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Rb^+ occlusion in renal ($Na^+ + K^+$)-ATPase characterized with a simple manual assay

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This paper describes properties of a simple manual assay for Rb^+ occlusion on renal ($Na^+ + K^+$)-ATPase. Rb^+ occlusion is measured by applying the enzyme plus Rb^+ (^{86}Rb) mixture to a Dowex-50 cation exchange column at 0°C, and eluting the enzyme with occluded Rb^+ using an ice-cold sucrose solution. The enzyme-Rb^+ complex is quite stable at 0°C. This method is useful for measuring Rb^+ occlusion under equilibrium binding conditions and slow rates of dissociation of the enzyme-Rb^+ complex. The stoichiometry of Rb^+ occluded per phosphorylation site is 2. Rb^+ saturation curves are strictly hyperbolic, suggesting that the two Rb^+ sites have very different affinities, one in the micromolar range and one in the tens of millimolar range. ATP shifts the Rb^+ saturation curves to the right (control $K_{0.5}$ 100–200 μM; plus ATP, $K_{0.5}$ 0.8–1.4 mM, in a 100 mM Tris-HCl medium, pH 7.0) and reduces the maximal level occluded (control approx. 4 nmol/mg; plus ATP approx. 3 nmol/mg protein). Thus, as expected, ATP shifts the $E_1 2Rb^+$-$E_2(2Rb^+)_{occ}$ equilibrium towards E_1. Sodium ions at concentrations of up to 30 mM compete with the rubidium ions, K_{Na} = 1.86 mM in the Tris-HCl medium. Na^+ at higher concentrations (30–100 mM) has an added non-competitive antagonistic effect. At room temperature, Rb^+ dissociates slowly from the enzyme, $k_{obs} = 0.08\ s^{-1}$, in the presence of either Rb^+ (20 mM) or Na, (100 mM). As expected, dissociation is greatly accelerated by ATP, the rate being to fast to be measured by this technique. ($Na^+ + K^+$)-ATPase proteolyzed selectively by chymotrypsin in a Na^+ medium, occludes Rb^+. For control and proteolyzed ($Na^+ + K^+$)-ATPase the Rb^+ saturation curves are similar and the rates of dissociation of the enzyme-Rb^+ complex are identical. The chymotryptic split appears to disrupt antagonistic interactions between cation and ATP binding domains, while the E_1-E_2 conformational transition of the unphosphorylated protein probably remains.

Introduction

The cell membrane ($Na^+ + K^+$)-ATPase actively transports three sodium and two potassium ions per cycle. During the transport cycle the ions become occluded. Sodium ions bound initially at the cytoplasmic surface to the E_1 form catalyze phosphorylation by ATP and became occluded in the form $E_1 \sim P(3Na^+)_{occ}$. Na^+ is released to the extracellular surface following the conformational transition $E_1P(3Na^+)_{occ} \rightarrow E_2P$. Potassium ions bound at extracellular sites on the $E_2 \sim P$ form catalyze hydrolysis of $E_2 \sim P$ and become occluded in the $E_2(2K^+)_{occ}$ form. Potassium ions are released at the cytoplasmic surface following the conformational change $E_2(2K^+)_{occ} \rightarrow E_1$. Potassium ions can also be occluded in $E_2(2\ K^+)_{occ}$

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following binding at the cytoplasmic surface in E_1 and reversal of the conformational transition, i.e., $E_1 + 2K \rightarrow E_2(2K^+)_{occ}$ [1].

Occlusion of ions has been studied thoroughly by Glynn and colleagues using apparatus which forces enzyme/$Rb^+(+{}^{86}Rb^+)$ or enzyme/$Na^+(+{}^{22}Na^+)$ mixtures through a Dowex-50 cation exchange column [2,3]. Thus, in practice 'occlusion' is an operative term referring to the cations which remain bound to the enzyme during the time it spends on the column. A minimum of 0.9 s is required for the Dowex to bind the free cation and isotope to a sufficient degree. In another development, Forbush [4] has described a rapid filtration apparatus which permits measurement of rapid rates of deocclusion of cations.

During experiments to covalently modify cation binding residues on $(Na^+ + K^+)$-ATPase, we felt the need for a convenient manual method permitting assay of Rb^+ occlusion in large numbers of samples (M. Shani and S. Karlish, unpublished data). The experiments in this paper describe the assay developed for this purpose and some of the features of Rb^+ occlusion observed. The assay involves elution of enzyme-bound Rb^+ on Dowex-50 cation exchange columns at 0 °C, a procedure which takes about 20 s. Its success depends on the great stability of the enzyme-Rb^+ complex at 0 °C. An advantage of relatively slow elution on Dowex-50 is that the full cation exchange capacity of the resin is utilized and hence the background of free cations (isotope) emerging from the column is extremely low.

