Biochimica et Biophysica Acta, 773 (1984) 262–270
Elsevier

BBA 72128

CHARACTERIZATION OF $(Na^+ + K^+)$-ATPase LIPOSOMES

II. EFFECT OF α-SUBUNIT DIGESTION ON INTRAMEMBRANE PARTICLE FORMATION AND Na^+,K^+-TRANSPORT

BEATRICE M. ANNER [a], H. PING TING-BEALL [b] and J. DAVID ROBERTSON [b]

[a] *Department of Pharmacology, Centre Médical Universitaire, CH-1211 Geneva 4 (Switzerland) and* [b] *Department of Anatomy, Duke University Medical Center, Durham, NC 27710 (U.S.A.)*

(Received December 6th, 1983)

Key words: $(Na^+ + K^+)$-ATPase; Liposome; Subunit modification; Trypsin digestion; Membrane reconstitution; Particle density; Freeze-fracture; (Rabbit kidney)

The effect of the protein structure of $(Na^+ + K^+)$-ATPase on its incorporation into liposome membranes was investigated as follows: the catalytic α-subunit of $(Na^+ + K^+)$-ATPase was split into low-molecular weight fragments by trypsin treatment and the digested enzyme was reconstituted at the same protein concentration as intact control enzyme. The reconstitution process was quantified by the average number of intramembrane particles appearing on concave and convex fracture faces after freeze-fracture of the $(Na^+ + K^+)$-ATPase liposomes. The number of intramembrane particles as well as their distribution on concave and convex fracture faces is not modified by the proteolysis. In contrast, the ATPase activity and the transport capacity of the $(Na^+ + K^+)$-ATPase decrease progessively with increasing incubation times in the presence of trypsin and are abolished when the original 100 000 molecular weight α-subunit is no longer visible by sodium dodecylsulfate gel electrophoresis. Apparently, functional $(Na^+ + K^+)$-ATPase with intact protein structure and digested, non functional enzyme consisting of fragments of the α-subunit reconstitute in the same manner and to the same extent as judged by freeze-fracture analysis. We conclude that, while trypsin treatment modifies the $(Na^+ + K^+)$-ATPase molecule in a functional sense, it appears not to modify its interaction with the bilayer in producing intramembrane particles. On the basis of our results, we propose a lipid-lipid interaction mechanism for reconstitution of $(Na^+ + K^+)$-ATPase.

Introduction

In the preceeding publication [1] we found that inhibition of $(Na^+ + K^+)$-ATPase with ouabain and vanadate had essentially no effect on the enzyme's ability to reconstitute. In constrast, intense detergent-treatment abolished the enzyme's capacity to reconstitute in lipid bilayers and to produce intramembrane particles [1]. We attribute this effect to a disruption of a lipid shell around the portions of the protein normally embedded in the lipid bilayer.

In view of these findings, we wondered whether intact protein structure is as important for reconstitution as the structure of the lipid shell around the protein. The best way to modify selectively the protein part of the $(Na^+ + K^+)$-ATPase molecule is proteolysis [2–4].

We therefore treated the $(Na^+ + K^+)$-ATPase molecules with trypsin and reconstituted the modified molecules into liposomes. We then determined vesicle size, intramembrane particle frequency and distribution with respect to concave and convex fracture faces as well as the Na^+,K^+-

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transport capacity of the reconstituted liposomes. We wished to know whether or not the fragmented α-subunits would produce a larger number of smaller intramembrane particles than the intact material. Thus we compared equal amounts of treated enzyme incorporated into liposome for intramembrane particle counts. We found that the Na^+,K^+-transport capacity of liposomes reconstituted with digested α-subunit is abolished but not the appearance and distribution of intramembrane particles.

Materials and Methods

Purified ($Na^+ + K^+$)-ATPase was obtained from the outer medulla of rabbit kidneys by the method of Jørgensen [5]. Pure egg phosphatidylcholine (PC) was purchased from Supelco. Trypsin from bovine pancreas (Type III) and soybean trypsin inhibitor were from Sigma.

