Hypothesis

A bar model for the pump and channel function of the reconstituted Na^+,K^+-ATPase

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Purified Na^+,K^+-ATPase is treated with trypsin. The altered enzyme is then reconstituted into liposomes and the change in active and passive Na^+,K^+-fluxes is recorded. Trypsin treatment transforms the slow passive Na^+,K^+-fluxes into leaks. The leak formation is correlated with the degree of proteolysis and the associated decrease in Na^+,K^+-ATPase activity. The active Na^+,K^+-transport capacity decreases in parallel with the passive transport. It is thus proposed that the Na^+,K^+-ATPase molecule primarily contains unspecific transmembrane tunnels that are rendered ion-selective by transverse bars of specific length (bar model).

Na^+,K^+-ATPase *Trypsin treatment* *Reconstitution* *Na^+,K^+-flux* *Membrane leak* *Bar model*

1. INTRODUCTION

The membrane Na^+,K^+-ATPase is responsible for the active Na^+,K^+-exchange across cell membranes [1]. The molecular mechanism of the ATP-driven transport process is not yet understood.

Some years ago, an original approach to dissect the transport process on a molecular level was designed [2]. The concept consists of altering specific parts of the Na^+,K^+-ATPase molecules by chemical modification, to incorporate the partially invalidated enzyme into liposomes, and to look for alterations in the Na^+,K^+-transport pattern.

While pursuing this line of research, I observed that leaks for Na^+ and K^+ appear in parallel with the chemical modification. The native enzyme seems to contain barriers or bars to limit the passive Na^+ and K^+ fluxes and perhaps also to mediate active fluxes after phosphorylation. The bars can be inactivated by treating the enzyme with trypsin. Although it is speculative, the bar model adequately explains the results described here.

2. MATERIALS AND METHODS

Purified Na^+,K^+-ATPase (EC 3.6.1.3) was obtained from the rabbit kidney outer medulla as in [3]. Pure phosphatidylcholine (egg) and phosphatidylserine were from Supelco, and trypsin from bovine pancreas type III and soybean trypsin inhibitor from Sigma. ATP was purchased from Boehringer and was transformed to the Tris-form by treatment with ion-exchange resin [4].

The Na^+,K^+-ATPase was reconstituted into single walled 900 Å liposomes [5] by a controllable step-by-step detergent-dialysis procedure [6]. The phosphatidylcholine was enriched with 20% phosphatidylserine. Graded trypsinolysis of Na^+,K^+-ATPase was performed as in [7]. Limited alkylation of SH groups by *N*-ethylmaleimide was done as in [8]. The liposomes were prepared in 'transport' solution which contained 50 mM NaCl, 50 mM RbCl or KCl, 5 mM $MgCl_2$, 30 mM histidine, 1 mM dithiothreitol, 1 mM EDTA (pH 7.15) with HCl.

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Passive or active Na^+,K^+-fluxes were measured as follows: 10 μl transport solution with or without 30 mM Tris–ATP was added to tubes containing 0.6 μCi $^{22}Na^+$ or 0.3 μCi $^{86}Rb^+$ (dried). One volume of the radioactive solution was then added to 2 vol. liposomes incubated at room temperature. To measure passive fluxes, the liposomes were incubated in the absence of ATP. To follow active Na^+-uptake, ATP was added together with $^{22}Na^+$. To determine active Rb^+ outflux, the liposomes were first loaded with $^{86}Rb^+$ and a concentrated ATP solution was added. The incubations were stopped by adding a 50-fold excess of stop-solution composed by 100 mM Tris and 30 mM imidazole (pH 7.15) with HCl. For incubation times below 30 s a short time uptake apparatus (Innovativ Medizin, Adliswil) was used. The internal vesicular isotope content was determined by washing 4 μl samples of the liposomes by passage through ice-cooled 15 cm Sephadex G-50 columns connected to a programmed fraction collector (Gilson).