Methods

$(Na^+ + K^+)$-ATPase was prepared from pig kidney by the simpler of the procedures developed by Jorgensen [5]. The membranes were suspended in a medium containing 250 mM sucrose, 25 mM histidine (pH 7.5), 1 mM EDTA (Tris), at a protein concentration of 3–5 mg/ml and stored at −70 °C. Prior to use the enzyme was dialyzed overnight against 1000 vols. of a solution containing 25 mM histidine (pH 7), 1 mM EDTA (Tris). $(Na^+ + K^+)$-ATPase activities and protein concentration were determined as described in Ref. 5. The spec. act. of preparations used in this study was between 10–20 units/mg protein.

Rb occlusion assay

30–50 μl of enzyme, containing 25–50 μg protein, was suspended in a medium of 100 mM Tris-HCl (pH 7.0) and was mixed at room temperature 20–25 °C with 10 μl of a reaction mixture containing 100 mM Tris-HCl (pH 7.0), RbCl up to 15 mM final concentration and ${}^{86}Rb^+$ $1–2 \cdot 10^6$ cpm per sample. After 5 min incubation at room temperature (usually in triplicate), 0.5 ml of ice-cold sucrose, 200 mM, was added, and the suspension was transferred immediately to ice-cold columns of Dowex-50 × 8, Tris form. The enzyme with bound Rb^+ was eluted with 1.5 ml of the sucrose solution. ${}^{86}Rb^+$ was counted by its Cherenkov radiation. Dowex (Tris form) was prepared from the Dowex (H) form as described in Ref. 6. The Dowex-50 was poured in Pasteur pipettes to a height of about 7 cm. The Dowex-50 was washed first with about 1 ml of bovine serum albumin 25 mg/ml, and then with 2 ml of sucrose, 200 mM. The $(Na^+ + K^+)$-ATPase was eluted essentially quantitatively from columns washed in this way, as detected by fluorescence of fluorescein isothiocyanate labelled $(Na^+ + K^+)$-ATPase [7], whereas significant losses of $(Na^+ + K^+)$-ATPase protein occurred if unwashed Dowex-50 columns were used. Occluded Rb^+ was expressed as nmol per mg protein.

Dissociation rates of occluded Rb^+ were determined as follows: enzyme was incubated with $Rb^+ + {}^{86}Rb^+$, about 2 mM as above. 200 μl of a solution at room temperature containing 100 mM Tris-HCl (pH 7) plus 20 mM RbCl (and 2 mM ATP where indicated) or 20 mM Tris-HCl (pH 7) plus 100 mM NaCl was rapidly added. At times between 2–600 s after mixing, 1 ml of an ice-cold solution of 200 mM sucrose was added and the suspension was transferred immediately to ice-cold Dowex-50 columns. The zero-time sample was obtained by adding the 1 ml of ice-cold sucrose prior to the 200 μl of dissociation medium.

For calculation of the dissociation rate in the medium containing 20 mM Rb^+ it is necessary to take into account that the specific activity of the ${}^{86}Rb^+$ is diluted only 10-fold so that about 10% of the Rb^+ bound at zero time remains bound at infinite time.

Phosphorylation

(a) From organic phosphate. The reaction mixtures were as follows: (1) 100–200 μg enzyme was incubated in triplicate for 1 h at room temperature in 100 μl of a medium containing 50 mM Tris-acetate (pH 7), 2 mM P_i (Tris) + $2–5 \cdot 10^5$ [^{32}P]P_i cpm per sample, 1 mM ouabain, 1 mM $MgCl_2$. 5 ml of an ice-cold solution containing 5% trichloroacetic acid, 50 mM inorganic phosphate (Tris) (solution A) was then added. After 30 min on ice, the denatured protein was filtered on a Whatman GF/B filter and the filter was washed with 100 ml of solution A. Background and non-specifically bound [^{32}P]P_i were estimated by adding the solution A to a suspension of enzyme in reaction 'mixture' lacking P_i + [^{32}P]P_i and adding the missing components after 30 min at 0 °C. After a further 60 min, the suspension was filtered. The filter was placed in a counting vial and scintillation fluid was added.