Reconstituted ($Na^+ + K^+$)-ATPase liposomes were prepared as described in the preceeding publication [1] and in Ref. 6. The ($Na^+ + K^+$)-ATPase was trypsin treated in the presence of NaCl or KCl as described by Jørgensen [3] except that the concentration of the enzyme and the trypsin/enzyme ratio were varied as indicated in the corresponding figure legends. Gel electrophoresis in the presence of SDS was performed according to the method of Weber and Osborne [7]. The morphology of the ($Na^+ + K^+$)-ATPase liposomes was determined as described in the preceeding publication. Protein concentrations were determined by the procedure of Lowry et al. [8].

The liposomes were prepared in a solution containing 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 30 mM imidazole or histidine buffer, 1 mM dithiothreitol and 1 mM EDTA adjusted to pH 7.15 by HCl. The passive transmembranous ion fluxes were measured as follows: 10 μl of solution (having the same composition as the solution in which the liposomes were suspended) were added to sealable conical plastic tubes containing 0.6 μCi ^{22}Na or 0.3 μCi ^{86}Rb. It was previously demonstrated that ^{86}Rb can be used as a convenient tracer for K^+ fluxes in this system (see Refs. 13, 15, 16, 17 in the preceeding publication [1]). One volume of the radioactive stock solution was then added to two volumes of liposomes and the mixture was incubated at room temperature. Active Na^+-influx into the liposomes was performed by adding 30 mM ATP to the radioactive stock solution, yielding a final ATP concentration of 10 mM. To follow active K^+-efflux, the liposomes were first equilibrated with ^{86}Rb and a concentrated ATP solution was added to the ^{86}Rb loaded liposomes to yield a final external ATP concentration of 10 mM. The ion fluxes were stopped by putting the samples on ice and adding a 50-fold excess of ice-cold stop solution containing 100 mM Tris and 30 mM imidazole buffer adjusted to pH 7.15 by HCl. The internal isotope content was determined as described in Ref. 6.

Results

Digestion of the α-subunit by trypsin

Fig. 1 shows the protein profiles of a control (a) and three trypsin-treated (b, c, d) soluble ($Na^+ + K^+$)-ATPase preparations (supernatant fractions) that were incorporated into liposomes. In preparation b about 65% of the α-subunit was digested. After a prolonged incubation in the presence of trypsin and NaCl (c) a trace of the α-subunit remained, whereas in KCl (d) the α-subunit was completely digested within 60 min. Whether the band remaining at the place of the β-subunit is only undigested β-subunit or contains in addition a newly formed, trypsin resistant 60000 mol. wt. fragment of the α-subunit is currently under investigation.

Effect of α-subunit digestion on intramembrane particle formation and distribution

When each of the three trypsin-treated ATPase preparations was reconstituted into liposomes neither the size distribution of the vesicles, nor their intramembrane particle content, nor the intramembrane particle distribution on concave and convex membrane fracture-faces, nor the form and size of the particles were significantly different from those observed with the reconstitued control preparation (Table I and Fig. 2). Thus, as far as these parameters are concerned, the liposomes containing the various digested forms of the α-subunit display the same ultrastructure as the liposomes reconstituted with native enzyme.

