Purification and Reconstitution of Na^+, K^+-Adenosinetriphosphatase

By

Douglas E. Mullinex

B.S., Kansas State University, 1986

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

Approved by:

David G. Nuttall

Major Professor

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Purification and Reconstitution of Sodium-Potassium Adenosinetriphosphatase

Chapter 1

Literature Review

Introduction

Cell membranes are made up of lipids and proteins. These components have several functions ranging from barrier functions to those of pumps and gates. The ability of membranes to act as barriers is what allows cells to remain distinct from other cells; the gates and pumps allow them to control their own environment to some degree. Sodium-potassium adenosinetriphosphatase, (Na^+, K^+-ATPase, EC 3.6.1.3), an integral protein found in eukaryotic surface membranes, is required for the maintenance of intracellular ion concentrations, primarily Na^+ and K^+.

Na^+, K^+-ATPase

The Na^+, K^+-ATPase and its molecular activity have been examined by several laboratories (1-7). The ATPase, commonly known as the Na^+-K^+ pump, consists of three subunits, the alpha subunit (MW 98,000-110,000 daltons), the beta subunit (MW 55,000 daltons), and the gamma subunit (MW 10,000 - 12,000 daltons)(8); a schematic drawing is shown in Figure 1. The alpha subunit appears to be the catalytic entity of the enzyme since the Na^+ binding site, the K^+ binding site, and the ATP binding site are all located on this subunit (9). The purpose of the glycosylated beta subunit is unknown; however, it must be present for activity. Based on studies involving the titration of active sites with radiolabeled molecules, the working unit of the protein appears to contain 2 alpha and 2 beta subunits (10). Little is known about the location of gamma subunit, or about its function.

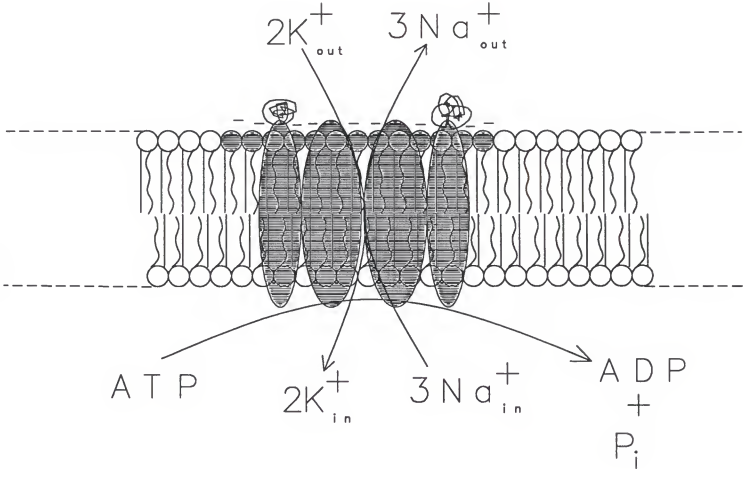
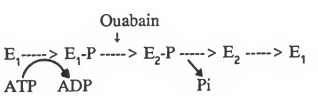

2K+ out
3Na+ out
ATP
2K+ in
3Na+ in
ADP
+
Pi

Figure 1. Diagrammatic representation of the structure and function of Na^+, K^+-ATPase. The larger (alpha) subunit of the ATPase contains the extracellular ouabain binding site, as well as the intracellular ATP binding site. The smaller (beta) subunit is glycosylated at the extracellular surface. Hydrolysis of 1 ATP is coupled to the outward transport of 3 Na^+ ions and inward transport of 2 K^+ ions. Negatively charged lipids (with black head groups) are shown at the extracellular face of the Na^+, K^+-ATPase lipid annulus.

The generally accepted kinetic scheme for the enzyme is as follows: 3 Na^+ bind on the intracellular side of the E_1 conformational state of the enzyme. An ATP-Mg^{2+} complex binds to the ATP binding site of the E_1 and is hydrolyzed, leaving a high energy acyl phosphate bond and the enzyme in the E_1-P state. This changes the conformation to the E_2-P state allowing release of the Na^+ into the extracellular fluid. The K^+ binding sites are now exposed and 2 K^+ ions bind to the extracellular side. This binding causes the acyl phosphate bond to hydrolyze, releasing a free phosphate and allowing the enzyme to return to the original E_1 state, releasing the bound K^+ on the intracellular side of the membrane.



Based on measurements of intravesicular pH in reconstituted vesicles, there is some evidence that protons are also transported into the cell during the cycle (11). This would imply that 3 Na^+ are exchanged for 2 K^+ and 1 H^+. Ouabain, a cardiac glycoside (Fig. 2), is generally believed to inhibit the enzyme at the step where E_1-P is converted to E_2-P. Other naturally-occurring inhibitors include nonesterified fatty acids and lysophospholipids (12).

Purification of Na^+, K^+-ATPase

Historically, several methods have been used to isolate and purify Na^+, K^+-ATPase (13-19). Tissues used have included kidney from various mammals, bovine brain, electric organ from Torpedo marmorata, anal gland from Squalus acanthias, nasal salt gland from Anas platyrhynchos, cardiac tissue from calf, and red blood cells (3,13-16,20-23). In the most commonly employed scheme, differential

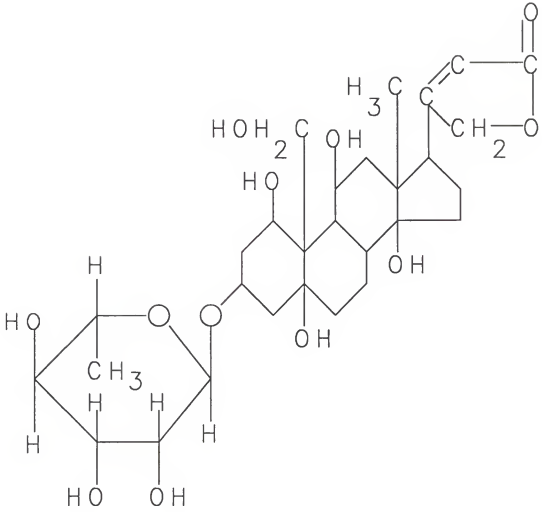

O
C
C
H 3 C
C
HOH 2 C
OH
CH 2 O
HO
OH
H
HO
O
O
OH
CH 3
H
H
H
H
HO
OH

Figure 2. The structure of ouabain, a potent inhibitor of Na^+, K^+-ATPase.

centrifugation is used to purify a microsomal fraction; Na^+, K^+-ATPase is then purified from other membrane proteins by one of two methods. One, known as "positive purification" involves the extraction of the ATPase from the membrane leaving other proteins and lipids behind (17). This method involves using a non-denaturing, nonionic detergent, such as Lubrol, and purification of the supernatant through several steps. The other method, known as "negative purification", involves eliminating all other proteins and leaving behind a membrane containing only lipid and Na^+, K^+-ATPase (14,18). This method involves a harsher detergent treatment, usually with SDS or deoxycholate. These techniques will be discussed in more detail and compared in Chapter 3.

Reconstitution of Na^+, K^+-ATPase

Reconstitution of proteins, particularly carrier proteins, has provided investigators with a powerful tool enabling them to study individual membrane components as never before. Reconstitution takes advantage of the hydrophobic nature of lipids and integral membrane proteins. Reconstitution involves the solubilization of proteins, often in the presence of lipids, by a detergent to create small detergent-lipid or detergent-protein complexes. The subsequent removal of the detergent by dialysis or dilution causes the lipids to aggregate forming bilayer vesicles. Proteins reconstituted into the bilayer membrane of a liposome produce a structure known as a proteoliposome. Proteoliposomes may be formed by several methods, including detergent dialysis, detergent dilution, sonication, freeze-thaw sonication, and gel filtration.

Detergent dialysis was the original method of reconstitution used (24-26). This method involves mixing the lipid, protein, and detergent together at the proper concentration and dialyzing for several days. The proteoliposomes "self-assemble" as the detergent is removed. Detergent dilution works in much the

same way but, instead of removal by dialysis the detergent is rapidly diluted to a concentration below its critical micelle concentration (cmc), causing the hydrophobic proteins and lipids to assemble (27).

Reconstitution by sonication simply involves the sonication of lipid and solubilized protein together for several minutes using a bath sonicator. The advantage to this method is that detergent inactivation of the enzyme is minimized. Disadvantages include exposure of the protein to sonication and thermal inactivation (27).

Freeze-thaw sonication consists of first preparing liposomes, usually by sonication, and then adding the desired protein followed immediately by freezing at dry ice temperatures. Thawing is then done slowly at room temperature. A very short period of sonication (30 sec) is then required. This method apparently fuses small liposomes into larger liposomes and as this occurs the protein is incorporated (27). Once again the primary advantage is the prevention of detergent inactivation.

Another method of reconstitution is by gel filtration. Detergent, protein, and lipid are added together and passed over a column matrix that will attract the detergent, causing the formation of liposomes and proteoliposomes. By controlling pore size of the column one can cause liposomes containing protein to elute in the void volume (28).

The reconstitution of Na^+, K^+-ATPase has primarily involved dialysis and freeze-thaw methods (19,27,29,30); however, other methods, such as gel filtration, have been used (31).

Lipid Interactions With Na^+, K^+-ATPase

Several investigators have examined the minimal lipid requirements for optimal Na^+, K^+-ATPase activity (2,20,32-35). This work, at least in the beginning,

centered on the minimal phospholipid requirements but has more recently included sulfatide and ganglioside requirements.

Phospholipids

One of the primary means of studying minimal lipid requirements has been to reconstitute the Na^+, K^+-ATPase in liposomes of known lipid content. Using this technique, Hilden and Hokin showed complete reactivation of Na^+ and K^+ transport in vesicles containing ATPase from the rectal gland of dogfish, and only one phospholipid, phosphatidylcholine (PC) (2). Their method involved preparation of vesicles using phospholipid, cholate, and a Na^+, K^+-ATPase preparation that included the endogenous membrane. Using a discontinuous sucrose gradient they were able to completely replace the endogenous phospholipids with phosphatidylcholine. The complete reactivation which they observed would indicate that the only required phospholipid is phosphatidylcholine.

Other researchers have found that phosphatidylcholine alone is not sufficient for full activity. These researchers indicate the need for a negatively charged phospholipid to obtain full activation (32,36-38). Kimelberg and Papahadjopoulos determined that phosphatidylserine (PS) or phosphatidylglycerol (PG) are required for full recovery of enzyme activity. Their experiments, using dispersed lipid added to solubilized ATPase purified from rabbit kidneys, showed a marked increase in activity when PS or PG was added; PS being the more effective of the two. The addition of PC resulted in little or no activation. Interestingly 3-sulfogalactosylceramide or sulfatide, a negatively charged glycosphingolipid (Fig. 3), activated the enzyme in only one of five trials (32). According to these investigators (32), the reason for the increases in activity in PS- and PG-containing

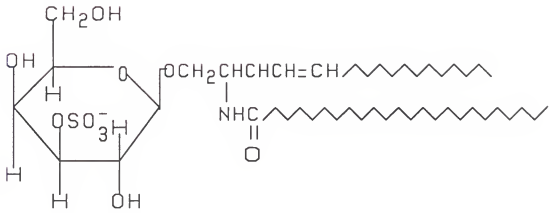

CH2OH
OH
O
OCH2CHCHCH=CH
NHC
O
OSO3H
H
H
OH

Figure 3. The structure of 3-Sulfogalactosylceramide (sulfatide).

samples is due to the significant discrimination shown by these two phospholipids for K^+ permeability over Na^+ permeability. They believe that PS, and PG in membranes lacking PS, may serve as a cation specific site for the ATPase.

