Preparation of Na^+,K^+-ATPase with Near Maximal Specific Activity and Phosphorylation Capacity: Evidence That the Reaction Mechanism Involves All of the Sites†

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ABSTRACT: The phosphorylation capacity of Na^+,K^+-ATPase preparations in common use is much less than expected on the basis of the molecular weight of the enzyme deduced from cDNA sequences. This has led to the popularity of half-of-the-sites or flip-flop models for the enzyme reaction mechanism. We have prepared Na^+,K^+-ATPase from nasal salt glands of salt-adapted ducks which has a phosphorylation capacity and specific activity near the theoretical maxima. Preparations with specific activities of >60 μmol (mg of protein)$^{-1}$ min^{-1} at 37 °C had phosphorylation capacities of >60 nmol/mg of protein, and the rate of turnover of the enzyme was 9690 min^{-1}, within the range reported for the enzyme from other sources. The fraction of the maximal specific activity of the enzyme compared well with the fraction of the protein on SDS−PAGE which was α and β chains, especially at the highest specific activity which indicates that all of the $\alpha\beta$ protomers are active. The gels of the most reactive preparations contained only α and β chains, but less active preparations contained a number of extraneous proteins. The major contaminant was actin. The preparation did not contain any protein which migrated in the molecular weight range of the γ subunit. The subunit composition of the enzyme was α_1 and β_1 only. This is the first report of a pure, homogeneous, fully active preparation of the protein. Reaction models which incorporate a half-of-the-sites or flip-flop mechanism do not apply to this enzyme.

Na^+,K^+-ATPase[1] (adensoine triphosphatase, EC 3.6.1.3), first identified by Skou (*1*), is the biochemical manifestation of the Na^+,K^+ pump which is responsible for the asymmetric distribution of Na^+ and K^+ across animal cell membranes. Early results of ion transport studies with intact cells showed that the movement of Na^+ out of the cell is coupled to the movement of K^+ in and both depend on the hydrolysis of ATP, and biochemical studies with enzyme preparations showed that Na^+ promotes phosphorylation from ATP and K^+ promotes dephosphorylation (*2*). The findings formed the basis of the Albers-Post model of the enzyme reaction mechanism which supposes that the enzyme exists in two major conformations, one of which can be phosphorylated by ATP and the other by inorganic phosphate, and that the reaction mechanism is ping-pong with respect to Na^+ and K^+. The model has been remarkably successful in accounting for the physiological, electrophysiological, and biochemical properties of the pump.

The enzyme is a heterodimer of an α subunit which contains the ligand binding sites and the phosphorylation site and which has an M_r of about 112 000 calculated from cDNA sequences (*3*−*5*) and a β subunit which is a glycoprotein with a protein molecular mass of about 35 kDa (*5*−*7*). Each $\alpha\beta$ protomer has a single phosphorylation site so that the maximal phosphorylation capacity of the enzyme should be 6.8 nmol of phosphate/mg of protein. Na^+,K^+-ATPase, both in situ and in purified preparations, has been found to turn over at a rate of 9000−10000 times/min at optimal substrate concentrations and 37 °C regardless of the specific activity of the specimen. From these values, the specific activity of the fully active enzyme should be 62−68 EU.

Soon after purified Na^+,K^+-ATPase preparations became available twenty-five years ago, it was found that maximal phosphorylation could not be achieved even with enzyme preparations which consisted entirely of α and β chains (*8*, *9*). About the same time, transport experiments were reported which seemed to show that Na^+−K^+ exchange is not ping-pong, but occurs simultaneously in a single step (*10*, *11*). These findings led to the popularity of reaction mechanisms (half-of-the-sites and flip-flop mechanisms) in which the functional unit is an $(\alpha\beta)_2$ diprotomer in which each protomer performs the steps of a transport cycle 180° out of phase with interaction between the protomers (*12*, *13*). It is now clear that, as originally proposed, transport of Na^+ and K^+ is via a ping-pong mechanism (*14*−*18*) so that one of the major supports of the half-of-the-sites mechanism was removed.

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[1] Abbreviations: Na^+,K^+-ATPase, Na^+- and K^+-dependent adenosine triphosphatase; SDS, sodium dodecyl sulfate; P_i, inorganic phosphate (ortho); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TCA, trichloroacetic acid; EU, enzyme unit, 1 EU = 1 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}; PAGE, polyacrylamide gel electrophoresis; PNPP, *p*-nitrophenyl phosphate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether.

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Reconciliation of the phosphorylation data with the Albers-Post model has been less successful. Most purified Na^+,K^+-ATPase preparations have specific activities that are lower than the theoretical maximum, and usually much lower. Although in rare cases an enzyme with a specific activity or binding capacity significantly greater than 50% of the maximum has been prepared (*19*), the procedure requires extraordinary skill and has not been reproduced in other hands (*20*). Recent careful attempts to prepare fully active enzyme have yielded preparations with specific activity and phosphorylation capacity that are slightly different from 50% of the theoretical maxima (*20*, *21*). The question of whether the enzyme mechanism involves half-of-the-sites or flip-flop steps persists (*22*).

The question will never be resolved by functional studies with inadequate enzyme preparations. The demonstration that it is possible to reproducibly prepare enzyme with near maximal specific activity and phosphorylation capacity alone will settle the issue. Here we demonstrate that it is possible to prepare such an enzyme in reasonable quantity.

EXPERIMENTAL PROCEDURES

Materials

Orthophosphate ($^{32}P_i$) was obtained from ICN as high-concentration H_3PO_4 in HCl-free H_2O (catalog no. 64014). [γ-^{32}P]ATP was purchased from Amersham (catalog no. PB.168). Gel electrophoresis molecular weight standards SDS-6H, SDS-7, and SDS-17S were purchased from Sigma, and biotinylated SDS−PAGE standards (low-, high-, and broad-range) were purchased from Bio-Rad. SDS was BDH brand Prod 44215. Immobilon-P was purchased from Millipore. 2F antibody against the α_1 subunit was a gift from D. Fambrough. Anti-rat α3 (6412) was a gift from R. Mercer. Anti-TED antibody against the α_3 subunit was a gift from T. Pressley. McB2 antibody against the α_2 subunit was a gift from K. Sweadner. JLA20 antibody against actin developed by J. J.-C. Lin and b4β4 and 24 antibodies against the β_1 subunit developed by D. Fambrough were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences at the University of Iowa (Iowa City, IA 52242). MA3-915 antibody against the α_3 subunit was purchased from Affinity BioReagents. All sucrose was ultrapure, enzyme grade and was obtained from varied sources. All other reagents were research grade.

