PURIFICATION AND PROPERTIES OF THE SODIUM–POTASSIUM TRANSPORT ADENOSINETRIPHOSPHATASE FROM THE RECTAL GLAND OF THE SPINY DOGFISH SQUALUS ACANTHIAS

Lowell E. Hokin

Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706

The (Na + K)–activated adenosinetriphosphatase (NaK ATPase) has been purified from Lubrol extracts of membranes from the rectal glands of Squalus acanthias. The specific activity of the purified enzyme is 2 to 3 times that previously reported by others after correction of their specific activities for detergent activation. The yield of the enzyme from the membranes is 70%. The enzyme is highly stable both at 0° and in the frozen state. Ninety-five percent of the enzyme consists of two subunits–the catalytic subunit with a MW of 97,000 and a glycoprotein with a MW of 55,000. At the last stage of purification the enzyme reverts to various membranous forms: the thickness of the membrane is about 80 Å; projections (probably the glycoprotein) of about 40 Å in diameter are seen at regular intervals.

INTRODUCTION

The (sodium + potassium)–activated adenosinetriphosphatase (ATP phosphohydrolase EC 3. 6. 1. 3) plays an important role in the coupled transports of Na and K across animal cell membranes and is a receptor for cardioactive drugs (1–3). Although considerable information about the role of this enzyme, hereafter referred to as the NaK ATPase, has accumulated since its discovery by Skou in 1957 (4), it has not been possible to date to completely purify this important enzyme from mammalian sources, although purifications up to 50% have probably been achieved (5–7).

The rectal glands of elasmobranchs secrete NaCl as a means of eliminating salt which accumulates from the sea. These glands have been reported to be rich in NaK ATPase and that from the spiny dogfish shark, Squalus acanthias, appeared to be the richest (8). Our laboratory therefore embarked on the purification of the NaK ATPase from the rectal glands, using modifications of purification procedures which had previously been worked out for the large-scale partial purification of the NaK ATPase from beef brain (5). We describe here the purification in high yield from membranes of dogfish rectal glands of an NaK ATPase complex of high stability in which 70% of the protein consists of the catalytic subunit of molecular weight 97,000 and 25% of the protein consists of a 55,000 molecular weight glycoprotein. Some properties of this enzyme are also described. Part of this work has recently been published in detail (9).

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RESULTS

Isolation of Membranes from Fresh Rectal Glands

Table I shows the isolation of membranes from homogenates of fresh rectal glands. A crude membrane fraction was obtained which contained about half of the units of the homogenate and a little over four times the specific activity of the homogenate. A purified membrane fraction was obtained which contained about one-third of the original enzyme activity in the homogenate and about ten times the specific activity.

Table I. Isolation of Crude and Purified Membranes from Fresh Rectal Glands[a]

Fraction	Total NaK ATPase (units)[b]	Total protein (mg)	Specific activity (units/mg protein)
Homogenate	75,500	1,830	41.3
Crude membranes	34,500	203	170
Purified membranes	24,000	60	400

[a]Rectal gland mince (13.4 gms) was obtained from glands from ten dogfish. The preparation of the membranes is described elsewhere (9).
[b]A unit is defined as μmoles of inorganic phosphate released per hour. (Reproduced by permission of the Journal of Biological Chemistry.)

Isolation of Membranes from Frozen Rectal Glands

The fresh rectal glands which were used in the experiment described in Table I were collected in the summer of 1971 at the Mount Desert Island Biological Laboratory, Salisbury Cover, Maine. It was obviously not feasible to routinely purify the NaK ATPase from fresh rectal glands, so isolation of enzyme from frozen glands was attempted. We found that if rectal glands were frozen over dry ice, shipped from Salisbury Cove to Madison on dry ice, and stored at -70° no loss in enzyme activity occurred on storage for over a year. However, the isolation of membranes with specific activities as high as those isolated from fresh glands has not been found possible, although the percentage yield of enzyme from the homogenate is about the same (Table II). Nevertheless, further purification steps led to enzyme which has identical specific activity and polacrylamide–SDS gel patterns, starting with membranes from either fresh or frozen glands (9). Thus, the routine use of

Table II. Isolation of Membranes from Frozen Rectal Glands[a]

Fraction	Total NaK ATPase (units)	Total protein (mg)	Specific activity (units/mg protein)
Homogenate	3,600	96	38
Crude membranes	2,400	22	110
Purified membranes	1,040	6.4	160

