Na^+ and the difference in binding shows a Na^+-dependent high-affinity binding of eosin (Figs. 6–8).

The difference in binding and affinity is, however, dependent on the cation concentration. With 2 mM of the cations the Na^+-dependent eosin binding is 2.2 nmol per mg protein, with a K_D of 0.9 μM. This is increased to 3 nmol per mg protein with 20 mM of

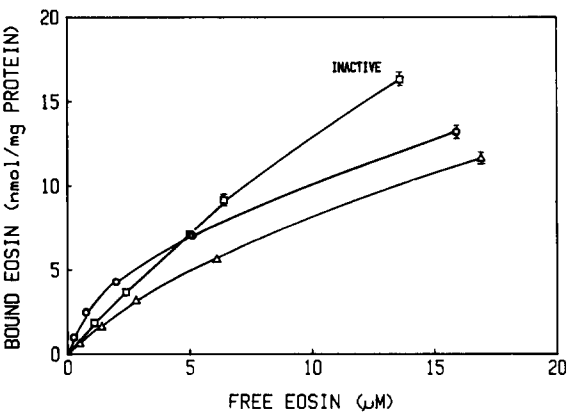


Fig. 8. The effect of heat-inactivation on the amount of eosin bound to $(Na^+ + K^+)$-ATPase in the presence of 150 mM Na^+ (o———o, □———□) and of 150 mM K^+ (Δ———Δ, □———□).

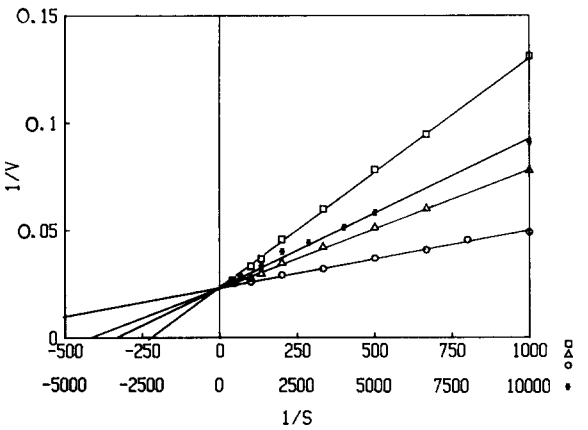


Fig. 9. The reciprocal catalytic activity ($1/v$) as a function of the reciprocal ATP-concentration ($1/S$) in the presence of 150 mM Na^+, 0.25 mM Mg^{2+} and with no (*———*), 2 (○———○), 5 (△———△) and 10 μM (□———□) eosin, 30 mM histidine buffer, pH 7.4, 37°C.

the cations and with a K_D of 0.25 μM. The high-affinity Na^+-dependent binding of eosin decreases by a further increase in the cation concentration to 2.2 nmol per mg protein with 150 mM Na^+ and with 500 mM Na^+. The K_D with 150 mM Na^+ is 0.45 μM and is increased to 0.9 μM with 500 mM. The values are read from the difference of the mean of 3–6 binding curves with Na^+ and with K^+, respectively, plotted in a Scatchard plot. For S.E. values see Figs. 6 and 8.

The enzyme has 2.1 nmol ^{32}P-labelling sites per mg protein. It means one high affinity eosin binding site per ^{32}P-labelling site with 2 mM Na^+, increasing to 1.4 with 20 mM and decreasing to one with 150 and 500 mM Na^+.

Heat inactivation (5 min at 65°C) of the enzyme eliminates the Na^+-dependent high-affinity binding and there is no difference in binding with Na^+ and K^+. The binding still increases with the cation concentration and is higher with the inactive than with the active enzyme in the presence of a given concentration of K^+. But with the low concentration of eosin the binding in the presence of Na^+ is lower with the inactive than with the active enzyme, Fig. 8.

Eosin-ATP interaction

The fluorescence in the presence of enzyme and Na^+ is decreased to the K^+ level by addition of ATP. With 30 nM eosin and 20 mM Na^+, 20 μM ATP (or ADP) eliminates the Na^+ effect. ATP also decreases the binding in the presence of Na^+ and tested with 100 μM ATP the eosin binding in the presence of 150 mM Na^+ becomes equal to the binding in the presence of 150 mM K^+. Due to problems with hydrolysis of ATP during the time it takes to do the binding experiments (the centrifugation) it is difficult to determine the effect of ATP on the binding. Instead, the mutual interaction between ATP and eosin has been tested in hydrolysis experiments in the presence of Na^+. With 150 mM Na^+ and optimum Mg^{2+} for hydrolysis, eosin inhibits hydrolysis of ATP but the effect can be overcome by an increase in ATP concentration. A double-reciprocal plot ($1/v$ against $1/[ATP]$) (Fig. 9) gives straight lines (tested with 2,5 and 10 μM eosin) which intercept at the same V but with a K_m which increases with the eosin concentration, suggesting that eosin is a competitive inhibitor for ATP. K_m for ATP is 0.3 μM without eosin, 1.2 μM with 2 μM eosin, 2.3 μM with 5 μM eosin and 4.6 μM with 10 μM eosin.