However, the intramembrane particles formed

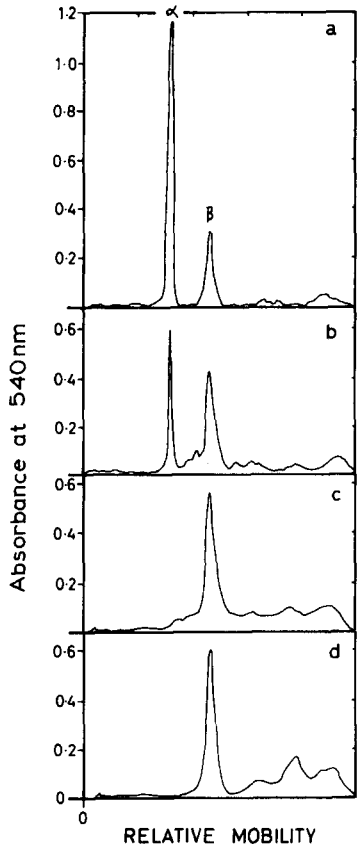

Fig. 1. Digestion of the α-subunit. Preparation (a) was incubated at a protein concentration of 200 μg protein/ml at 37°C in 150 mM NaCl, 25 mM imidazole buffer (pH 7.5), 100 μg trypsin/mg protein and 4 μg trypsin inhibitor/μg trypsin for 60 min. Preparations (b) and (c) were incubated in the same conditions, except that trypsin inhibitor was added after a 20 (b) and 60 (c) min incubation period in the presence of trypsin. Preparation (d) was incubated for 60 min under the same conditions, but in the presence of 150 mM KCl instead of NaCl. The treated enzymes were washed as described in Ref. 4. They were then solibilized in a 1% cholate solution as described in Ref. 7 and aliquots of the 100000 × *g* supernatants containing 20 μg protein were used for SDS-polyacrylamide gel electrophoresis. The remaining supernatants were processed further to liposome series 142. The Coomassie blue stained protein profiles were obtained in a ZK 4 Zeiss gel scanner accessory fitted to a Zeiss PMQ spectrophotometer. The appatent molecular weights were 106000 for the large α-subunit and 68000 for the β-subunit as compared to molecular weight markers of BDH Chemicals Ltd.

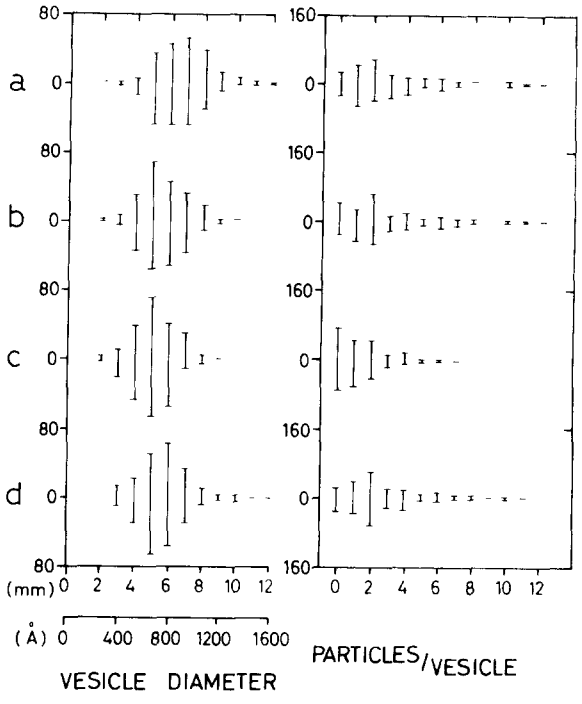

Fig. 2. Histograms of vesicle size and particle distribution of liposomes reconstituted with trypsinized ($Na^+ + K^+$)-ATPase. Liposomes were reconstituted as described in Ref. 7 with ($Na^+ + K^+$)-ATPase treated for 60 min with trypsin plus trypsin inhibitor (a) or with trypsin alone for 10 (b), 20 (c) or 60 (d) min before reconstitution. The incubation conditions are indicated in the legend to Fig. 1, the protein profile in Fig. 1 and the transport properties of the enzymes in Fig. 4. Aliquots of the liposomes were processed for electron microscopy as described under Materials and Methods and 200 convex fracture faces (above zero line) and 200 concave fracture faces (below zero line) were analyzed for vesicle size and particle distribution.

by ($Na^+ + K^+$)-ATPase with completely digested α-subunits are not functional with regard to active ion transport. In fact they are not only inactive, but present leak pathways. The number of passively entrapped potassium ions and of actively incorporated sodium ions decrease proportionally to the degree of proteolysis and the associated drop in ATPase activity (Table I and Ref. 9).

Effect of α-subunit digestion on Na^+,K^+-transport

Experiments were performed to test the effect of trypsin inhibitor alone, trypsin inhibitor plus trypsin or trypsin alone on the transport properties of ($Na^+ + K^+$)-ATPase. The enzyme was first pretreated in these three conditions and was then reconstituted at equal amounts of protein. Pre-