3. A Na^+,K^+-LEAK INDUCED BY TRYPSIN TREATMENT

It has been shown previously that the reconstituted Na^+,K^+-ATPase forms a K^+-selective cation channel [9,10]. The passive channel is 2–3-fold more permeable for K^+ than for Na^+ at equal $[Na^+]$ and $[K^+]$ and this selectivity as well as the transport rate depend on the active transport capacity of the enzyme [11].

In the present work it was attempted to abolish artificially the passive K^+/Na^+ discrimination by treating the Na^+,K^+-ATPase with trypsin before reconstitution into liposomes. We controlled by freeze fracture analysis that the proteolysed Na^+,K^+-ATPase retains its ability to reconstitute (Anner, B.M., Ting-Beall, H.P. and Robertson, J.D., in preparation).

Fig.1 illustrates that the passive K^+-flux exceeds the passive Na^+-flux when liposomes have been reconstituted with control Na^+,K^+-ATPase that has been incubated with trypsin plus trypsin inhibitor (upper curves). In contrast, when the enzyme has been incubated in the presence of trypsin alone, the K^+/Na^+ discrimination is lost (lower curve). In addition, the absolute amounts of both Na^+ and K^+ contained in the liposomes are lower

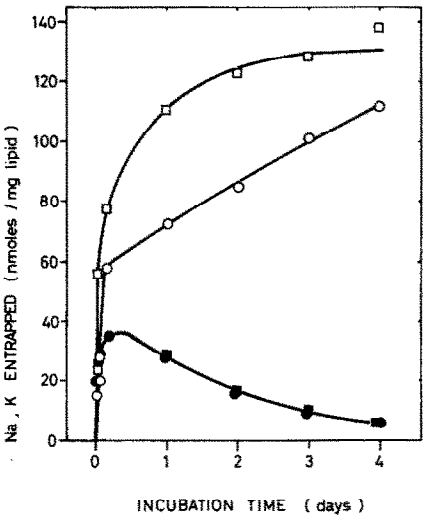

Fig.1. Na^+,K^+-leak induced by trypsin treatment. Na^+,K^+-ATPase of spec. act. 1415 μmol.mg^{-1}.h^{-1} was diluted in a solution containing 150 mM NaCl, 25 mM imidazole (pH 7.5 with HCl) to 200 μg protein/ml and was then incubated at 37°C for 20 min in the presence of 16 μg trypsin/ml with or without 64 μg trypsin inhibitor/ml. In the presence of a mixture of trypsin and trypsin inhibitor (control) the Na^+,K^+-ATPase activity was decreased to 770 μmol.mg^{-1}.h^{-1} and with trypsin alone to 376 μmol.mg^{-1}.h^{-1}. The treated enzymes were then reconstituted into liposomes. The Na (○, ●) and K (□, ■) content of liposomes reconstituted with control (○, □) and trypsin treated (●, ■). Na^+,K^+-ATPase was determined after gel filtration. For details of the procedures used see section 2.

than in the control liposomes. Apparently, the trypsin treatment produces leaks at strategic points in the protein structure. The leak is so large that the reconstituted Na^+,K^+-ATPase is now incapable of holding back the internal Na^+ and K^+ during the wash in the Sephadex column. Prolonged incubation at room temperature accentuates the leak (fig.1), presumably by further weakening the bars that have been attacked by trypsin. A similar effect is observed when the Na^+,K^+-ATPase has been pretreated with 1 mM vanadate before reconstitution or if 1 mM vanadate is added to the finished liposomes (not shown). Thus, the gel filtration procedure can be used to detect leaks in the liposomes because the true passive fluxes mediated by the intact Na^+,K^+-ATPase are so slow that no loss of internal Na^+ and K^+ occurs during gel filtration [6].