ATP effect of eosin

An opening of the high-affinity site for eosin by Na^+ and with competition between eosin and ATP suggests that the eosin site is the ATP binding site on the system. Support for this would be provided were eosin also to have an ATP effect, and as seen from the following this seems to be the case. Under non-hydrolyzing conditions, ATP or ATP analogues increase the rate of release of K^+ from a K^+-form of the system (E_2K) [4–6] and in the presence of a given concentration of K^+ [7,8] and of $K^+ + Na^+$ [9–12] the distribution of the enzyme molecules between the K^+-form and the Na^+-form is shifted towards the Na^+-form by addition of ATP [9–12]. The shift can be observed as a decrease in the Na^+ concentration necessary for activation of hydrolysis in the presence of K^+ [12] and also as a decrease in the concentration of Na^+ necessary in the presence of K^+ for protection against inactivation by an SH reagent such as *N*-ethylmaleimide [11].

An increase in the ATP concentration from 1.5 μM to 10 and 50 μM shifts the activation by Na^+ towards lower values (Fig. 10A); $Na^+ + K^+$ is kept constant at 150 mM. The activity is given as percentage of the activity with the optimum Na^+/K^+ ratio, and increases from 93 μmol P_i/mg protein per h with

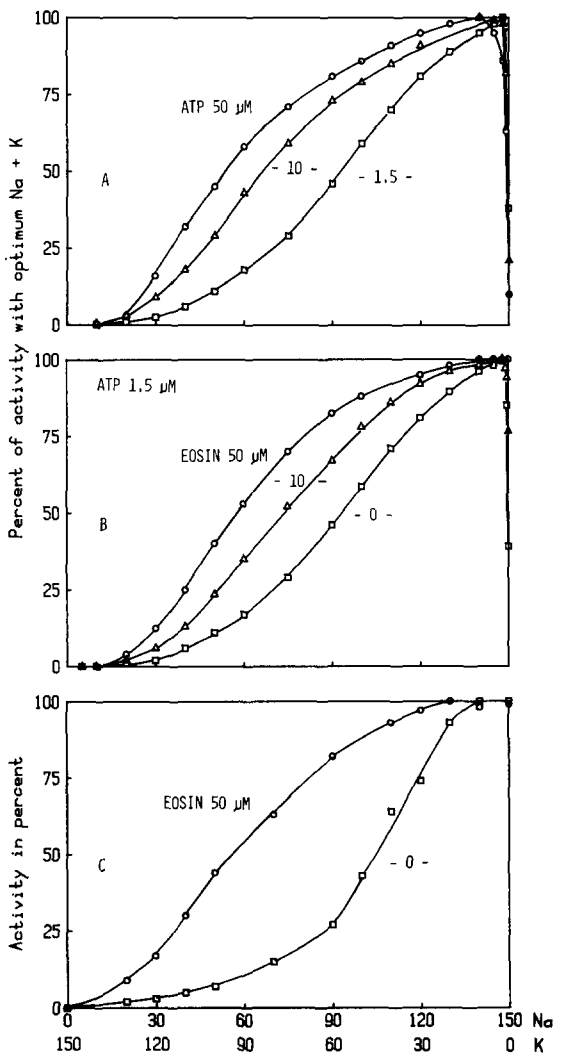


Fig. 10. A. The effect of an increase in the ATP concentration from 1.5 μM to 10 and to 50 μM on the Na^+/K^+ ratio for activation of the hydrolysis. B. The effect of 10 and 50 μM eosin on the Na^+/K^+ ratio for activation of hydrolysis of 1.5 μM ATP. C. The effect of 50 μM eosin on the Na^+/K^+ ratio for Na^+ protection against inactivation by *N*-ethylmaleimide. In A and B the activity was tested with the different Na^+/K^+ ratios in a 30 mM histidine buffer, pH 7.4, 37°C and with 0.1 mM Mg^{2+} in the presence of 1.5 μM ATP/0.25 mM Mg^{2+} with 10 μM ATP and 0.5 mM Mg^{3+} with 50 μM ATP. The activity at the different Na^+/K^+ ratios is given as the percentage of the activity with the optimum Na^+/K^+ ratio. In C the enzyme was preincubated for 4 min at 37°C in 30 mM histidine, pH 7.4, with different Na^+/K^+ ratios and with 0.1 mM *N*-ethylmaleimide ± 50 μM eosin. After preincubation, the reaction with *N*-ethylmaleimide was stopped by addition of mercaptoethanol and the activity tested in 130 mM Na^+/20 mM K^+/4 mM Mg^{2+}/3 mM ATP 30 mM histidine pH 7.4, 37°C. The effect of Na^+ in the preincubation medium on the protection against inactivation by *N*-ethylmaleimide is expressed as the activity after preincubation with ($Na^+ + K^+$) minus the activity after preincubation with 150 mM K^+ and is given in percent of the activity after preincubation with 150 mM Na^+ minus the activity after preincubation with 150 mM K^+. In the experiments with eosin it is the activities after addition of 50 μM eosin to the preincubation medium. The activity after preincubation with 150 mM K^+ (0%) is 378 ± 12 (n = 3) and with 150 mM Na^+ (100%) 639 ± 31 (n = 3) μmol P_i/mg protein per h. With 50 μM eosin the values are 650 ± 22 (n = 3) with 150 mM K^+ (0%) and 975 ± 8 (n = 3) μmol P_i/mg protein per h with 150 mM Na^+ (100%).

1.5 μM ATP to 193 with 10 μM ATP and to 394 with 50 μM ATP. The Na^+/K^+ ratio for half-maximum activation is decreased from 95 : 55 (95 ± 1.9 (n = 3)) with 1.5 μM ATP to 67 : 83 (67 ± 0.3 (n = 3)) with 10 μM ATP and to 54 : 96 (54 ± 0.9 (n = 3)) with 50 μM ATP.

