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Interaction of kidney (Na^+–K^+)-ATPase with phospholipid model membrane systems

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SUMMARY

The transport-related, Na^+, K^+-activated ATPase from kidney can be solubilized by treatment with the non-ionic detergent, Lubrol. The Lubrol-solubilized enzyme does not require phosphatidylserine for activation.

This enzyme preparation binds to positively charged, but not to negatively charged, liposomes. It also binds to sphingomyelin liposomes which are formally neutral in charge, but not to lecithin. The bound enzyme can be extracted with solutions of high ionic strength.

To further understand the nature of specific lipid–protein interactions in biological membranes, we have investigated the association of various proteins with the well known "liposomal" model membrane systems[1-3]. Bovine serum albumin was shown to bind to phospholipid membrane systems at a pH value below its isoelectric point[1]. This binding was dependent on the ionic strength, the membrane charge and the protein conformation, and appeared to evidence both electrostatic and hydrophobic character. Spectrin, a membrane protein from erythrocytes, was found to bind both to negatively and positively charged liposomes; its mode of binding appeared to be primarily hydrophobic in nature[2]. In an attempt to extrapolate this approach to a functionally more interesting protein, we chose to investigate whether phospholipid liposomes would bind the transport-related (Na^+–K^+)-ATPase.

Redwood *et al.*[4] reported a decrease in membrane resistance upon addition of a bacterial ATPase to a model lipid bilayer system. However, this system makes it difficult to study the nature of the membrane–enzyme complex and to directly measure any binding which might occur. It was felt that demonstration of such binding might lead to further insight regarding the nature of the interaction of the enzyme with membranes *in*

vivo as well as providing a highly interesting lipoprotein model membrane system. This paper describes such experiments and, in addition, presents some points relevant to the extraction and purification of the transport ATPase from kidney.

In early experiments, we prepared (Na^+–K^+)-ATPase from frozen young rabbit kidneys (Pel Freeze Biochemicals) following the method of Tanaka and Strickland[5] as modified by Towle and Copenhaver[6]. Generally, the enzyme obtained after $(NH_4)_2SO_4$ fractionation was of reasonably good activity (2.9 μmoles P/mg protein per h) and was stabilized in 50 mM Tris buffer (pH 7.2 at 3 °C) containing 20% glycerol (v/v). We verified that the enzyme prepared by this procedure is Na^+, K^+ activated, ouabain inhibited, and dependent on added phosphatidylserine for activity. However, we found this preparation technique difficult to repeat, frequently resulting in drastic losses of activity, and we therefore investigated other methods for preparation of the solubilized enzyme.

Hokin and his coworkers[7,8] have solubilized brain ATPase with the non-ionic detergent, Lubrol. After further experimentation, a modification of the method of Uesugi *et al.*[7] was adopted and this method reproducibly gave us a Lubrol-soluble enzyme of good activity from kidney microsomes.

Enzyme activity in the Lubrol extracts was determined in duplicate tubes by the following method[6,9]: To 1.0 ml reaction mixture containing 80 mM imidazole–HCl (pH 7.0) 2.4 mM $MgCl_2$, 2.4 mM Na_2ATP, 20 mM KCl and 100 mM NaCl, 0.5 or 1.0 ml of protein sample was added to start the reaction. After 20 min at 37 °C, the reaction was stopped by adding 2.0 ml of 5% cold trichloroacetic acid (in chloroform–methanol, 1:1, v/v) with vigorous mixing. Phosphate was determined on 1.0 ml of the supernatant by the Fiske and SubbaRow method[10]. Protein in all the samples was determined by the Lowry method[11], as modified by Medzihradsky[9].

Positively charged liposomes were prepared by suspending 24.0 mg lecithin, 1.8 mg cholesterol and 2.4 mg stearylamine in 2.0 ml buffer (pH 7.0 buffer, used throughout the following experiments, contained 20 mM imidazole–HCl), 40 mM NaCl and 1 mM EDTA. To test the binding of the enzyme, 1.0 ml of enzyme was incubated with 2.0 ml of the above liposomes for 30 min at 3 °C. The suspension was then centrifuged at 100 000 × *g* for 30 min. The supernatant was separated with a pipette and analysed for the unbound enzyme. The pellet was washed once, suspended in 2.0 ml of the same buffer and analysed for the bound enzyme. In later experiments, in order to extract the bound enzyme, the pellet was washed and incubated with 2.0 ml of 1.1 M or 2.0 M NaCl–20 mM imidazole–HCl (pH 7.0)–1.0 mM EDTA for 30 min at 3 °C. The suspension was centrifuged at 100 000 × *g* for 30 min. This pellet was suspended in 2.0 ml of buffer, and the supernatant and pellet analysed for ATPase activity to determine the displaced and the bound enzyme.