Physical studies of Na^+, K^+-ATPase and its interactions with lipids are consistent with the idea that negatively charged lipids may be involved with ATPase function. Esmann and coworkers, using electron spin resonance (ESR), have studied the interaction of lipids with the Na^+, K^+-ATPase (39). They determined that Na^+, K^+-ATPase from the rectal gland of Squalus acanthias interacted with lipids in the following order: cardiolipin > phosphatidylserine > phosphatidic acid > phosphatidylglycerol = phosphatidylcholine = phosphatidylethanolamine = androstanol. This work demonstrated that a negatively charged lipid may be found in the boundary lipid layer surrounding the ATPase. Cardiolipin does not occur in eukaryotic plasma membranes and PS is only found on the inner half of the plasma membrane (43). Thus these lipids are probably not physiologically relevant as modulators of cation specificity at the cell surface. However, the indication is that negatively charged phospholipids may be located preferentially next to the ATPase. However, the fact that PG shows no preference indicates that something other than electrostatic charge may also be involved, since PS and PG have the same net charge. Other effects involved may be hydrogen bonding, head group hydration, or other chemical effects. Despite these other factors, the evidence implicating PS as the preferred phospholipid, further suggests that there is a role for a negatively charged lipid in the normal function of Na^+, K^+-ATPase.

In the same study, Esmann and colleagues demonstrated the presence of a motionally restricted spin-labeled component in Na^+, K^+-ATPase membranes. This component was calculated to contain approximately 66 lipid sites per Na^+,

K^+-ATPase molecule. Previous lipid-protein titrations had yielded a figure of 63 lipid sites per molecule (40). If one considers that the functional moiety of the ATPase is a dimer, Esmann et al. has calculated that between 57 to 72 lipid molecules would be required to form an annulus around a Na^+, K^+-ATPase dimer. Thus the experimental data are consistent with the theoretical calculation implying a lipid annulus of 50-70 phospholipids surrounding the ATPase.

Sulfatides

Karlsson has hypothesized that sulfatide molecules, located as an annulus around the ATPase, act as a cofactor in Na^+, K^+-ATPase function by providing a high affinity site for the attraction of the needed K^+ ions (9). Sulfatide is a likely candidate for the cofactor lipid for several reasons. It has a negative charge, it is restricted to the outer side of plasma membranes, sulfate groups show a strong electrostatic binding of K^+ over Na^+ ions (9), and Na^+, K^+-ATPase activity and sulfatide in biomembranes have a definite correlative relationship (Fig. 4, ref. 20).

Much of the work directly correlating sulfatide content with Na^+, K^+-ATPase activity and sodium flux has been done by Zambrano and coworkers (22,33,34,41). These workers also demonstrated that porcine ATPase activity can be inhibited by sulfatide hydrolysis (41). More recently, these workers examined the role of sulfatide in K^+ activated phosphatase activity, the reaction which causes the conformational change from E_2-P to E_1 (34). Using arylsulphatase purified from porcine kidney cortex to hydrolyze sulfatide, it was demonstrated that as sulfatide was hydrolyzed, K^+-dependent phosphatase activity decreased to a point where almost all (98%) activity was eliminated. This effect was apparent even at very high K^+ concentrations (100 mM). The same effect was shown, though to a smaller degree, if sulfatide was microdispersed in the assay medium before the addition of arylsulfatase. However, if sulfatide was added after arylsulfatase treatment, then reactivation occurred up to a level of 90%.

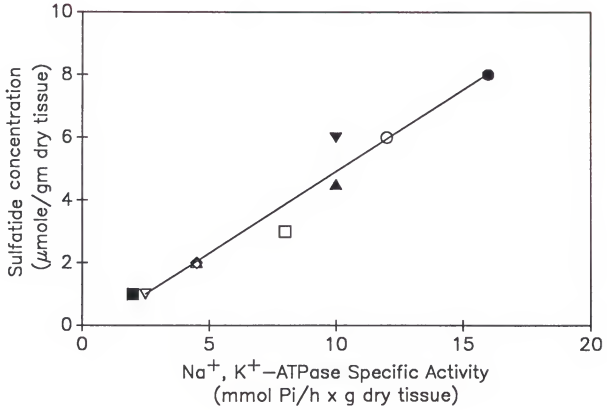

Sulfatide concentration
(μmole/gm dry tissue)
10
8
6
4
2
0
0
5
10
15
20
Na+, K+–ATPase Specific Activity
(mmol Pi/h x g dry tissue)

Figure 4. The relationship between Na^+, K^+-ATPase activity and sulfatide concentration in various tissues. Note the data for erythrocytes has been multiplied to keep all the points within the same range (from Karlsson, 1976). Open circles, salt gland of herring gull; closed circles, salt gland of eider duck; open triangles, domestic duck on freshwater; closed triangles, domestic duck on saltwater; open squares, rectal gland of spiny dogfish; closed squares, electric organ of Torpedo marmota; open inverted triangles, bovine kidney medulla; closed inverted triangles, bovine brain gray matter; open diamond, human erythrocytes. Least squares linear regression analysis (solid line) gave a regression value (r) of 0.98.

It is important to note that the investigators performed identical experiments with various phospholipids in an attempt to show reactivation of the K^+-dependent phosphatase activity and were not successful. While this is not surprising, due to the specificity of the enzyme arylsulfatase, it does suggest that sulfatide, rather than phospholipid, is the required lipid for K^+ activated phosphatase activity.

The same laboratory has also examined the role of sulfatide in phosphoenzyme formation, E_1 to E_1-P, and in ouabain binding (33). In this report they demonstrated, by measuring steady state phosphorylation, the inability of arylsulfatase to inhibit formation of the phosphoenzyme. However, once the phosphorylated enzyme was formed it was unable to complete the cycle in the presence of arylsulfatase. Addition of sulfatide to the assay medium following arylsulfatase treatment allowed dephosphorylation to occur. Addition of phospholipid was not effective.

Inhibition of ouabain binding was also examined (33). The addition of arylsulfatase inhibited ouabain binding up to 96%. Again the addition of sulfatide to the medium reversed the effect; if added before arylsulfatase then 47% of binding was preserved, if added after arylsulfatase treatment then 28% of binding was reactivated. The role of sulfatide in ouabain binding is not clearly understood. Data from this work and previous work would suggest that the role of sulfatide in Na^+, K^+-ATPase activity occurs at the step immediately following the location in the cycle where ouabain is believed to act. While this may not be a problem in understanding the normal activity of the Na^+, K^+-ATPase, it could play a very important role in future pharmacological studies.

Additional biophysical evidence implicating sulfatide in Na^+, K^+ transport is provided by Rintoul and Welti, who examined the formation of sulfatide

domains in liposomes containing dielaidoylphosphatidylcholine (DEPC). These workers determined that monovalent cations, in physiological concentrations, have an effect on lipid domain formation in mixed sulfatide/PC vesicles (42). Using steady-state fluorescence anisotropy, found that a sulfatide domain had a higher transition temperature in 100 mM K^+ than in a similar concentration of Na^+. This evidence clearly shows an effect of K^+ on sulfatide distribution in the model membranes. The authors also demonstrate that this effect is only seen with hydroxy fatty acid-containing sulfatides.

Gangliosides

Ganglioside interactions with Na^+, K^+-ATPase have also been examined, since gangliosides are also negatively charged glycosphingolipids occurring on the outer leaflet of plasma membranes (35). Leon *et al.* show an activation of rat brain ATPase activity after incubation with several gangliosides, including G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, with G_{M1} being the most effective, causing an activation of 43% when compared to the original activity. They also examined the binding of G_{M1} which was compared with enzyme activity. The results indicate that only bound G_{M1} activated the ATPase. The amount needed for maximal activation was fairly small indicating that the G_{M1} was binding in specific sites, perhaps in regions containing the Na^+, K^+-ATPase.

Discussion

Na^+, K^+-ATPase has been actively studied for several years. In that time much information on the physical properties as well as the molecular mechanism of the ATPase itself has been accumulated. The interests of this laboratory, however, and the focus of this thesis, are not the protein itself, but rather the interaction of the protein with the lipids surrounding it. Specifically, we are interested in whether the protein requires particular lipids for its normal activity.

Research by others in this area has led to conflicting conclusions. Phospholipids, the most common lipids found in membranes, have attracted the attention of several researchers. Researchers who have examined the specific requirements for phospholipids have come to different conclusions. These discrepancies can perhaps best be explained by methodology. In determining that PC was enough to establish full activation, Hilden and Hokin showed 100% replacement of endogenous phospholipid with added PC. However, they did not examine glycolipid content in the PC liposomes. It is entirely possible that sulfatide or a gangliosides was present thus allowing for the activation seen. The best candidate for a phospholipid activator of the ATPase appears to be PS. However, this lipid is not found on the outer leaflet of eukaryotic cells (43). This would seem to eliminate it as a possibility in any type of a model that involves a cofactor attracting K^+ ions and there is no evidence suggesting a Na^+ cofactor moiety on the inside of the cell. So while PS may be an excellent cofactor in reconstituted membranes it would appear to be an unlikely candidate for the role of a physiological activator.

Sulfatide and GM_1 are both negatively charged lipids found on the outer leaflet exclusively. Both have been shown to cause activation of the Na^+, K^+-ATPase. However, sulfatide has been examined the most thoroughly and the data indicate it to be the most logical choice to examine more fully.

Experimental questions to be examined include the following: Do PC vesicles containing Na^+, K^+-ATPase and sulfatide show more ATPase activity than vesicles lacking sulfatide? Is the acyl chain composition of sulfatide (nonhydroxy vs hydroxy) important? Are these effects temperature dependent? What is the effect of sulfatide on the K^+ concentration optimum?

This thesis deals with the first question; does sulfatide concentration have an effect on Na^+, K^+-ATPase activity. We have examined this question by isolating microsomes from mammalian kidneys, and purifying Na^+, K^+-ATPase from them with the use of detergent. The purified ATPase was solubilized with another detergent treatment and reconstituted into vesicles containing various lipids. These studies indicate that sulfatide may act as an activator of Na^+, K^+-ATPase in eukaryotic cells.