The effect on the Na^+/K^+ ratio can be obtained by adding eosin instead of raising the ATP concentration. With 1.5 μM ATP the Na^+/K^+ ratio for half-maximum Na^+ activation is decreased from 95 : 55 (95 ± 2 (n = 3)) to 73 : 77 (73 ± 0.9 (n = 3)) by 10 μM eosin and to 60 : 90 (60 ± 1.5 (n = 3)) by 50 μM eosin (Fig. 10B). It is about the same effect as was obtained by ATP at the same molar concentrations. Eosin does not increase the hydrolysis as ATP does, but decreases the activity from 93 μmol P_i/mg protein per h to 15 with 10 μM eosin and to 2.5 with 50 μM eosin. The activity is lower at all the Na^+/K^+ values.

That the effect of eosin is not secondary to the decrease in rat of hydrolysis is seen from Fig. 10C, which shows that eosin without ATP decreases the Na^+/K^+ ratio for protection against inactivation by N-ethylmaleimide. Na^+/K^+ for half maximum protection is decreased from 105 : 45 (105 ± 1.5 (n = 3)) without eosin to 58 : 92 (58 ± 1 (n = 3)) with 50 μM eosin. This effect is also obtained by ATP (compare Fig. 10 with Fig. 11 in Ref. 11).

Discussion

The present experiments suggest that the previously observed effect of K^+ and of Na^+ on the fluorescence of eosin maleimide-labelled enzyme [1] is due

to a difference in binding of the non-covalently bound mercaptoethanol-reacted eosin maleimide which was left after the washing procedure.

There are two different bindings of eosin to the enzyme. The one is seen in the presence of K^+, the affinity is low and the binding is not reflected by an increase in fluorescence, at least not at the low concentration of eosin which can be tested. As this binding increases with heat inactivation it seems to be related to a certain structure of the system, but it is not possible from the present experiments to tell if it is a binding to the enzyme molecules in the preparation or to other components (the enzyme preparation is 55–60% pure).

Above this low-affinity binding (in the following the background binding) there is a binding of eosin to a site with high affinity induced by Na^+ and with eosin bound in an environment with a low polarity. It is not possible to tell if this eosin site also exists in the presence of K^+ but with a low affinity and hidden in the background binding. Alternatively, it is selectively opened by Na^+.

The effects of K^+, ATP and of heat inactivation on the high-affinity binding suggest that it is specific for the $(Na^+ + K^+)$-ATPase. Between one and two high affinity binding sites for eosin per ^{32}P-labelling site at lower Na^+ concentrations, decreasing to one at 150 mM Na^+ or at higher Na^+ concentrations, suggest that at a low Na^+ concentration there is a mixture of enzyme molecules with one and two high affinity binding sites per ^{32}P-labelling site. This suggests a dual effect of Na^+: at low concentrations Na^+ opens both sites (effect of Na^+ on internal sites?), but with higher concentrations one of the sites is closed (effect of Na^+ on external sites, i.e., a combined effect of Na^+ on internal and on external sites?). Another possibility is that the closing of the one site is not a specific Na^+ effect but an ionic strength or salt effect.