4. SIMULTANEOUS DECREASE OF ACTIVE Na^+-TRANSPORT AND PASSIVE K^+-TRANSPORT BY PROTEOLYSIS

When Na^+,K^+-ATPase is incubated in the presence of trypsin and NaCl, the catalytic subunit and the Na^+,K^+-ATPase activity gradually disappear ([7] and unpublished).

Aliquots of Na^+,K^+-ATPase were incubated in the presence of trypsin and NaCl for 5–60 min. The different trypsin treated enzymes were then reconstituted into liposomes. The Na^+,K^+-ATPase-liposomes were then incubated in the presence of $^{86}Rb^+$ without ATP for 24 h (passive K^+-flux) or in the presence of $^{22}Na^+$ and ATP for 30 min (active Na^+-transport); i.e., until the respective fluxes had reached a plateau [9].

Fig.2 shows the correlation between the passively entrapped K^+ and the actively accumulated Na^+ with the Na^+,K^+-ATPase activity that was present after the trypsin treatment. Clearly, the internal pools of both ions decrease simultaneously with decreasing ATPase activity; i.e., increasing proteolysis.

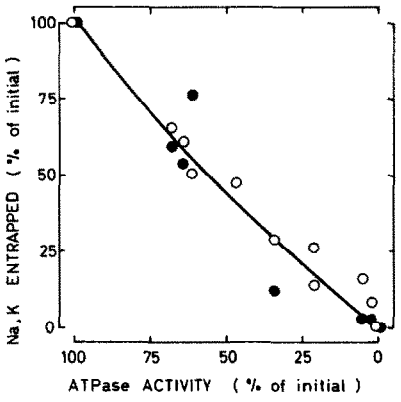

Fig.2. Simultaneous decrease of active Na^+-transport and passive K^+-transport by proteolysis. Na^+,K^+-ATPase preparations with spec. act. 1300–2280 $\mu mol.mg^{-1}.h^{-1}$ were trypsin treated as in fig.1, except that the incubation times were varied from 5–60 min. Aliquots of the trypsin-treated enzymes were used to determine: (1) the decrease in Na^+,K^+-ATPase activity by the linked-enzymes as in [16]; (2) the protein profile indicating the degree of proteolysis by sodium dodecyl sulfate–gel electrophoresis as in [17] (not shown); (3) the amount of actively accumulated Na^+ (●) and the amount of passively entrapped K^+ (○) remaining in the liposomes after gel filtration, determined as in section 2.

5. EVIDENCE FOR A LABILE Na^+-TRANSPORT FRACTION

We had reported that the purified Na^+,K^+-ATPase contains a fraction of Na^+-uptake that occurs when the internal K^+-pool has been extruded; i.e., without K^+-antiport [9,12,13]. This extra Na^+-transport fraction is labile and can be selectively inactivated by limited treatment with trypsin [2,14]. In those experiments the modified enzyme had been treated only slightly with trypsin and no proteolytic split was visible after separation of the enzyme subunits by sodium dodecyl sulfate–gel electrophoresis [7]. The control enzyme was incubated with trypsin inhibitor alone which does not affect the ATPase activity nor the Na^+-transport capacity.

We recently discovered that incubation of Na^+,K^+-ATPase with a mixture of trypsin and trypsin inhibitor produces an irreversible inhibition of the Na^+,K^+-ATPase activity ranging from 35–56% within a 20 min incubation. The inhibition does not further increase even when the incubation time is increased to 3 h. No proteolytic split (i.e., no appearance of low-M_r fragments) is visible after separation of the Na^+,K^+-ATPase subunits by gel electrophoresis (not shown). A Na^+,K^+-ATPase preparation that had been inhibited by 35% by incubation with trypsin plus trypsin inhibitor was reconstituted into liposomes in parallel with control enzyme that had been incubated with trypsin inhibitor alone.

