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Occlusion of Rb^+ by detergent-solubilized ($Na^+ + K^+$)-ATPase from shark salt glands

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Occlusion of Rb^+ by $C_{12}E_8$-solubilized ($Na^+ + K^+$)-ATPase from shark salt glands has been measured. The rate of de-occlusion at room temperature is about 1 s^{-1}, which is the same as for the membrane-bound enzyme. The amount of Rb^+ occluded is 3 moles Rb^+ per mole membrane-bound shark enzyme, whereas only about 2 moles Rb^+ are occluded by the $C_{12}E_8$-solubilized enzyme.

Introduction

In 1972 Post et al. [1] suggested that K^+ (or congeners of K^+) became occluded in the E_2 form of the ($Na^+ + K^+$)-ATPase as a part of the hydrolysis cycle. A direct demonstration of the occlusion was given in 1979 by Beaugé and Glynn [2], who showed that ($Na^+ + K^+$)-ATPase in the presence of Rb^+ (and absence of Na^+ and nucleotides) trapped some of the Rb^+ in such a way, that the free Rb^+ could be removed before the trapped (or occluded) Rb^+ was released from the enzyme. The presence of nucleotides (e.g. 2 mM ADP) reduced the amount of Rb^+ occluded considerably, which suggested that it is the E_2 state that occludes Rb^+ and that Rb^+ is released (de-occluded) when the enzyme is turned into the E_1 state. Further work has given information on the influence of nucleotides on the rate of de-occlusion, and a stoichiometry of 3 moles Rb^+ occluded per mole of enzyme was found [3].

The rate of the de-occlusion of Rb^+ ions has also been measured with a new method developed by Forbush [4]. The rate of de-occlusion was measured in the presence of physiological concentrations of cations and nucleotide, and was found to be about the same as the turnover rate of the ($Na^+ + K^+$)-ATPase hydrolysis cycle, indicating a rate-limiting role of the de-occlusion step, i.e. the E_2–E_1 conformational change (see Ref. 4).

The above mentioned findings strongly suggest a role for the Rb^+ occluded state in the hydrolysis and possibly the transport cycle of the membrane bound ($Na^+ + K^+$)-ATPase.

The purpose of the present paper is to investigate whether the $C_{12}E_8$-solubilized, fully hydrolytically active ($Na^+ + K^+$)-ATPase also can occlude Rb^+. The kinetic properties of the soluble enzyme resemble that of the membrane-bound enzyme to a large extent [5], and the $C_{12}E_8$-solubilized enzyme can also be incorporated in artificial vesicles to give full Na^+/K^+ transport activity [6]. Transport activity cannot be demonstrated in the solubilized state, but detection of Rb^+ occlusion is technically possible. Occlusion of cations by the ($Na^+ + K^+$)-ATPase is interpreted as being a necessary step for the translocation of the cation. It is therefore of interest to see whether cations are occluded by the enzyme also when the protein is 'uncoupled' from the membrane, i.e. in the solubilized state.

The results show that the solubilized enzyme can occlude Rb^+, and the rate of de-occlusion is about the same as for the membrane-bound en-

zyme (about 1 s^{-1} at 23°C). However, the stoichiometry is only about 2 moles Rb^+ per mole enzyme in the solubilized state, whereas the membrane-bound shark enzyme occludes 3 moles Rb^+ per mole, the latter figure being in agreement with the occlusion in the membrane bound kidney enzyme [3].

The findings suggest either that the α-β protomer * of ($Na^+ + K^+$)-ATPase cannot occlude Rb^+, whereas the α-β diprotomer behaves as the membrane-bound enzyme (and occludes Rb^+) or that one of the Rb^+ occlusion sites on the membrane-bound enzyme is lost upon solubilization (or the affinity for the site is decreased markedly).

Methods

Preparation of membrane-bound enzyme. ($Na^+ + K^+$)-ATPase is prepared from salt glands of the spiny dogfish (*Squalus acanthias*) as previously described, omitting saponin in the preparative procedure. The enzyme fraction used is the 220 000 × *g* pellet (see Ref. 7) with a specific ($Na^+ + K^+$)-ATPase activity of about 1100 μmol/mg protein per h. The amount of α- and β-subunit is about 50% of the total protein, as quantitated from SDS-poly acrylamide gel electrophoresis. Enzymatic activities and protein assays of the membrane bound enzyme are performed as previously described [7].