It would be of interest to know if the dual effect of Na^+ on eosin binding is related to the dual effect on the activation of the hydrolysis by Na^+ without K^+ [13–15] and to the effect on the ratio between the ADP and the K^+-sensitive phosphoenzyme of an increase in the concentration of Na^+ [16–18].

The conformation of the enzyme with Na^+ thus differs from the conformation of the enzyme with K^+ by a site or sites for eosin with a high affinity and with a low polarity.

In the experiments with eosin-maleimide-treated enzyme it was observed that titration with Na^+ gives a bell-shaped fluorescence change curve [1]. Na^+ up to 20 mM increases the fluorescence and upon further increase in the Na^+ concentration the fluorescence again decreases. The present experiments suggest that this is due to the sum of the two effects of Na^+, the 'bell-shaped' effect on the number of eosin molecules bound and the 'bell-shaped' effect on the affinity. In the Na^+ fluorescence titration experiments, the concentration of mercaptoethanol-reacted eosin maleimide was below the K_D value.

The disappearance of the high-affinity binding site when the enzyme is heat inactivated suggests that it requires an organized structure.

High-affinity binding of eosin to a site on the enzyme which is opened by Na^+, closed by K^+, and which is eliminated by ATP and with a competition between ATP and eosin for hydrolysis suggests that the high-affinity binding site for eosin seen with 150 mM Na^+ is an ATP binding site of the system.

The K_D for eosin, 0.45 μM (23°C, 150 mM Na^+) is close to the K_D for ATP, 0.12–0.2 μM (0°C) [9,10], and to the K_m for ATP of 0.3 μM (37°C) observed in the hydrolysis experiments.

The effect of ATP which leads to a decrease in the Na^+/K^+ ratio for half-maximum Na^+ activation of hydrolysis of ATP seems to be due to a decrease in pK values of the system and can be mimicked by an increase in pH [1]. There is no effect of addition of 10 μM and 50 μM eosin on pH of the test solution, indicating that the 'ATP effect' of eosin must be on the system and suggesting that eosin as well as ATP can decrease pK values in the system of importance for the Na^+/K^+ affinity ratio.

Eosin has about the same affinity for the enzyme system as ATP and on a molar basis the 'ATP effect' of eosin on the Na^+/K^+ ratio is about the same as of ATP (compare Fig. 10A and B). This suggests that the eosin effect is due to a binding of eosin to the site at which ATP exerts its effect and not to an effect of the low-affinity background binding of eosin.

The 'ATP effect' of eosin on the Na^+/K^+ ratio is also seen under non-hydrolysing conditions (protection against *N*-ethylmaleimide) and can therefore not follow from eosin inhibition of hydrolysis. As the 'ATP effect' of eosin on the Na^+/K^+ ratio is seen on that fraction of the enzyme molecules which is not

inhibited by eosin but which hydrolyses ATP, these enzyme molecules must react both with eosin and with ATP in each turnover cycle. If it is correct that the 'ATP effect' of eosin is on an ATP site, this means either that there must be consecutive reactions with eosin and ATP at this site or that there are two ATP sites in the system. It then follows that, without eosin, the ATP which has the effect on the Na^+/K^+ ratio (K^+ release) is not the ATP which is hydrolyzed, i.e., there are two different effects of ATP in a turnover cycle in the presence of $Na^+ + K^+$: one effect which is of importance for the release of incoming K^+ and uptake of outgoing Na^+, and another which leads to an activation by external K^+ of the hydrolysis [1,12]. The view about two ATP effects is supported by inactivation studies which show two different effects of ATP [19–21].

Binding experiments show one high-affinity ATP binding site per ^{32}P-labelling site [9,10]. With one ^{32}P-labelling site per two α-chains [22,23] and with two α-chains per enzyme molecule [23–30] it means that there is one ATP or one eosin binding site per enzyme molecule. Is the explanation of two ATP effects, then, that there are two different steps in the reaction, two different conformations in which ATP can enter and leave, one which is related to the release of incoming K^+ and another to the activation by external K^+ of the hydrolysis [1,12]. Or are there two different sites for ATP but only one ATP bound at a time – half-of-the-sites reactivity [31,32] – or is the affinity of the second site so low that it is difficult to measure with the methods to hand?

Or is it the concept of one ^{32}P-labelling site per α-chains which is incorrect? Is there, as suggested from recent experiments, one ^{32}P-labelling site [33] or one ATP-binding site [34] per α-chain, i.e., two ATP-binding sites per $(\alpha\beta)_2$ molecule?

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