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PURIFICATION AND CHARACTERIZATION OF ($Na^+ + K^+$)-ATPase

VIII. ALTERED Na^+ : K^+ TRANSPORT RATIO IN VESICLES RECONSTITUTED WITH PURIFIED ($Na^+ + K^+$)-ATPase THAT HAS BEEN SELECTIVELY MODIFIED WITH TRYPSIN IN PRESENCE OF NaCl

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Summary

1. Using the cholate dialysis technique for reconstitution of ($Na^+ + K^+$)-ATPase into phospholipid vesicles we have examined the transport properties of purified ($Na^+ + K^+$)-ATPase from the outer renal medulla after selective modification of its protein by a single tryptic split.

2. After this digestion in NaCl medium a stable preparation is obtained with intact ATP binding and phosphorylation sites, but with defective transition from E_1P to E_2P and K^+-dependent dephosphorylation and phosphatase reactions.

3. After reconstitution, the active transport of Na^+ in vesicles containing the trypsinized ($Na^+ + K^+$)-ATPase is 30—40% of control, whereas the active transport of K^+ is close to or identical to that in control. Passive fluxes of Na^+ and K^+ are identical in the two preparations. The reduction in Na^+ : K^+ transport ratio may be due to interference with the binding of Na^+ or with the conformational change accompanying the translocation of Na^+.

4. The residual active Na^+ transport in the trypsinized preparation is resistant to vanadate. The capacity for binding of [^{48}V]vanadate is the same in the two preparations (3.4—3.5 nmol · mg^{-1} protein) but the dissociation constant is much higher for the trypsinized (K_D = 32 nM) than for the control ($Na^+ + K^+$)-ATPase (K_D = 4.5 nM). The lower affinity of the trypsinized preparation to vanadate may reflect the abnormal transformation from E_1P to E_2P of the pro-

tein with inadequate exposure of the phosphate site which may also be the site for binding of vanadate.

Introduction

After reconstitution into phospholipid vesicles, $(Na^+ + K^+)$-ATPase couples hydrolysis of ATP to active transport of Na^+ and K^+ with a ratio close to $3Na^+ : 2K^+$ [1—3]. In an attempt to establish true structure-function relationships for the protein parts of this pump machinery, we have examined the cation transport after reconstitution of selectively modified $(Na^+ + K^+)$-ATPase into phospholipid vesicles.

After graded tryptic modification of the Na^+-form of the protein to a $(Na^+ + K^+)$-ATPase activity of 40%, a stable enzyme preparation can be obtained with an almost intact number of ATP binding and phosphorylation sites, but with a defect in the transformation from E_1P to E_2P and the dephosphorylation reaction and a K^+-phosphatase activity which is 15—20% of control [4—6]. Vesicles containing this preparation (TENa-vesicles) or control preparations of the purified $(Na^+ + K^+)$-ATPase were formed and both the active and the passive transport of Na^+ ($^{22}Na^+$) and K^+ ($^{86}Rb^+$) were measured. The results revealed a selective transport defect for Na^+ in vesicles containing the modified preparation with a decrease in $Na^+ : K^+$ transport ratio from about $3Na^+ : 2K^+$ in control vesicles to $1—2Na^+ : 2K^+$ in TENa-vesicles.

To characterize further the abnormal transport of Na^+ we examined the kinetics of the activation by external Na^+ and the effects of vanadate on the transport. Vanadate acts like a phosphate analog and binds with high affinity to the cytoplasmic portion of $(Na^+ + K^+)$-ATPase [7] which faces outward in the transport vesicles. The ATPase activity of the trypsinized enzyme is insensitive to vanadate [8]. As K^+-dependent dephosphorylation and phosphatase are reduced in the trypsinized enzyme [6], we studied vanadate inhibition of cation transport and [^{48}V]vanadate binding to see if the abnormal transport of Na^+ is due to damage to the site from which phosphate is released during hydrolysis.