[a]In this run 2 gms of dogfish rectal glands were used (before mincing). The method of isolation of membranes is as described elsewhere (9). Crude membranes refers to the loose pellet after centrifugation in 250 ml bottles at 19,600 × g for 2 hrs. Purified membranes refers to the loose pellet after centrifugation of a suspension of crude membranes in 50 ml tubes at 24,500 × g for 30 min. (Reproduced by permission of the Journal of Biological Chemistry.)

membranes from frozen glands proved to be feasible. The membranes isolated from either frozen or fresh rectal glands were stable at -70° for over a year and they lost no activity after thawing when stored for two weeks at 0°.

Purification of NaK ATPase from Membranes from Frozen Glands

The purification steps which were found effective in purifying the NaK ATPase from microsomes from beef brain cortex were 1) treatment of the microsomes with NaI by the method of Nakao et al. (10), 2) extraction of the NaI-treated microsomes with the nonionic detergent, Lubrol WX, 3) removal of free Lubrol and inert protein by zonal centrifugation, and 4) a novel ammonium sulfate fractionation (5). A systematic study of NaI treatment of membranes from the rectal glands indicated that this step could not be adapted to purification of the NaK ATPase from rectal glands. The other steps were highly effective, however. Table III shows the purification of the NaK ATPase from the membrane fraction isolated from frozen glands. Lubrol extraction increased the specific

Table III. Purification of the NaK ATPase from Membranes Isolated from Frozen Rectal Glands of the Dogfish[a]

Fraction	Total NaK ATPase (units)	Total protein (mg)	Specific activity (units/mg protein)
Membranes	41,100	367	112
Lubrol extract	76,400	176	434
Zonal pool	47,100	–	–
Zonal concentrate	44,600	70	637
Ammonium sulfate enzyme (cushion prep)	29,000	19.3	1,510

[a]The purification procedure is described elsewhere (9). An accurate protein could not be done on the zonal pool because of interference by sucrose but could be done on the zonal concentrate after removal of sucrose in the Diaflo ultrafiltration cell. Cushion prep refers to centrifugations of the ammonium sulfate enzyme on to a 50% sucrose cushion. (Reproduced by permission of the Journal of Biological Chemistry.)

activity of the enzyme four-fold. Half of this increase in specific activity was due to activation of the enzyme, as has been observed with Lubrol and other detergents at appropriate concentration by other workers (11). This activation was removed by the zonal centrifugation step which removes free Lubrol. Interestingly, Lubrol did not activate the NaK ATPase in membranes isolated from fresh glands. About a 15-fold purification was achieved, as shown in Table III, in a 70% yield. In this experiment the enzyme, which reverted to the membranous form at the last stage of purification, was centrifuged against a 50% sucrose cushion, since some inactivation was observed if the enzyme was pelleted by standard centrifugation. This method of harvesting the enzyme led to lower yields, since not all of the enzyme collected on the sucrose cushion. On average 20 to 30 mg of purified enzyme protein could be obtained from ten rectal glands, which weigh 1–2 gms each. The purified enzyme was stable for months on storage at -70°, and lost no activity on storage at 0° for ten days.

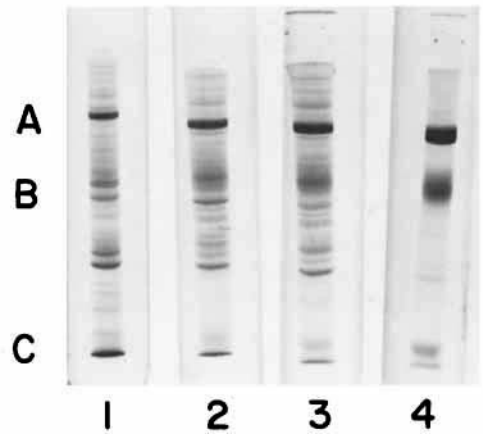


Fig. 1. SDS–polyacrylamide gel electrophoresis of rectal gland NaK ATPase at successive stages of purification. (1) Membranes from frozen glands. (2) Lubrol extract membranes. (3) Zonal enzyme. (4) Ammonium sulfate enzyme. SDS–polyacrylamide gel electrophoresis and staining with Coomassie blue were carried out as described elsewhere (9). Twenty μg of protein were applied to each gel. (A) Catalytic subunit band. (B) Glycoprotein. (C) Protein associated with tracking dye. In this run some of the protein normally associated with the tracking dye ran slightly above. The staining at the tracking dye position in the ammonium sulfate gel in this run is due to nonprotein staining material in the gel system. The stacking gel had detached from the running gel in gels 1 and 2. (Reproduced by permission of the Journal of Biological Chemistry.)

