RECONSTITUTION OF AN ATP-DEPENDENT SODIUM PUMP WITH AN ATPase FROM ELECTRIC EEL AND PURE PHOSPHOLIPIDS

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SUMMARY

An ATP-dependent sodium pump was reconstituted with an ATPase preparation from the electric eel and purified phospholipids. Vesicles reconstituted by the sonication procedure catalyzed Na^+ translocation at a rate 10 to 20 times higher than vesicles reconstituted by the cholate–dialysis procedure. A small but reproducible stimulation of Na^+ transport by valinomycin in the presence of K^+ was observed.

Biological pumps are embedded in membranes of considerable complexity containing a multitude of proteins and phospholipids. For the assessment of the minimum requirements for function the approach of resolution and reconstitution has been successful. In the case of the reconstituted Ca^{++} pump (1), the Ca^{++}-ATPase (molecular weight of about 100,000) together with a proteolipid (molecular weight of about 12,000) are required for the reconstitution of an active pump (2). The complex phospholipid composition of the natural sarcoplasmic reticulum membrane is not required for the effective translocation of Ca^{++} in liposomes, since phosphatidylethanolamine alone suffices as the only phospholipid present in the reconstituted liposomes (3). In the case of the proton pump of mitochondria both protein and phospholipid requirements are much more complex involving at least 8 polypeptide chains (cf 4) and 2 phospholipids (5,6).

The Na^+ pump of the plasma membrane was successfully reconstituted in several laboratories including our own by the cholate-dialysis procedure which was first described for the reconstitution of the mitochondrial proton pump (5). The Na^+K^+ ATPase from dog kidney (7) and from the rectal gland of

the dog fish (8) incorporated into liposomes, catalyzed Na^+ translocation dependent on externally added ATP. Thus these reconstituted vesicles are "inside out" with the ATP side of the pump on the outside and the ouabain side on the inside. The consequence of this reconstitution is that Na^+ translocation as well as ATP hydrolysis by the properly oriented ATPase is insensitive to ouabain added from the outside.

We have now found that reconstitution of the ATPase from electric eel by the sonication procedure (9) yields vesicles that catalyze ATP-dependent Na^+ transport at a rate which is 10 to 20 times higher than that of vesicles reconstituted by the cholate-dialysis procedure.

Materials and Methods

Egg phosphatidylcholine (chromatographically pure) was purchased from Grand Island Biological Co. Phosphatidylethanolamine was purified from soybean phospholipids as described previously (10). The ATPase activity of electric eel was purified by Dr. Y. Kuriki by a modification of the procedure of Albers et al (11). The specific activity was 8 to 10 μmoles ATP hydrolyzed/min/mg protein. When an ATP regenerating system was used for assay, the activity was about 20 μmoles/min/mg protein.

Reconstitution was usually performed as follows: 4 mg of phospholipids were freed of organic solvent by drying with a stream of nitrogen. About 200 μg of enzyme was added in 0.2 ml of a buffered (pH 7.4) solution containing 100 mM KP_i, 40 mM NaP_i, 2 mM $MgSO_4$ 83 mM sucrose, 3.3 mM β mercaptoethanol and 10 mM imidazole. The last three ingredients were components of the stock enzyme solution. This mixture was exposed to sonication as described (9) for 30 min except that the sonication bath water was kept at about 5° by addition of ice at intervals

Na^+ translocation was measured as follows: to 60 μl of $^{22}NaCl$ in the same buffer used above were added 5 μl of either 0.2 m Tris ATP or H_2O. After a few minutes equilibration at 30°, the reconstituted vesicles (50 μl) were added and the mixture incubated at 30°. After 2 min, a 100 μl sample was removed and placed on a Dowex 50 column with a void volume of about 1.2 ml. With 0.5 ml of

0.25 M sucrose added drop-wise, the vesicles were allowed to enter the column and the eluate was discarded. Then the vesicles were eluted with 3 ml of 0.25 M sucrose. A sample (1 ml) of this fraction was dried and counted in a Nuclear-Chicago flow counter. To correct for quenching by sucrose the ^{22}Na standards were counted after drying samples in 1 ml of 0.25 M sucrose.

Results

Method of reconstitution of ATP-dependent Na^+ pump - Early attempts of the reconstitution of the Na^+K^+ pump in our laboratory with the cholate-dialysis procedure yielded vesicles with Na^+ translocation activities of 10 nmoles/min/mg or less, similar to those obtained by others (5,6). An alternative method that avoids the use of detergent is the sonication procedure (9) which had proved to be much more effective with bacteriorhodopsin. As shown in Table I, the Na^+ pump can also be reconstituted by this procedure although prolonged sonication (30 min) was required for optimal results. In this particular experiment, sonication for 5 or 10 minutes gave relatively high values. However, considerable variability was observed with these short sonication periods, depending on the fluid volume, shape of test tubes and energy output of the instrument. The 30

Table I

Effect of sonication time on Na^+ translocation by reconstituted vesicles

Reconstitution was performed as described under Materials and Methods with a 4:1 ratio of phosphatidylethanolamine and phosphatidylcholine.

Sonication exposure in minutes	$^{22}Na^+$ translocation natoms/min/mg protein		
	- ATP	+ ATP	Δ
5	80	175	98
10	71	158	87
20	61	206	145
30	69	248	179
60	61	212	151

minute sonication procedure, which yielded translucent vesicles, was quite reproducible. One hour sonication gave vesicles with consistently lower activity. It is important to obtain high values for ATP-dependent Na^+ transport because the control values without ATP were relatively high and made it more difficult to obtain quantitative evaluation of the active transport.