The enzyme preparation used here is about 50% pure as estimated from the content of α- and β-peptides. The molar concentration is proportional to the phosphorylation capacity, which is about 2.0 nmol/mg protein. Both these numbers are in agreement with the measured specific activity (see above), which is about half of the highest obtained (see Ref. 7 for a discussion). The concentration of vanadate and ATP-binding sites is about the same as the concentration of phosphorylation sites, this being consistent with a half pure preparation. The molar activity is about 9000 min^{-1}, which indicates that all the enzyme in the preparation is active.

* The word protomer will be used to denote the α-β heterodimer consisting of one α-subunit (M_r 104000) and one β-subunit (M_r 40000). A diprotomer will thus consist of 2 α- and 2 β-subunits.

Solubilization in $C_{12}E_8$. The solubilized enzyme is prepared in principle as previously described [5]: 1/10 volume of $C_{12}E_8$ in water was added at 4°C to a buffered solution containing the membrane-bound enzyme, giving final concentrations of 15 mM histidine-HCl (pH 7.0 at 23°C), 0.5 mM EDTA (Tris salt), 0.24 mg protein/ml and $C_{12}E_8$ between 0.25 and 20 mg/ml (see below). At $C_{12}E_8$/protein weight ratios above 1 all the ($Na^+ + K^+$)-ATPase activity is solubilized (see Ref. 5), i.e. remains in the supernatant after 30 min at full speed in a Beckman Airfuge. In experiments with ADP present, the nucleotide (Tris salt) is added (to a final concentration of 2 mM) before the detergent.

Measurement of enzymatic activity of the solubilized enzyme. The ($Na^+ + K^+$)-ATPase activity of the $C_{12}E_8$ solubilized enzyme is assayed in the presence of 1 mg $C_{12}E_{10}$/ml at 23°C, as described in Ref. 5.

Detection of occluded Rb^+. The technique used is essentially as described by Glynn and Richards [3]. The carboxylic resin Bio-Rad Bio-Rex 70 is equilibrated in 100 mM Tris (pH 7.0) and packed to a bed volume of 0.5 ml in disposable syringes (see Ref. 3). Before application of a membranous enzyme suspension, the column was washed with 1 ml of a 100 mM Tris buffer (pH 7.0) containing 0.5 mM EDTA. If solubilized enzyme was to be assayed the washing buffer also contained 0.2 mg $C_{12}E_8$/ml.

The solubilized, or membrane-bound, enzyme is equilibrated with $^{86}RbCl$ at the desired rubidium concentration for 2–4 min at room temperature before about 400 μl is forced through the column. The speed of the piston forcing the solution through the column is determined by a stepping motor. The flow rates used in the present experiments are adjusted to allow the enzyme suspension to be in contact with the resin between 0.38 s and 1.8 s, calculated as given by Glynn and Richards [3].

The amount of $^{86}Rb^+$ emerging from the column was measured and the amount of protein determined by the method of Lowry et al. [8] as modified by Peterson [9]. The data presented in Figs. 1–4 are obtained as the net occlusion, i.e. the difference between the amount of occluded Rb^+ in the absence and in the presence of 2 mM ADP

(see Ref. 3). The Rb^+ emerging with the enzyme in the presence of ADP (i.e. the non-specific binding) was about 20% of the 'specific' binding, with the membrane bound enzyme and this value was reduced to about 5% with the solubilized enzyme.

The change of ionic strength that occur when the enzyme suspension is forced through the column (equilibrated in 100 mM Tris (pH 7.0) and 0.5 mM EDTA) gives rise to a small decrease in pH in the enzyme suspension, about 0.5 pH units. It was, however, checked that the specific ($Na^+ + K^+$)-ATPase activity of both membrane-bound and solubilized enzyme did not decrease after passing the resin. Another control experiment showed that the concentration of $C_{12}E_8$ in the column had no effect (between 0.1 and 2 mg/ml) on the rate of the de-occlusion of Rb^+; therefore, 0.2 mg $C_{12}E_8$/ml was used (see above).