Disc Gel Electrophoresis of the Rectal Gland NaK ATPase at Various Stages of Purification

Figure 1 shows the protein patterns on polyacrylamide–SDS gel electrophoresis of NaK ATPase preparations at successive stages of purification. As purification proceeded a subunit in the same location as that previously identified as the catalytic subunit of the NaK ATPase (5) increased markedly in intensity. Another protein which uniquely ran as a broad fuzzy band and contained carbohydrate (see below) also increased in intensity. The position of the tracking dye is indicated by a sharp band at the bottommost part of the gel. In this particular run no protein was associated with this band at the final stage of purification, the staining being due to impurities in the gel which ran with the tracking dye (a similar band was obtained with blank gels). In other runs a small amount of protein was associated with the tracking dye. In this preparation this protein ran just above the position of the tracking dye.

Identification of the Catalytic Subunit by Polyacrylamide–SDS Gel Electrophoresis of ^{32}P Labeled NaK ATPase

The NaK ATPase is phosphorylated when incubated with ATP, Mg, and Na and dephosphorylated on addition of K (12–14). The phosphorylated intermediate appears to be an acyl phosphate (15, 16), apparently on the γ–carboxyl of a glutamyl residue (17). Measurement of the increment in radioactivity between enzyme incubated with [γ–^{32}P] ATP, Mg, and K and enzyme incubated with [γ–^{32}P] ATP, Mg, and Na gives the level of phosphorylated protein. Incubation of purified shark rectal gland NaK ATPase at optimal protein concentrations (20 μg/ml) with [γ–^{32}P] ATP and Mg with Na or with K gave an increment of radioactivity in the protein as high as 4,080 pmoles of acyl phosphate per mg

protein (18). This is twice the highest level of phosphorylated protein reported previously (5, 19). When the purified NaK ATPase was incubated with [^{32}P] ATP and Mg and with either Na or K, solubilized in SDS at 0°, and electrophoresed on SDS–polyacrylamide gels at 4°, a radioactive peak was found associated with the main protein on the gel (9). The radioactive peak appeared only under conditions of phosphorylation of the NaK ATPase (Na but not K present) and not under conditions of dephosphorylation (K but not Na present). This identifies the major band on the gel as the catalytic subunit of the NaK ATPase, as had been done previously for the NaK ATPase from beef brain (5) and other sources (19–22). The solubilization and electrophoresis in the cold gave a much higher yield of radioactivity associated with the catalytic subunit (9) than was obtained previously (5).

Carbohydrate Components of the Purified NaK ATPase

Cell membranes contain glycoproteins with the oligosaccharides projecting from the outer surface (23). Figure 2 shows the staining of polyacrylamide gels with periodic acid–Schiff reagent. The protein with a molecular weight of 55,000 gave a positive reaction. The 97,000 molecular weight protein, in spite of its much stronger intensity for protein staining, did not stain with PAS. One can conclude that the 55,000 MW protein is probably a glycoprotein. Another PAS staining band ran slightly ahead of the tracking dye. It is presumably glycolipid since it does not correspond to any protein band and glycolipids have been reported to run in this general area on SDS–polyacrylamide electrophoresis (23). Recently, Kyte (24) reported the presence of sialic acid, glucosamine,