Methods

Na^+,K^+-ATPase Preparations. Microsomal and purified Na^+,K^+-ATPase were prepared from the nasal salt glands of salt-adapted Pekin ducks following a protocol based on those of Hopkins et al. (*23*) and Jorgensen (*24*). Two-week-old white Pekin ducks were fed standard duck mash and given 1% NaCl as their only water source for about 10−13 days. The ducks were euthanized, and the supraorbital salt-secreting glands were dissected free of adherent connective tissue, submerged in liquid N_2, and transferred to a tared beaker containing ice-cold homogenization buffer [0.25 M sucrose/20 mM Tris/1 mM EDTA (pH 7.5)]. The glands were kept ice-cold and minced finely with scissors. The minced glands were suspended at 10 mL/g of tissue in ice-cold homogenization buffer. The suspension was homogenized with a Polytron P10 apparatus with a PT20A generator set at 4.5 for five periods of 15 s separated by 2 min on ice. The homogenate was passed through three layers of cheesecloth and the filtrate centrifuged for 15 min at 6500 rpm (average of 3300*g*) in a Sorval SS34 rotor. The supernatant was removed and saved and the pellet resuspended in 10 mL of homogenization buffer by two strokes of an ice-cold Potter homogenizer at 1300 rpm. The suspension was centrifuged as described above, and the supernatants were combined and centrifuged at 17 000 rpm (average of 22500*g*) in a Sorval SS34 rotor for 1.5 h. The resultant pellet was resuspended using a buffer stream generated with a pipet to a concentration of ∼10−15 mg/mL in homogenization buffer (∼5 mL of buffer when starting with 12 ducks). The resuspended microsomes were quick-frozen in liquid N_2 and stored at −80 °C.

Na^+,K^+-ATPase was purified from the microsomes by partial extraction with SDS. Salt gland microsomes were incubated at 20 °C at a concentration of 1.4 mg/mL in an extraction medium with the following composition: 2.3 mM EDTA, 3.0 mM Na_2ATP, and 19.3 mM Tris (pH 7.5). SDS was added to the stirred solution at a rate of 0.19−0.67 mg/min depending on the final incubation volume such that the time for SDS addition was between 10 and 25 min in length. This was most conveniently done by using a stock 2 mg/mL SDS solution and a metered syringe pump. The final incubation conditions were 1 mg/mL protein, 0.55 mg/mL SDS, 1.7 mM EDTA, 2.2 mM Na_2ATP, and 14 mM Tris (pH 7.5) (∼25 mM sucrose carried over with microsomes). This mixture was incubated for 40 min at 20 °C. The incubate (2−5 mL) was subsequently layered over a step sucrose gradient formed in a Beckman SW27 Ultraclear centrifuge tube with the following sucrose solutions (w/v) in 1 mM EDTA and 20 mM Tris (pH 7.5): 4.1 mL at 50%, 12.4 mL at 29.4%, 9.9 mL at 15%, and 6.6 mL at 10%. The gradients were centrifuged in a Beckman SW27 rotor at 25 000 rpm (average of 85000*g*) for 7−16 h at 4 °C. Two major bands were visible at the end of the centrifugation. The band at the 29.4%−50% interface was removed. The sucrose concentration of the removed band was reduced to <10% by adding 20 mM Tris, 1 mM EDTA buffer (pH 7.5), and the diluted suspension was centrifuged for 1 h at 50 000 rpm (average of 170000*g*) in a Beckman Type 65 rotor at 4 °C. The resultant supernatant was discarded, and the pellets were carefully resuspended with limited foaming using a buffer stream from a pipet in a minimal volume of homogenization buffer at 4 °C. The samples were quick-frozen in liquid N_2 and stored at −80 °C. Starting with 12 ducks, we typically obtained 3−4 mg of purified Na^+,K^+-ATPase at a concentration of 1−3 mg/mL.

Na^+,K^+-ATPase Activity Assay. Na^+,K^+-ATPase activity was routinely measured using an NADH-coupled assay. The assay medium contained 40 mM NaCl, 20 mM KCl, 1.5 mM trisodium phosphoenolpyruvate, 5 mM Na_2ATP, 5 mM $MgCl_2$, 0.2 mM NADH, 5 mM EGTA, 15 units/mL pyruvate kinase, 36 units/mL lactate dehydrogenase, and 10 mM Tris (pH 7.5). Activity was assayed by monitoring the loss of OD_{340} of the assay medium after addition of the ATPase preparation to a thermostatically controlled cell in a Hewlett-Packard 8452 diode array spectrophotometer. ATPase activity was also assayed by directly following the amount of P_i

released from ATP hydrolysis using a colormetric assay based upon that of Peterson (*25*). The methods agreed within ±10%. The maximal ATPase activity from salt gland microsomes was obtained after incubating the microsomes (0.2 μg/μL) in 0.5% saponin (10 min at room temperature) to increase the membrane permeability to substrates prior to adding the microsomes to the assay medium (additional saponin was not added to the assay medium). The maximal ATPase activity from purified Na^+,K^+-ATPase did not require saponin. Unless otherwise indicated, activity is expressed in EU where 1 EU is 1 μmol of ATP hydrolyzed per minute per milligram of protein at 37 °C. The protein concentration was determined using the Peterson (*26*) modification of the Lowry assay. Recrystallized BSA was used as the standard, and the BSA concentration was verified by absorption spectroscopy using an extinction coefficient $\epsilon^{1mg/mL}$ of 0.667 at 279 nm.