Bibliography

1. Marasco, W.A., Becker, K.M., Feltner, D.E., Brown, C.S., Ward, P.A., and Nairn, R. (1985) *Biochemistry* **24**, 2227-2236

2. Hilden, S., and Hokin, L. (1976) *Biochem. Biophys. Res. Commun.* **69**, 521-527

3. Karlish, S.J.D., and Kempner, E.S. (1984) *Biochim. Biophys. Acta* **776**, 288-298.

4. Marcus, M.M., Apell, H.-J., Roudna, M., Schwendener, R.A., Weder, H.-G., and Lauger, P. (1986) *Biochim. Biophys. Acta* **854**, 270-278

5. Kirley, T.L., Lane, L.K., and Wallick, E.T. (1986) *J. Biol. Chem.* **261**, 4525-4528

6. Yoda, A., and Yoda, S. (1982) *Mol. Pharmacol.* **22**, 693-699

7. Garrahan, P.J., Rossi, R.C., and Rega, A.F. (1982) *Ann. NY Acad. Sci.* **402**, 239-251

8. Collins, J.H., and Leszyk, J. (1987) *Biochemistry* **26**, 8665-8668

9. Karlsson, K.-A. (1976) in *Structure of Biological Membranes* (Abrahamsson, S., and Pascher, I., eds) pp. 245-275, Plenum Press, NY

10. Anner, B.M. (1985) *Biochim. Biophys. Acta* **822**, 319-334

11. Jacobs, R.E., and White, S.H. (1987) *Biochemistry* **26**, 6127-6134

12. Kelly, R.A., O'Hara, D.S., Mitch, W.E., and Smith, T.W. (1986) *J. Biol. Chem.* **25**, 11704-11711

13. Hopkins, B.E., Wagner, H., Jr., and Smith, T.W. (1976) *J. Biol. Chem.* **251**, 4365-4371

14. Jorgensen, P.L. (1974) *Meth. Enzymol.* **32**, 277-290

15. Yoda, A., and Yoda, S. (1981) *Anal. Biochem.* **110**, 82

16. Matsui, H., and Schwartz, A. (1966) *Biochim. Biophys. Acta* **128**, 380-390

17. Hokin, L.E. (1977) in *Biochemistry of Membrane Transport, FEBS Symposium No. 42* (Semenza, G., and Carafoli, E., eds) pp. 374-380, Springer-Verlag, Berlin

18. Lane, L.K., Copenhaver, J.H., Jr., Lindenmayer, G.F., and Schwartz, A. (1973) *J. Biol. Chem.* **248**, 7197-7200

19. Anner, B.M., Lane, L.K., Schwartz, A., and Pitts, B.J.R. (1977) *Biochim. Biophys. Acta* **467**, 340-345

20. Hansson, G.C., Heilbronn, E., Karlsson, K.A., and Samuelsson, B.E. (1979) *J. Lipid Res.* **20**, 509-18.

21. Jorgensen, P.L. (1974) *Biochim. Biophys. Acta* **356**, 36-52

22. Zambrano, F., Morales, M., Fuentes, N., and Rojas, M. (1981) *J. Membr. Biol.* **63**, 71-5.

23. Anner, B.M. (1982) in *Transport in Biomembranes: Model Systems and Reconstitution* (Antolini, R., Gliozzi, a., and Gorio, A, eds) pp. 1590-168, Raven Press, NY

24. Kagawa, Y., and Racker, E. (1971) *J. Biol. Chem.* **246**, 5477

25. Kagawa, Y., Kandrach, A., and Racker, E. (1973) *J. Biol. Chem.* **248**, 676

26. Racker, E., and Kandrach, A. (1973) *J. Biol. Chem.* **248**, 5841

27. Hokin, L.E. (1981) *J. Membr. Biol.* **60**, 77-94

28. Spector, M., O'Neal, S., and Racker, E. (1980) *J. Biol. Chem.* **255**, 8370-8373

29. Chin, G., and Forgac, M. (1984) *J. Biol. Chem.* **259**, 5255-5263

30. Brotherus, J.R., Jacobsen, L., and Jorgensen, P.L. (1983) *Biochim. Biophys. Acta* **731**, 290-303

31. Yoda, A., Clark, A.W., and Yoda, S. (1984) *Biochim. Biophys. Acta* **778**, 332-340

32. Kimelberg, H.K., and Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta* **282**, 277-292

33. Jedlicki, A., and Zambrano, F. (1985) *Arch. Biochem. Biophys.* **238**, 558-564

34. Gonzalez, E., and Zambrano, F. (1983) *Biochim. Biophys. Acta* **728**, 66-72.

35. Leon, A., Facci, L., Toffano, G., Sonnino, S., and Tettamanti, G. (1981) *J. Neurochem.* **37**, 350-357.

36. Taniguchi, K., and Tonomura, Y. (1971) *J. Biochem. (Tokyo)* **69**, 543-548

37. Fenster, L.J., and Copenhauer, J.H. (1967) *Biochim. Biophys. Acta* **137**, 406

38. Israel, Y. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D., ed) pp. 529, Prentice Hall, New York

39. Esman, M., Watts, A., and Marsh, D. (1985) *Biochemistry* **24**, 1386-1393

40. Brotherus, J.R., Griffith, O.H., Brotherus, M.O., Jost, P.C., Silvius, J.R., and Hokin, L.E. (1981) *Biochemistry* **20**, 5261-5267

41. Gonzalez, M., Morales, M., and Zambrano, F. (1979) *J. Membr. Biol.* **51**, 347-359.

42. Rintoul, D.A., and Welti, R. (1989) *Biochemistry* **28**, 26-31

43. Rothman, J.E., and Lenard, J. (1977) *Science* **195**, 743-753

Chapter 2

Methods and Materials

Reagents, salts, and buffers, unless otherwise stated, were obtained from Sigma Chemical Company, St. Louis, MO..

Isolation of microsomes

Isolation of microsomes containing Na^+, K^+-ATPase was carried out using the method of Jorgensen (1). All procedures were done at 0°-4° C. Sheep kidneys were obtained from the Department of Animal Science, Kansas State University, Manhattan KS or from Pel Freez (Rogers, Ark). It is important to note that microsomes prepared from frozen kidneys had similar specific activities when compared to microsomes prepared from fresh kidneys. Kidneys from Pel Freez were shipped on dry ice and were frozen immediately at -70° C upon receipt. Immediately before use, kidneys were brought to 0°-4°C by thawing in ice cold buffer containing 0.25 M sucrose and 30 mM histidine at pH 7.2 (20°). The kidneys were cut into small transverse sections and the dark outer medulla was removed, cut into small chunks and placed in buffer on ice. The tissue was then weighed and homogenized with seven strokes of a tight fitting teflon pestle in a 55 ml glass homogenizer; 10 ml of buffer were added per gram of tissue. The homogenate was centrifuged at 6000 x g for 15 minutes (6000 rpm in a GSA rotor) using a Sorvall RC-5 superspeed centrifuge. The supernatant was saved on ice and the pellet was resuspended, homogenized again and centrifuged as before. Once again the supernatant was saved and the pellet resuspended in the 30-40 ml and homogenized before being placed in a Parr nitrogen cavitation bomb for 20 minutes at 750 psi. After cavitation, the lysate was centrifuged as before and the supernatant saved. The combined supernatants were centrifuged at 48,000 x g in a

Beckman Ti-60 rotor for 1 h. The pellets were resuspended at a protein concentration of approximately 6 mg/ml in 25 mM imidazole-HCl, 1mM Tris-EDTA with 10% sucrose and placed in a freezer at -70° C. The ATPase specific activity of this preparation was typically around 30-40 μmol P_i/mg per hr.

TCA Precipitation

Samples to be assayed for protein content were precipitated with trichloroacetic acid (TCA) by adding equal amounts of sample and 50% TCA in a 1 ml microfuge tube. After incubation for at least 1 hour samples were centrifuged at 15,800 x g for 15 minutes in a Tomy microcentrifuge (model # MC-150). Supernatant was removed and the pellets were gently washed with 5% TCA. Centrifugation and washing were repeated 3 times with the final resuspension in 1% SDS in 0.8 N NaOH. Pellets were then dissolved by heating at 60° C in a water bath and assayed.

Protein Assay

Protein was assayed by the Peterson modification of the Lowry method (2).

Na^+-K^+ ATPase Activity

Na^+-K^+ ATPase activity was measured by the method of Schimmel, *et al.* (3). The assay buffer consisted of 60 mM Imidazole/HCl, 220 mM NaCl, 30 mM KCl, 10 mM sodium azide, 1 mM EGTA, and 8 mM $MgCl_2$. 25 μl of buffer was added to 6x50 mm tubes. Each sample involved 2 tubes. 10 μl of 5 mM ouabain in buffer was added to one of the tubes; 10 μl of buffer was added to the other. Sample (1-5 μg of protein) was then added to all tubes. Water was then added to bring the total volume to 55 μl. Samples were preincubated for 5 minutes at 37° C to allow ouabain binding. The assay was initiated by adding 5 μl of 30 mM ATP, and mixed vigorously. Samples were then incubated once again for 5 minutes at 37 ° C. The assay was terminated by the addition of 50 μl of 10% TCA.

Phosphate Assay for ATPase Activity

Samples from the previous ATPase assay were subjected to phosphate determination by a modification of the method of Muszbek (4).

Determination of Active Na^+, K^+-ATPase with Anthroylouabain Binding

Anthroylouabain (AO) binding measurements were obtained using the method of Fortes (5) using a Spex Fluorolog Fluorometer with an excitation wavelength of 369 nm (bandpass 10 nm) and an emission wavelength of 485 nm (bandpass 20 nm). The assay buffer consisted of 50 mM Tris, 1 mM EDTA, 4 mM $MgCl_2$, 3 mM Na_2HPO_4 (pH 7.0). The anthroylouabain was stored as a 2 mM stock solution in ethanol at 4°C and diluted to 20, 40, and 200 μM with absolute ethanol before use.

Fluorescence of AO in the absence of ATPase was measured in the following manner. 3 ml of buffer was added to a fluorescence cuvette and the temperature was allowed to stabilize at 37°C. 1.0 μl of 200 μM anthroylouabain was added and 6 measurements were recorded. Successive additions of 1.0 μl of anthroylouabain were added until a total of 5 μl (final concentration 3.33×10^{-1} μM) had been added. 5.0 μl were then added, followed by the addition of 10 μl. Six measurements were recorded after each addition.

Buffer plus 50-300 μg of SDS-purified ATPase (see Chapter 3) in a total volume of 3 ml, was equilibrated to 37°C. Aliquots (1.0 μl) of 20 μM anthroylouabain were added until a total of 10 μl had been added. Following each addition, a minimum of 5 min was allowed for binding before 6 measurements were recorded. Successive additions of 4 μl, 5 μl, and 10 μl of 200 μM anthroylouabain were then added, each followed by a 5 min incubation and recording of 6 measurements.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by polyacrylamide gel electrophoresis as described by Chrambach (6). Bands were visualized using silver nitrate as described by Andrews (7).

Bibliography

1. Jorgensen, Peter L. (1974) *Meth. Enzymol.* **32**, 277-290

2. Peterson, G.L. (1977) *Anal. Biochem.* **83**, 346-356

3. Forbush, B. 3d., Kaplan, J.H., and Hoffman, J.F. (1978) *Biochemistry* **17**, 3667-3676

4. Muszbek, L., Szabo, T., and Fesus, L. (1977) *Anal. Biochem.* **77**, 286-288

5. Fortes, P.A.G. (1096) *Anal. Biochem.* **158**, 454-462

6. Chrambach, A. (1985) *The practice of quantitative gel electrophoresis*, VCH Publishers, Deerfield Beach, FL

7. Andrews, A.T. (1981) *Electrophoresis: Theory, techniques, and biochemical and clinical applications*, Clarendon Press, Oxford

Chapter 3

PURIFICATION OF Na^+-K^+ ATPase

Introduction

As stated earlier, there are several methods suitable for the purification of Na^+, K^+-ATPase from microsomal membranes. Common to all of these methods is the use of a detergent to isolate the Na^+, K^+-ATPase from other membrane proteins. However, enzyme activity is easily lost due to conformational changes caused by detergents. Our experience has shown that prior to any attempt at enzyme purification, experiments must be carried out to determine the detergent concentration that gives maximum protein yield with minimal loss of activity for each particular method. Even when using established protocols each laboratory should investigate this aspect of purification before proceeding so that maximal specific activity as well as maximum yield may be obtained.