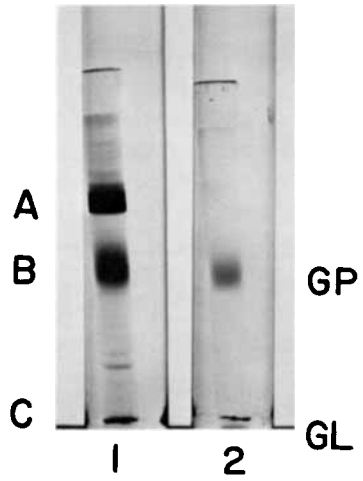


Fig. 2. Periodic acid–Schiff staining of SDS–polyacrylamide gels after electrophoresis of purified NaK ATPase. SDS–polyacrylamide gel electrophoresis and staining were carried out as described elsewhere (9). Gel No. 1 was stained with Coomassie blue. Gel No. 2 was stained with periodic acid–Schiff reagent. Considerably more enzyme was run (50 μg) than required for protein staining in order to show up the carbohydrate stain. This accounts for the appearance of weak bands of other proteins. The periodic acid–Schiff staining band at the bottom of the gel is probably glycolipid, based on its position and its failure to stain for protein. The position of the tracking dye is marked with draftsman's ink. (A) Catalytic subunit. (B) Glycoprotein. (C) Protein at position of tracking dye. GP–glycoprotein stained with periodic acid–Schiff reagent. GL–glycolipid stained with periodic acid–Schiff reagent. (Reproduced by permission of the Journal of Biological Chemistry.)

galactosamine, and neutral sugar in acid hydrolysates of a protein which is presumably the same as the glycoprotein reported here and which was isolated from a purified NaK ATPase from the outer medulla of the kidney.

Chromatography of SDS–Solubilized Purified NaK ATPase on Sephadex G–150

Figure 3 shows a typical elution profile of SDS–solubilized ammonium sulfate enzyme on Sephadex G–150 in the presence of 0.1% SDS. Three peaks of 280 nm absorbing material are seen. Disc gel electrophoresis of protein from the three peaks are also shown. Disc gel electrophoresis of fractions from the first peak showed it to contain only the 97,000 molecular weight catalytic subunit. The second peak contained primarily the 55,000 molecular weight glycoprotein with some contamination of the early fractions with the 97,000 molecular weight subunit. The third peak contained Coomassie blue

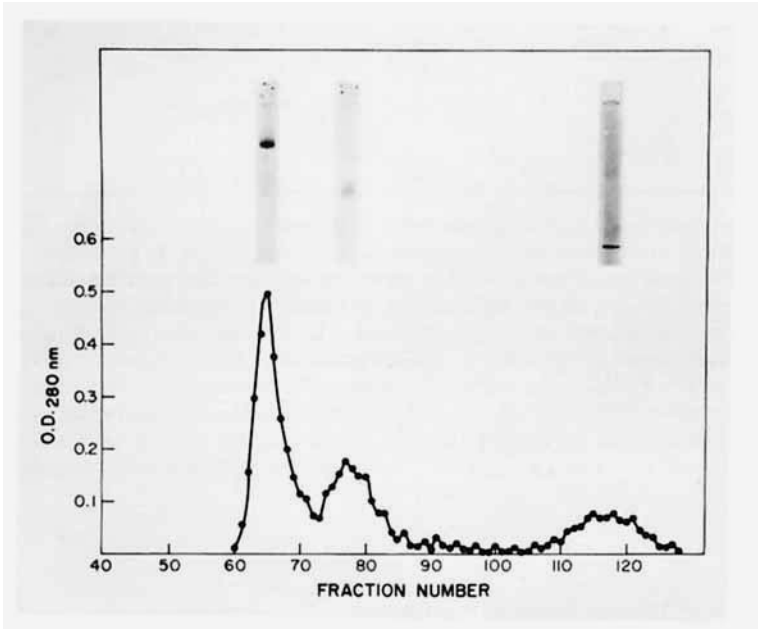


Fig. 3. Chromatography of SDS–solubilized ammonium sulfate enzyme on Sephadex G–150. Ammonium sulfate enzyme was solubilized in SDS and chromatographed on Sephadex G–150 in 0.1% SDS as described elsewhere (9). Based on the Lowry method, 10.7 mg of protein was applied to the column and 9.3 mg was recovered. Disc gel electropherograms of aliquots of selected fractions are shown above the peaks. (Reproduced by permission of the Journal of Biological Chemistry.)

staining material which ran with the tracking dye. These three peaks correspond to the three major protein bands seen on SDS–polyacrylamide gel electrophoresis of the purified enzyme. On the basis of their electrophoretic patterns fractions were pooled, and protein was determined by the method of Lowry et al. (25) before and after dialysis and lyophilization. Much of the 280 nm absorbing material in the third peak, although non-dialyzable, was not protein, as measured by the Lowry method (see below). Since both UV absorption at 280 nm and the Lowry method are based primarily on tyrosine content of protein, the discrepancy must be due to nonproteinaceous 280 nm absorbing material running with the third peak.