Results and Discussion

1. Occlusion of Rb^+ by the membrane-bound shark rectal glands ($Na^+ + K^+$)-ATPase

The membrane-bound ($Na^+ + K^+$)-ATPase from shark rectal glands is able to occlude Rb^+ in the absence of Na^+ and nucleotides in the same manner as previously shown for the kidney enzyme (see Refs. 2 and 3).

The rate of de-occlusion of Rb^+ at room temperature is about 1.2 s^{-1} (see Fig. 1, curve A) which is a 10-fold higher rate than has been observed with the kidney enzyme [3]. The higher rate of de-occlusion in the shark enzyme is to be expected, since the rate of E_2–E_1 conformational change monitored by eosin fluorescence changes at room temperature also is 10-fold higher with the shark enzyme than with the kidney enzyme (compare Refs. 10 and 3).

The affinity for Rb^+ is determined as described by Glynn and Richards [3] and a dissociation constant of about 15 μM for the membrane bound shark enzyme is obtained (data not shown). Note that the affinity is determined in a 15 mM histidine buffer, and not in the 100 mM Tris buffer used by Glynn and Richards. The reason for this is that 100 mM Tris (pH 7.0) turns the shark enzyme in the E_1 form and thereby counteracts the effect of Rb^+. In practice the (apparent) affinity for Rb^+ becomes so low in 100 mM Tris that almost no occlusion is observed with 100 μM Rb^+.

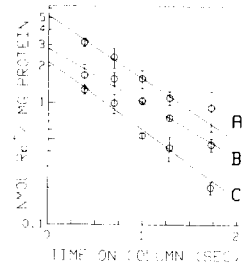

Fig. 1. Three experiments to measure the rate of release of Rb^+ from the occluded state. Each set of data points shows the amount of Rb^+ carried through the column by the enzyme as a function of time spent in contact with the resin. ($Na^+ + K^+$)-ATPase (0.24 mg/ml) was incubated for 2–4 min at room temperature in 15 mM histidine (pH 7.0), 0.5 mM EDTA and 100 μM $^{86}Rb^+$ (chloride salt). The incubation medium also contained 0 (Curve A), 0.50 (Curve B) or 2.50 mg $C_{12}E_8$/ml (Curve C). Each point represents the mean (±S.E.) of 3–5 determinations of the difference between the amount of Rb^+ carried through the column with no ADP in the incubation medium and with 2 mM ADP in the incubation medium. The lines are fitted to single exponentials of the form $Y_t = Y_0 \cdot \exp(-kt)$ with $Y_0 = 5.19$ nmol/mg and $k = 1.19$ s^{-1} for curve A, $Y_0 = 2.8$ nmol/mg and $k = 1.03$ s^{-1} for curve B and $Y_0 = 2.17$ nmol/mg and $k = 1.29$ s^{-1} for curve C.

The maximal binding of Rb^+ to the membrane bound enzyme was calculated from the amount of Rb^+ bound at 100 μM Rb^+ (about 5.1 nmol/mg protein, Fig. 1) and extrapolating to saturating Rb^+. This gives a value of about 5.7 nmol/mg protein, equivalent to about 3 moles Rb^+ per mole of enzyme (i.e. per mole of phosphorylation sites). This value is in good agreement with the value of 2.9 moles per mole of kidney enzyme measured by Glynn and Richards [3].

Control experiments showed that (a) the Rb^+ occlusion was counteracted by Na^+ as previously described [3], (b) that the pH of the incubation medium in the range 6.5 to 7.5 had no (or a very small) effect on the rate of de-occlusion, and (c) that the membrane-bound enzyme emerging from the column had the same specific ($Na^+ + K^+$)-ATPase activity and polypeptide composition as before passage of the resin.

In summary the properties of membrane-bound shark ($Na^+ + K^+$)-ATPase resembles that of the kidney enzyme to a large extent, the major difference being the (expected) higher rate of de-occlusion.