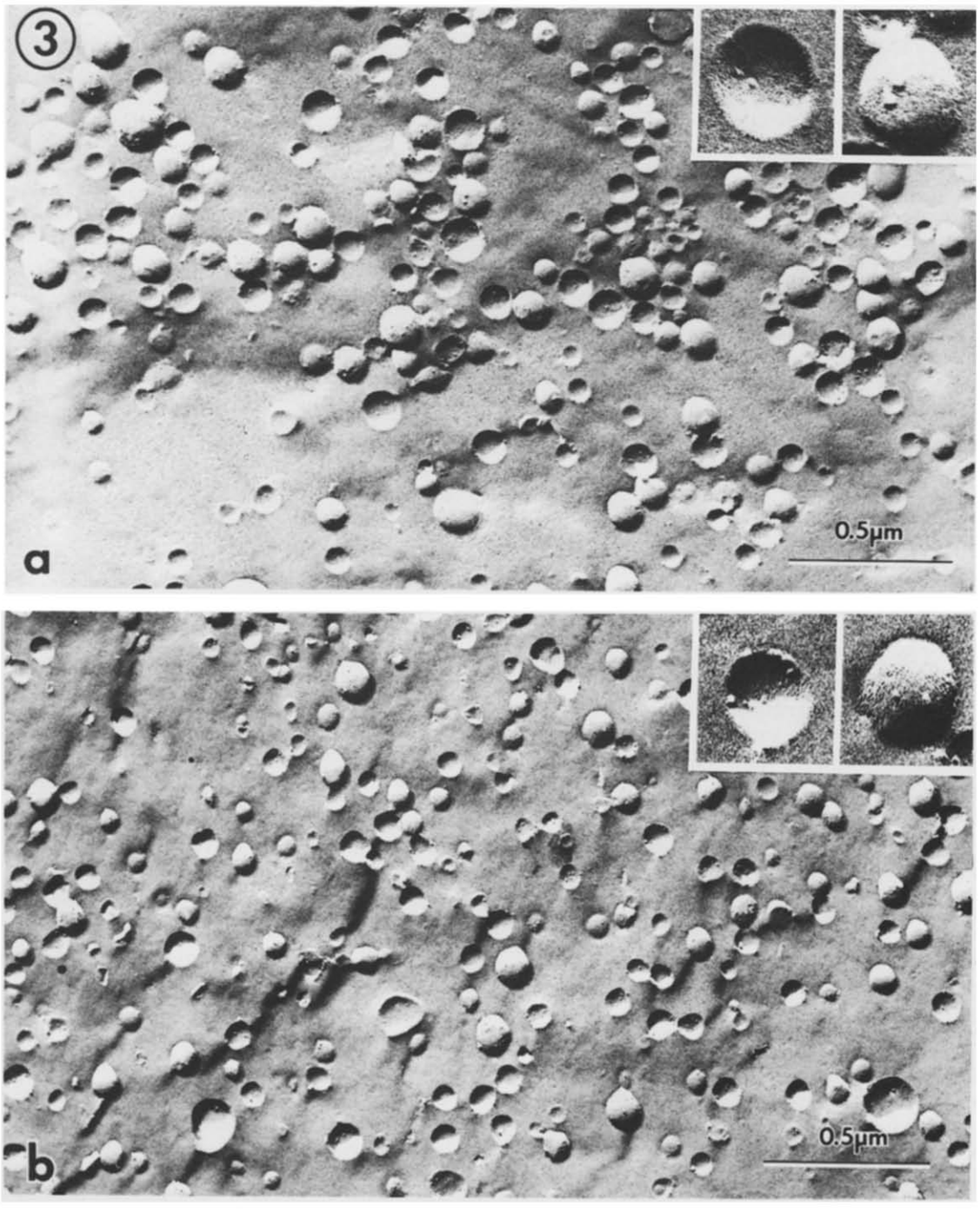

Fig. 3. Electron micrographs of freeze-fractured ($Na^+ + K^+$)-ATPase liposomes. Preparation (a) was reconstituted with intact ($Na^+ + K^+$)-ATPase and preparation (b) with ($Na^+ + K^+$)-ATPase that was digested with trypsin in the presence of KCl until the 106000 mol. wt. α-subunit had disappeared (compare Fig. 1d for gel pattern). Magnification $\times 75000$. The insets, which were printed at $3\times$ the magnification of the main figures, show the convex and concave fractured faces. The direction of shadowing is from below.