Table IV. Protein Composition at Various Stages of Purification of NaK ATPase from Membranes from Frozen and Fresh Glands[a]

Fraction	Scans of Coomassie blue stained gels			
	Catalytic subunit	Glycoprotein	Protein at tracking position	Other
From frozen glands				
Membranes	18.1	8.2	16.0	57.7
Lubrol extract	29.5	14.8	14.7	41.0
Zonal concentrate	35.7	16.8	17.4	30.1
Ammonium sulfate	72.5	19.0	8.5	–
From fresh glands				
Membranes	24.4	11.5	21.0	43.1
Lubrol extract	30.3	9.6	14.8	45.3
Zonal concentrate	44.0	20.0	28.7	7.3
Ammonium sulfate	71.5	19.9	8.6	–
	Chromatography on Sephadex G–150			
From frozen glands				
Ammonium sulfate	66.0	28.7	5.1	–

[a]In the case of gel electrophoresis 2.6 units (μmoles Pi/hr) of enzyme were run on duplicate gels for each fraction, stained with Coomassie blue, and scanned. In all fractions except the ammonium sulfate fraction the glycoprotein peak was drawn in pencil to correct for superimposed protein peaks running in the same region. The peaks were cut out, weighed, and the percentages calculated. Weights of peaks from duplicate gels agreed within 10%. In the case of Sephadex G–150 chromatography, fractions were obtained from the chromatogram shown in Fig. 3; The catalytic subunit peak (fractions 61–74), the glycoprotein peak (fractions 75–108), and the tracking dye protein (fractions 109–124), were identified by gel electrophoresis, were pooled, dialyzed, lyophilized, and protein determined by the Lowry method. Recovery from the column was 87%. SDS–Polyacrylamide electropherograms are shown for selected fractions from each peak (Fig. 3). (Reproduced by permission of the Journal of Biological Chemistry.)

Protein Composition at Various Stages of Purification

The results of scanning of Coomassie blue stained gels at various stages of purification of membranes from both fresh and frozen glands are shown in Table IV. The percentage composition of the ammonium sulfate enzyme determined by chromatography of SDS–solubilized enzyme on Sephadex G–150 is also given. Scanning of gels gave the following composition for the three proteins in the ammonium sulfate enzyme: catalytic subunit, 72.5%; glycoprotein, 19%; and protein at the position of the tracking dye, 8.5%. The percentage compositions of the ammonium sulfate enzymes purified from membranes from either fresh or frozen membranes were essentially identical. The percentage composition obtained by chromatography on Sephadex G–150 was: catalytic subunit, 66%; glycoprotein, 28.7%; protein in the position of the tracing dye, 5.1%. For a given quantity of each of the two major proteins isolated on Sephadex G–150 and determined by the Lowry method, the same area was obtained on scanning of Coomassie blue stained gels in which the proteins were run individually, indicating that the stained proteins had the same extinction coefficient. The fact that the results of scanning of Coomassie blue

stained gels and chromatography on Sephadex G–150 were in reasonably good agreement, although based on quite different methods of protein separation and detection, gives considerable validity to the percentages of the various proteins.