2. Occlusion of Rb^+ by $C_{12}E_8$-solubilized shark enzyme

Solubilization of the membrane bound ($Na^+ + K^+$)-ATPase was carried out as previously described [5]. It was ensured by centrifugation that all of the ($Na^+ + K^+$)-ATPase activity was solubilized by the amount of detergent added in the presence of the Rb^+ used (usually 100 μM). Note that the detergent was added before the Rb^+. A set of experiments showed that the $C_{12}E_8$ concentration in the column had no effect (in the range tested 0.1–2 mg/ml) on the rate of de-occlusion or amount of occluded Rb^+, and 0.2 mg $C_{12}E_8$/ml was used routinely in the column (the detergent/protein ratio at the incubation step, on the other hand, proved very critical to the amount of Rb^+ occluded, see below).

The rate of de-occlusion of Rb^+ from $C_{12}E_8$-solubilized enzyme is determined from Fig. 1 (curve B) to be about 1.0 s^{-1}, which is about the same as for the membrane-bound enzyme. The effect of an increase in the detergent/protein ratio at the incubation step from 2 g/g (curve B) to 10 g/g (curve C) is also shown in Fig. 1. A slight increase in the rate of de-occlusion is observed (from 1.0 to 1.3 s^{-1}) and a decrease in the maximal binding is also seen.

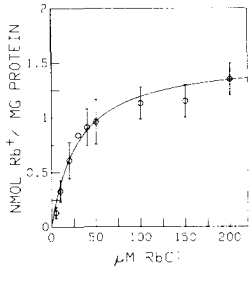

Fig. 2. The amount of Rb^+ occluded as a function of the Rb^+ concentration in the incubation medium. ($Na^+ + K^+$)-ATPase (0.24 mg/ml) was incubated 2–4 min at room temperature in 15 mM histidine (pH 7.0), 0.5 mM EDTA, 0.50 mg $C_{12}E_8$/ml, the Rb^+ concentrations shown on the figure and 0 or 2 mM ADP. About 0.4 ml was forced through the column at a flow rate such that the enzyme spent about 0.5 s in contact with the resin. The amount of specific Rb^+-binding was calculated after subtraction of the ADP-'blank' and is given in the figure for each Rb^+ concentration. Each point represents the mean ($\pm$S.D.) of three determinations. The continuous line is drawn according to the Michaelis-Menten type equation $Y = Y_{max}/(1 + K_m/(Rb^+))$, where Y_{max} is 1.55 nmol/mg protein and K_m is 30 μM.

The observation that the rate of de-occlusion (interpreted as the rate of the E_2–E_1 transition) of the solubilized enzyme is about the same as the membrane-bound enzyme is in agreement with the also similar rates of eosin fluorescence changes (e.g. Fig. 2 in Ref. 5).

The dissociation constant for Rb^+ in the presence of $C_{12}E_8$ is about 30μM, as estimated from Fig. 2. The curve drawn in Fig. 2 is based on a simple hyperbolic saturation. The quality of the data does not allow for a more detailed kinetic analysis such as that given by Glynn and Richards (cf. Fig. 7 in Ref. 3).

An increase in the detergent/protein ratio from 2 g/g shown in Fig. 2 to 10 g/g (not shown) did not change the apparent affinity for Rb^+, although the lower binding at the higher detergent/protein ratios gave more scatter on the data points.

3. The effect of $C_{12}E_8$ on the ($Na^+ + K^+$)-ATPase activity and Rb^+ occlusion

The $C_{12}E_8$/protein weight ratio must be carefully controlled to avoid inactivation (e.g. Fig. 6 in Ref. 5). At weight ratios above 6 g/g a irreversible inactivation occurs (at low ionic strength). The purpose of the present experiments is to see whether the Rb^+ occlusion disappears together with the overall ($Na^+ + K^+$)-ATPase activity, or whether it is more resistant to detergent inactivation such as the vanadate binding ability is [5].

The inactivation of the ($Na^+ + K^+$)-ATPase activity is shown in Fig. 3 (open symbols). The activity is irreversibly lost in the interval 1.5–6.0 mg $C_{12}E_8$/ml equivalent to detergent/protein weight ratios of 6–25 g/g. Note that the enzymatic activity is measured in the presence of $C_{12}E_{10}$, which allows for an (otherwise inactivating) excess of $C_{12}E_8$ in the test solution (see Ref. 5).