Phosphorylation of Na^+,K^+-ATPase by P_i. Na^+,K^+-ATPase was phosphorylated with orthophophorus-32 ($^{32}P_i$). High-concentration, carrier-free $^{32}P_i$ was treated to remove radioactive contaminants which have a high affinity for biological membranes (*27*). Two methods were used for this purpose. One method was similar to that described previously (*28*). Briefly, 1 volume of washed human red cell membranes was added to 2 volumes of 80 mM Tris, 0.8 mM EGTA, and 4 mM $^{32}P_i$ (200 Ci/mol) (pH 7.2) and the mixture incubated for 15 min at room temperature. The suspension was centrifuged at 200000*g* for 30 min at 4 °C, and the supernatant was transferred to another centrifuge tube containing 1 volume of washed red cell membranes. The incubation and centrifugation were repeated, and the resultant supernatant was removed and used the same day as the $^{32}P_i$ source for phosphorylations. Alternatively, radioactive contaminants were removed by paper chromatography. Typically, to obtain 1 mL of $^{32}P_i$ that was suitable for phosphorylation, 300 μCi of high-concentration $^{32}P_i$ (≥100 μCi/μL) was spotted 1 cm from the bottom of a 1.3 cm × 11.4 cm strip of Whatman 3MM filter paper. If necessary, the spot was applied in multiple aliquots not exceeding 1 μL per aliquot. The spot was allowed to dry for 10 min, and then the strip was lowered into an 18 mm × 150 mm glass tube containing 1 mL of eluant buffer [40 mM Tris, 0.2 mM EGTA, and 2 mM Tris-phosphate (pH 7.2)]. The top of the tube was covered with a Petri dish cover and the chromatograph allowed to develop until the solvent front reached within 2 mm of the top of the paper strip (~30 min). The strip was removed from the tube, and the top 1.3 cm was cut off and placed in a 2 mL screw top microfuge tube. One milliliter of eluant buffer was added to the tube and the tube incubated at room temperature for 30 min with vortexing every 10 min. The eluant buffer was removed form the microfuge tube and passed through a Nalgene 4 mm, 0.2 μm cellulose acetate syringe filter. The resultant $^{32}P_i$ solution (~100−200 Ci/mol) was used directly or isotopically diluted with $^{31}P_i$ as needed. Using the same size paper strip, the method worked equally well for smaller amounts of $^{32}P_i$ and extracting the excised 1.3 cm top of the strip with 500 μL of buffer. The resultant $^{32}P_i$ solution was generally used immediately but proved to be satisfactory for phosphorylations for 2−3 days after purification. The total phosphate concentration of all $^{32}P_i$ solutions was determined against a standard curve using acidic ammonium molybdate and Fiske & Subbarow reagent following the method of Peterson (*25*) without the addition of SDS.

Unless otherwise indicated, the usual reaction for phosphorylation by $^{32}P_i$ was conducted in a microfuge tube containing 100 μL of 3 mM $MgCl_2$, 0.5 mM ouabain, 0.5 mM $^{32}P_i$ (100−200 Ci/mol), 40 mM Tris (pH 7.2), and a Na^+,K^+-ATPase preparation at a protein concentration of 50 μg/mL. The extent of nonspecific phosphate incorporation was measured in parallel in tubes containing the same amount of protein and $^{32}P_i$ but no $MgCl_2$ or ouabain and with the addition of 1 mM EDTA. The tubes were incubated for 10 min at room temperature. The reaction was quenched with 1 mL of ice-cold, freshly prepared 10% TCA containing 0.2 M H_3PO_4. Sodium deoxycholate (100 μL, 0.15%) was added to each tube, and the tubes were vortexed and incubated on ice for 10 min. The membranes were centrifuged for 5 min in an Eppendorf microfuge at 4 °C and washed five times with 1 mL of ice-cold 5% TCA and 0.1 M H_3PO_4. The resultant pellets were dissolved in 100 μL of 1% SDS and 0.1 N NaOH. The tubes containing the dissolved pellets were subsequently treated with 100 μL of 0.1 N HCl, dissolved in 1 mL of Packard Ultima Gold LSC-Cocktail, and counted in a Packard 1900CA liquid scintillation counter. The total exposure time of the sample to TCA was recorded and used to correct the data for dephosphorylation which occurs in the presence of TCA. Control studies determined that at ice-cold temperatures the preparation was dephosphorylated in the presence of TCA with a rate constant of ~0.0007 min^{-1}. The correction was usually on the order of 5−7% and never more than 10%. Turnover numbers were determined by simultaneously measuring the ATPase activity of an aliquot of the Na^+,K^+-ATPase preparation used in the phosphorylation study.

Polyacrylamide Gel Electrophoresis and Immunoblots. Gel electrophoresis was conducted using varied gel systems as indicated in the figure legends. In all gels, except that of Figure 10, we used the discontinuous buffer system of Laemmli (*29*) and had stacking gels of 5% and resolving gels of either 7.5 or 10% polyacrylamide as indicated in the figure legends. All samples were reduced by incubation for 1 h at room temperature in 1% SDS and in either 5% 2-mercaptoethanol or 50 mM dithiothreitol. Heating the purified Na^+,K^+-ATPase tended to produce nondissociable aggregates. Gels were blotted onto Immobilon-P using an LKB Multiphor II semidry apparatus. Immunostaining was performed using various primary antibodies and the biotinylated conjugate of appropriate anti-mouse IgG or anti-rabbit IgG. Streptavidin-conjugated alkaline phosphatase and BCIP/NBT phosphatase substrate were used for color development.

RESULTS

The Preparation. The salt gland microsomes are comprised mainly of vesicles with diameters of ~0.1−0.2 μm as shown Figure 1, an electron micrograph of a suspension of negatively stained microsomes. Protein within the microsome suspension is not homogeneously distributed, as shown in Figure 2. When we centrifuged the microsome suspension over a linear sucrose gradient, we found that peak ATPase activity migrated with a density corresponding to ~34% sucrose (w/w) (ρ ~ 1.148 g/mL). The maximum protein

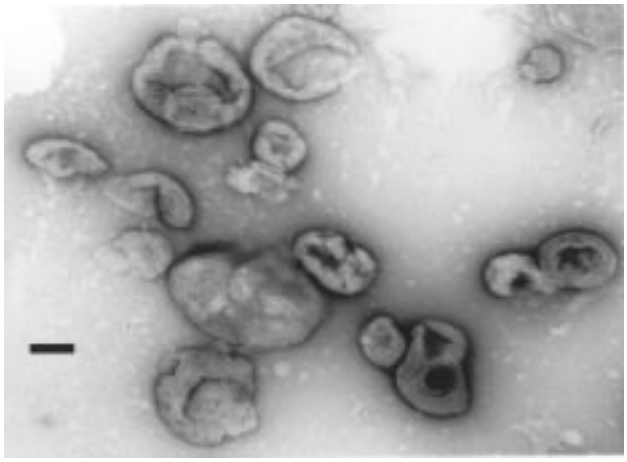

FIGURE 1: Electron micrograph of duck salt gland microsomes. Microsomes were attached to a Formvar-coated copper grid, negatively stained in 1% uranyl acetate, and viewed under an 80 kV beam at a magnification of 40000×. The bar is 0.1 μm long.