Effect of phospholipid composition of Na^+ translocation - As shown in Table II, vesicles reconstituted with an excess of phosphatidylethanolamine showed the highest transport activity, catalyzing about 0.2 μmoles Na^+/min/mg protein. With this particular preparation of soybean phosphatidylethanolamine (average of 2 experiments) inclusion of small amounts of phosphatidylcholine caused some decrease in the transport rate. With other preparations the presence of phosphatidylcholine, in amounts of 10 to 20% of total phospholipids, gave optimal results. Most experiments were therefore performed with vesicles reconstituted with a 4:1 phosphatidylethanolamine : phosphatidylcholine ratio. Inclusion of an acidic phospholipid such as phosphatidylserine at various concentrations did not improve the Na^+ transport rates.

Table II

Effect of phospholipid composition on Na^+ translocation in reconstituted liposomes

Reconstitutions and assays were performed as described under Materials and Methods.

Phospholipid composition	$^{22}Na^+$ translocation natoms/min/mg protein		
	- ATP	+ ATP	Δ
Phosphatidylcholine (PC)	55	98	43
PC:PE (1:1)	56	130	74
PC:PE (1:2)	69	141	72
PC:PE (1:3)	76	187	111
PC:PE (1:4)	72	226	154
Phosphatidylethanolamine (PE)	95	295	200

Table III

Effect of ionophores and inhibitors on the reconstituted Na^+ pump

Reconstitution and assays were performed as described under Materials and Methods. In experiment 1, valinomycin and the inhibitors were added to the assay mixture. In experiment 2, gramicidin and ouabain were added prior to sonication.

Additions	$^{22}Na^+$ translocation natoms/min/mg protein		
	- ATP	+ ATP	Δ
Exp. 1			
None	177	439	262
+ 0.2 µg valinomycin	220	586	366
+ 10 µg rutamycin	233	415	182
+ 2 mM ouabain (external)	248	443	195
Exp. 2			
None	180	436	256
+ 0.2 µg valinomycin	245	553	308
+ 5 µg gramicidin (internal)	360	360	0
+ 2 mM ouabain (internal)	178	159	0

Time course and effect of ionophores and of ouabain on Na^+ translocation - Gramicidin (5 µg) added during reconstitution greatly increased the control values of Na^+ uptake in the absence of ATP, and obliterated the active transport (Table III). Ouabain present at 2 mM during sonication also obliterated active Na^+ transport, but inhibited only slightly when added to the assay mixture. Rutamycin at the concentration tested was slightly inhibitory. Valinomycin (0.2 µg) consistently increased the rate of Na^+ translocation. A time course of the rate of Na^+ translocation is shown in Fig. 1 in the presence and absence of valinomycin. As in the case of the Ca^{++} pump (1) the rate of ion uptake is rapid for a few minutes and falls off quite sharply after 5 minutes. A stimulatory effect of valinomycin was more pronounced during the first 5 minutes of Na^+ uptake, the extent of stimulation varying between 20% to 50% in different experiments.

trophoresis, only a single protein band was observed as shown in Fig. 4 (A). By comparing its mobility with those of marker protein (bovine serum albumin, monomer of bovine liver catalase, ovalbumin and bovine carboxypeptidase A), an apparent molecular weight of about 46 kilodaltons was estimated for P-$450_{11\beta}$. As also in Fig. 4, when a mixture of equal amounts of P-$450_{11\beta}$ and P-450_{scc} was run under the same conditions, two distinct bands were separated clearly. When the preparation was carboxymethylated in the presence of 6 M guanidine-HCl and 100 mM 2-mercaptoethanol, the sedimentation equilibrium data gave a molecular weight of 43 kilodaltons which compared with the value of P-450_{scc} (46 kilodaltons).

The present study provides direct evidences that separate cytochrome P-450 species catalyzes the cholesterol side-chain cleavage and steroid 11β-hydroxylation, respectively. P-$450_{11\beta}$ and P-450_{scc} are similar in physico-chemical properties such as in spectral properties and in molecular size per heme, but are different in properties with respect to its specificity either in substrate binding or in catalytic activity. During purification and storage P-$450_{11\beta}$ is less soluble than P-450_{scc} and requires more hydrophobic environment such as in the presence of a non-ionic detergent viz., Tweens, Triton X-100 or Emulgen 220. This evidence may suggest possible difference in hydrophobic nature of the two hemoproteins.

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(usually less than 5% in our hands), with most of the ouabain-sensitive ATPase activity remaining unincorporated. On the other hand, we have found that efficiency of Na^+ translocation in the reconstituted vesicles measured by the Na^+/ATP ratio in the presence of ouabain was high (over 3). Thus the question of the possible involvement of a proteolipid in the operation of the Na^+ pump must remain unanswered until further resolution of the Na^+K^+ ATPase preparation can be achieved. All preparations thus far tested contain a component that moves rapidly in SDS acrylamide gels and may in fact be a proteolipid. This problem is of considerable biological interest, particularly in view of the demonstration of an inefficient Na^+K^+ pump in Ehrlich ascites tumor cells (14).

Finally, attention should be drawn to the method of analysis of Na^+ translocation. The procedure using an ion exchange column was developed for reconstituted liposomes (15) which yield variable results by the millipore filtration method. The procedure is suitable for separation of anions (with Dowex formate) and for larger particles including mitochondria and cells.

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