The Rb^+-occlusion decreases from about 1.4 nmol/mg protein at the low $C_{12}E_8$/protein ratios to no specific binding at high $C_{12}E_8$/protein ratios (measured at 100 μM Rb^+, see legend to Fig. 3). The major part of the decrease takes place between 2 and 5 mg $C_{12}E_8$/ml, i.e. the effect of the detergent is in the same concentration range as that giving inactivation (see above). Note that the decrease in binding at high $C_{12}E_8$/protein ratios cannot be explained by an increase in rate of

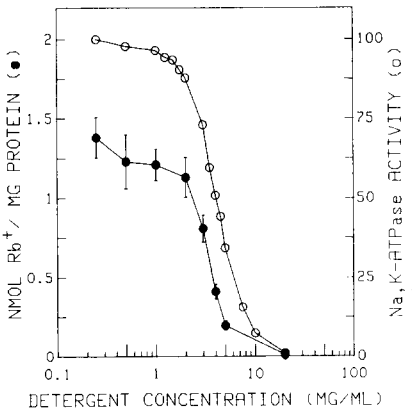

Fig. 3. The effect of $C_{12}E_8$ on Rb^+-occlusion and $(Na^+ + K^+)$-ATPase activity. $(Na^+ + K^+)$-ATPase (0.24 mg/ml) was incubated for 2–4 min at room temperature in 15 mM histidine (pH 7.0), 0.5 mM EDTA, 100 μM RbCl, the $C_{12}E_8$ concentration indicated in the figure, and 0 or 2 mM ADP. About 0.4 ml was forced through the column at a flow rate such that the enzyme spent about 0.5 s in contact with the resin. The amount of specific Rb^+ binding was calculated of subtraction after the amount occluded in the presence of 2 mM ADP, and is given as the mean ($\pm$S.D.) for 4–6 determinations (filled symbols). The specific $(Na^+ + K^+)$-ATPase activity (measured in the presence of $C_{12}E_{10}$, see Methods) is given in % of the specific activity of the membrane bound enzyme at the same temperature (open symbols).

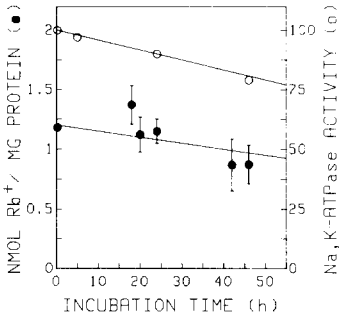

Fig. 4. The effect of incubation at 20°C on the Rb^+-occlusion and the $(Na^+ + K^+)$-ATPase activity. $(Na^+ + K^+)$-ATPase (0.24 mg/ml) was incubated at 23°C in 15 mM histidine (pH 7.0), 0.5 mM EDTA, 100 μM RbCl, 0.5 mg $C_{12}E_8$/ml with 0 or 15 mM NaCl. At the time points shown the $(Na^+ + K^+)$-ATPase activity was measured (open symbols) and is given in % of the activity at time zero. For measurement of occlusion, about 0.4 ml was forced through the column at a flow rate such that the enzyme spent about 0.5 s in contact with the resin. The amount of specific Rb^+ binding was calculated after subtraction of the amount occluded in the presence of 15 mM NaCl, and is given as the mean ($\pm$S.D.) for three determinations (open symbols). Note that the amount of nonspecific Rb^+ binding (i.e. in the presence of Na^+ or ADP) increased from typically 0.2 nmol/mg at time zero to 3–4 nmol/mg at 48 h. This large increase in background makes the measurement of the specific binding more difficult.

de-occlusion, since the rate is increased only slightly by an increase in the $C_{12}E_8$/protein ratio (cf. Fig. 1). Since the affinity also is unchanged by an increase in the detergent/protein ratio (see above) the simplest explanation of the findings in Fig. 3 is that the amount of Rb^+ occluded decreased by the increase in detergent concentration.