Table I presents data indicating that the kidney enzyme can be purified and solubilized by this technique. As indicated in this table, the kidney microsomal enzyme is solubilized by Lubrol, but repeated extractions with the detergent led to solubilization of additional enzyme, and in fact after the fourth extraction the solubilized enzyme has higher specific activity than any of the earlier extracts. The assays with diluted enzyme

TABLE I

SPECIFIC ACTIVITY OF (Na^+–K^+)-ATPase AT DIFFERENT STAGES OF EXTRACTION FROM RABBIT KIDNEYS

Fraction	*Total activity (μmoles P/h ×10⁻³)*	*Total protein (mg)*	*Specific activity (μmoles P/mg protein per h)*
Microsomes	1140.0	750.0	16.0
NaI-treated microsomes	762.0	250.0	32.1
Lubrol extract I	10.3	24.0	4.5
Lubrol extract II	22.1	29.0	8.2
Lubrol extract III	19.3	25.0	8.3
Lubrol extract IV	9.0	7.3	13.0
Diluted Lubrol extract II*	93.6	34.0	29.2
Diluted Lubrol extract III*	98.4	27.0	38.4

* Diluted 10 × with deionized water.

demonstrate inhibition of the enzyme by high concentrations of Lubrol as reported earlier[7]. Further work showed that the Lubrol-extracted enzyme from kidney microsomes is the characteristic, Mg^{2+}, Na^+, K^+-dependent, ouabain-inhibited enzyme. However, this preparation was not dependent on phosphatidylserine for activity (Table II), although we had verified that the deoxycholate-solubilized enzyme[6] from kidney does require phosphatidylserine.

TABLE II

LACK OF PHOSPHATIDYLSERINE REQUIREMENT FOR LUBROL-SOLUBILIZED KIDNEY ATPase

Concentration of phosphatidylserine (mg/ml)	*Ouabain presence (10 mM)*	*Total activity (μmoles P/mg protein per h)*	*Ouabain-sensitive ATPase (μmoles P/mg protein per h)*
3.0	–	2772	1701
3.0	+	1071	
None	–	3024	1890
None	+	1134	

Experiments attempting to demonstrate and study binding of the enzyme to phospholipid liposomes were not successful initially. However, it was found that tight binding occurred when the liposomes were constituted so as to carry a net positive charge. Table III summarizes these experiments. Binding of both the deoxycholate-extracted enzyme and the Lubrol-solubilized enzyme was found with the stearylamine–lecithin–cholesterol liposomes. Very little binding could be demonstrated with liposomes of either neutral or net negative charge[3]. In addition, however, it is clear from the data that sphingomyelin liposomes, or liposomes containing sphingomyelin, have some affinity for

TABLE III

BINDING OF OUABAIN-SENSITIVE ATPase TO LIPOSOMAL PREPARATIONS

*Liposome composition**	*Total enzyme activity***	
	Lipid bound	*soluble*
No lipid (control)***		17.4
Lecithin (12.0): cholesterol (0.9): stearylamine (1.2)***	10.8	0.0
Lecithin (12.0): cholesterol (0.9): phosphatidylserine (3.4)***	0.6	15.6
Lecithin (12.0): cholesterol (0.9): sphingomyelin (3.1)***	6.6	12.0
Sphingomyelin (30.0)***	4.8	9.6
Lecithin (12.0): cholesterol (0.9): stearylamine (1.2)†	87.6	1.2
No lipid (control)†	–	109.2

* Given as a suspension of mg lipid/ml of buffer.

** Expressed as total ouabain-sensitive μmoles P/mg protein per h for a given enzyme preparation under the standard assay conditions.

***Enzyme prepared as described by Towle and Copenhaver[7].

†Enzyme extracted as described by Uesugi *et al.*[4].

the enzyme. This is in distinct contrast to liposomes containing only phosphatidylcholine, even though the ionic moieties in these two phospholipids are identical.

Experiments attempting to extract the bound enzyme from liposomes with solutions of high ionic strength are shown in Table IV. It was found that about 50% of the ouabain-sensitive ATPase could be extracted from liposomes with 1.1 M NaCl and up

TABLE IV

DISPLACEMENT OF LIPOSOME-BOUND ATPase BY EXTRACTION WITH SOLUTIONS OF HIGH IONIC STRENGTH*

The enzyme was extracted as described by Uesugi *et al.*[4].