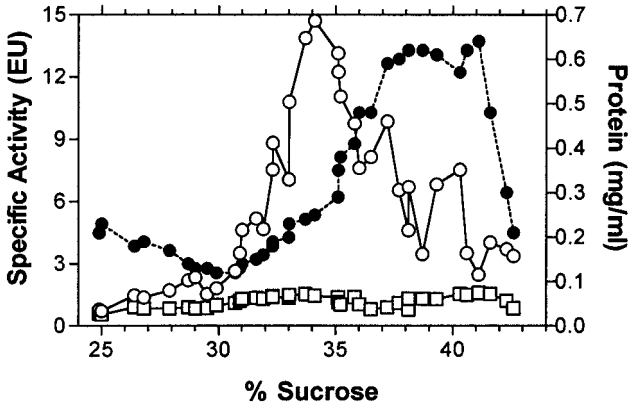

FIGURE 2: Salt gland microsomes were fractionated over a linear sucrose gradient. Prior to ATPase activity measurements, the samples were incubated with 0.5% saponin for 10 min at room temperature. ATPase activity was measured at 37 °C as described in Methods. White circles (○) represent the specific activity of each fraction, white squares (□) the specific activity in the presence of 0.1 mM ouabain, and black circles (●) the protein concentration in each fraction.

concentration peaked at a density corresponding to ~39% sucrose ($\rho \sim 1.173$ g/mL). More than 90% of the ATPase activity in the peak fractions was inhibited after incubation for 10 min with 0.1 mM ouabain. Maximal Na^+,K^+-ATPase activity in the peak fractions of gradients from six different preparations ranged from 8 to 18 EU.

The salt gland microsome suspension is partially clarified after extraction with SDS as described in Methods. This suspension is passed over a sucrose gradient as described in Methods, and the predominant proportion of the material is collected at the cushion interface. A small and variable amount of material floats above the interface. Although we have not throughly characterized this less dense material, we find that its specific activity is much smaller (<30 EU) than that of the denser fraction (≥60 EU). During the early phases of protocol development, we found that we could improve the specific activity of the resultant purified Na^+,K^+-ATPase if we prefractionated the microsomes over a sucrose gradient as described in the legend of Figure 2 and used the microsome fraction with the highest specific activity for the subsequent SDS extraction step. The protocol as described

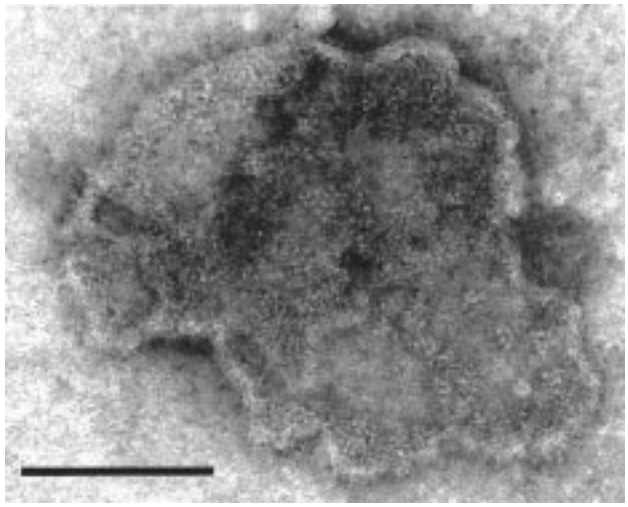

FIGURE 3: Electron micrograph of purified Na^+,K^+-ATPase preparation. A purified Na^+,K^+-ATPase preparation was adhered to a Formvar-coated copper grid, negatively stained in 1% uranyl acetate, and viewed under an 80 kV beam at a magnification of 200000×. The bar is 0.1 μm long.

in Methods now routinely yields Na^+,K^+-ATPase with specific activities of >60 EU without prior fractionation of the microsome suspension. All of the ATPase activity in the purified preparation can be inhibited by ouabain. Figure 3 shows an electron micrograph of the purified Na^+,K^+-ATPase preparation resulting from extraction of salt gland microsomes with SDS. This preparation does not contain clearly defined vesicles but rather membrane fragments that are a more heterogeneous mix of sizes compared with the microsome suspension, although fragments with diameters of 0.1−0.2 μm prevail within the preparation. The distribution of particles within the membrane fragments is similar to that described in preparations from other sources (*30*). The particles appear to be associated in close contact, forming patches of random aggregates separated by particle-free areas.

The protein composition of the varied salt gland preparations was analyzed by polyacrylamide gel electrophoresis (Figure 4). The same amount of total protein was applied to each lane of the gel. Lane 1 contained a sample of salt gland microsomes, lane 2 a sample of salt gland microsomes taken from the peak activity region of a sucrose fractionation similar to that of Figure 2, lane 3 a sample of purified Na^+,K^+-ATPase with a specific activity of 42 EU, and lane 4 a sample of purified Na^+,K^+-ATPase with a specific activity of 62 EU. In all lanes, the α subunit ($M_r \sim 100\,000$) and the β subunit ($M_r \sim 58\,000$) of the Na^+,K^+-ATPase are the predominant bands. Comparing the contents of lanes 1 and 2 reveals a subtle but reproducible removal of non-ATPase bands. When a similar analysis was carried out on all of the fractions from a sucrose gradient purification of microsomes, such as in Figure 2, we observed a continuum in the relative amounts of non-ATPase bands compared with those of the α and β subunits where the high-density, high-protein fractions tended to have greater relative amounts of non-ATPase components. The band migrating at $M_r \sim 45\,000$ became particularly prominent in the high-protein, low-specific activity fractions. We also see the presence of this band in lane 3 which contained purified Na^+,K^+-ATPase with a lower specific activity. The high-specific activity preparation in lane 4 does not have this band. We found that the

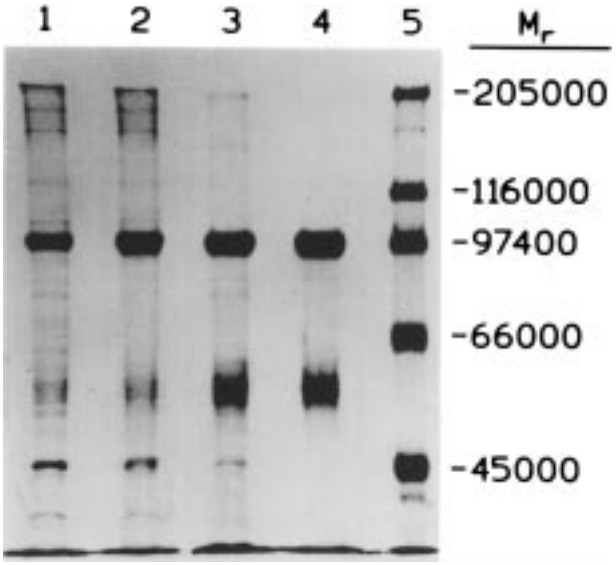

FIGURE 4: Polyacrylamide gel electrophoresis of various salt gland preparations on a 7.5% Laemmli gel: lane 1, salt gland microsomes; lane 2, high-activity microsomes purified using a linear sucrose gradient; lane 3, SDS-extracted, purified Na^+,K^+-ATPase, with a specific activity of ~42 EU; and lane 4, SDS-extracted, purified Na^+,K^+-ATPase, with a specific activity of ~62 EU. All lanes had the same total protein and were stained with Coomassie blue. All activities were measured at 37 °C. Lane 5 contained molecular weight standards.