In our laboratory we have used two different methods to examine this question. The first was to purify the enzyme as described by the original authors, using various detergent concentrations. Immediately following detergent incubation samples were assayed for protein and Na^+, K^+-ATPase activity as described earlier. The second method of determining the proper detergent concentration involved the use of anthroylouabain. Anthroylouabain fluorescence is an excellent method of measuring "potential" ATPase activity (1). This assay allows one to determine the amount of phosphorylated enzyme in a preparation since ouabain, and its derivatives, will only bind to phosphorylated (active) enzyme. One can then estimate the amount of active enzyme since, theoretically at least, phosphorylation indicates enzyme that is active. Binding of anthroylouabain to the ATPase causes an increase in quantum yield when compared to free anthroylouabain (1). As increasing amounts of anthroylouabain are added and the available binding sites are saturated the rate of increase in the fluor

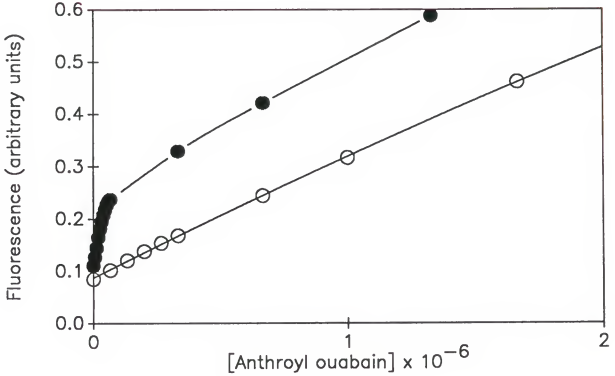

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fluorescence (arbitrary units)
0
1
2
[Anthroyl ouabain] x 10^{-6}

Figure 5. Titration of Na^+, K^+-ATPase with anthroylouabain (AO). Sequential additions of AO were made to cuvettes in the absence of (open circles) and in the presence of (closed circles) 248 μg of ATPase in buffer as described in Chapter 2. AO bound to protein is determined by the point on the graph where the data derived from the sample containing ATPase changes slope to that found in the absence of ATPase. AO binds to the protien with a stoichiometry of 1:1, therefore, the amount of AO bound is equal to the amount of phosphorylated ATPase.

decreases. The point at which the slope changes to that found in the absence of protein can be used to calculate the amount of active protein (Fig. 5).

Our own experiments have indicated several interesting facts about anthroylouabain binding, and its relationship with detergent concentration and specific activity. Figure 6 shows the effects of differing detergent/protein ratios on Na^+, K^+-ATPase activity. This figure clearly shows that a detergent/protein ratio in the range of 0.5 to 0.55 (mg/mg) gives optimal specific activity. However, interpreting the data seen in Figure 7, involving anthroylouabain binding, presents more difficulty. These data, taken from 3 separate experiments, indicate that as the detergent concentration is increased, anthroylouabain binding increases. One would expect, therefore, that specific activity would also increase. However, it has already been shown (Fig.6) that specific activity begins to decrease after a SDS/protein ratio of 0.55 (mg/mg) is reached. Therefore, anthroylouabain binding and ATPase specific activity may not necessarily equivalent. Before determining this for certain, however, it would be interesting to determine if the activity of the SDS purified ATPase in the samples with decreasing specific activity can be reconstituted by dialysis or detergent dilution, if so it may indicate that the protein has not been denatured, just delipidated.

One can also use anthroylouabain to determine the purity of the preparation being examined. The binding data shown in Fig. 7 are presented as nmol/mg of protein. If one divides that number by 6.33 (the inverse of the molecular weight of 1 nmol of Na^+, K^+-ATPase), the resulting number is the percentage of protein in the preparation that is pure phosphorylated ATPase. Our own anthroylouabain binding results indicate that we the SDS-treated ATPase was 20-40% pure. More recent data (Fig. 8) indicate that this percentage may be higher.

Na+, K+–ATPase specific activity
(μmol Pi/mg/hr)
400
300
200
100
0
0.2
0.3
0.4
0.5
0.6
0.7
0.8
SDS/Protein (mg/mg)

Figure 6. Scatter plot demonstrating the relationship between Na^+, K^+-ATPase activity and SDS/protein ratio. Samples were incubated in buffer containing various amounts of SDS as described in Chapter 3. Samples were assayed for protein and Na^+, K^+-ATPase activity as described in Chapter 2. Open circles represent data from experiment 1, closed circles from experiment 2.

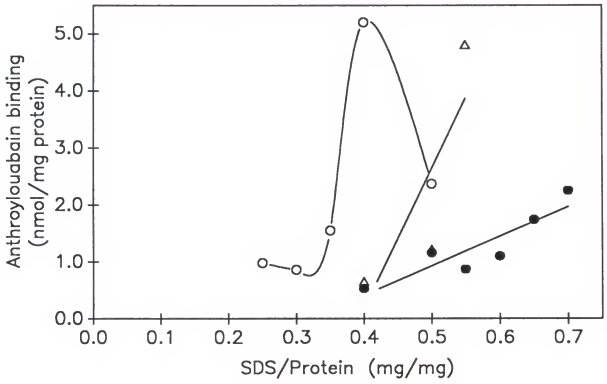

Anthroyluabain binding
(nmol/mg protein)
5.0
4.0
3.0
2.0
1.0
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
SDS/Protein (mg/mg)

Figure 7. Scatter plot of SDS/protein ratio vs AO binding. Experimental data from 3 separate experiments are shown. The open circles represent data from experiment 1, the closed circles represent data from experiment 2, and the open triangles represent data from experiment 3. AO binding was determined as described in Chapter 3. Protein was determined as described in Chapter 2.

NaI Method

Two methods of purification were attempted. Both methods attempted involved "negative" purification, i.e. removal of other membrane components. The first method involved purification of the ATPase using NaI and glycerol (2).

In this procedure microsomes were prepared as described previously. Frozen microsomes were thawed and pelleted at 30,000 rpm (48,000 x g) in a Beckman Ti-60 rotor for 1 hour. They were then resuspended in 1mM EDTA at a protein concentration of 5 mg/ml. The protein was added to 3 M NaI in 15mM EDTA, 7.5mM $MgCl_2$, 120 mM Tris pH 8.3 at 4° C to give a final NaI concentration of 1 M and stirred gently on ice for 10 min. After stirring, the solution was diluted to 0.5 M NaI by the addition of a buffer containing 25 mM imidazole, 0.4 M NaCl, 40 mM KCl, and 1 mM Tris-EDTA, pH 6.9 (medium II) and centrifuged for 45 minutes at 30,000 x g. Following centrifugation the pellet was washed very gently in medium II to eliminate traces of NaI solution and frozen at -70° C in medium II at a protein concentration of approximately 8 mg/ml.

To solubilize the protein, the NaI treated protein was thawed; a 5% solution of sodium-deoxycholate was added dropwise until a ratio of 0.65 mg of detergent/mg of protein was achieved. This solution was incubated on ice with occasional stirring for 30 min, at which time it was centrifuged for 1 h at 32,500 rpm (100,000 x g) in a Beckman SW 50.1 rotor in a Beckman model L5-50 ultracentrifuge. Following centrifugation the supernatant was removed and 0.25 volumes of glycerol were added slowly. The solution was allowed to stir for 15 min at which time an equal volume of 25 mM imidazole, 1 mM Tris-EDTA, pH 7.1 was added; incubation was allowed to continue for an additional 45 min. The sample was then centrifuged at 32,500 rpm (100,000 x g) for 2.5 hours in the same apparatus as before. The resulting pellets were resuspended in medium II at a

Table 1. Flow chart of ATPase purification using the NaI method.

	Trial	Trial 2	Trial 3
Microsomes			
Total protein (mg)	83.2	278.0	114.0
Specific Activity (μmol P_i/mg per hr)	17.2	15.2	22.0
Total units (μmol P_i/hr)	1436.1	4218.0	2508.0
NaI treated enzyme			
Total protein (mg)	37.1	72.1	44.0
Specific Activity (μmol P_i/mg per hr)	44.6	24.3	55.0
Total units (μmol P_i/hr)	1654.0	1750.0	2411.0
Deoxycholate solubilized			
Total protein (mg)	15.5	------	14.5
Specific Activity (μmol P_i/mg per hr)	47.9	------	23.3
Total units (μmol P_i/hr)	726.0	------	338.0
Glycerol treated enzyme			
Total units	3.6	8.9	4.7
Specific Activity (μmol P_i/mg per hr)	164.7	150.0	172.0
Total units (μmol P_i/hr)	592.9	1332.0	806.0

Protein was purified from microsomes using the methods described in Chapter 3. Samples were assayed for protein and ATPase activity as described in Chapter 2.

concentration of 5-8 mg/ml. Specific activity of this preparation was typically 150-175 μmol free phosphate/mg of protein per hour (μmol P_i/mg/hr). Table 1 shows the yield and specific activity at each step through a typical purification procedure.

SDS Method

The other method involved purification of the Na^+, K^+-ATPase using the detergent sodium dodecyl sulfate (SDS) followed by isolation of membranes on sucrose gradients (3). After thawing, microsomes were added to a buffer containing 25 mM imidazole, 2 mM EDTA, 3 mM ATP, pH 7.5 at 20° C, at a concentration of approximately 2.0 mg protein/ml of buffer. SDS (10% w/v in the above buffer) was added dropwise to give a final concentration of 0.5 mg SDS/mg of protein. The solution was stirred gently for 30 min at 16° C.

Sucrose gradients were prepared in 25 x 89 mm Ultra-Clear tubes (Beckman Instruments, Inc.). Gradients contained 5 ml of 39.5% (w/v), 10 ml of 29.4% (w/v), 7 ml of 15% (w/v), and 4.5 ml of 10% (w/v) sucrose in 25 mM imidazole, 2 mM EDTA, pH 7.5 at 20° C. 10 ml of sample was layered on top of each of the gradients. Tubes were then placed in a Beckman SW-27 rotor and centrifuged overnight at 25,000 rpm at 4° C. Following centrifugation the sample was collected in the band above the 38.5% gradient. The sample was then diluted in freezing buffer (described in Ch. 2), and centrifuged for 1 h at 47,000 rpm in a Ti-50 rotor. The pellets were resuspended in 700 μl of freezing buffer. Aliquots were assayed for protein concentration and Na^+, K^+-ATPase activity. The protein suspension was stored at -70° C. Typical specific activities ranged from 275-375 μmoles P_i/mg per hr. Table 2 shows the protein yields and specific activities through a typical procedure, including freeze-thaw reconstitution. The SDS-PAGE profile of the protein at various steps in this purification protocol is seen in Figure 8.

Table 2. Flow chart of Na^+, K^+-ATPase purification using the SDS method and including freeze-thaw reconstitution.

Source	Total Protein (mg)	Specific Activity (μmol P_i/mg/hr)	Total Units (μmol P_i/hr)
Whole cell homogenate (100%)	526.0 (100%)	6.6	3455.8
Microsomes (103%)	96.0 (18.2%)	37.0	3552.0
SDS-purified ATPase	1.6 (0.3%)	377.0	603.0 (17%)
Na^+ cholate solubilized (1.5%)	0.32 (6 x 10^{-3}%)	161.0	51.5
Freeze-thaw vesicles (3.0%)	0.35 (7 x 10^{-3}%)	300.0	105.0

Flow chart of ATPase purification using SDS including freeze-thaw reconstitution in bovine brain phospholipid. Numbers in parentheses indicate the percentage of the original protein or activity remaining in each step of the procedure. Samples were assayed for protein and ATPase activity as described in Chapter 2.