Extraction medium	*Fraction*	*Total enzyme activity (μM P/h)*	*Total protein (mg)*	*Specific activity (μM P/mg protein per h)*
None	Stock enzyme solution	2205	2.43	9.5
Buffer	Unbound enzyme	252	2.07	1.2
	Bound enzyme	936	1.08	9.1
	Extracted enzyme	24	0.08	3.4
Buffered 1.1 M NaCl	Unbound enzyme	144	2.04	0.74
	Bound enzyme	528	0.39	14.2
	Extracted enzyme	492	0.55	9.4
Buffered 2.0 M NaCl	Unbound enzyme	198	2.07	1.0
	Bound enzyme	421	0.32	14.0
	Extracted enzyme	888	0.70	13.3

* ATPase bound to stearylamine liposomes as in Table IV.

to 80% with 2.0 M NaCl. In each case, the specific activity of the extracted enzyme was somewhat increased over that of the buffered control or stock enzyme solution.

In regard to the use of the non-ionic detergent, Lubrol, for solubilization of the kidney ATPase, it should be noted that the requirement for phosphatidylserine is lost when this detergent is used. Because of this fact, we suggest that Lubrol probably solubilizes the enzyme and phosphatidylserine together in an active complex, while deoxycholate separates the two. Further, it appears that the required phospholipid is then incorporated into the liposome–ATPase complex when the deoxycholate-solubilized enzyme is bound to the model membranes. This suggests that the phosphatidylserine site on the enzyme is not buried or otherwise altered by binding of the enzyme to liposomes.

These studies, then, indicate that while it is possible to demonstrate tight binding of a transport-related ATPase to liposomes, the strongest interaction depends on the presence of a non-physiological lipid with net positive charge, *i.e.* stearylamine. Since there are no known naturally occurring lipids which are positively charged at physiological pH, it is possible that the interaction reported here might be of little physiological significance, simply reflecting an electrostatic interaction between particles of opposite charge. Additionally, in the case of the deoxycholate-solubilized enzyme it might be argued that the detergent simply lends negative charge to the enzyme particle and that these additional negative sites create loci for binding to positively charged liposome surfaces. Since it is not known how many molecules of detergent are bound per enzyme molecule, it is difficult to estimate the significance of such a contribution by the detergent. However, the fact that enzyme solubilized by the non-ionic detergent, Lubrol, also binds only to positive liposomes suggests that the binding is probably due to charged loci on the enzyme molecule itself. It should be noted that, if nothing else, this system represents a lipoprotein membrane with the bound protein catalyzing a reaction of great significance to the natural function of cellular membranes, so that it offers an opportunity to study the action of ATPase as it is bound at a lipid–water interface.

In addition, the ability of sphingomyelin to bind the enzyme suggests a physiological rationale for the data. Since the ionic moieties of sphingomyelin and lecithin are the same, the ability of the former to bind the ATPase must result from a different molecular configuration in the phospholipids at the liposome surface. In studies of surface potentials of phospholipids, Shah and Schulman[12] found that sphingomyelin generated a surface potential indicative of a net positive charge at the lipid–water interface. Our data suggest that such a positively charged surface would then lead to binding of the transport ATPase. This, of course, further suggests the possibility that sphingomyelin in natural cellular membranes could conceivably be related to the incorporation of transport ATPase into the cellular membranes. Although highly speculative, this notion does suggest a specific membrane function for sphingomyelin. Furthermore, there is some indication that sphingomyelin is present in highest concentrations in tissues with high ion transport activity, *e.g.* the nervous system[13], as is the transport ATPase.

In regard to the observation of Redwood *et al.*[4] that a bacterial ATPase can increase the electric conductance of a phosphatidylcholine bilayer, our data suggest that

such effects may be due either to a transient interaction of the protein at the lipid–water interface, or to specific molecular properties of the *Streptococcus fecalis* enzyme which are different from those of the kidney ATPase and which allow it to bind to a neutral bilayer. It is not possible to demonstrate direct formation of a complex in the simple bilayer system used by Redwood *et al.*[4] and it is conceivable that effects on ion permeability could be induced by alterations in the membrane properties, *e.g.* by disruption of ionic or water structures at the membrane interface, without actual formation of a stable lipoprotein complex. Since the transport enzyme is tightly membrane bound *in situ*, direct demonstration of such a complex is an important step in studying such reconstructed lipoprotein membranes.

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