TABLE I

EFFECT OF α-SUBUNIT DIGESTION ON Na^+,K^+-TRANSPORT,VESICLE SIZE AND INTRAMEMBRANE PARTICLE DISTRIBUTION

To prepare liposome series 124, ($Na^+ + K^+$)-ATPase of a specific acitivity of 2284 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ was incubated at a protein concentration of 200 μg/ml at 37°C in 150 mM NaCl, 25 mM imidazole buffer (pH 7.50) with 100 μg trypsin/mg protein with (a) or without (b, c, d) 4 μg trypsin inhibitor/μg trypsin for 60 (a), 10 (b), 20 (c) and 60 (d) min. For liposome series 142, the initial specific activity was 1634 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ and the incubation times were 60 (a), 20 (b), 60 (c) and 60 (d) min. The incubation with trypsin was stopped by adding trypsin inhibitor and the enzymes were washed as described (4). Preparation 142 d was incubated in 150 mM KCl instead of 150 mM NaCl. The specific activity of the enzymes treated with trypsin with (a) or without inhibitor (b, c, d) for series 124 were: 1077 (a), 655 (b), 38 (c) and 12 (d) $\mu mol \cdot mg^{-1} \cdot h^{-1}$. The different enzymes were then reconstituted into liposomes and analyzed as described under Materials and Methods.

Series No.	Entrapped cation (‰ of external)		Average vesicle diameter (Å) [a]	Particles per fracture face [a]		Particles per vesicle
	K(−ATP)	Na(+ATP)		Convex	Concave	
124 a	33.0	30.52	797 ± 12	2.11 ± 0.12	2.72 ± 0.12	4.83
b	16.74	23.23	812 ± 11	2.07 ± 0.12	2.87 ± 0.13	4.94
c	15.58	25.76	828 ± 12	1.77 ± 0.10	2.04 ± 0.16	3.81
d	4.25	7.92	734 ± 11	1.26 ± 0.07	4.71 ± 0.14	5.97
142 a	12.76	35.22	870 ± 14	2.60 ± 0.11	2.50 ± 0.15	5.10
b	3.91	4.25	751 ± 12	2.40 ± 0.17	2.56 ± 0.16	4.96
c	2.16	0.77	681 ± 12	1.30 ± 0.10	1.30 ± 0.10	2.60
d	1.11	1.01	755 ± 13	2.67 ± 0.16	2.44 ± 0.15	5.11

[a] Mean ± S.E. ($n = 200$).

treatment with trypsin inhibitor alone did not reduce the ATPase activity of the isolated enzyme and, as expected, displays the highest Na^+,K^+-transport activity (Fig. 4). In contrast, when the enzyme was incubated with a mixture of trypsin inhibitor and trypsin, the ATPase activity of the isolated enzyme was decreased from an initial specific activity of 2284 to 1077 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ in preparation 124 (decrease of 53%), from 1634 to 712 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ in preparation 142 (decrease of 56%), and from 1300 to 847 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ in preparation 149 (decrease of 35%).

Fig. 4 presents the Na^+,K^+-transport in liposomes reconstituted with enzyme (preparation 149) that has been pretreated with trypsin plus trypsin inhibitor. The protein profile of the treated enzyme showed no evidence of proteolysis (Fig. 1a) and no esterase activity was detected by the *p*-toluene sulfonyl-L-arginine methyl esterase assay [10]. The active Na^+-transport capacity, however, is reduced by 35% while the active K^+-transport capacity is unchanged. The ATPase activity of the reconstituted enzyme is also decreased by 35%, i.e. there is a quantitative correlation between the enzyme activity before reconstitution and the Na^+-transport capacity after reconstitution.

This correlation confirms our previous report of a 40–60% reduction of the ($Na^+ + K^+$)-ATPase activity produced by brief incubation in the presence of trypsin and an associated 40–60% selective reduction of the Na^+-transport capacity after reconstitution of the modified enzyme into liposomes [11,12]. Likewise, no proteolytic split could be seen after gel electrophoresis of the subunits [13].

The same decrease in ATPase activity and Na^+-transport can be produced, as we show in the present work, by simple incubation of the ($Na^+ + K^+$)-ATPase with trypsin plus inhibitor. In either condition, we obtain a selective reduction of the Na^+-transport capacity which is exactly proportional to the decrease in ATPase activity before reconstitution.