Experimental

$(Na^+ + K^+)$-ATPase with specific activities of 18—36 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein and 2—3.7 nmol ATP or ouabain binding sites/mg protein was obtained from the outer medulla of pig kidney by incubation of a microsomal fraction with sodium dodecyl sulfate (SDS) and ATP followed by an isopycnic zonal centrifugation [9]. Digestion with trypsin in presence of 150 mM NaCl to 40% of control $(Na^+ + K^+)$-ATPase activity and wash of the membranes by centrifugation was done as before [5]. Vesicles were prepared following a modification of the previous procedure [3]. Aliquots containing 500 μg of $(Na^+ + K^+)$-ATPase protein were suspended in 250 μl of 1% sodium cholate in buffer A: 20 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 50 mM choline chloride, 1 mM cysteine chloride, 1 mM EDTA, 30 mM imidazole, pH 7.1 (20° C). The suspension was centrifuged for 15 min at 100 000 $\times g$ and the supernatant was mixed by

sahking in a Vortex with 4—5 mg lecithin dissolved in 250 μl of a 1% solution of sodium cholate in buffer A. Vesicles were formed during dialysis for 90 h against buffer A in 6 mm dialysis tubing (Union Carbide). For transport measurements after equilibration with isotope (Figs. 1 and 2) the vesicles are incubated at 24°C for 60 min in buffer A and equilibrated with either $^{22}Na^+$ (0.5 μCi/100 μl) or $^{86}Rb^+$ (4 μCi/100 μl) for 270 min at 24°C. For measurements of unidirectional fluxes of Na^+ (Figs. 3 and 5) the vesicles were incubated at 24°C for 5—15 min and transport was started by adding ATP and $^{22}Na^+$ simultaneously. The Tris salt of ATP was prepared from the disodium salt (Sigma) using a Dowex W-X2 resin which is known to remove contaminant vanadate [10]. After addition of ATP to final concentrations between 30 μM and 6 mM, duplicate samples of 10—20 μl were taken out at the times indicated in the graphs and passed through Sephadex G-50 columns (1 × 25 cm) with buffer A. The radioactivity eluting with the liposomes in the void volume was determined by scintillation counting. Intravesicular cation concentrations were calculated as before [3] assuming that vesicle volumes remained constant or the data were processed to nmol cation transport/mg protein.

For determination of vanadate binding, aliquots containing 85 μg ($Na^+ + K^+$)-ATPase protein (0.3 nmol) were mixed in centrifuge tubes at 22°C with 0.1 μCi [^{48}V]vanadate (Amersham) in 1 ml containing unlabelled vanadate in concentrations between 0.05 μM and 2 μM, 3 mM $MgCl_2$, 20 mM KCl, and 25 mM imidazole, pH 7.5. To estimate unbound vanadate in the sediments, 10 mM unlabelled vanadate was added to two tubes. After incubation for 15 min at 22°C the tubes were counted in a Packard γ-counter and centrifuged for 90 min at 50 000 rev./min in a Beckman type 65 rotor. The supernatants were removed and remaining droplets were wiped off. After counting the sediments were dissolved in 200 μl 1 N NaOH with heating for 30 min at 60°C and samples were taken out for protein determination as before [5]. Bound vanadate was calculated from the radioactivity in the pellets, and free vanadate from the total radioactivity in the tubes minus that in the pellets or from the counts in the supernatant.

Results

Active transport

Externally added ATP generated active uptake of Na^+ ($^{22}Na^+$) into the vesicles and extrusion of K^+ ($^{86}Rb^+$) from the intravesicular space of vesicles reconstituted with either the control or the trypsinized preparation. The tryptic digestion seems therefore not to interfere with the reconstitution of the ($Na^+ + K^+$)-ATPase into liposomes. The results in Fig. 1 are representative for a series of experiments. It is seen that the increase of intravesicular concentration of Na^+ in TENa-vesicles was 1/3 of that in control vesicles, and that the decrease in intravesicular concentration of K^+ was the same in the two preparations. The Na^+ : K^+ transport ratio for control vesicles was close to $3Na^+$: $2K^+$, whereas for TEN-vesicles the ratio was about $1Na^+$: $2K^+$. Thus, active transport of Na^+ into TENa-vesicles was lower than outward active transport of K^+. In a series of similar experiments with 24 measurements on eigth different batches of control and trypsinized preparations the concentration gradients were mea-