(b) From ATP. 50–100 μg enzyme was suspended in triplicate at 0 °C in 125 μl of a medium containing 100 mM choline chloride, 25 mM histidine (pH 7.0), 20 mM NaCl, 3 mM $MgCl_2$, 10 μM ATP + [γ-^{32}P]ATP, about $2 \cdot 10^5$ cpm per sample. After 30 s at 0 °C, the reaction was stopped with 5 ml of an ice-cold solution containing 5% perchloric acid, 5 mM ATP, 5 mM P_i. After 30 min, the suspension was filtered on a GF/B filter and washed with 30 ml of trichloroacetic acid 5% (solution B). Background and non-specific binding was determined by incubating enzyme with the solution B solution for 30 min prior to addition of the ATP + [γ-^{32}P]ATP.

It has been found in this laboratory that in [γ-^{32}P]ATP obtained from Amersham International, not all of the ^{32}P is in the γ position. The fraction in the γ position was estimated in each batch by determining the fraction of ^{32}P released from [^{32}P]ATP when incubated with a large excess of the $(Na^+ + K^+)$-ATPase in a reaction medium optimal for expression of $(Na^+ + K^+)$-ATPase activity, and incubating at 37 °C until all the ATP had been hydrolyzed. This was performed according to a semimicro ATPase assay in which the [^{32}P]molybdate complex is extracted into isobutanol, as described in Ref. 8. For determination of the total radioactivity, the [^{32}P]ATP was boiled with 1 M HCl for 30 min and then extracted as usual. In different batches, only between 60–90% of the total radioactivity was extractable into isobutanol, after incubation with $(Na^+ + K^+)$-ATPase, and was therefore derived from the γ position in [γ-^{32}P]ATP.

Digestion with chymotrypsin

This was performed in conditions slightly different from those described by Jorgensen [9]. $(Na^+ + K^+)$-ATPase (450 μg) suspended in a medium containing 15 mM histidine, (pH 7.5), 1 mM NaCl was incubated for 1 h at 37 °C with 70 μg of α-chymotrypsin. At the end of this period, proteolysis was effectively stopped by diluting the enzyme chymotrypsin mixture 3-fold with an ice-cold solution containing 100 mM choline chloride, 25 mM histidine and this suspension was kept on ice until use. Control enzyme was also incubated as above, without chymotrypsin. An aliquot of proteolyzed enzyme was also kept for determination of $(Na^+ + K^+)$-ATPase activity, which was inhibited 90–100% in different experiments.

Computations

Calculations were performed with an Apple II E computer. Kinetic parameters of hyperbolic saturation curves K_m and B_{max} were fitted by least squares to the experimental points using the programme HYPMIC. Rate constants of the exponential phase of dissociation curves were obtained by linear regression.

Materials

ATP (vanadate-free) α-chymotrypsin, Dowex 50 × 8 and bovine serum albumin, fraction V, were purchased from Sigma Chemicals, St. Louis, MO, U.S.A. [γ-^{32}P]ATP and $^{86}Rb^+$ were obtained from the Amersham International, Amersham, U.K. [^{32}P]P_i was obtained from the Israel Atomic Energy Commission, Dimona, Israel.

Results

The basis of the Rb^+ occlusion measurement can be seen from the experiment in Fig. 1, showing dissociation of prebound $^{86}Rb^+$ at 0 °C or, room temperature and the effect of ATP on dissociation. $Rb^+ + {}^{86}Rb^+$ (1.3 mM) was first mixed

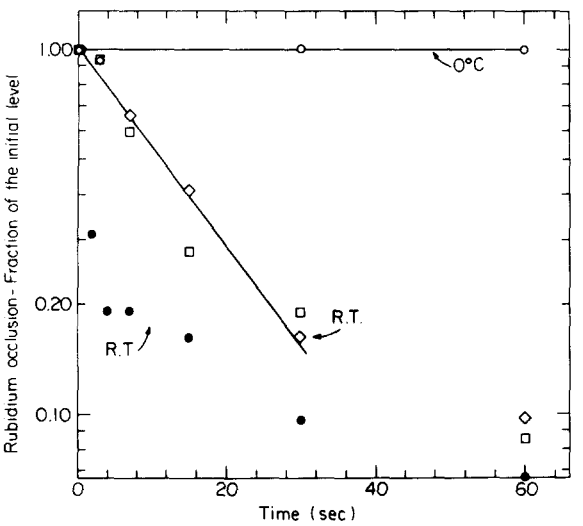

Fig. 1. Rate of dissociation of occluded Rb^+ at 0 °C and room temperature. Effect of ATP. Dissociation rates at room temperature were performed as described in the methods. Dissociation media: ◇ 20 mM RbCl, 100 mM Tris (pH 7) (□); 100 mM NaCl, 20 mM Tris-HCl (pH 7). (●), 20 mM RbCl + 2 mM ATP (Tris), 100 mM Tris-HCl (pH 7). The dissociation rate at 0 °C (○) was performed slightly differently in that the enzyme 126 μg was mixed initially with $Rb^+ + {}^{86}Rb^+$, 1.3 mM in a total volume of 150 μl. After 5 min incubation at room temperature, the suspension was diluted 17.6-fold with 2.5 ml of ice-cold sucrose 200 mM and at times from 30 s to 20 min, aliquots of 500 μl (24 μg protein) were removed to the Dowex-50 columns.