Fig.3a illustrates the selective decrease of the Na^+-transport capacity caused by pretreatment of the Na^+,K^+-ATPase with trypsin plus trypsin inhibitor. The Na^+-transport of the treated enzyme reaches a plateau within about 5 min after the ATP addition whereas the native enzyme attains a Na^+-transport plateau within 15 min after the ATP addition (fig.3a). The plateau of the pretreated Na^+,K^+-ATPase is ~35% lower than the control plateau; i.e., the decrease in Na^+-transport capacity corresponds to the decrease in ATPase activity present before reconstitution. This correlation confirms our earlier results where we also reported a direct correlation between the 40–50% reduction of the

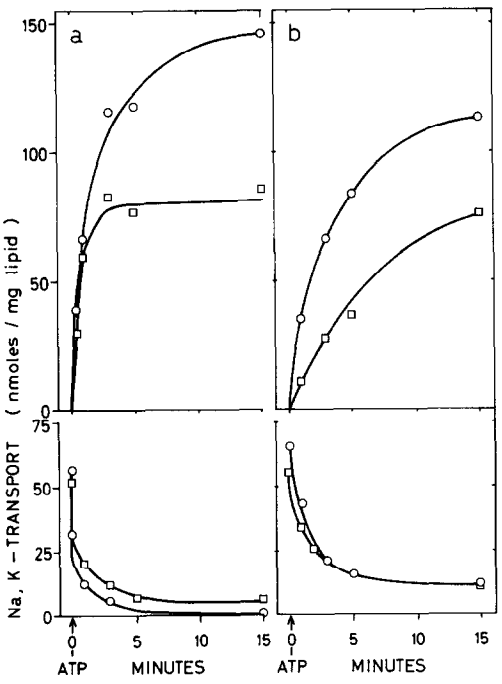

Fig.3. Evidence for a labile Na^+-transport fraction. For the experiment shown in fig.3a, the Na^+,K^+-ATPase was incubated before reconstitution at 100 μg protein/ml in the presence of 10 μg trypsin/ml (□) or in the presence of 10 μg trypsin/ml plus 40 μg/ml trypsin inhibitor (○) in a solution containing 150 mM NaCl and 25 mM imidazole (pH 7.5) with HCl at 37°C for 20 min. For the experiment shown in fig.3b, the Na^+,K^+-ATPase was treated as in [8], in the presence of 10 mM *N*-ethylmaleimide followed by the addition of 30 mM β-mercaptoethanol (□) or in the presence of 30 mM β-mercaptoethanol alone (control) (○) for 60 min at 30°C in a solution containing 5 mM Tris (pH 7.5 with HCl), 2 mM KCl and 3 mM Tris–ATP. The trypsin or *N*-ethylmaleimide-treated enzymes were then reconstituted into liposomes and the active Na^+-uptake (ascending curves) and the active K^+-extrusion (descending curves) were determined as in section 2.

Na^+,K^+-ATPase activity by the limited trypsin treatment and the Na^+-transport capacity present after reconstitution without noticeable change in the total K^+-transport capacity [2].

A selective reduction of the Na^+-transport capacity, although with different kinetics, was also obtained when the Na^+,K^+-ATPase had been pretreated in specific conditions with *N*-ethylmaleimide (fig.3b). This result confirms our experiments where *N*-ethylmaleimide was added to the finished Na^+,K^+-ATPase liposomes [15].

We do not know whether the inhibitory action of the trypsin and trypsin inhibitor mixture on the Na^+,K^+-ATPase activity and the Na^+-transport capacity is a bulk effect or is caused by a limited residual proteolytic activity that produces a split so close to the terminus of the catalytic subunit that no fragments are seen after gel electrophoresis. Regardless of the precise mechanism of action, the two chemical modifications used in the present work show that a labile fraction of the Na^+-transport capacity of the Na^+,K^+-ATPase can be inactivated without changing much the overall K^+-transport capacity. The fact that a very slight chemical modification is sufficient to remove the extra Na^+-transport suggests that this transport mechanism may be located in a superficial region of the Na^+,K^+-ATPase molecule.