In contrast, when the ($Na^+ + K^+$)-ATPase was incubated with trypsin in conditions which split the α-subunit to low molecular weight fragments visible by gel electrophoresis in the presence of SDS, the Na^+- and K^+-transport capacity de-

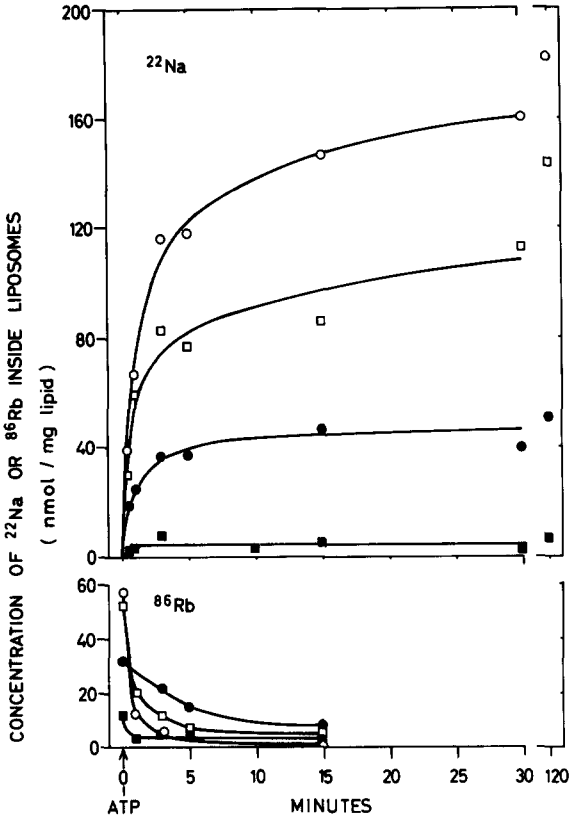

Fig. 4. Na^+,K^+-transport of liposomes reconstituted with $(Na^+ + K^+)$-ATPase that was pretreated with trypsin inhibitor with or without trypsin or with trypsin alone. Before reconstitution. $(Na^+ + K^+)$-ATPase of initial specific activity of 1300 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ was incubated at a protein concentration of 100 μg/ml in the conditions described by Jørgensen, i.e. 150 mM NaCl, 25 mM imidazole buffer (pH 7.5 at 37°C) in the presence of 40 μg trypsin inhibitor/ml (○), in the presence of 40 μg trypsin inhibitor plus 10 μg trypsin for 20 min (□), in the presence of 10 μg trypsin followed by the addition of 40 μg trypsin inhibitor after 10 min (●) or 20 min (■). The specific activity of the treated enzymes was 1300 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ for the enzyme incubated with a mixture of trypsin inhibitor and trypsin, and 544 and 434 for the enzymes incubated in the presence of trypsin alone for 10 min and 20 min, respectively. The treated enzymes were then reconstituted in parallel by the method described in Ref. 7 and the transport was measured as explained under Materials and Methods. The upper graph is the ^{22}Na uptake and the bottom graph presents the $^{86}Rb[K]$ efflux.