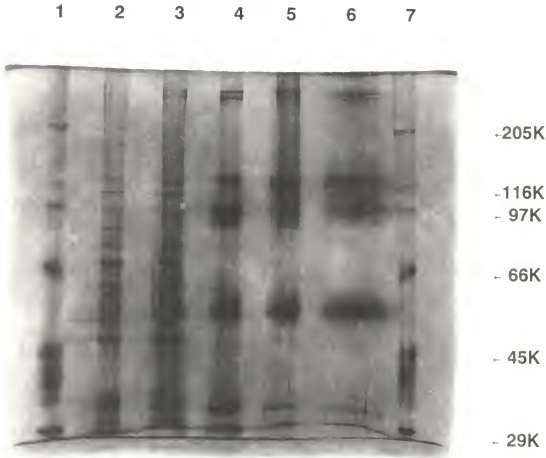

1
2
3
4
5
6
7
205K
116K
97K
66K
45K
29K

Figure 8. SDS-PAGE of Na^+, K^+-ATPase purified using the Jorgenson (SDS) method, and reconstituted using the freeze-thaw method of reconstitution. 6 μg of protein was applied to each lane, electrophoresis and silver staining was performed as described in Chapter 2. Migration positions of marker proteins of designated molecular weights are shown in Lanes 1 and 7. Lane 2-whole cell homogenate; Lane 3-microsomes; Lane 4-SDS purified Na^+, K^+-ATPase; Lane 5-Na^+ cholate solubilized Na^+, K^+-ATPase; Lane 6-reconstituted vesicles containing Na^+, K^+-ATPase.

Discussion

Both methods of Na^+, K^+-ATPase purification gave preparations that showed increases in specific activity when compared to microsomal specific activities. Published reports using sheep kidney outer medulla had indicated a specific activity of 140 μmol P_i/mg per hr could be expected when using the NaI method (4). We were able to obtain preparations with a specific activity of 175 μmoles P_i/mg per hour. However, even with this increase, the NaI method of purification does not give results that can be compared favorably to the specific activity achieved when using the SDS method of purification.

Bibliography

1. Fortes, P.A.G. (1096) *Anal. Biochem.* **158,** 454-462

2. Lane, L.K., Copenhaver, J.H., Jr., Lindenmayer, G.F., and Schwartz, A. (1973) *J. Biol. Chem.* **248,** 7197-7200

3. Jorgensen, P.L. (1974) *Meth. Enzymol.* **32,** 277-290

4. Anner, B.M., Lane, L.K., Schwartz, A., and Pitts, B.J.R. (1977) *Biochim. Biophys. Acta* **467,** 340-345

Chapter 4

Reconstitution of the Na^+, K^+-ATPase

Introduction

Successful reconstitution of the Na^+, K^+-ATPase was originally reported in the literature in 1974 (1,2). Since that time several laboratories have successfully reconstituted this enzyme, using a variety of methods (for examples see references 3-9). Despite the number of laboratories demonstrating successful reconstitution, there are several potential problems that can arise in any attempt to reconstitute this or any other enzyme. The primary problem, as in purification, is inactivation of the protein caused by high detergent concentrations.

Specific Problems in Na^+, K^+-ATPase Reconstitution

Although detergents are a necessary component of the solubilization step in reconstitution, their effect on the enzyme must be monitored carefully. This can be difficult since solubilization of the protein removes it from its lipid surroundings; it is believed that this alone can cause inactivation of the protein. Therefore, one must either reconstitute the protein in membrane vesicles to check for a final activity, or one must find an alternative method to assess for activity. One simple way to assess detergent inactivation is to dialyze the solubilization mix in an appropriate buffer allowing the native lipids to reincorporate with the protein. In our experiments we attempted both reconstitution with native lipids and the anthroylouabain binding assay (10).

We proceeded to use several detergents in an attempt to solubilize the Na^+, K^+-ATPase for reconstitution. As expected, the type of detergent used, as well as the detergent to protein ratios influenced the amount of protein solubilized, as is seen in Table 3. Sodium cholate, the detergent eventually used in the freeze-thaw reconstitutions, solubilized approximately 34% of the initial protein at a detergent

to protein ratio of 8 mg/mg, the amount used in these reconstitutions.

The initial detergent investigated, octaethyleneglycol dodecylmonoether ($C_{12}E_8$) was abandoned when it proved difficult to remove. Attempts to remove this detergent included dialysis, dilution, and incubation of the detergent treated protein with Bio-Beads SM-2 (Bio-Rad, Richmond, CA), a styrene-divinyl-benzene polymer known to absorb $C_{12}E_8$ (11). Other detergents tested included CHAPS, Na^+ cholate, Na^+ deoxycholate, and octylglucoside. To determine the minimal amounts of CHAPS and octylglucoside needed for solubilization we measured light scattering of SDS-treated membranes at 450 nm at increasing detergent concentrations using a Spex Fluorolog (Metuchen, NJ). In this assay, additions of detergent caused a decrease in intensity that eventually reached a baseline level. The detergent/protein ratio that corresponded to the initial point on the baseline was considered the minimal detergent/protein ratio needed for complete solubilization.

Assessment of Successful Reconstitution

The objective of reconstitution is to incorporate active Na^+, K^+-ATPase molecules into tightly sealed vesicles composed of the appropriate lipids. After examination of successful reconstitution protocols, Anner (12) listed the following criteria for reconstitution. First, all vesicles must contain at least one functional transport molecule. This necessarily means an excess of enzyme must be added allowing for the probability that there will be free enzyme in the resulting solution. Hence, one might expect that, even if vesicles are sealed, some ouabain inhibitable ATPase activity will be seen due to the presence of free enzyme. Another criterion for successful reconstitution is an internal K^+ content of zero in the vesicles following the addition of ATP. If vesicles are tight, the addition of extravesicular ATP clears the vesicles of internal K^+ quickly since the internal

volume of the vesicles typically contains about 8000 K^+ ions (12). This allows for approximately 100 sec of pumping if there only one pump per vesicle. A third criterion for tight vesicles is electron microscopic examination. The number of particles per vesicle is used as an indicator of the "degree of" reconstitution.

The parameters of a successful reconstitution of purified Na^+, K^+-ATPase according to Hokin (13) are shown in Table 4. These parameters can be easily tested in the laboratory, after elimination of free enzyme in the solution by centrifugation. According to these criteria, ouabain should not inhibit reconstituted enzyme, the direction and kinetics of ion movement should be demonstrable, ATP must be required for transport, and vanadate should inhibit transport.

Table 3. Protein yield following detergent solubilization.

Detergent	Detergent/protein (mg/mg)	% Solubilized Range	Average
$C_{12}E_8$	2.5	26-67	44.8
CHAPS	6.0	42-71	57.3
Octylglucoside	13.0	39	39.0
Sodium cholate	5.0	19-26	22.3
	8.0	31-38	34.0
	10.0	52	52.0

Percentage of protein recovered following detergent solubilization. Samples were solubilized as described for each detergent in Chapter 4. Protein was assayed as described in Chapter 2.

In our attempts to demonstrate closed vesicles we have primarily been concerned with ouabain inhibition. We have assumed that the Na^+, K^+-ATPase molecules in our vesicles are inserted randomly. Thus we assume that half of the ATPase molecules will have accessible ouabain binding sites; the other half of the inserted enzyme molecules will have their phosphorylation sites exposed (see Fig. 9). A sealed vesicle is not permeable to ATP or ouabain, thus, the ATPase molecules with phosphorylation sites on the outside, (the "inverted" proteins) release free phosphate, until the intercellular K^+ is depleted, producing a small amount of measurable ATPase activity. If ouabain is added it will bind to the available sites (on the proteins inserted "right side out"), but will not affect the inverted molecules; thus the ATPase activity will appear the same in the absence or presence of ouabain. If valinomycin, a K^+ specific ionophore, is added to the enzyme mix, then K^+ is able to enter the vesicles, while ouabain should still be excluded. Therefore, the measurable ATPase activity will increase, but ouabain inhibition will still be minimal. In contrast, the ionophore alamethicin allows all components of the assay mix to enter the vesicles (14), thus ATPase activity and ouabain inhibition should be higher in assays with alamethicin.

Table 4. Parameters of the Na^+-K^+ pump reconstituted from purified Na^+, K^+-ATPase (from Hokin 1979)

---

1. Ouabain-inhibitable Na^+ and K^+ transport dependent on external MgATP.

2. Transports of Na^+ and K^+ in opposite directions to those in cells.

3. Transports of Na^+ and K^+ coupled (omission of external Na^+ blocks K^+ exit).

4. Stoichiometry of Na^+ transport to K^+ transport 3:2.

5. Ouabain inhibits Na^+ and K^+ transport only from the inside (K^+ side)

6. Gradients approaching the physiological obtained for Na^+ and K^+.

7. Effectiveness of various nucleoside triphosphates for Na^+, K^+-ATPase activity and for reconstituted Na^+-K^+ transport parallel each other.

8. Ouabain-inhibitable exchange diffusion of Na^+ demonstrable in the presence of ATP and in the absence of K^+.

9. Ouabain-inhibitable exchange diffusion of K^+ demonstrable in the presence of ATP and P_i in the absence of Na^+.

10. Na^+ and K^+ transport inhibited by external vanadate.

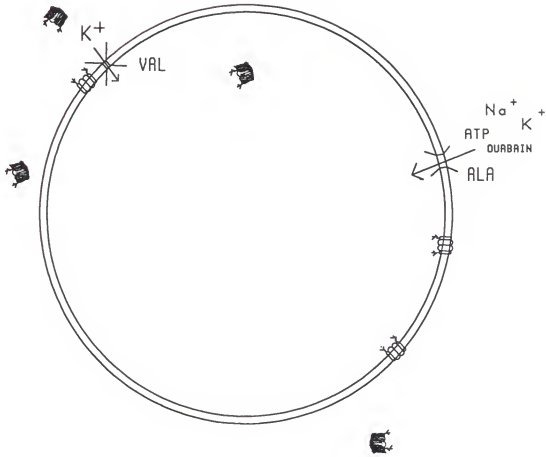

K+
VAL
Na+
K+
ATP
OUABAIN
ALA

Figure 9. Diagrammatic representation of a proteoliposome. The Na^+, K^+-ATPase is shown as a dimer, both in the liposome bilayer and in solution. The soluble enzyme is depicted as being surrounded by detergent molecules, but with both the ouabain-binding and ATP-binding sites exposed to the aqueous medium. Val = valinomycin, a K^+ ionophore. Ala = alamethicin. ATP = adenosine triphosphate.

We attempted several methods of reconstitution including freeze-thaw, dialysis, gel filtration, and dialysis following gel filtration. We were able to eventually demonstrate successful reconstitution using dialysis and the freeze-thaw method.

Reconstitution by Gel Filtration

Reconstitution by gel filtration involves the removal of the detergent by the column matrix. In our experiments we used a modified version of the method of Yoda *et al*. All work was done either on ice or in a cold room at 4° C. The Sephadex G-75 (Sigma Chemical Company, St.Louis, MO) column (1.5 x 27 cm) was equilibrated with several volumes of 100 mM NaCl, 25 mM histidine, 1 mM EDTA, 0.1% sodium azide, pH 7.0. Following equilibration with buffer the column was washed with lipid vesicles in order to saturate the non-specific binding sites. 100 mg of egg PC, dissolved in methanol, was dried under a stream of nitrogen. The dried lipid was suspended in 130 mM NaCl, 25 mM histidine, 1 mM EDTA, pH 7.0 (Buffer L) by sonication in a VibraCell bath sonicator (Sonics and Materials, Danbury, CT) for 30 min under N_2. 80 ml of Buffer L then was added to give a final lipid concentration of 1 mg/ml. Several column volumes (50 -60 ml) of the lipid solution were then passed over the column, followed by several volumes of buffer without lipid until the eluant became clear.