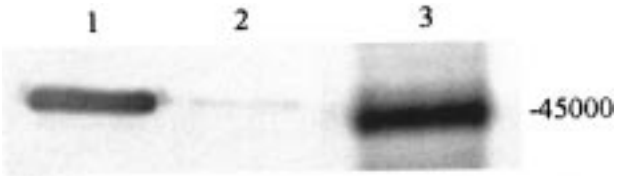

FIGURE 5: Actin is a nonpump protein in the duck salt gland preparations. A salt gland microsome suspension and a purified Na^+,K^+-ATPase preparation were subjected to SDS−PAGE over a 10% Laemmli slab minigel (8 cm × 7 cm × 0.15 cm), blotted onto Immobilon-P, and immunostained for actin using antibody JLA20 as the primary antibody, goat anti-mouse IgM biotin conjugate as the secondary antibody, and a streptavidin-linked alkaline phosphatase BCIP/NBT color development system. The 45 000 Da region of the blot is shown. Lane 1 contained 8 μg of duck salt gland microsomes. Lane 2 contained 8 μg of purified Na^+,K^+-ATPase. Lane 3 contained biotinylated ovalbumin, with an MW of 45 000 Da. The purified Na^+,K^+-ATPase used in this study had a specific activity of ~65 EU and did not have a visible band in the 45 000 Da region in Coomassie-stained minigels of 8 μg of protein. The Coomassie-stained gel of the microsome preparation was similar to that of lane 1 of Figure 4.

appearance of this band was well-correlated with preparations of purified Na^+,K^+-ATPase having lower specific activities. Using actin specific monoclonal antibodies, we determined that actin is present in our preparation and that it migrates to the same location as the 45 000 Da contaminant. Preparations which contain more of the contaminant exhibit a heavier immunostaining for actin (Figure 5). We therefore believe that the 45 000 Da contaminant is probably actin.

These data suggested to us that the presence of cytoskeletal proteins is a major determinant of the specific activity of the purified Na^+,K^+-ATPase from duck salt glands. To test this hypothesis, gels were run on preparations with varied specific activities and stained with Coomassie blue. The bands and regions of the gel lanes were excised, and the

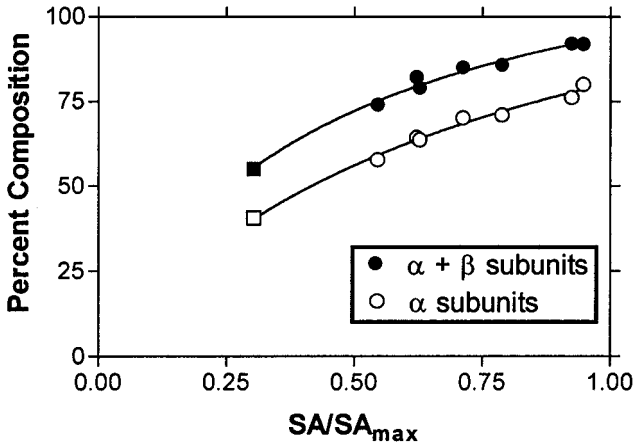

FIGURE 6: Specific activity is correlated with the fraction of total protein which is Na^+,K^+-ATPase. Preparations of purified Na^+,K^+-ATPase were resolved on polyacrylamide electrophoresis gels and stained with Coomassie blue. The protein bands were excised, and Coomassie blue stain was extracted as described by Michalak et al. (*74*). The optical absorbances at 560 nm of the extracted α and β subunits were divided by the total absorbance of all bands within the gel lane and used to determine the percentage of the total protein that could be attributed to the Na^+,K^+-ATPase subunits. The circles represent data from purified Na^+,K^+-ATPase preparations and the squares from a salt gland microsome suspension. The black symbols represent the percent composition that can be attributed to the sum of the α and β bands. The white symbols represent the percent of total protein that can be attributed to the α subunit. Na^+,K^+-ATPase activity was measured as described in Methods. The specific activity (SA) of each preparation was divided by the theoretical maximum specific activity (SA_{max}) of 66 EU to give the fraction of maximum specific activity measured for each preparation.

absorbance of the Coomassie stain was extracted from each piece that was measured. The fraction of total absorbance of the Coomassie stain in each lane which could be attributed to the absorbance of the bands from the α and β subunits was determined. Figure 6 shows the comparison of eight preparations with varied specific activities. The fraction of Coomassie staining that can be attributed to the sum of the α and β subunit regions is represented by the black symbols, and the white symbols represent the fraction of Coomassie staining that can be attributed only to the α subunit. The α subunit only data are presented because the diffuse staining of the β region made precise demarcation of the β band at times difficult. The circles are data from purified Na^+,K^+-ATPase preparations obtained during a 3 year period. The squares are from a high-activity salt gland microsome preparation. To clarify the relationship between fractional protein composition and specific activity, the specific activities of the preparations are expressed as a fraction of the maximum theoretical specific activity of 66 EU based upon ATPase turnover rates and discussed below. The purified preparations exhibit a good correlation between the specific activity of the preparation and the protein fraction that can be attributed to the Na^+,K^+-ATPase subunits. For the highest-activity preparations, the fraction of the protein due to α and β subunits is nearly identical to the fractional specific activity. Even the microsome preparation, which contains higher levels of non-ATPase proteins and for which precise excising of the subunit bands is more difficult, exhibits a close correlation between the amount of non-ATPase protein and fractional specific activity. The data suggest that this preparation produces Na^+,K^+-ATPase in which the specific