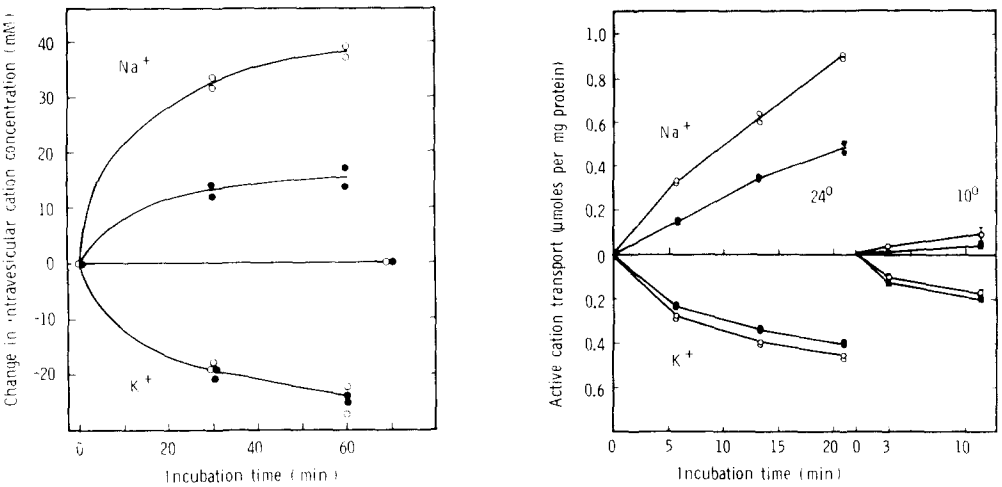

Fig. 1. Active transport of Na^+ ($^{22}Na^+$) and K^+ ($^{86}Rb^+$) in vesicles reconstituted with trypsinized (•) and control (○) ($Na^+ + K^+$)-ATPase. Vesicles containing control or modified enzyme were formed in parallel batches in buffered solutions containing 20 mM NaCl, 50 mM KCl and 5 mM $MgCl_2$. Active transport was initiated by adding 6 mM ATP to aliquots of the vesicle suspension. Details of reconstitution and transport assay are described under Experimental. The change in cation concentration was calculated from the radioactivity in vesicles incubated with ATP minus the activity in vesicles incubated without ATP.

Fig. 2. Transport rates for Na^+ ($^{22}Na^+$) and K^+ ($^{86}Rb^+$) in vesicles containing trypsinized (•) and control enzyme (○) at two temperatures, 24°C (left) and 10°C (right) with a low concentration of ATP (30 μM) in the medium. Procedure and ion concentrations as in Fig. 1 and under Experimental. ATP concentration was kept constant at 30 μM by adding 2 mM phospho*enol*pyruvate and pyruvate kinase, 0.1 mg/ml. Data at 10°C are average values ± S.E. of four measurements. Transport was calculated from the difference in radioactivity between vesicles incubated with ATP and vesicles incubated without ATP.

sured from 10 to 120 min after addition of ATP. The concentration gradients for Na^+ varied from 31 to 97 mM in control and from 12 to 47 mM in TENa-vesicles. The gradients for K^+ fell in the range of 15—30 mM for both control and TENa-vesicles. On an average the gradient for Na^+ in TENa-liposomes was 41 ± 2% of control (S.E., n = 24) whereas the gradient for K^+ was 97 ± 3% of control (S.E., n = 19). In these experiments the data were processed to cation concentration without correcting for changes in vesicle volume. The results were essentially the same when expressed in nmol cation/mg enzyme protein (cf. Figs. 2, 3 and 5).

It is seen from Fig. 1 that the vesicular content of K^+ reached a level after 20—30 min of incubation with ATP. The explanation for this could be that a balance is reached between active efflux and passive influx. However, the vesicle equilibrate slowly with K^+ (Fig. 4) and the initial rate of active efflux is high (Fig. 2). The level could therefore also reflect that there is a limited intravesicular pool of K^+ available for outward ATP-dependent transport. Exhaustion of this pool could result in masking of a difference in rate of transport of K^+ between TENa-vesicles and control. The purpose of the experiment in Fig. 2 was therefore to examine if an altered K^+ transport occurring in the early phase of extrusion is overlooked in experiments similar to that in Fig. 1. Short incubation times were used and the turnover rate/pump site was reduced by lowering ATP concentration and temperature. Almost linear rates of Na^+

transport were obtained. The net rate of influx of Na^+ into TENa-vesicles was 40—45% of the rate in control vesicles, both at 24°C and at 10°C. As the passive leaks were identical (Fig. 4) these results show that the active transport of Na^+ into TENa-vesicles in either condition was smaller than outward transport of K^+. The curves for transport of K^+ began to level off already after 3 or 5 min, although the measurements were made at apparent intravesicular concentrations of K^+, 35—45 mM, which could not be rate limiting for active transport. At 24°C the rate of K^+ transport in TENa-vesicles was 22% lower than in control, whereas at 10°C the rate of K^+ transport was 20% higher in TENa-vesicles than in control. Thus, on the average the experiment did not reveal differences in active K^+ transport.