with the enzyme at room temperature for 5 min. For the dissociation at 0 °C the suspension was then diluted 17.6-fold with an ice-cold sucrose solution and at different times aliquots were removed to the Dowex-50 columns. Despite the large dilution, ^{86}Rb bound at room temperature did not dissociate at 0 °C during the 60 s shown or indeed during 20 min (not shown). The bound Rb^+ is therefore referred to as 'occluded'. To expose the dissociation rate at room temperature the enzyme/$Rb^+ + {}^{86}Rb^+$ mixture was diluted with 200 μl of medium at room temperature, containing a high concentration of Rb^+ or Na^+, and at the times indicated the suspension was diluted 6-fold with the ice-cold sucrose solution. About 80% of the Rb^+ dissociated exponentially and slowly with a rate-constant of 0.08 s^{-1}. This rate and lack of effect of Rb^+ or Na^+ on the dissociation are quite similar to results in the literature [2]. About 20% of the $^{86}Rb^+$ dissociated much more slowly. When ATP was present in the Rb^+ dissociation medium about 80% of the bound $^{86}Rb^+$ dissociated, as expected, at a rate too rapid to be detected by this method. Again about 20% of the radioactivity dissociated much more slowly. The very slow phase of dissociation could be thought to represent egress of Rb^+ trapped within a population of vesicles. This possibility, however is unlikely because no apparent occlusion of $^{22}Na^+$ was detectable as might have been expected if the ions were being trapped within vesicles.

Fig. 2 shows curves of Rb^+ occlusion as a function of Rb^+ concentrations up to 10 mM, in the absence or presence of ATP. In the absence of ATP the maximal amount of Rb^+ occluded was usually found to be between 3–4 nmol per mg protein. This Rb^+ was entirely associated with the $(Na^+ + K^+)$-ATPase protein, since preincubation of the enzyme with ouabain and Mg^{2+} completely prevented the Rb^+ occlusion (not shown). In the experiment of Fig. 2, and several other similar ones, strictly hyperbolic saturation of Rb^+ occlusion was observed with $K_{0.5}$ of 100–200 μM Rb^+. Fitted values of the maximal level and $K_{0.5}$ in this experiment were 3.99 ± 0.09 nmol/mg protein and 149 ± 16 μM, respectively. In a medium containing 120 mM choline chloride instead of Tris-HCl the apparent affinity for Rb^+ was much higher,

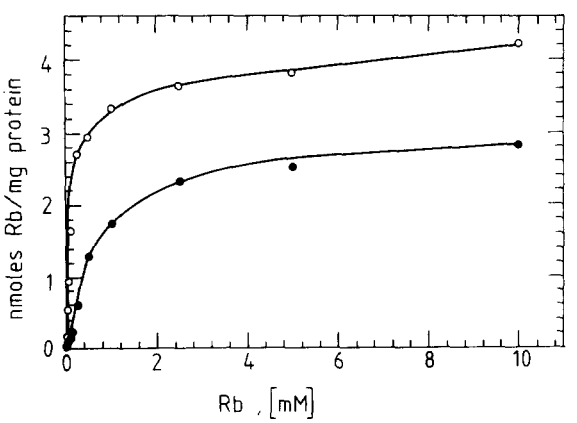

Fig. 2. Occlusion of Rb^+ at different Rb^+ concentration in the absence or presence of ATP. Enzyme (47 μg) was incubated in the standard Tris-HCl medium in triplicate with 10 μM–10 mM RbCl + $^{86}Rb^+$, in the absence or presence of 9.6 mM ATP (Tris) and occlusion was measured as described in Methods. ○, control; ●, 9.6 mM + ATP.

$K_{0.5} \approx 20$ μM (see Fig. 4). Both Tris and choline are known to have 'Na^+-like' effects [10], but Tris seems to have a much stronger effect than choline under our conditions. The presence of ATP at 9.6 mM strongly inhibited Rb^+ occlusion at low Rb^+ concentrations, but was much less effective in a high concentration range. Thus, the major effect of ATP was to displace the saturation curve to the right with a $K_{0.5}$ (fitted) of 775–76 μM, while the maximal level of occlusion was reduced to 3.05 ± 0.08 nmol/mg protein in this experiment.