6. THE BAR MODEL

These results, in conjunction with our previous results, suggest that the Na^+,K^+-ATPase molecule contains leaks or tunnels that are partially closed by transverse bars. The role of the bars is to reduce selectively the diameter of the tunnel for Na^+ or K^+ (fig.4a). Considering that about twice as much K^+ as Na^+ flow passively across the Na^+,K^+-ATPase containing liposome membrane [10], the simplest assumption is that two passive tunnels exist in the Na^+,K^+-ATPase molecule; only one is selective for Na^+, but both are selective for K^+ (fig.4a). When the Na^+,K^+-ATPase has been trypsin treated before reconstitution, the ion-selective bars are inactivated and both Na^+ and K^+ have now equal probability to traverse the membrane. The inactivation of the bars renders the tunnels so leaky that the internal Na^+ and K^+ leak out of the liposomes during gel filtration.

The question now arises whether the same bars that regulate the passive transport are also involved in the active transport. The finding that both the passive K^+-transport and the active Na^+-transport decrease simultaneously when the Na^+,K^+-ATPase has been proteolysed before reconstitution could indicate that both processes are mediated by structures of similar trypsin sensitivity. On the other hand, the correlation could be indirect in the sense that a passive Na^+,K^+-tunnel opened by proteolysis could pre-

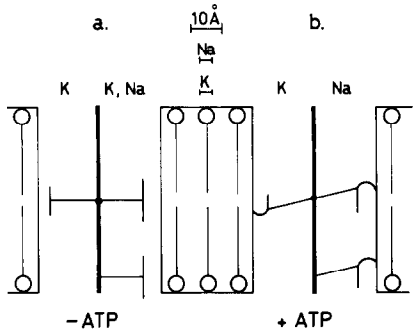

Fig.4. The bar model. (a) The bar model for the channel function of the reconstituted Na^+,K^+-ATPase. The vertical holes across the membrane indicate the tunnels contained in the Na^+,K^+-ATPase molecule. The horizontal lines within the tunnels indicate the hypothetical bars reducing the tunnel diameter selectively for K^+ (both tunnels) or for Na^+ (right tunnel). (b) The bar model for the pump function of the reconstituted Na^+,K^+-ATPase. Phosphorylation of the enzyme may lead to the formation of a specific, oriented ion binding site for K^+ (left tunnel) or for Na^+ (right tunnel). The coupled $Na^+:K^+$ exchange bars are protected within the tunnel. The labile, exclusive Na^+-transport bar may be located closer to the intracellular tunnel entry. The scale of the scheme is hypothetical.

sent a short circuit for a still intact active Na^+-transport. However, the fact that the Na^+,K^+-ATPase activity decreases with the same kinetics as the active Na^+-transport capacity renders the first possibility (i.e., common bars for active and passive transport) more likely because in the second case the Na^+,K^+-ATPase activity should be unchanged or increased. Therefore, we tentatively use the same bars for mediated passive (fig.4a) and active (fig.4b) transport.

Upon phosphorylation of the enzyme by ATP, the bars in the K^+-tunnel may form a K^+-selective binding site opposed to the ATP side and the Na^+-bars in the Na^+-tunnel may form a Na^+-selective binding site on the ATP side (fig.4b). The collision of the ions with their respective binding sites could provide kinetic energy to the bars and move them in the direction of the impact. The bars are moving until the ions are able to slip on the other side. When the ions are released the bars move back to close the pore.

The fraction of the Na^+-transport that occurs apparently without K^+-antiport and that is easily inactivated by slight chemical modification of the Na^+,K^+-ATPase molecule is probably mediated by a hypothetical bar that is located rather superficially; i.e., close to the entry of the tunnel (fig.4b). In contrast, the $Na^+:K^+$ exchange process is more resistant to chemical modification and may thus be effectuated by protected bars that are located deeper within the tunnel.

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