The selective reduction in active Na^+ transport in TENa-vesicles could suggest that binding sites of Na^+ are damaged. Active transport of Na^+ is a sigmoid-shaped function of the cytoplasmic concentration of Na^+ in cells (see Refs. 11 and 12). Reduction in the number of Na^+ bound to the enzyme might alter the shape of this curve from an S shape to a hyperbolic shape. To examine this possibility we measured active transport of Na^+ as a function of extravesicular concentration of Na^+, since only pumps with the cytoplasmic aspect of the molecule facing outward are utilized in the reconstituted vesicles.

In the experiment in Fig. 3 initial tracer fluxes of Na^+ ($^{22}Na^+$) from outside to inside the vesicles were measured with and without ATP present. The extravesicular concentration of Na^+ was varied from 0 to 10 mM and the concentration of K^+ was kept constant on both sides of the membrane. Also in these conditions the active transport of Na^+ in TENa-vesicles was only 1/3 of control. The activation curves are not complete but it is obvious that the first part of the S-shaped curve is retained in TENa-vesicles. The experiment did therefore not provide kinetic evidence for a reduced number of Na^+ bound to the enzyme after the trypsin treatment in presence of NaCl.

Passive transport

In view of the observation of a selective decrease in active transport of Na^+ it was important to determine if passive ion permeabilities of the membrane in TENa-vesicles were different from control. The tracer equilibration curves in Fig. 4 show that the passive permeabilities for $^{22}Na^+$ and $^{86}Rb^+$ were identical for control and TENa-vesicles. In agreement with previous observations [2,3] the half-time for equilibration was close to 45 min for both isotopes. The altered rate of active transport of Na^+ in TENa-vesicles was therefore not due to a selective increase in permeability to Na^+ of the vesicular membrane containing the trypsinized enzyme.

Vanadate inhibition

Vanadate from the outside inhibited the active transport in control vesicles with a $K_{1/2}$ of 0.4—0.5 μM (Fig. 5). Inhibition of the residual active transport of Na^+ in TENa-vesicles required 3—4-fold higher concentrations. The $K_{1/2}$ for inhibition by vanadate of the remaining ($Na^+ + K^+$)-ATPase and K^+-phosphatase activities was also increased about 3—4-fold by the treatment with trypsin in presence of NaCl (not shown).