Fig. 3 shows the effect of increasing Na^+ concentrations at a fixed Rb^+ of 2.05 mM, in a medium of fixed total salt concentration. As expected, increasing the Na^+ decreased the Rb^+ occlusion (Fig. 3A). For simple competitive inhibition between Na^+ and Rb^+ one would expect the replot in Fig. 3B of $E_2(Rb^+)_{occ} : E_2(Rb^+)_{occ}$ ($+Na^+$) versus Na^+, to be linear. This was found to be the case up to about 30 mM Na^+, but at higher concentrations the plot shows an upward curvature. Plots of the square or cube root of the

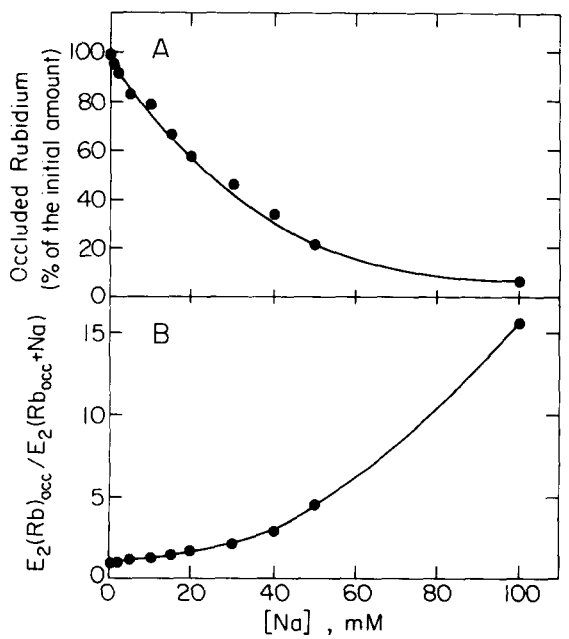

Fig. 3. Effect of different Na^+ concentrations on Rb^+ occlusion at a fixed Rb^+ concentration. Enzyme (20 μg) was incubated with 2.05 mM $Rb^+ + {}^{86}Rb^+$ in a medium containing NaCl plus Tris-HCl (pH 7.0) at a total concentration of 150 mM. The NaCl concentration was varied between 0 and 100 mM and the Tris-HCl concentration was varied appropriately.

value $E_2(Rb^+)_{occ} : E_2(Rb^+)_{occ}$ ($+Na$) versus Na^+ were not linear, over the whole Na^+ concentration range. Therefore, one cannot explain this behaviour by assuming simple competition at multiple sites of equal affinity for Rb^+ and Na^+, respectively. Assuming simple competition at one site up to 30 mM Na^+, it is easy to show that the slope of the linear portion is given by $(K_R/(K_R + Rb^+)) \cdot 1/K_{Na^+}$, where K_R is the apparent half saturation constant in the absence of Na^+ (150 μM in Fig. 2). K_{Na^+} is the inhibition constant for Na^+, and Rb^+ is the fixed Rb^+ concentration (2.05 mM). Hence the K_{Na^+} can be calculated and is found to equal 1.86 mM. The behaviour at Na^+ concentrations higher than 30 mM suggests that an added antagonistic effects comes into play, as discussed further below.

Table I summarizes estimates of the stoichiometry of Rb^+ occluded per phosphorylation site, using either orthophosphate in the presence of ouabain or ATP plus Na^+ and Mg^{2+}. The average stoichiometry from all of these figures is 1.98 ± 0.1 Rb^+ per phosphoenzyme. We have observed that when levels of phosphorylation from P_i and ATP are compared several days after dialysis of the enzyme, rather than immediately, the phosphoenzyme level from ATP (1–1.5 nmol/mg protein) is usually lower than that from phosphate which is invariably 1.5–2.5 nmol/mg protein. With freshly prepared and dialyzed enzyme, as in experiment 23986 in Table I, the phosphoenzyme levels from ATP or P_i are not significantly different. Since the level of occluded Rb^+ is invariably 3–4 nmol/mg of enzyme, irrespective of the age, one can obtain spuriously high values of the Rb^+- to -phosphoenzyme ratio when it is measured several days after dialysis using ATP + Na^+/Mg^+ for phosphorylation.