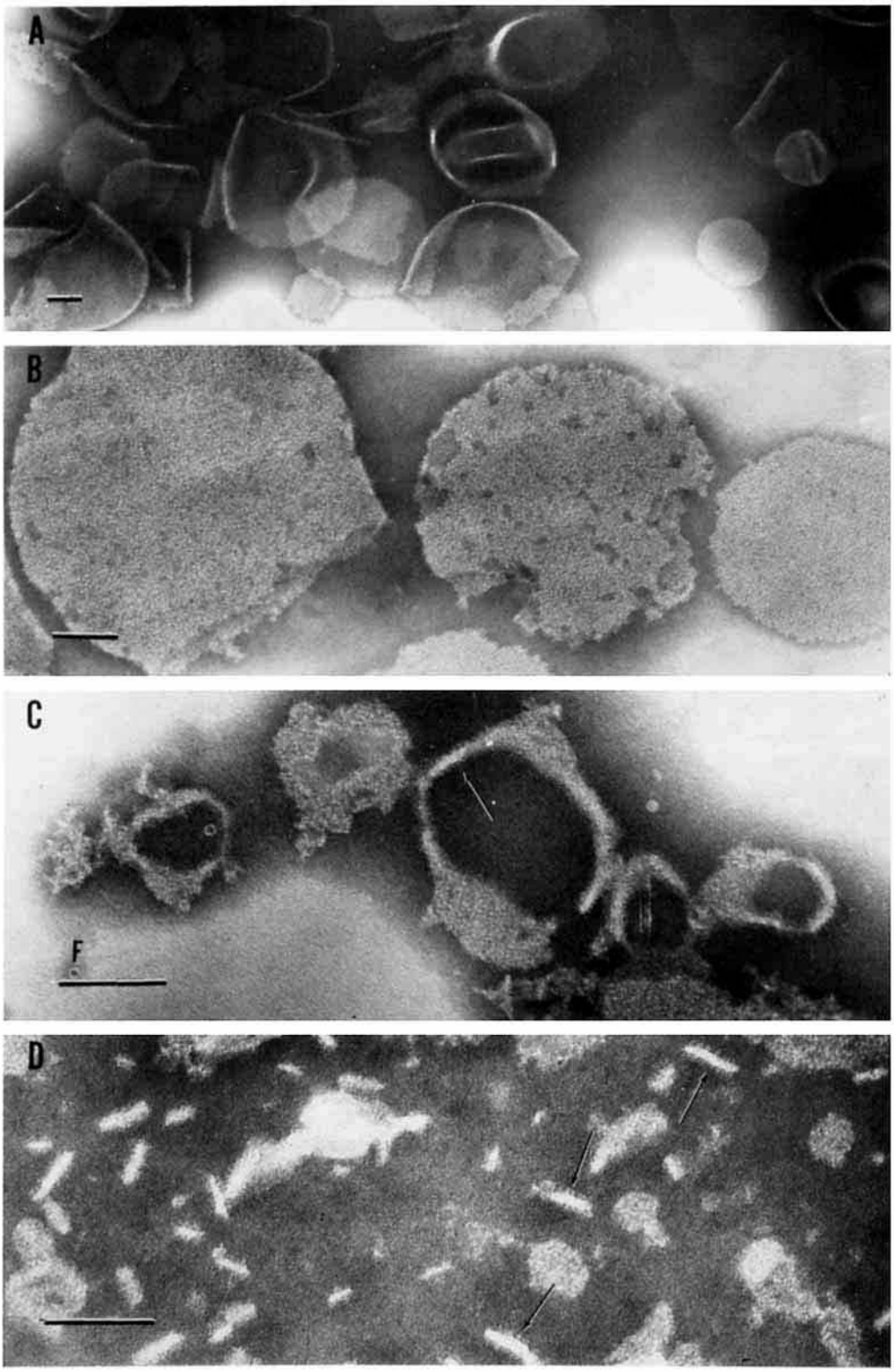
It was difficult to determine whether the molar ratio of the catalytic subunit to the glycoprotein was absolutely constant at each stage of purification because it was necessary to pencil in the glycoprotein peak at purification steps up to the ammonium sulfate fractionation due to the presence of other protein peaks in the glycoprotein region. This procedure may have led to an overestimation of the glycoprotein peak. A constant molar ratio between the two proteins is a condition which must be fulfilled if the two proteins are integral components of the NaK ATPase. However, from visual examination of Fig. 1 and the data in Table IV, it is clear that the two proteins did enrich more or less in parallel on purification.

Electron Micrographs of Purified Shark Rectal Gland NaK ATPase

The purified NaK ATPase from the rectal gland of the shark assumed different forms when it was precipitated with ammonium sulfate (Fig. 4). One form of the enzyme was vesicular (Fig. 4A and B). Some of the vesicles appeared to be intact. Others were broken; this may have occurred upon drying within the layer of phosphotungstic acid. A surface view of the vesicular membrane which reconstituted during ammonium sulfate treatment (Fig. 4B) gives a suggestion of subunit packing with the units having a diameter of 55 Å. These particles may be the same as those seen as projections from the rods and rings which were the other forms of the enzyme (Fig. 4C and D). These latter two forms were present in variable amounts along with the vesicular form. Because of their favorable orientation within the layer of negative stain, the rods and rings provide a more detailed picture of the organization of the NaK ATPase. Similar to what was observed with the beef brain enzyme, the rods and rings are composed of an electron translucent region which excludes the negative stain and which is about 80 Å in thickness and which is very likely hydrophobic (Fig. 4C and D). Attached to these rods and rings are small projections ranging in diameter from 35 to 55 Å and which are seen on both sides of the electron translucent rods and rings.