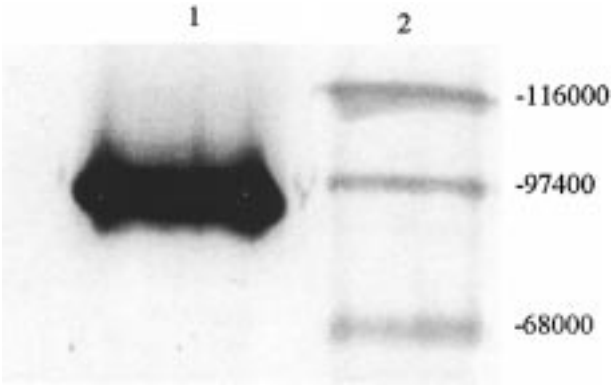

FIGURE 7: Purified Na^+,K^+-ATPase immunostains strongly for the α_1 isoform. A 0.25 μg sample of purified Na^+,K^+-ATPase was applied to a 6 mm wide well on a minigel electrophoresis unit and resolved using a 10% Laemmli gel. The protein was blotted onto Immobilon-P and treated with primary antibody 2F which is specific to the α_1 isoform of the Na^+,K^+-ATPase. The blot was treated with secondary antibody goat anti-mouse IgG biotin conjugate and stained with the alkaline phosphatase system described in Methods. Lane 1 contained the Na^+,K^+-ATPase sample. Lane 2 contained biotinylated molecular weight markers.

activity of the enzyme units is relatively constant between preparations and the measured specific activity in large part reflects the degree of purification from non-ATPase proteins.

The Preparation Contains α_1 and β_1 Subunits and No γ Subunits. Coomassie blue-stained gels of our most active preparations usually contain only α and β subunit bands. Occasionally, other very minor high-molecular weight bands are discernible under careful inspection. We hypothesize that the occasional faint high-molecular weight bands observed in Coomassie blue-stained gels of high-activity preparations are probably due to some aggregation of the ATPase protein during preparation for electrophoresis since this protein has a tendency to aggregate when severely denatured, as mentioned in Methods. The purified Na^+,K^+-ATPase reacts strongly with monoclonal antibodies that are specific to the α_1 subunit isoform. In a minigel format, $\leq$0.25 μg of the protein applied to a 6 mm wide well was sufficient to give a very strong immunostain (Figure 7). At higher loads, we see some immunostaining in the regions of the high-molecular weight bands mentioned above, supporting our hypothesis that these bands represent aggregates of the ATPase. We have carried out two-dimensional electrophoresis of purified ATPase in which we excised a lane of electrophoresed protein and subsequently layered that lane onto another gel, electrophoretically resolved the protein in the second dimension, and blotted and immunostained the resultant gel for the α_1 subunit. We found that some of the high-molecular mass protein migrated in the second dimension as a 100 000 Da protein which stains for the α_1 subunit (data not shown). These observations also argue that the high-molecular weight bands contain aggregated α subunits.

The purified Na^+,K^+-ATPase reacts weakly with antibodies against the α_2 subunit isoform. We observed no detectable reaction of anti-α_2 with $\leq$2 μg of ATPase. Figure 8 shows an immunostain of 2, 5, 10, and 20 μg of purified Na^+,K^+-ATPase with anti-α_2. Also shown is the immunostain of 2 μg of an SDS-extracted duck brain homogenate. Comparing the stain intensities indicated that 2 μg of duck brain preparation was stained with about the same intensity as that of 10 μg of purified Na^+,K^+-ATPase. The purified Na^+,K^+-ATPase had a specific activity of 54, and the brain preparation had a specific activity of 5.8 which could be inhibited by 90% by ouabain. If we assume that 100% of the brain preparation was α_2 and that the brain and salt gland Na^+,K^+-ATPases have the same turnover rate, than on the basis of the relative staining intensities we calculate that <2% of the purified salt gland Na^+,K^+-ATPase is α_2. We do not know the isoform makeup of our duck brain preparation which originates from a whole brain homogenate. However, we may safely assume that the brain preparation was not 100% α_2 and that therefore the salt gland preparation is probably <1% α_2. At these levels, we believe the most probable explanation of our blot data is that there was some cross-reactivity between the high levels of blotted α_1 in the salt gland preparation and the anti-α_2 antibody and that the purified Na^+,K^+-ATPase contains no α_2.

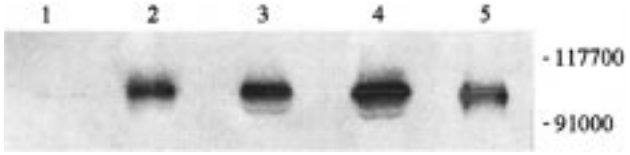

FIGURE 8: Purified salt gland Na^+,K^+-ATPase immunostains weakly for the α_2 isoform. Various amounts of purified salt gland Na^+,K^+-ATPase and a sample of semipurified duck brain Na^+,K^+-ATPase were electrophoresed on a 7.5% Laemmli standard sized gel (12 cm $\times$ 11 cm $\times$ 0.15 cm, 9 mm lane). The gel was blotted onto Immobilon-P and immunostained with α_2 specific Mcb2 primary antibody as described in Methods. Lanes 1−4 contained 2, 5, 10, and 20 μg of salt gland Na^+,K^+-ATPase, respectively. Lane 5 contained 2 μg of duck brain Na^+,K^+-ATPase.

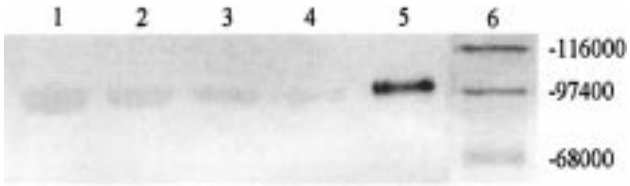

FIGURE 9: Purified salt gland Na^+,K^+-ATPase does not immunostain for the α_3 isoform. Various amounts of purified salt gland Na^+,K^+-ATPase and a sample of semipurified duck brain Na^+,K^+-ATPase were electrophoresed on a 7.5% Laemmli minisized gel (8 cm $\times$ 5 cm $\times$ 0.15 cm, 5 mm lane). The gel was blotted onto Immobilon-P and immunostained with α_3 specific anti-TED primary antibody as described in Methods. Lanes 1−4 contained 8, 4, 2, and 1 μg of salt gland Na^+,K^+-ATPase, respectively. Lane 5 contained 1 μg of duck brain Na^+,K^+-ATPase. Lane 6 contained biotinylated molecular weight standards. The shadowy bands seen in lanes 1−4 reflect a nonspecific interaction due to the high protein load.