Selective proteolysis of ($Na^+ + K^+$)-ATPase is an important tool for detecting conformational states of the protein and dissecting structure-function relations [9,11]. In a Na^+ medium chymotrypsin splits the α-chain highly selectively between Leu-266, and Ala-267 [12], and produces a modified enzyme which does not hydrolyze ATP and has several properties suggesting it is blocked in an E_1 conformation. The chymotryptically split enzyme has been used to study Na^+ occlusion [3]. Somewhat paradoxically, it has also been found

TABLE I

STOICHIOMETRY OF Rb^+ OCCLUSION

Expt. No.	Phosphoenzyme with P_i/ouabain (nmol/mg protein ± S.E.)	Phosphorylation with ATP/Na^+ (nmol/mg protein ± S.E.)	Occluded Rb^+ (nmol/mg protein ± S.E.)	Occluded Rb^+ phosphoenzyme (± S.E.)
20586	2.5 ±0.152	–	4.62±0.4	1.83±0.19
9786	1.73±0.02	–	2.85±0.10	1.65±0.06
9986	1.88±0.05	–	4.05±0.38	2.15±0.21
23985	1.82±0.05	1.72±0.062	2.86±0.1	2.12±0.08
				2.17±0.02

that the modified enzyme will also occlude Rb^+ ions but in an ATP-insensitive manner [13]. Figs. 4 and 5 confirm that the modified enzyme occludes Rb^+ and demonstrate features of that occlusion. Fig. 4 shows Rb^+ saturation curves in choline media, of control ($Na^+ + K^+$)-ATPase and enzyme chymotryptically split to the point of losing 100% of hydrolytic activity. The maximal level of Rb^+ occlusion is lower by about 30% than in control. The fitted half-saturation constants for Rb^+ are not very different, 21.8 ± 3.2 μM in control and 9.9 ± 3 μM for chymotryptic enzyme. The small reductions in maximal level and $K_{0.5}$ for Rb^+ occlusion in modified enzyme may well be the result of secondary proteolytic splits, because no reduction in the maximal level or $K_{0.5}$ was observed until proteolysis had proceeded to the point where over 50% of ATPase activity was lost (not shown). Finally, Fig. 5 shows that the rate of dissociation of occluded Rb^+ from control and the chymotryptically split enzyme is essentially the same, calculated from semi-logarithmic plots to equal 0.063 s^{-1} in this experiment. As discussed below, the results of Figs. 4 and 5 do not favour

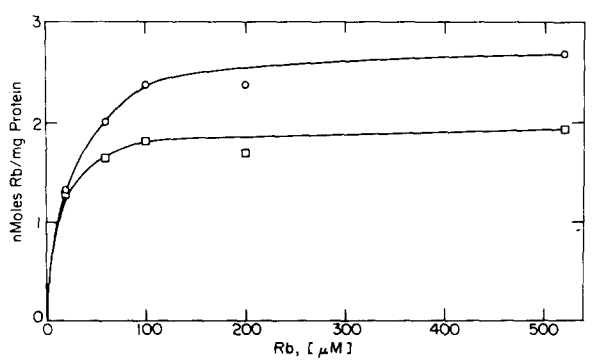

Fig. 4. Occlusion of Rb^+ at different Rb^+ concentrations on control and chymotryptically digested ($Na^+ + K^+$)-ATPase. For this measurement, control and chymotryptically digested ($Na^+ + K^+$)-ATPase (46 μg) were incubated with RbCl + $^{86}Rb^+$ (20–520 μM) in a medium containing 120 mM choline chloride, 12 mM histidine (pH 7.4). The specific ($Na^+ + K^+$)-ATPase activities of the control and modified enzyme were 13 units/mg and zero, respectively. ○, control; □, chymotryptically split.

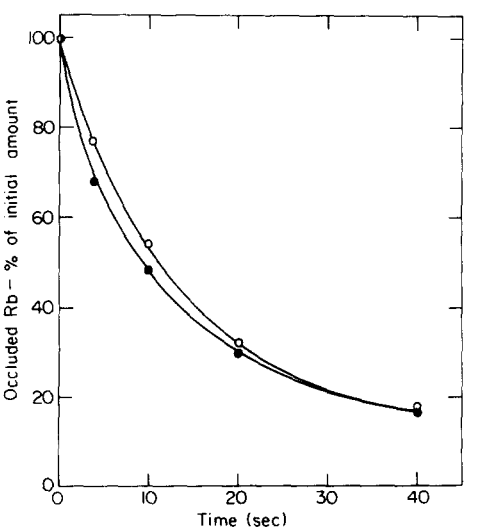

Fig. 5. Rate of dissociation of Rb^+ from control and chymotryptically digested ($Na^+ + K^+$)-ATPase. Control and chymotryptically digested ($Na^+ + K^+$)-ATPase (44 μg) were incubated in the choline chloride medium of Fig. 4 containing 0.52 mM $Rb^+ + {}^{86}Rb^+$. After 5 min, 200 μl of the dissociation medium containing 120 mM choline chloride, 12 mM histidine (pH 7.4) and 20 mM RbCl was added and at times 4–40 s, 1 ml of 200 mM ice-cold sucrose solution was added to halt de-occlusion. ○, control; ●, chymotryptically split.