The projections appear to have taken up phosphotungstate more readily than the central core, suggesting that the former may be more hydrophilic. This would be compatible with their being composed of the smaller molecular weight glycoprotein seen on polyacrylamide

Fig. 4. Various morphological forms which the rectal gland NaK ATPase assumes on purification by ammonium sulfate fractionation. The specimens were examined by negative contrast. Each line represents 100 mμ. (A and B) Precipitation of the NaK ATPase results in the formation of membrane vesicles. Many of the vesicles are completely enclosed as evidenced by examination of thin sections [see Fig. 9 in Uesugi et al. (5)], while others are broken, possibly as a result of specimen preparation. On surface view of the vesicles, 4B, there is a suggestion of periodicity with particles having a diameter of about 45 Å. (C) Ring-like forms of the isolated NaK ATPase are also present in the ammonium sulfate preparations. They are frequently associated with elements which are obviously vesicular in nature. There is no evidence to indicate that the ring and rod forms, (D), are basically different from the membrane-like form. Ferritin (F) with a diameter of 110 Å is present as an internal marker. The small subunits (arrow) seen in favorable orientation are about 55 Å in diameter. The electron translucent region which forms the membrane has a thickness of about 80 Å. (D) Rod-like aggregates of the enzyme are a third form which is assumed during precipitation of the NaK ATPase with ammonium sulfate. Small globular units (arrows) are observed on both sides of the translucent, membrane-forming region of the rods. (A) × 44,400. (B) × 88,000. (C) × 144,000. (D) × 144,000. (Reproduced by permission of the Journal of Biological Chemistry.)


A
B
C
F
D

gels. Carbohydrate components of glycoproteins are known to project from the outer surface of membranes (23).

In different preparations of ammonium sulfate enzyme one or the other of the morphological forms usually predominated, and conditions for controlling the particular morphological form the enzyme takes on ammonium sulfate fractionation have not been worked out. In all instances, however, it is obvious that the enzyme has reverted to a membranous form. Attemps to separate various morphological forms on continuous and discontinuous sucrose gradients were only partially successful, but these experiments did indicate that all morphological forms had essentially the same specific activity for the NaK ATPase.

DISCUSSION

Rectal glands of elasmobranchs secrete salt in high concentrations, and this affords a mechanism for elimination of NaCl from the animal. The gills of these fish, in contrast to those of teleosts, are poorly equipped for elimination of salt. In view of their high capacity to secrete NaCl one would predict that rectal glands would be rich in NaK ATPase, and this has in fact been found to be the case (8). In the work reported here homogenates of this gland were richer by an order of magnitude in NaK ATPase than homogenates of beef brain cortex (5), which is a rich mammalian source of the enzyme.

Other rich sources of the enzyme are the outer medulla of the mammalian kidney (7, 26) and the electric organ of the electric eel (13, 27). The former generally gives low yields of NaK ATPase (7, 26), and the high specific activities of the enzyme from this organ are usually obtained only after activation of membranous preparations with deoxycholate (26) or assaying in the presence of this detergent (6). Membranes obtained from the electric organ are comparable in purity to the membranes obtained from the rectal gland of the dogfish, but, unfortunately, the enzyme from the former is highly unstable, particularly at mammalian temperatures (28). On the other hand, the enzyme from the rectal gland of the dogfish is extremely stable. The rectal gland is thus probably the most advantageous source available to date for purification of the NaK ATPase.

As shown here membranes from both fresh and frozen rectal glands can be prepared in reasonably good yields. The specific activity of the membranes from fresh glands is at the lower end of the range of specific activities of the partially purified enzyme from beef brain cortex, which was estimated to be 25 to 50% pure (5). The nonionic detergent, Lubrol WX, extracts the NaK ATPase from the membranes quantitatively and somewhat selectively, giving a two-fold purification. In the case of membranes from frozen but not from fresh glands there is a further doubling in specific activity due to activation. Further purification by zonal centrifugation and ammonium sulfate fractionation gives a preparation with a specific activity of 1,500 μmoles Pi/mg protein/hr, which is two to three times higher than the highest specific activities reported earlier by us (5) and by Kyte (7). This specific activity is comparable to that reported by Jorgensen et al. (6), but their assays were carried out in the presence of a concentration of deoxycholate which activated their enzyme five-fold (6, 26). No gel patterns were given. With the rectal gland enzyme reported here, a two-fold activation occurred on treatment of membranes from frozen glands with Lubrol; there was no activation of the enzyme by Lubrol with membranes from fresh glands. The activation was removed during purification since idential specific activities and gel patterns were obtained when enzyme from membranes from fresh or frozen glands was purified. The activation appears to be removed when the enzyme is separated