We also tested the purified Na^+,K^+-ATPase preparation for the presence of α_3 isoforms. Earlier studies of the duck salt gland Na^+,K^+-ATPase preparation indicated that the preparation did not contain α_3 isoforms (*31*). However, the antibodies used in that study (*32*) were raised against the N-terminus of rat α_3, and we determined that they do not react with duck α_3 as evidenced by their failure to react against our duck brain preparation, although they did react against a rat brain preparation (data not shown). Similar results were also obtained with a commercially available anti-α_3 raised against rabbit (BioAffinity Reagents antibody MA3-915). We obtained another anti-α_3 as a gift from T. Pressley (anti-TED). This antibody had already been shown to react against chicken brain (*33*). Figure 9 shows a Western blot of a minigel of 1 μg of our duck brain Na^+,K^+-ATPase

Table 1: N-Terminal Sequence of Duck Salt Na^+,K^+-ATPase α and β Subunits[a]

α-subunit:	G	R	D	K	Y	E	P	T	A	T	S	E	H	G	A	K	K	K	K[b]	G				
				5						10					15					20				
β-subunit:	A	R	G	K	A	K	D	G	D	G	N	W	K	K	F	I	W	N	S	E	K	K	E	L	L	G
				5						10					15					20					25

[a] Amino acid sequence analysis was performed by A. Khatri, Director, Biopolymers Core Facility, Massachusetts General Hospital, Boston, MA 02114. [b] Questionable step, most probable amino acid.

preparation and 1−8 μg of purified duck salt gland Na^+,K^+-ATPase all immunostained using anti-TED as the primary antibody. Anti-TED reacts strongly with the duck brain Na^+,K^+-ATPase preparation, but there was no specific reaction with up to 8 μg of purified salt gland Na^+,K^+-ATPase. These data conclusively demonstrate that duck α_3 does not exist in the purified salt gland Na^+,K^+-ATPase. Taken together, the above data demonstrate that the salt gland Na^+,K^+-ATPase is an α_1 subunit isomer. We have immunostained our preparation with antibody 24 and b4β4 raised against the β_1 subunit. Both antibodies weakly stained our preparation in the region corresponding to the β subunit.

We have recently sequenced the first 20 and 26 N-terminal amino acids of the α and β subunits, respectively (Table 1). Our data confirm earlier results (*34*) that indicated the duck nasal salt gland is composed of α_1 and β_1 subunits. We could find no evidence within the amino acid sequence data to support a minor population of other isoforms.

Some preparations of Na^+,K^+-ATPase contain a γ subunit in addition to the α and β subunits (*35*, *36*). The putative γ subunit does not appear to be associated with all the Na^+,K^+-ATPase preparations but is only present in preparations derived from select tissues and species. The γ subunit is reported to be a low-molecular weight protein ($M_r \sim 10000-12000$). To test for the presence of the γ subunit in our purified Na^+,K^+-ATPase preparation, we analyzed our preparation using the gel electrophoresis method of Schägger and von Jagow (*37*) which is specifically designed to resolve low-molecular weight proteins. Figure 10 shows the results of analysis of our preparation for low-molecular weight proteins. A very large sample, enough to contain ≥6 μg of a 10 000 Da protein if it existed in an equimolar ratio with respect to the α and β subunits, was applied to the gel. No protein was detected in the $M_r = 10000-12000$ region of the γ subunit. In addition to the α and β subunits, minor bands were observed at ∼45 000 and 32 000 and a very faint band at 20 000. Silver staining of this gel did not reveal any other bands. On the basis of this analysis, we conclude that this purified Na^+,K^+-ATPase preparation does not have a γ subunit.

The Extent of Phosphorylation of the Duck Salt Gland Na^+,K^+-ATPase Is Proportional to the Specific Activity and Corresponds to One Phosphate Bound per αβ Unit. The above data demonstrate that the specific activity of this preparation is closely correlated with the protein purity of the preparation. An important question to ask of this preparation is whether the high specific activity of this preparation reflects a highly pure, fully active protein, or an unusually high turnover rate for the ATPase. The dependence of the ATPase activity on ATP concentration is illustrated in Figure 11. Analysis of six preparations yielded half-maximal ATPase activities occurring at 550 ± 200 μM ATP with a maximal activity at 65 ± 6 EU.

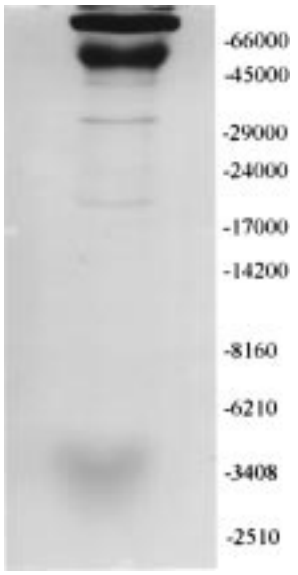

FIGURE 10: Purified Na^+,K^+-ATPase did not contain any protein bands in the region ascribed to the γ subunit. A 100 μg sample of purified Na^+,K^+-ATPase was electrophoresed over a thick, large format (12 cm × 11 cm × 0.3 cm, 9 mm lane) 16.5% acrylamide gel prepared by the method of Schägger and von Jagow (*37*). Molecular weight standards SDS-7 and SDS-17S obtained from Sigma were used to cover the M_r range of 66000−2510. The gel was stained with Coomassie blue.

The enzyme can be phosphorylated by incubating the ATPase in the presence of inorganic phosphate (P_i) and Mg^{2+}. If this incubation is carried out in the presence of ouabain, the enzyme is forced into a conformation which is favorable for phosphorylation so that all active units can be phosphorylated. This is illustrated in Figure 12 which shows the extent of highly cooperative phosphoenzyme formation (E−P) as a function of $MgCl_2$ concentration in the presence of 0.5 mM P_i and 0.5 mM ouabain. In an analogous experiment, E−P formation was essentially constant (6.5 ± 0.3 nmol/mg of protein) when phosphorylations were carried out in the presence of 3 mM $MgCl_2$ and 0.5 mM ouabain and the P_i concentration was varied from 0.07 to 1.2 mM. Accordingly, phosphorylation by inorganic phosphate can be used to measure the concentration of active units in a Na^+,K^+-ATPase suspension. If the specific activity of the

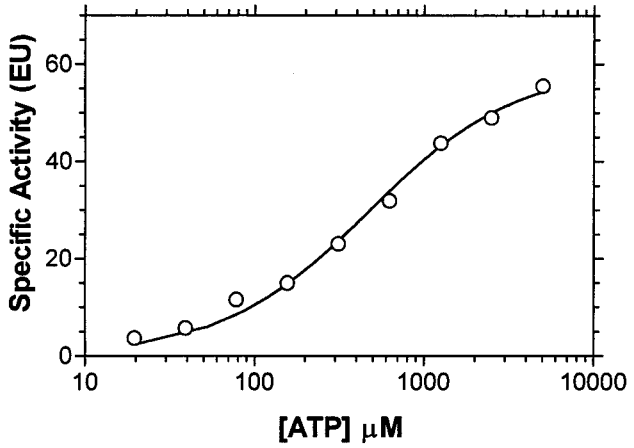

FIGURE 11: ATPase activity was dependent upon ATP concentration. The ATPase activity of purified Na^+,K^+-ATPase was measured at 37 °C as described in Methods at varied ATP concentrations. In the above experiment, 50% activity occurred at an ATP concentration of 470 μM, and the maximum activity was 60 ± 4 EU.