the notion that chymotrypsin stabilizes the E_1 form of the unphosphorylated protein.

Discussion

The utility of the present Rb^+ occlusion assay lies mainly in its simplicity and convenience. It cannot, of course replace, the flow equipment used to follow fast rates of Rb^+ dissociation but it provides accurate data in equilibrium binding conditions, and reasonable estimates of slow dissociation rates. The observed characteristics of Rb^+ occlusion are, in comparable experiments, largely the same as reported in the literature [2,13]. New information obtained concerns the stoichiometry of Rb^+ occlusion, saturation by Rb^+ up to high concentrations and competition with Na^+, and properties of Rb^+ occlusion in chymotrypticaly split $(Na^+ + K^+)$-ATPase. The simplicity of this assay is also proving to be of great benefit in current experiments to detect covalent modification of Rb^+ binding sites using carbodiimides (M. Shani and S. Karlish, unpublished data).

The question of stoichiometry of Rb^+ occlusion has been problematic. The present result of two rubidium ions occluded per phosphoenzyme (Table I) is lower than the value reported by Glynn and Richards of three rubidium ions occluded per phosphorylation or ouabain binding site [2], but is the same, two rubidium/site, as estimated from equilibrium binding experiments [14,15]. The major difference in results between the present experiments and those of Glynn and Richards lies in the estimates of site concentrations. The amounts of Rb^+ occluded per mg protein are very similar in the two studies. We have noted two problems leading to underestimates of phosphoenzyme levels determined with ATP. Firstly the ^{32}P in commercial [γ-^{32}P]ATP is not entirely in the γ position, the fraction varying from 90 to as low as 60% in different batches (see Methods). Secondly, ATP-dependent phosphorylation is lost more quickly than P_i-dependent phosphorylation of Rb^+ occlusion upon ageing. These factors could explain the differences in ATP-dependent phosphorylation (but not, of course, the P_i-dependent estimates of site concentration).

Most of the features of Rb^+ occlusion can be understood in terms of the following scheme, developed previously to explain effects of K^+ on conformational states [16] and transport of Rb^+ in reconstituted vesicles [17,18].

$$Rb^+_{cyt} + E_1 \underset{K_H}{\rightleftharpoons} E_1Rb^+_{cyt} \underset{K_L}{\rightleftharpoons} E_1 2Rb^+ \underset{K_C}{\rightleftharpoons} E_2(2Rb^+)_{occ} \rightleftharpoons E_2 2Rb^+$$

$$\rightleftharpoons E_2 + 2Rb^+_{exc}$$

Two Rb ions bound on the E_1 form, at cytoplasm oriented sites, become occluded in the conformational transition to $E_2(Rb^+)_{occ}$ and can be released from E_2 at the extracellular surface. K_H and K_L are intrinsic binding constants. K_C is the conformational transition equilibrium constant. Because our experiments are performed in a high ionic strength medium, the state of the protein in the absence of Rb^+ is E_1 and Rb^+ occlusion involves the transition to $E_2(2Rb^+)_{occ}$ which is the exclusive form at high concentration levels of Rb^+ [10]. A striking feature of the $Rb^+(K)$ dependence of conformational transitions [7], of passive Rb^+ transport in vesicles [18] and of Rb^+ occlusion [2] itself, is that the Rb^+ saturation curves are strictly hyperbolic, despite the fact that two rubidium ions are occluded per enzyme molecule. This can be explained [18] by assuming that the first intrinsic dissociation constant K_H is very much lower than K_L and that at the lowest available concentrations of Rb^+ (approx. 10 μM), the high affinity site is already occupied. The $K_{0.5}$ for Rb^+ is then equal to $K_L/K_C + 1$. K_L is estimated to equal 20–50 mM from fluorescence, and transport and occlusion experiments, while K_C in the absence of ATP has a value in the range 100–1000 [7,2,16,19]. K_H must be in the micromolar range. Thus, the occlusion experiments provide further evidence for a high affinity Rb^+ site on E_1, in addition to the low affinity site.