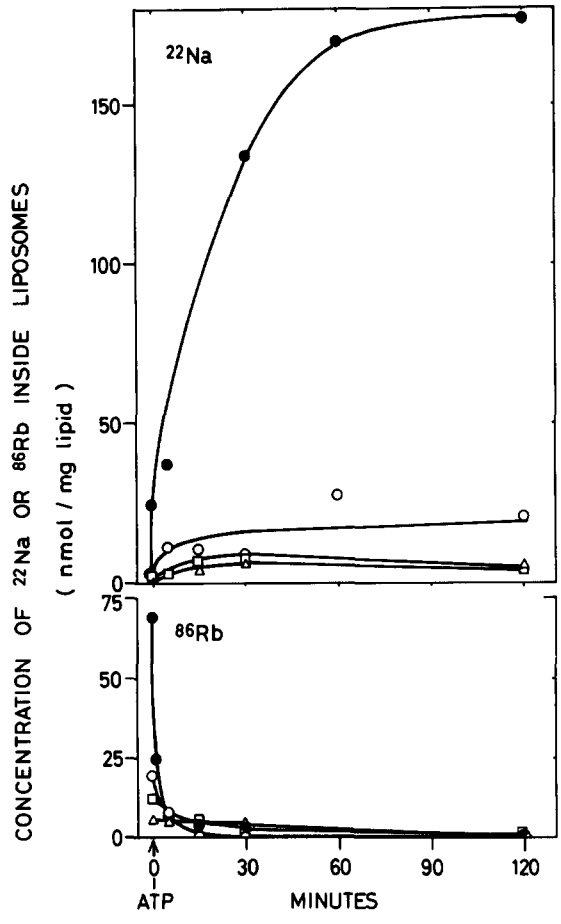

Fig. 5. Na^+,K^+-transport of liposomes reconstituted with $(Na^+ + K^+)$-ATPase that was pretreated with trypsin plus inhibitor or with trypsin alone. Before reconstitution, $(Na^+ + K^+)$-ATPase of a specific activity of 1634 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ was incubated in the conditions described in the legend for Fig. 4 in the presence of trypsin plus inhibitor for 60 min (●) or in the presence of trypsin alone for 20 (○) and for 60 min (□) or for 60 min with 150 mM KCl instead of NaCl (△). The incubations in trypsin alone were stopped by the addition of trypsin inhibitor. The resulting specific activities were 712 (●), 240 (○), 38 (□) and 12 (△) $\mu mol \cdot mg^{-1} \cdot h^{-1}$. The treated enzymes were then reconstitued in parallel by the method described in Ref. 7 and the transport was measured as explained under Materials and Methods. The upper graph is the ^{22}Na uptake and the bottom graph presents the $^{86}Rb[K]$ efflux.

crease in parallel (Figs. 4, 5). Such a trypsin effect on the active Na^+,K^+-transport was also observed by Karlish and Pick [14] by adding trypsin externally to liposomes reconstituted with $(Na^+ + K^+)$-ATPase. Their control liposomes were incubated with trypsin plus trypsin inhibitor, i.e. in the condition which produces, as shown in the present work, a selective decrease in the active Na^+-transport capacity. This may explain why the Na^+/K^+ transport ratio of their control preparation is 1 : 1

and not 3 : 2 as in their native liposomes.

Taken together, the results suggest that trypsin has two distinct effects on the $(Na^+ + K^+)$-ATPase protein. The first effect does not produce a visible proteolytic split of the α-subunit (Fig. 1) but reduces the ATPase activity and the Na^+-transport capacity by about 50% (Fig. 4). The second action splits the α-subunit to low mol wt fragments (Fig. 1) with concomitant loss of ATPase activity, Na^+-transport, as well as K^+-transport (Figs. 4, 5).

Discussion

Leak formation by α-subunit digestion

The trypsin-treatment reduces the internal K^+-pool and the active transport capacity. We believe that the decrease in entrapped cations is caused by leaky $(Na^+ + K^+)$-ATPase molecules. Accordingly, the liposome population containing the digested, leaky $(Na^+ + K^+)$-ATPase molecules would loose its internal cation pool during passage of the liposomes through the Sephadex column. The residual K^+-pool would then be entrapped in vesicles containing only intact pump molecules. Prolonged incubation of altered liposones in the presence of external ^{86}Rb does not augment the labeling of the residual internal K^+-pool (unpublished results and Ref. 9). This finding rules out the possibility that trypsin-treatment could decrease the permeability of the enzyme molecules and thereby slow down isotope exchange.

To bring additional evidence for the leak-theory, a defined number of pump molecules was transferred to planar bilayers by fusion of our well characterized $(Na^+ + K^+)$-ATPase-liposome preparations [15]. The $(Na^+ + K^+)$-ATPase molecules formed single channels in the bilayer of 40 to 50 pS conductance. The cation flux rate in such a channel is largely sufficient to eliminate the few thousand internal cations per vesicle during passage through the gel column.

We found a direct correlation between the ATPase activity present after graded proteolysis and the transport activity appearing after reconstitution of the altered enzyme preparation in liposomes. The correlation is not influenced by the number of $(Na^+ + K^+)$-ATPase molecules incorporated per vesicle. If the enzyme molecules would be randomly distributed amongst the vesicle population, the probability that each vesicle would receive a leak would augment with increasing protein concentrations. The observation that this is not the case implies that the leaky molecules reconstitute in a grouped manner.