The purified ATPase was prepared for reconstitution by centrifuging the previously prepared microsomes in a Beckman Airfuge at 100,000 x g for 15 min at 4° C. The pellet was resuspended and incubated in 400 μl of solubilization buffer containing 500 mM NaCl, 25 mM histidine, 1 mM EDTA, pH 7.0 for 30 min at 15° C. Also included in the solubilization buffer was the detergent CHAPS. Preliminary light scattering experiments (described above) indicated that the optimal ratio of detergent/protein was 7 mg/mg. The lipid to be used in

the reconstitution was dried under nitrogen; initial experiments utilized 1.3 μmol DEPC, and 0.25 μmol sulfatide. Solubilization buffer (600 μl) containing CHAPS at the same concentration as was used for the protein solubilization was added to solubilize the lipid completely. The protein, solubilized and centrifuged, was added to the solubilized lipid and mixed thoroughly. The resulting mixture was allowed to incubate for 15 min on ice at which time it was placed on the column. The vesicles were collected as the cloudy fraction eluting at the void volume and assayed for protein and ATPase activity as described earlier. The reconstituted vesicles resulting from this procedure gave specific activities of approximately 20-30 μmoles P_i/mg per hr. The ouabain inhibited activities, as well as the specific activities were similar to those of the original microsomes. The addition of valinomycin showed no effect.

Due to the low specific activities obtained using this procedure, several variables were changed. The concentration of the detergent used to dissolve the lipid was decreased until eventually no detergent was added at this step. Cholesterol was added to the lipid mixture at a ratio of 50:8 (PC:cholesterol, mole:mole), since it had been included in the original method (5). To eliminate the possibility that residual detergent was affecting the enzyme, we dialyzed the vesicles in 25 mM histidine, 1 mM EDTA, pH 7.0 for periods ranging from 24-72 hr. This had no effect on the resulting specific activities, even when the vesicles were assayed in the presence of alamethicin; however, at this time the alamethicin concentration and incubation times had not been optimized. We also varied lipid concentration, both increasing and decreasing the lipid/protein ratios. Finally, we tested several other detergents including octyl glucoside, Na^+ deoxycholate, Na^+ cholate, and $C_{12}E_8$. None of these experiments were considered successful since the addition of ionophores made no difference in the specific ATPase activities of

the reconstituted vesicles and the activity was still ouabain-inhibitable. Therefore, this method was abandoned.

Dialysis

Two different dialysis protocols were utilized. The initial method was modeled after the procedure of Anner *et al.* (4) The solubilization and dialysis media were essentially the same (solution A) with the exception that detergent was present in the solubilization buffer. This buffer contained 20 mM NaCl, 5 mM $MgCl_2$, 50 mM choline chloride, 1 mM EDTA, 1 mM cysteine, and 30 mM imidazole-HCl, pH 7.0. The enzyme preparations used included ATPase preparations prepared by both the SDS and NaI methods as described earlier.

Purified enzyme (2 mg) was solubilized in 1 ml of solution A containing 10 mg Na^+ cholate; this was centrifuged in a Beckman Airfuge at 100,000 x g for 15 min. The supernatant was added to 1 ml of solution A containing 40 mg PC and 10 mg Na^+ cholate, the mixture was dialyzed vs. 2 liters of solution A for 24 h. Specific activities of the ATPase in vesicles formed in this way were approximately half that of the purified enzyme preparation.

The second method of vesicle formation by dialysis was originally described by Marcus *et al.* (6). This method used a buffer (buffer H) which contained 30 mM imidazole, 1 mM cysteine, 1 mM EDTA, and 5 mM $MgSO_4$ pH 7.2. The adjustment of pH was done with H_2SO_4 since this buffer uses SO_4^{2-} as an anion to avoid passive anion permeation. The enzyme preparation was purified using the SDS method described earlier.

Purified enzyme was centrifuged in a Beckman Airfuge for 30 min at 100,000 x g. The protein (1 mg) was resuspended in 0.5 ml of buffer H with 8 mg of Na^+ cholate/mg of protein plus 70 mM K_2SO_4 and 5 mM Na_2SO_4. The sample was vortexed for 30 seconds before centrifugation in the Airfuge for 15 minutes at

100,000 x g to remove undissolved material. Lipid (8 μmol) to be used for reconstitution (phospholipid or PC/sulfatide mixtures) was dried under nitrogen. The lipid was dissolved in 400 μl of 1% Na^+-cholate in methanol. The methanol was then evaporated under nitrogen and the lipid was resuspended in 400 μl of buffer H.

Vesicles were formed by combining the solubilized protein with the resuspended lipid and mixing gently. The sample was then dialyzed for 60 hours at 4° C vs. Buffer H with 70 mM K_2SO_4 and 5 mM Na_2SO_4 added. Initial experiments yielded vesicles with ATPase specific activities between 95-110 μmoles P_i/mg per hr. We have recently, using increased amounts of alamethicin, been able to obtain specific activities between 250-300 μmoles P_i/mg per hr which is comparable to the specific activity of the purified enzyme preparations.

Freeze-thaw Reconstitution

The final method of proteoliposome formation which we attempted involved freezing and thawing preformed vesicles in the presence of protein. This method has the advantage that the protein is subjected to minimal additional detergent after the SDS solubilization. This procedure is also faster than dialysis since it can be done in a few hours, thereby reducing the amount of time the protein may be exposed to unfavorable temperatures. The procedure we used was modified from Brotherus *et al.* (3).

The buffers used in this procedure are known as buffers A and C. Buffer A is composed of 300 mM KCl, 20 mM Tes, 2 mM dithiothreitol, 2 mM Tris-EDTA, pH 7.5. Buffer C is simply buffer A diluted 1:1 with water.

16.25 μmol lipid was dried under a stream of nitrogen, resuspended in 1 ml of buffer C and sonicated in a VibraCell bath sonicator for 30 min under nitrogen. One mg of SDS-treated Na^+, K^+-ATPase was centrifuged in a Beckman Airfuge

for 30 minutes to 1 hr at 100,000 x g. The pellets were resuspended in 250 μl of buffer A to give a protein concentration of 4 mg/ml. Na^+ cholate was added to this solution by adding 250 μl of solution (32 mg/ml of detergent in 40% glycerol) to give a final detergent/protein ratio of 8/1. The protein was incubated for 20 minutes on ice before centrifugation in an Airfuge for 10 min at 100,000 x g. The yield was approximately 34% of the original protein (Table 3).

The solubilized protein (200 μl) was then added to the preformed sonicated liposomes (600 μl) and frozen in a dry ice-acetone mixture. The mixture was thawed at room temperature and the proteoliposomes were sonicated for 30 seconds. Samples were then assayed for protein and Na^+, K^+-ATPase activity. Depending on the lipid composition of the preformed liposomes, specific activities in the reconstituted vesicles ranged from very low, (less than 20 μmol P_i/mg per hr), to values equivalent to those of the purified enzyme.

The effects of varying amounts of alamethicin in freeze-thaw vesicles are seen in Table 5. Clearly, increasing amounts of alamethicin in the assay mix resulted in increasing ouabain inhibitable specific activities. Other experiments indicated that increasing the alamethicin to protein ratio above 2.0 mg/mg resulted in no further increase in ATPase activity (data not shown). We therefore have determined an optimal ratio of 1.8-2.0 mg alamethicin/mg protein in our reconstituted vesicles. This ratio may vary with different methods of reconstitution depending on the amount of lipid used in each method. For example, our laboratory has demonstrated that a concentration of .5 mg alamethicin/mg protein is optimal in an assay of the EGF receptor kinase in shed vesicles from A431 cells (Song, W. personal communication).

Table 5. Effects of alamethicin on ouabain-inhibitable Na^+, K^+-ATPase activity.

Alamethicin/Protein (mg/mg)	Alamethicin/Phospholipid (mol/mol)	Na^+, K^+-ATPase activity (μmol P_i/mg per hr)
0.0	0	92.0
0.3	0.19	198.0
0.6	0.38	205.0
1.2	0.76	215.0
1.8	1.15	227.0

Vesicles (bovine brain phospholipid) were formed using the freeze-thaw method described in Chapter 4. Alamethicin was added to the ATPase assay mix, including vesicles, and allowed to incubate for 20 min on ice before proceeding as described in Chapter 2. Vesicles contained approximately 12 mg of phospholipid /150 μg of protein. Molar ratios were calculated using an average molecular weight of 850 for the bovine brain phospholipid and 165 kD for the protein.

Discussion

All of these procedures have been used successfully by other laboratories to reconstitute Na^+-K^+ ATPase. Despite this, we were only successful with 2 methods, the freeze-thaw technique and dialysis as described by Marcus *et al.* There are several possible reasons for this. Firstly, reconstitution is not always an exact science. In our hands, the same method yielded different results at times. This could be due to slight differences in temperature, detergent concentrations, specific activity of initial enzyme preparation, or other factors that could cause enzyme inactivation. However, as experiments were repeated and we became more familiar with the preparation procedures, our results became more consistent. This can be said for all procedures involved from protein purification to reconstitution.

There is another potential reason for the apparent failure of our early reconstitution experiments, and that is our inability to demonstrate high specific activity, due to less than optimal assay procedures. It is in fact possible that all of these methods may have yielded preparations containing reconstituted ATPase, but we were unable to "see" it. The basis for this statement is the belated discovery that increasing the alamethicin concentrations enabled us to enhance the specific activity in both the dialyzed vesicles and the freeze-thaw proteoliposomes (Table 5). It was discovered that the measured ATPase activity increased as the amount of alamethicin was increased until a ratio of 1.75-2.0 mg of alamethicin/mg of protein was reached. The difference in specific activity ranged from 92 μmol P_i/mg per hr with no alamethicin to 227 μmol P_i/mg per hr with 1.8 mg of alamethicin/mg of protein. This effect was demonstrated in both freeze-thaw vesicles and dialyzed vesicles with a decrease in activity seen in the dialyzed vesicles if the alamethicin/protein ratio was increased above 2 mg/mg.

This decrease in activity was probably due to the ethanol in which the alamethicin was dissolved in. This indicates that all methods of reconstitution described earlier should be examined once again using these high alamethicin/protein ratios in the assay mix.