Sodium ions at concentrations of up to 30 mM appear to compete with Rb^+, as expected. Presumably competition is at the lower-affinity Rb^+ binding site. The calculated K_{Na^+} is 1.86 mM (see Results). At concentrations higher than 30 mM, Na^+ is more effective than might be expected on the basis of simple competition. This additional low-affinity effect of Na^+ could be related to a *TRANS* effect of Na^+ which we have described recently in vesicles [18]. With vesicles, high concentration of Na^+ at the extracellular surface in-

duce 'allosterically' a transition from hyperbolic to sigmoid activation by Rb^+ of its fluxes at the cytoplasmic surface and concurrently an increase in the affinity for competition by Na^+ at the cytoplasmic surface. This *TRANS* effect can be explained by a change in the relative affinities for Rb^+ and Na^+ at the two cytoplasmic Rb^+ sites, as discussed in detail by Karlish and Stein [18].

The effects of ATP observed in Figs. 1 and 2 are largely what one might have expected knowing that ATP displaces the $E_1 2Rb^+$-$E_2 2Rb^+_{occ}$ equilibrium towards the E_1 form [16,19,20]. In Fig. 2, ATP displayed the Rb^+ activation curve to the right but somewhat unexpectedly the maximal level of occlusion was as much as 75% of that in the absence of ATP. Probably the simplest explanation would be that even at saturating Rb^+ and ATP concentrations the E_1Rb^+-$E_2(Rb^+)_{occ}$ equilibrium is still poised towards $E_2(Rb^+)_{occ}$, (although much less than in the absence of ATP), and the complex $E_2(2Rb^+)_{occ} \cdot$ ATP is sufficiently stable at 0 °C so as not to dissociate to $E_1 + Rb^+$ in the 20 s or so which the enzyme spends on the Dowex column. From the ratio of $K_{0.5}$ for Rb^+ without ATP, 0.1–0.2 mM to that with ATP, 0.8–1.4 mM the ATP would appear to have shifted the equilibrium about 5–10 fold towards E_1 under the conditions of this assay.

The maximal rate of $E_2(Rb^+) \rightarrow E_1$ in the presence of ATP is 18 s^{-1} at room temperature [21]. Therefore, it was surprising, in the experiment of Fig. 1, that a fraction (about 20%) of the occluded Rb^+ dissociated slowly in the presence of ATP. It seems conceivable that this small fraction represent pump molecules which bind and occlude Rb^+, but do not bind ATP. That is, they are partially denatured or proteolyzed.

Chymotrypsin is an important tool in dissecting structure-function relations because the split in the Na^+ medium between Leu-266 and Ala-267 is highly selective. The modified $(Na^+ + K^+)$-ATPase does not hydrolyze ATP and does not display K^+-dependent responses of fluorescence probes or the antagonistic effects of K^+ on ATP binding and ATP on K^+ binding or occlusion which are characteristic of the E_1-E_2 conformational transition of the unphosphorylated protein [9]. Conversely, ATP is bound tightly, the enzyme undergoes Na^+-dependent phosphorylation [9] and Na^+ is occluded [3]. These features led to the conclusion that the modified enzyme is locked in an E_1 state and E_1-E_2 conformational transitions are totally blocked [9]. The recent finding that rubidium ions are occluded by the modified enzyme, but in an ATP-insensitive fashion [13], might, therefore, be taken to imply that occlusion of rubidium ion occurs on an E_1 form without necessity of the E_1-E_2 conformational transition. However, as pointed out by Glynn et al. [13] several findings suggest that the chymotryptic enzyme can adopt an E_2 state. These include ouabain sensitivity of Rb^+ occlusion, demonstration of an $E_2(K^+)_{occ}$ pattern of tryptic digestion of the chymotryptically modified $(Na^+ + K^+)$-ATPase [23] and passive Rb^+ fluxes. The results in Figs. 4 and 5 showing similar Rb^+ saturation curves and essentially identical rates of deocclusion, obviously strengthen the view that the same rate-limiting step $E_2(Rb^+)_{occ} \rightarrow E_1$ and conformational transitions are operative in the control and modified enzyme. An alternative explanation of the lack of the usual antagonism between K^+ and ATP or K^+-induced fluorescence responses could be that the chymotryptic split has disrupted the normal interactions between cation and nucleotide binding sites. An attractive implication of this hypothesis is that cation binding and transport-linked conformational changes, and ATP binding sites, may be in quite separate domains of the protein and their normal interactions are transmitted along the polypeptide backbone.

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