Determination of [^{48}V]vanadate binding to control and trypsinized (Na^+ +

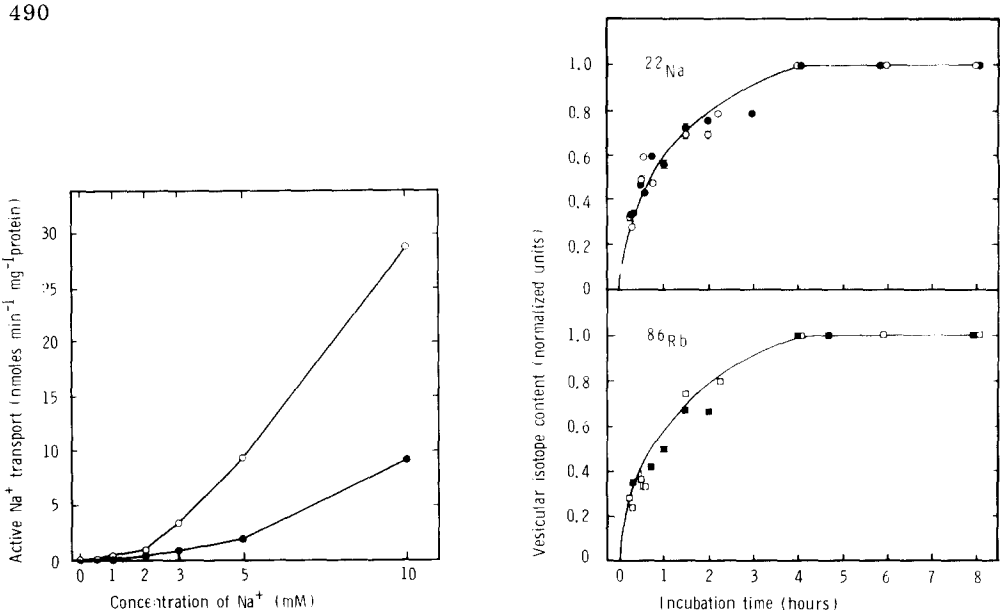

Fig. 3. Active influx of Na^+ ($^{22}Na^+$) into TENa-vesicles (●) and control vesicles (○) as a function of the concentration of Na^+ in the incubation medium. Vesicles were prepared as in Fig. 1 except that NaCl in the dialysis buffer was replaced by choline chloride. The vesicles were mixed with buffer A containing from 0 to 20 mM NaCl and from 20 to 0 mM choline chloride to obtain the final concentrations of NaCl on the abscissa. 1 min later $^{22}Na^+$ (0.5 μCi/100 μl) was added with ATP to a final concentration of 3 mM. After incubation for 5 min at 24°C radioactivity in the vesicles was determined after separation on Sephadex G-50 columns as in Fig. 1. Transport was calculated from the radioactivity in vesicles incubated with ATP minus that in vesicles incubated without ATP.

Fig. 4. Time course for loading of vesicles with $^{22}Na^+$ or $^{86}Rb^+$. Vesicles were prepared as in Fig. 1 and incubated at 24°C. At zero time the isotopes were added to vesicles containing trypsinized (●, ■) or control (○, □) ($Na^+ + K^+$)-ATPase, and vesicular isotope content was determined at the times indicated on the abscissa. The content of isotope was expressed as a fraction of isotope content in the vesicles at equilibrium.

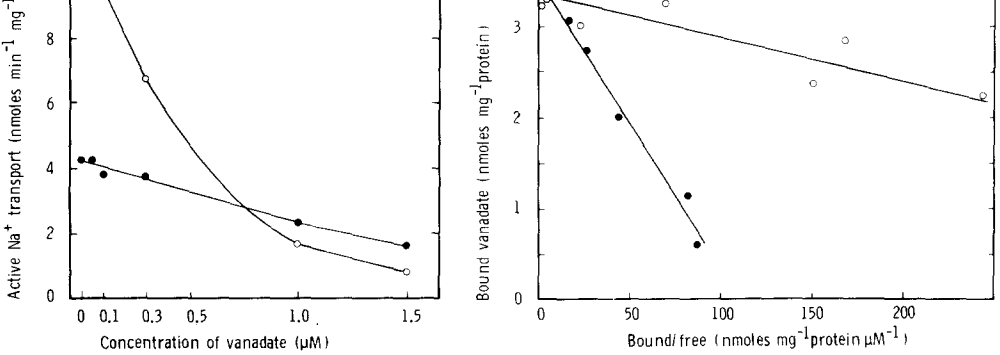

Fig. 5. Vanadate inhibition of active Na^+ transport in TENa-vesicles (●) or control (○) vesicles. After incubation with vanadate (0.05—1.5 μM) for 15 min at 24°C in buffer A, transport was initiated by adding $^{22}Na^+$ and ATP to a final concentration of 3 mM. Intravesicular activity of $^{22}Na^+$ was determined after incubation for 5 min at 24°C and transport was calculated from the radioactivity in vesicles incubated with ATP minus the activity in vesicles incubated without ATP.

Fig. 6. Scatchard plots of vanadate binding to trypsinized (●) and control (○) preparations of purified ($Na^+ + K^+$)-ATPase. Procedure as described under Experimental. Binding capacities and dissociation constants as calculated by the least-square method were 3.4 ± 0.1 nmol · mg^{-1} protein and 4.5 ± 0.9 nM for control and 3.5 ± 0.1 nmol · mg^{-1} protein and 32 ± 2 nM for the trypsinized preparation.