Normal intramembrane particle formation despite α-subunit digestion

The trypsin-treatment does not affect the enzyme's ability to reconstitute. The resulting intramembrane particles are essentially the same of size and distribution as those formed by the intact molecules. This implies that the fragments produced by splitting of the α-subunit do not separate when they are reconstituted. Since the number of particles and their size remain the same, it appears that the hydrophobic polypeptide segments remain associated not only with themselves, but also with the β-subunit. If this were not the case we should at least see some increase in the number of particles yielded by a given quantity of enzyme and perhaps these particles should also be smaller. The hydrophobic fragments could indeed form separate polypeptide-detergent micelles and then reconstitute as multiple smaller particles. Apparently, the boundary lipid layer surrounding the $\alpha_2\beta_2$ dimer stays there and prevents the separation of the polypeptide fragments throughout the detergent solubilization and reconstitution process. Deamer and Yamanaka [16] treated sarcoplasmic reticulum with the proteolytic Nagarase enzyme and found no modification of the intramembrane particle pattern in this situation.

The nature of intramembrane particles in liposomes

It is believed that intramembrane particles represent protein molecules traversing or embedded within the lipid bilayer. Often a rather literal interpretation is made equating the intramembrane particle directly with polypeptide. However, the problem is more complex. For instance in the case of bacteriorhodopsin, it has been possible to make a direct correlation between observed individual imtramembrane particles and three transverse bacteriorhodopsin molecules [17]. The fracture plane seems to depart from the center of the bilayer to run around groups of three bacteriorhodopsin molecules leaving them intact in

the PF face. It thus appears that in general the fracture plane does not break polypeptide chains. Recent work by Pinto da Silva and his group by the 'fracture-label' technique applied to erythrocyte membranes supports the idea that the fracture plane tends to deviate around transverse protein molecules leaving the whole molecule within one or the other fracture face [18]. In the case of the native ($Na^+ + K^+$)-ATPase molecule, where there is a pronounced structural asymmetry (19–21], it seems reasonable to suppose such an asymmetrical fracture plane. The fact that we find the same number of intramembrane particles on the convex and concave fracture faces of liposomes would then indicate a random orientation of the ($Na^+ + K^+$)-ATPase molecules.

One might expect that each intramembrane particle would have a corresponding pit in the opposite fracture face. However, we have seen no pits in any of our preparations. The failure to demonstrate pits to correspond to intramembrane particles has puzzled freeze-fracture electron microscopists for many years [22]. It is commonly stated that it is very easy to obliterate pits by contamination and not so easy to obliterate intramembrane particles. There are other explantions, such as the notion that the intramembrane particle does not represent the protein molecule and its associated lipid directly but rather a disturbance in the lipid bilayer caused by the presence of the protein leading to selective decoration of the fracture face in the region of the disturbance [23]. This mechanism would produce intramembrane particles but not pits and would also provide an explanation for the relatively large size of intramembrane particles which often exceeds estimates of the mass of the protein that could be involved.

A lipid-lipid interaction mechanism for reconstitution of ($Na^+ + K^+$)-ATPase

In visualizing the mechanism whereby the ($Na^+ + K^+$)-ATPase liposomes form we may regard the enzyme molecules as condensation centers for precipitation of the phospholipid molecules from solution. Thus, it seems reasonable to suppose that the phospholipid molecules would attach to the parts of the enzyme molecule that are already coated with a shell of lipid from the native membrane. A protein-associated lipid shell of 50 to 60 molecules per $\alpha_2\beta_2$ dimer has been demonstrated by electron spin resonance [24,25]. If we assume that this enzyme phospholipid retains its essential bilayer configuration, with the detergent molecules providing an interface between the carbon chains and the water, the newly added phospholipid molecules would be expected to replace detergent molecules and attach themselves to the enzyme lipids with the carbon chains and head groups lining up with their counterparts so as to form an extending bilayer.

Acknowledgement

Mrs. Marlis Moosmayer provided outstanding assistance with regard to ($Na^+ + K^+$)-ATPase purification, liposome preparation and transport measurement. The work was supported by grant No. 3.155-0.81 of the Swiss National Science Foundation to B.M.A and the U.S. National Institute of Health GM-27804 and GM/AM-28224 to H.P.T.-B. and J.D.R., respectively.

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