The amount of Rb^+ occluded in the solubilized enzyme is lower than in the membrane-bound enzyme. From the Rb^+ affinity (Fig. 2) and the rate of de-occlusion (Fig. 1, curve B), the maximal amount of Rb^+ occluded can be calculated to about 3.7 nmol/mg, equivalent to about 2 moles Rb^+ per mole enzyme.

The solubilized enzyme is stable for prolonged periods even at room temperature. Fig. 4 shows an experiment in which the $(Na^+ + K^+)$-ATPase activity and the Rb^+ occlusion was followed for 48 h. There is a slight decrease in the enzymatic activity (to about 80%) and also a decrease in the amount of Rb^+ occluded (see below).

Solubilized enzyme did not lose activity within 96 h when stored at 4°C, and in agreement with this there was no change in the amount of Rb^+ occluded.

The reason for performing the experiment shown in Fig. 4 is that a time-dependent aggregation of the solubilized enzyme has been observed previously (cf. Ref. 11). It was therefore of interest to see whether the amount of Rb^+ occluded changed with time, but as seen from Fig. 4, there seems to be no change except for the parallel decrease of $(Na^+ + K^+)$-ATPase activity and Rb^+ occlusion.

4. Possible reasons for the lower amount of occluded Rb^+ with $C_{12}E_8$

The properties of Rb^+ occlusion by the $C_{12}E_8$-solubilized enzyme are the same as those of the membrane bound enzyme, except for the lower amount of Rb^+ occluded.

Since the soluble enzyme is fully active, the decrease in binding must be attributed to a change in structure, which does not affect overall ATP

hydrolysis. The two explanations given below seem plausible.

(a) An effect of the protomer / diprotomer ratio

The $C_{12}E_8$-solubilized shark $(Na^+ + K^+)$-ATPase is a mixture of protomers and diprotomers, as suggested by gel filtration experiments of shark enzyme in the presence of $C_{12}E_8$ [11]. A number of observations on the $C_{12}E_8$-solubilized kidney enzyme also suggest a mixture of protomers and diprotomers [12–15], although the solubilized kidney enzyme seems to be predominantly on the more labile protomeric form. Both the protomer and the diprotomer of the shark enzyme seems to have catalytic activity (cf. Fig. 3A in Ref. 11), although the oligomeric structure has not been determined under ATP-hydrolysing conditions.

It seems plausible that the active structure of the membrane bound enzyme is the diprotomer, evidence coming from kinetic observations (see, for example, Ref. 16) and from structural information [17–19]. Since the membrane bound enzyme occludes 3 moles Rb^+/mole enzyme and the solubilized enzyme only 1.8 moles/mole, a simple explanation could be that the solubilized protomer does not occlude Rb^+. The solubilized enzyme should thus consist of about 60% diprotomer and 40% protomer at the lower detergent/protein ratios. In agreement with this the Rb^+ occlusion is abolished at high $C_{12}E_8$/protein ratios (cf. Fig. 2), where the enzyme is predominantly on the protomeric form [20].

(b) A change in the number or affinity of the Rb^+ sites

The three Rb^+ sites on the membrane bound enzyme appear to be heterogeneous in the absence of Tris (see for example Fig. 1 in Ref. 21). An explanation for the lower Rb^+ occlusion in the presence of $C_{12}E_8$ could therefore be that the affinity for Rb^+ at one of the three sites is decreased by the detergent to a value too low to be measured with the present method. In this case the protomer must be assumed to be able to occlude Rb^+ (and possibly transport cations). The decrease in Rb^+ occlusion at high detergent/protein ratios is thus not related to protomerization of the enzyme (cf. Fig. 3), but to another process leading to inactivation.

Conclusion

The present experiments show that the $C_{12}E_8$-solubilized $(Na^+ + K^+)$-ATPase is able to occlude Rb^+ in the same manner as the membrane-bound enzyme. If the ability of occlude Rb^+ is taken as a necessary step in the translocation reaction, then the present experiments show that this step in the catalytic cycle is preserved in the solubilized state, i.e. in the absence of most of the lipids from the native membrane. This suggests, together with other observations (see, for example, Ref. 5), that the solubilized shark $(Na^+ + K^+)$-ATPase has all the catalytic features of the membrane-bound enzyme.

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