Bibliography

1. Goldin, S.M., and Tong, S.W. (1974) *J. Biol. Chem.* **249**, 5907-5915

2. Hilden, S., Rhee, H.M., and Hokin, L.E. (1974) *J. Biol. Chem.* **249**, 7432-7440

3. Brotherus, J.R., Jacobsen, L., and Jorgensen, P.L. (1983) *Biochim. Biophys. Acta* **731**, 290-303

4. Anner, B.M., Lane, L.K., Schwartz, A., and Pitts, B.J.R. (1977) *Biochim. Biophys. Acta* **467**, 340-345

5. Yoda, A., Clark, A.W., and Yoda, S. (1984) *Biochim. Biophys. Acta* **778**, 332-340

6. Marcus, M.M., Apell, H.-J., Roudna, M., Schwendener, R.A., Weder, H.-G., and Lauger, P. (1986) *Biochim. Biophys. Acta* **854**, 270-278

7. Chin, G., and Forgac, M. (1984) *J. Biol. Chem.* **259**, 5255-5263

8. Goldin, S.M., Forgac, M., and Chin, G. (1988) *Meth. Enzymol.* **156**, 127-155

9. Hilden, S., and Hokin, L. (1976) *Biochem. Biophys. Res. Commun.* **69**, 521-527

10. Moczydowlski, E.G., and Fortes, P.A.G. (1980) *Biochemistry* **19**, 969-977

11. Jorgensen, P.L. (1974) *Biochim. Biophys. Acta* **356**, 36-52

12. Anner, B.M. (1982) in *Transport in Biomembranes: Model Systems and Reconstitution* (Antolini, R., Gliozzi, a., and Gorio, A, eds) pp. 1590-168, Raven Press, NY

13. Hokin, L.E. (1979) in *Membrane Bioenergetics* (Lee, C.P., Schatz, G.P., and Ernster, L., eds) pp. 281-296, Addison-Wesley Publishing Co., Inc., Reading, Mass

14. Albe, K.R., Witkin, H.J., Kelley, L.K., and Smith, C.H. (1983) *Exp. Cell Res.* **147**, 167-176

Chapter 5

Sulfatide dependence

Methods

The methods used to examine sulfatide dependence involved freeze-thaw reconstitution as described earlier. Differences between experiments included variations in the lipid compositions of the preformed liposomes and the initial source of the enzyme.

Sulfatide with Phosphatidylcholine

The vesicles composed of differing mole to mole ratios of sulfatide and DOPC ranging from 0-25% sulfatide. The sulfatide was obtained from Sigma Chemical company, St. Louis MO (catalog # S-1006), while the DOPC was purchased from Avanti Polar Lipids, Birmingham, AL.,(catalog # 850375). The source of enzyme was microsomes obtained from the outer medulla of canine kidneys (Pel-Freez, Rogers, Ark.) and prepared as described for sheep kidneys in Chapter 2. ATPase was prepared by treatment of the microsomes with 0.5 mg/mg (detergent/protein) SDS as described above.

Sulfatide with Mixed Phospholipids

These experiments were done as described above for the sulfatide with PC experiments with the exception that bovine brain phospholipids, prepared in this laboratory and containing 26% PS was used (1). The enzyme source for these experiments was the outer medulla of sheep kidneys as described in Chapter 2. ATPase was prepared by treatment of the microsomes with 0.5 mg/mg (detergent/protein) SDS as described above.

Results

Sulfatide with Phosphatidylcholine

In these experiments we wanted to examine the effect of sulfatide on ATPase activity in PC vesicles. The results are shown in Figure 10. These data clearly indicate that ATPase specific activity increased as the sulfatide level increased. In fact, in vesicles with 25 mole% sulfatide the ouabain-inhibitable ATPase specific activity assayed without valinomycin or alamethicin is close to 50% of the specific activity in the purified protein. In vesicles with no added sulfatide the specific activity is only 14% of the ouabain-inhibitable specific activity of the purified enzyme.

Sulfatide with Mixed Phospholipids

The purpose of this experiment was to determine if increasing the amounts of sulfatide could cause increasing amounts of enzyme activation in reconstituted vesicles even if PS was present. The results indicate that this does occur (Table 6). The first experiment shown indicates that with no added sulfatide the reconstituted vesicles have a specific activity similar to the purified enzyme. However, in vesicles containing 10 mole% sulfatide, the ATPase activity increases to approximately 172% over that of the purified enzyme. Increasing the sulfatide to 20 mole% had little additional effect. However, at other times the increase to 20% sulfatide did show an effect. This occurs in the second experiment shown in Table 6. In this experiment, the vesicles containing only phospholipid and no sulfatide have a low specific activity when compared to the SDS purified enzyme. Addition of up to 20 mol% sulfatide, however, resulted in an increase in ouabain inhibitable ATPase activity when assayed in the presence of alamethicin.

750
700
650
600
550
500
450
400
350
300
0
5
10
15
20
25
30
Sulfatide in DOPC (mole %)
Na+, K+–ATPase specific activity
(μmol Pi/mg/hr)

Figure 10. The effects of increasing amounts of sulfatide in DOPC vesicles on Na^+, K^+-ATPase activity. Vesicles were prepared using DOPC and varying amounts of sulfatide using the freeze-thaw method described in Chapter 4. Na^+, K^+-ATPase source was canine kidneys. Samples were assayed as described in Chapter 2.

Table 6. Effect of Sulfatide on Na^+, K^+-ATPase activity in bovine brain phospholipid vesicles

mole % Sulfatide	Specific Activity (μmol P_i/mg per hr)
Trial 1	
0	90.0
10	156.0
20	160.0
SDS purified ATPase	90.0
Trial 2	
0	10.0
10	40.0
20	54.0
SDS purified ATPase	61.0

Vesicles were formed from brain phospholipids as described in Chapter 3. Vesicles were assayed for protein and Na^+, K^+-ATPase activity as described in Chapter 2 in the presence of 2 mg of alamethicin/mg of protein.

Discussion

The results seen in these reconstitution experiments, whether the vesicles included sulfatide with mixed phospholipids or with PC only, indicate that sulfatide has the capability to enhance ATPase activity. However, in vesicles containing mixed phospholipids, the presence of PS can possibly also cause activation. This phospholipid has previously been shown to cause full activation of the Na^+, K^+-ATPase (2-5). However, our experiments indicate that sulfatide can cause additional activation even in the presence of substantial amounts of PS. The specific activities of the reconstituted vesicles varied from experiment to experiment. This may be due to partial inactivation, indicated by the low specific activity, of the SDS purified enzyme used in the second experiment. However, an increase in ATPase specific activity is seen in all cases when sulfatide is present, even though the maximal activation may occur at either 10 mole% or 20 mole% sulfatide depending on the experiment. Therefore this set of data supports the hypothesis that sulfatide may be involved as an activator of Na^+,K^+-ATPase.

The results from the experiments with proteoliposomes containing sulfatide and PC strongly suggest that sulfatide can act as a lipid cofactor. It is clear from the results shown in Fig. 10 that as sulfatide concentration is increased, Na^+, K^+-ATPase activity likewise increases. Results from this experiment indicate that 20 mole% sulfatide restores ATPase activity to a level that is approximately 50% of that found with the purified enzyme. However, since these vesicles were assayed without ionophore, these increases in activity are possibly not due to reconstituted ATPase but to free ATPase present in the preparation.

These experiments suggest that sulfatide can act as a potential activator, but they do not show that it is the only lipid cofactor involved. However, many other investigators have also implicated sulfatide as the potential cofactor.

Zambrano and coworkers have shown that sulfatide is involved in Na^+, K^+-ATPase activity, sodium flux and more specifically the dephosphorylation of the phosphoenzyme (6-9). Lipid-lipid interaction experiments of Rintoul and Welti indicate that sulfatide interacts more strongly with K^+ than with Na^+ in vesicles (10). The work of Hansson and others (11) has shown the quantitative relationship of sulfatide concentration and Na^+, K^+-ATPase activity in several tissues. All of these results when considered together strongly suggest that sulfatide is the lipid cofactor for activation of the Na^+, K^+-ATPase as suggested by the proposed model.

Bibliography

1. Scaglione, B.A., and Rintoul, D.A. (1989) *Invest. Ophthalmol. Vis. Sci.* **30**, 961-966

2. Kimelberg, H.K., and Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta* **282**, 277-292

3. Taniguchi, K., and Tonomura, Y. (1971) *J. Biochem. (Tokyo)* **69**, 543-548

4. Fenster, L.J., and Copenhauer, J.H. (1967) *Biochim. Biophys. Acta* **137**, 406

5. Israel, Y. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D., ed) pp. 529, Prentice Hall, New York

6. Gonzalez, M., Morales, M., and Zambrano, F. (1979) *J. Membr. Biol.* **51**, 347-359.

7. Zambrano, F., Morales, M., Fuentes, N., and Rojas, M. (1981) *J. Membr. Biol.* **63**, 71-5.

8. Gonzalez, E., and Zambrano, F. (1983) *Biochim. Biophys. Acta* **728**, 66-72.

9. Jedlicki, A., and Zambrano, F. (1985) *Arch. Biochem. Biophys.* **238**, 558-564

10. Rintoul, D.A., and Welti, R. (1989) *Biochemistry* **28**, 26-31

11. Hansson, G.C., Heilbronn, E., Karlsson, K.A., and Samuelsson, B.E. (1979) *J. Lipid Res.* **20**, 509-18.

Chapter 7

Immunological Methods

Introduction

Monoclonal antibodies directed at sulfatide were produced in order to determine if antibody binding to the sulfate group on the sulfatide would influence Na^+, K^+-ATPase activity in any way. At present, few experiments have been performed using these antibodies, but the examination of this question will continue.

Cell Cultures

All cell culture work was done in a sterile environment inside a Labguard laminar flow hood. Hybridoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), (HyClone Laboratories, Logan, Utah) and 5% of a cocktail containing 100x Nonessential Amino Acids, 100x vitamins, 20 mM sodium pyruvate, 100x amino acids, and 40 mM glutamine (all obtained from Flow laboratories, McLean, VA.). The cell maintenance media also included $NaHCO_3$ at a final concentration of 0.375% and 0.01 mM HEPES.

For some experiments the hybridoma cells were cultivated in serum free media. This medium consisted of RPMI 1640 medium containing additional L-glutamine (300 μg/ml), Na-pyruvate (110 μg/ml, HEPES (15 mM). To this medium, the following compounds were added: transferrin (5 μg/ml), insulin (10 μg/ml), ethanolamine (20 μM), linoleic acid (5 μg/ml), fatty acid free bovine albumin (1 mg/ml), ascorbic acid (3 μg/ml), hydrocortisone (2 ng/ml), $MnCl_2$ (0.5 nM), $(NH_4)_6Mo_7O_{24}$ (0.5 nM). Cells were maintained in a 37° incubator in a 5% CO_2 atmosphere.

Cells were diluted 3 times weekly in fresh media (1 part cells: 9 parts fresh media). Cells were transferred weekly to a fresh 25 cm^2 flask (Falcon, Oxnard, CA). Spent media was centrifuged briefly to remove cells and stored at -20°C.

Production of Hybridomas

Mice (RBF/Dn) were a gift from Dr. William Fortner, Kansas State University. Fox-NY cells, a myeloma cell line, were obtained from Dr. Robert Phillips, Kansas State University School of Veterinary Medicine. The antigen was prepared by combining 2.2 μmol of sulfatide, 4.4 μmol of PC, and 44 μmol of cholesterol. The resulting solution was dried down under nitrogen and resuspended in 0.5 ml of ethanol. The mixture was once again dried down, resuspended in sterile saline, and stored at 4° C. The final molar ratio was 1:2:20 (sulfatide:PC:cholesterol) (1).

Mice were immunized 4 times at 2 week intervals using 250 μl of a 1:1 mixture of antigen and Freund's complete adjuvant. The mixture was injected into the intraperitoneal space.