K^+)-ATPase (Fig. 6) showed that the number of vanadate binding sites were the same in the two preparations. The trypsinized enzyme bound vanadate with much lower affinity (K_{diss} = 32 nM) than the control (K_{diss} = 4.5 nM). The maximum vanadate binding capacity for the two preparations was 3.4 and 3.5 nmol · mg^{-1} protein. This corresponds to binding of 1 mol of vanadate/mol of binding sites for ATP or per 2 mol of large polypeptide chain with molecular weight close to 100 000 (cf. Ref. 5).

Discussion

Both control and modified ($Na^+ + K^+$)-ATPase was incorporated into vesicles in a reproducible manner by the procedure employed in the present work. Comparison of their ($Na^+ + K^+$)-transport patterns showed that the active Na^+ transport in vesicles containing trypsinized enzyme is reduced whereas active K^+ transport appears to be unchanged. The residual active Na^+ transport in TENa-vesicles responds poorly to vanadate and the affinity of the trypsinized ($Na^+ + K^+$)-ATPase for binding of vanadate is reduced.

The selective reduction in active Na^+ transport in TENa-vesicles is also observed when the intravesicular concentration of K^+ is not rate limiting and the change is the same whether the transport of Na^+ is measured after equilibration with isotope or as unidirectional flux of tracer. Initial velocities of K^+ transport were not obtained. However, in all conditions tested in our experiments, the rate of transport of Na^+ in TENa-vesicles is lower than the rate of transport of K^+ and the observed rates of active transport of K^+ are close to or identical to those in control vesicles. This in conjunction with the identity of the passive fluxes in the two preparations serve as internal controls of the experiments as any methodological variation would have to affect selectively the active transport of Na^+ to explain the results. The possibility of an altered orientation of the trypsinized ($Na^+ + K^+$)-ATPase in the vesicles or of incomplete reconstitution appears also to be excluded by these observations.

It is therefore reasonable to conclude that the lower ratio of $Na^+ : K^+$ transport in TENa-vesicles is due to an altered cation stoichiometry of the trypsinized ($Na^+ + K^+$)-ATPase. A similar change in $Na^+ : K^+$ transport ratio following inhibition or modification of the protein of ($Na^+ + K^+$)-ATPase has apparently not been observed before [11,12].

Vanadate in low concentrations inhibits the active Na^+ transport catalyzed by the purified ($Na^+ + K^+$)-ATPase from the outer renal medulla. The insensivity of the residual active transport in TENa-vesicles is not due to disappearance of vanadate binding sites but to a reduction in affinity of the sites to vanadate. Vanadate is assumed to bind to the site where phosphate is released during ATP hydrolysis [7]. The lower affinity for vanadate is thus in agreement with the weak effect of K^+ on the rate constant for dephosphorylation and the low K^+-phosphatase activity and seems to be another reflection of the slower and abnormal transition from E_1P to E_2P of the large chain in the trypsinized enzyme [6].

Together with previous data [4] the results show that both the catalytic defects [5,6] and the reduced Na^+ transport are caused by the tryptic split in the protein of the purified ($Na^+ + K^+$-ATPase. The mechanism of the defective

transport remains to be elucidated. Possibly, the cleavage could interfere with Na^+ binding or with the translocation process subsequent to loading of cation sites. Either possibility is compatible with the impaired transition from E_1P to E_2P. The split in the protein reduces active Na^+ transport in parallel to the ($Na^+ + K^+$)-ATPase activity, whereas ATP binding and phosphorylation are preserved along with the apparently intact transport of K^+. It seems therefore that the active transport of K^+ can be related to the ability to bind ATP, whereas the active transport of Na^+ rather depends on the ability to transform the phosphoenzyme into E_2P and to split phosphate from the protein. As the transport of K^+ appears to be unchanged it will be of interest to learn if the energy yield from ATP is altered by the tryptic split. Determination of the energy coupling of the abnormal ($Na^+ + K^+$)-transport will require separation of transport vesicles from unreconstituted ($Na^+ + K^+$)-ATPase to ensure that all ATP splitting is coupled to transport.

Tryptic modification of catalytic areas and binding sites at the membrane surface does not affect the lipid-associated segments of the catalytic subunit [13] which may form hydrophobic bonds with lipids and thus determine the reconstitution into vesicles. Other procedures for protein modification may also preserve the ability to reconstitute into vesicles. The combination of selective protein modification of the purified ($Na^+ + K^+$)-ATPase and measurement of cation transport in vesicles may therefore form a powerful tool in future studies of the molecular mechanism of this important transport process.

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