from free Lubrol by zonal centrifugation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a progressive elimination of protein bands on purification. At the last stage of purification, scanning of Coomassie blue stained gels showed that 73% of the protein could be accounted for by a 97,000 molecular weight protein and 19% by a 55,000 molecular weight glycoprotein. The corresponding percentages obtained by chromatography of SDS–solubilized ammonium sulfate enzyme on Sephadex G–150 were 66% and 29%. The 97,000 molecular weight protein was shown to be the catalytic subunit of the NaK ATPase by labeling it with ^{32}P by incubation with [γ–^{32}P] ATP, Na, and Mg. This molecular weight agrees closely with that previously reported by us (5) and by others (19–22). Much higher yields of radioactivity associated with this subunit have been achieved here by rapid solubilization of the protein with SDS and electrophoresis at 0 to 4°.

The purity of the present preparation rests heavily on the question as to whether the 55,000 molecular weight glycoprotein is a subunit of the NaK ATPase or is a contaminant (probably a cell membrane component) which binds firmly to the catalytic subunit and is not removed by all solubilization and purification procedures which have so far been tried. Kyte (7) favored the former view primarily because the ratio of his 84,000 molecular weight protein to his 55,000 molecular protein was constant in different fractions on chromatography of a deoxycholate "solubilized" partially purified enzyme on Sepharose 2B. However, it is likely that the two proteins were part of a membrane fragment under these conditions (the apparent molecular weight of the material was in the millions). One would expect a constant ratio on chromatography on Sepharose 2B if the fragment remained intact. Several lines of evidence presented here could be interpreted to mean that the catalytic subunit and the glycoprotein are both subunits of the enzyme. First, within the limits of the methods both proteins appeared to enrich in parallel as the NaK ATPase was purified. Second, at the final stage of purification the two subunits are seen in association in the form of membranous vesicles, rods, and rings, with the rods being 80 Å in diameter. Subunits project from the rods and rings at regular intervals; these projections range in diameter from 35 to 55 Å, and based on their affinity for phosphotungstic acid appear to be more hydrophilic. The size of the projections and their greater hydrophilicity are compatible with their being the glycoprotein component; projection of the carbohydrate moiety from the surface of the membrane is consonant with current views of membrane structure (23). It should also be mentioned that Kepner and Macey (29) calculated from their X-ray inactivation studies the diameter of the NaK ATPase to be 85 Å (assuming a spherical molecule).

Based on chromatography on Sephadex G–150 on scans of Coomassie blue stained polyacrylamide gels, the present preparation is 90 to 95% pure if both the catalytic subunit and the glycoprotein are components of the enzyme and 66–72% pure if only the catalytic subunit is a component of the enzyme. In either case the purity of the present preparation appears to be the highest so far reported in the literature. This is further supported by the very high level of phosphorylation of the protein on incubation with [γ–^{32}P] ATP, Na, and Mg, namely, 4,080 pmoles/mg protein (18). This level of phosphorylation is 2 to 3 times higher than the highest levels reported previously (5, 19). It is generally accepted that the level of phosphorylation is proportional to the number of NaK ATPase molecules. This is based on the fact that the level of phosphorylation parallels rather closely the specific activity of the enzyme (30) and the number of ouabain molecules bound (31) when different NaK ATPase preparations are studied. The level of phosphorylation of the present enzyme and the corresponding specific activity

give a turnover number of 6,300 min^{-1}, which is within the range of turnover numbers for a large number of NaK ATPase preparations (30).

The rectal gland enzyme is remarkably stable in the membranous form and in the purified form, both on freezing and on storage at 0° in the unfrozen state. This is a fortunate circumstance since it will permit a variety of studies of the purified enzyme.

It is of interest that negatively stained vesicular forms of the purified NaK ATPase have an almost identical structure on electron microscopy to that of purified cytochrome oxidase (32). Vesicles with projections of about the same diameter covering the surface of the vesicles are seen in both preparations. The rod and ring forms of the NaK ATPase reported here also resemble closely the reconstituted oligomycin-sensitive mitochondrial ATPase (32). These may be fairly common morphologies for purified and/or reconstituted membrane enzymes.

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