Fusion

Fusion of myeloma cells with spleen cells was done as follows. The media used in the cell fusion procedures consisted of DMEM with 0.02 mM HEPES. Prior to cell fusion, feeder cells were prepared in four 24 well cluster plates (Costar, Cambridge, MA) in the following manner. A C3H mouse was sacrificed by ether inhalation, secured on its back and its abdomen was exposed. The skin was removed from the abdomen with care taken not to puncture the skin. The peritoneal cavity membrane was lifted gently with sterile forceps and 5 ml of Hank's Balanced Salt Solution (HBSS) was injected into the peritoneal cavity. The cavity was then gently massaged in order to wash cells into the cavity. A sterile Pasteur pipette was gently inserted into the cavity and as much fluid was

removed as possible. The fluid was centrifuged in a table top centrifuge for 8 minutes at 1000 rpm. The supernatant was then aspirated off and the pellet was resuspended in 1 ml of Mac Sel-DMEM which was composed of 76 ml of DMEM, 15 ml of FBS, 7 ml of the previously described cocktail and 1 ml of 100x AAT selection media. The cells were counted using a hemocytometer and diluted to a concentration of 6×10^3 cells/ml; 1 ml of this suspension was added to each of the wells in four 24 well cluster plates.

Four days after the final injection the spleens were removed, placed in a small petri dish containing 5 ml of HBSS, and transferred to a laminar flow hood where all subsequent activities were carried out. Using sterile forceps, the spleen was transferred to another petri dish containing 5 ml of sterile HBSS. Cells were gently teased out of the spleen capsule using a sterilized bent needle. The capsule was discarded and the cells were transferred to a sterile 15 ml centrifuge tube. HBSS (5 ml) was then used to wash out the petri dish. This wash solution was added to the centrifuge tube. The cell suspension was then pipetted several times in order to break up cell clumps. Cells were incubated on ice for 10 minutes before being placed in a 50 ml centrifuge tube (Corning Laboratory, Corning, NY), and centrifuged in a Sorvall GLC-1 tabletop centrifuge for 8 minutes at 1000 rpm. Supernatant was then aspirated off and the spleen cell pellet was resuspended in 5 ml of DMEM.

Actively growing myeloma cells were centrifuged for 8 minutes at 1000 rpm in a tabletop centrifuge and resuspended in 5 ml of DMEM. Spleen cells and myeloma cells were mixed at a ratio of 2:1 (5 ml:2.5 ml) in a 50 ml centrifuge tube. The tube was then filled with DMEM, and centrifuged for 8 minutes at 800 rpm. The supernatant was aspirated off and the pellet dislodged by gentle tapping. The cells were then placed in a 37° C water bath for 1 minute. 1 ml of

sterile fusion media containing 1.88 ml of Polyethyleneglycol (PEG), 1.82 of DMEM, and 0.3 ml of DMSO was added dropwise over a 1 minute time period while shaking gently. Over the next 3 min 2 ml of DMEM, previously warmed to 37° C, was added while the tube was shaken continuously. Over the next 3 min, 7 ml of DMEM containing 15% fetal bovine serum was added also with continuous shaking. Cells were transferred to a bottle containing 37°C Fusion Select DMEM which was composed of 66 ml of DMEM, 15 ml FBS, 7 ml of the previously described cocktail and 1 ml of 100X AAT selection media. Cells were plated in the four 24 well plates containing feeder cells at 1 ml/ well. One well was plated with myeloma cells + Fusion Select DMEM to ensure that myeloma cells were sensitive to the selective medium. Plates were placed in the incubator and left untouched for 10 days at which time they were checked for growth. If growth was seen, wells were checked for the presence of specific antibody.

ELISA

Supernatants from wells showing growth were analyzed by ELISA (Enzyme Linked ImmunoSorbent Assay). This assay involves binding the antigen of interest to plate, incubating it with the putative antibody, and addition of a second labeled antibody which binds to the first antibody if it is present.

An ethanol/water solution (1/1) was used to dissolve sulfatide for binding. A solution containing 10 μg of sulfatide was placed in each well of a 96 well cluster plate (Costar, Cambridge, MA). The plates were incubated at 37°C overnight. After incubation, wells were washed 3 times with a buffer containing 10 mM Tris-HCl and 0.05% Tween 20, pH 8.0; 100 μl of supernatant from the hybridoma cells to be examined was added to each well and incubated for 4 hours at 37°C. Wells were then washed as before and 100 μl of an anti-mouse antibody (conjugated to alkaline phosphatase, diluted 1/250 in water), was added. The

plates were then incubated for 1 hour at 37°C before washing 3 times with buffer and 2 times with glass distilled water. 100 μl of alkaline phosphatase substrate was then added, plates were incubated for 20-30 minutes at 37° C before absorbance was read at 405 nm.

Isolation of Monoclonal Antibodies

Colonies that reacted positively in the ELISA for sulfatide were cloned by limiting dilution to produce monoclonal colonies. This was done by diluting the appropriate cultures to a final concentration of 230 live cells in 4.6 ml of conditioned DMEM, prepared as described earlier. 0.1 ml of this cell suspension was added to 36 wells. 4.0 ml of used DMEM was added to the remaining 1.0 ml; an additional 36 wells were plated with 0.1 ml of this suspension. 1.4 ml of used media was added to the remaining cells, and 0.1 ml of this suspension was added to the remaining 24 wells. The plates were then placed in a 37°C incubator.

On days 5 and 12 post- dilution cells were fed with 2 drops of medium with a Pasteur pipette. On day 12 and again on day 14 plates were examined for growth and the supernatants from the dilution with no growth in 35%-40% of the wells were examined by ELISA. The dilution procedure was repeated with cells from antibody positive wells. Wells that again showed 60%-65% growth and tested positive were considered to be producers of monoclonal antibodies directed against sulfatide.

Antibody Inhibition

Results

Attempts were made to determine the effect of sulfatide directed monoclonal antibody on the activity of purified Na^+, K^+-ATPase. To prevent possible interaction from antibody contained in FBS, the antibodies used were

generated in serum-free media. Cell culture supernatants, (20 ml collected during regular feedings) were then dialyzed vs. 4 liters of ATPase assay buffer for 2 days. As a control, cell cultures supernatants from hybridomas producing either no antibody or antibody against lactosylceramide were dialyzed and used in ATPase inhibition experiments. Small aliquots of the dialyzed sulfatide antibody, when added to the assay medium appeared to activate the SDS purified enzyme. However, larger amounts resulted in some inactivation (Table 7). Additionally, the specific activities increased in all cases including controls containing serum-free medium with no antibody. These experiments were attempted several times and in all cases the same result was seen. Only in experiment 1 was the antisulfatide antibody effective in reducing the specific activity to below that of the original purified enzyme. These experiments are therefore inconclusive.

Discussion

The results of the antibody inhibition experiments are confusing at best. The initial increase may be explained in the fact that the BSA present in the media may be binding free fatty acids present in the ATPase preparation. This would cause an increase in specific activity since free fatty acids are known inhibitors of the enzyme (2). This could be examined by using BSA in the ATPase assay buffer and measuring the activity of purified enzyme using this buffer. These experiments are being planned. However, the decrease seen is harder to explain and at the moment we have no explanation.

Bibliography

1. Hofstetter, W., Bologa, L., Wetterwald, A., Z'graggen, A., Blaser, K., and Herschkowitz, N. (1984) *J. Neurosci. Res.* **11**, 341-350

2. Kelly, R.A., O'Hara, D.S., Mitch, W.E., and Smith, T.W. (1986) *J. Biol. Chem.* **25**, 11704-11711

Table 7. Monoclonal antibody effect on SDS purified Na^+, K^+-ATPase

Trial 1	Trial 2	Trial 3	
Media control			
(10 μl)	-----	-----	260.0
(20 μl)	266.0	245.0	227.0
Non-producing hybridoma			
(10 μl)	-----	-----	292.0
(20 μl)	266.0	244.0	212.0
Anti-Sulfatide hybridoma			
(10 μl)	280.0	318.0	295.0
(20 μl)	236.0	235.0	240.0
SDS-purified enzyme (control)	253.0	201.0	194.0

Effect of monoclonal antibodies, directed against sulfatide, on the Na^+, K^+-ATPase activity of reconstituted vesicles. Vesicles were formed from bovine brain phospholipid using the freeze-thaw method described in Chapter 4. Vesicles were assayed for protein and Na^+, K^+-ATPase activity as described in Chapter 2 in the presence of 2 mg alamethicin/mg of protein as described in Table 5. Antibodies were included in ATPase assay buffer and preincubated with alamethicin and ouabain (see chapter 7).

Perspectives

In the introduction I stated that this thesis would examine the influence of sulfatide on Na^+, K^+-ATPase activity. I believe we have determined that sulfatide has a positive effect on reconstituted ATPase activity. The data in Chapter 5 show that increases in sulfatide content in vesicles cause an increase in Na^+, K^+-ATPase activity. The data also suggest that this "activation" occurs regardless of the phospholipid composition in the vesicles. However, before answering this question, much work was done in attempts to purify and reconstitute the ATPase. The most successful methods included SDS purification at a detergent to protein ratio of 0.5 (mg/mg), freeze-thaw reconstitution using sodium cholate to solubilize the SDS-treated enzyme at a detergent to protein concentration of 8.0 (mg/mg), and assaying reconstituted vesicles in the presence of alamethicin at a ratio of 2 mg alamethicin/mg of protein. Other methods of reconstitution that have shown promise include both dialysis protocols described in Chapter 4, though these methods need to be reexamined using the proper alamethicin concentration.

In spite of this work many questions still remain to be answered. We have not determined if monoclonal antibodies, directed against sulfatide, will inhibit ATPase activity. We have not examined the possibility that sulfatides containing non-hydroxy fatty acids might influence ATPase activity differently than hydroxy fatty acid containing sulfatides. We have also not determined the effects of temperature or K^+ concentration in regards to ATPase activity with various sulfatide concentrations.

This thesis, therefore, has answered only one of several questions regarding sulfatide influence on Na^+, K^+-ATPase. Hopefully, however, the work done on

purification and reconstitution as well as the data from experiments examining the effects of different sulfatide concentration on ATPase activity will aid others as they attempt to answer these questions.

Acknowledgements

It's impossible to thank all of the people who have helped me in my years at Kansas State. There have been many friends who have made my stay here enjoyable, and I will look back at this time in my life with fondness.

I would especially like to thank Dave Rintoul for allowing me to work in his laboratory for the past few years. He has shown remarkable patience at many times in the past, and I feel I have benefited from his tutelage. I would also like to thank the other members of my committee, Jim Guikema and Ruth Welti, for their suggestions and helpful comments. I would especially like to thank Ruth for her motivational speeches, even when I didn't need them. I would also like to thank Drs. Consigli and Fortner and their respective laboratories for their advice and openness.

I would also like to express my thanks to Syed Ameenuddin, Wenxia Song, and Beth Scaglione for their help and tolerance.

Finally, I would like to thank my wife Pam and my children P.J., Courtney, and Deven who have supported me for the past 3 years.

This work was supported by grants from the American Heart Association, Kansas Affiliate, Inc.

Purification and Reconstitution of Na^+, K^+-Adenosinetriphosphatase

By

Douglas E. Mullinex

B.S., Kansas State University, 1986

An Abstract of

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

Purification and Reconstitution of Na^+, K^+-Adenosinetriphosphatase.

Methods for the isolation and purification of Na^+-K^+ Adenosinetriphosphatase (Na^+, K^+-ATPase EC 3.6.1.3) were attempted and selected. The chosen method involved isolation of microsomes by differential centrifugation and purification using the detergent sodium dodecyl sulfate. Purified ATPase was then reconstituted in characterized vesicles and activity was examined. Results indicate that the glycosphingolipid 3-sulfogalactosylceramide (sulfatide) may be involved in normal ATPase function in eukaryotic cells.