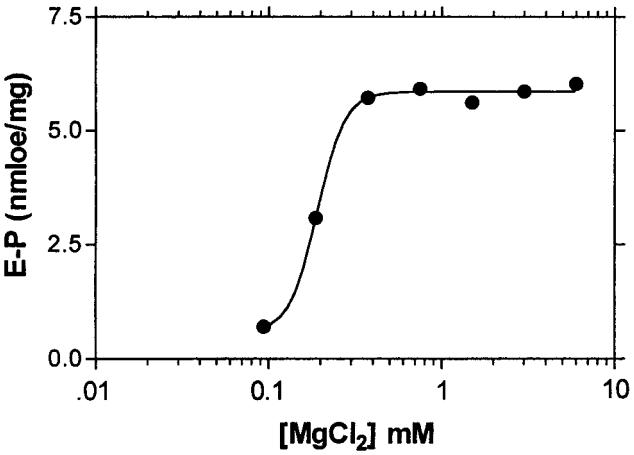

FIGURE 12: Phosphoenzyme (E−P) formation from inorganic phosphate (P_i) was dependent upon $MgCl_2$ concentration. Purified Na^+,K^+-ATPase was phosphorylated with $^{32}P_i$ in the presence of ouabain at varied $MgCl_2$ concentrations as described in Methods. Half-maximal phosphorylation occurred at a $MgCl_2$ concentration of 0.2 mM. The maximal extent of phosphorylation was 5.9 ± 0.2 nmol/mg. The data points are the means of triplicate determinations.

preparation is also known, the turnover number for the preparation can be calculated. Figure 13 summarizes the results of turnover number determinations for numerous salt gland Na^+,K^+-ATPase preparations. The extent of phosphorylation is linearly correlated with the specific activity of the preparation, and the slope of this linear correlation corresponds to a turnover number of 9690 ± 340 cycles/min at 37 °C, which is consistent with turnover numbers usually reported for this enzyme from other tissues (9000−10000 min^{-1}). It should be noted that this linear relationship is a good fit to both the purified Na^+,K^+-ATPase and the precursor microsomal preparation, suggesting that enzyme efficiency is maintained during the purification procedure. Our highest-specific activity preparations yield ≥6 nmol of E−P/mg of protein. This value corresponds to one phosphate incorporated per 147 000 Da $\alpha\beta$ diprotomer. These data along with those of Figure 6 indicate that virtually all the $\alpha\beta$ units in this preparation are active and that the variation in the specific activity reflects the variation in the purity of the preparation as opposed to a variation in the native state

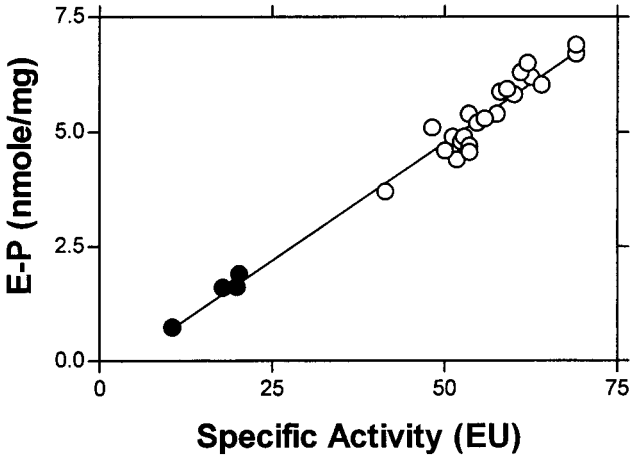

FIGURE 13: The level of phosphoenzyme is linearly related to the specific activity of the Na^+,K^+-ATPase preparation. Purified Na^+,K^+-ATPase and duck salt gland microsomes with varied specific activities were phosphorylated with $^{32}P_i$ as described in Methods. ATPase activities of the preparations were assayed at 37 °C as described in Methods. All data points are the means of triplicate determinations. The white circles (○) represent data collected from purified Na^+,K^+-ATPase. The black circles (●) represent data collected from salt gland microsomes. The data were fit with a linear regression. The reciprocal of the slope of the linear regression yielded a turnover number of 9690 ± 341 cycles/min. If an $\alpha\beta$ protomer molecular weight of 147 000 Da is assumed, this corresponds to a maximal theoretical specific activity of 66 ± 2 EU.

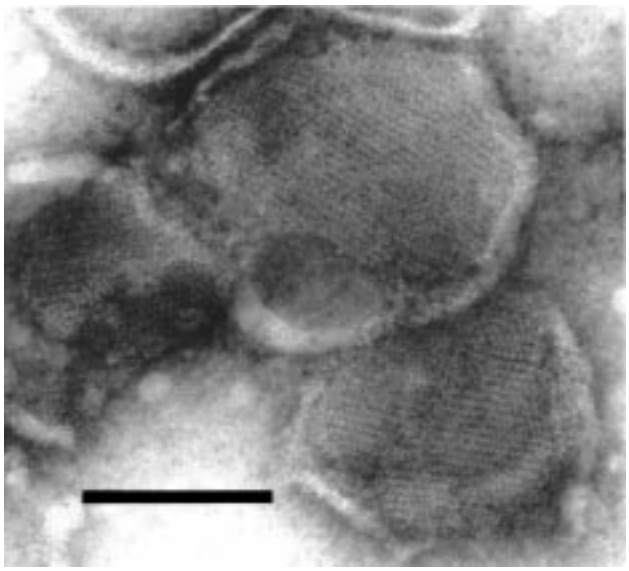

FIGURE 14: Electron micrograph of purified duck salt gland Na^+,K^+-ATPase after incubation in vanadate-containing media. Purified Na^+,K^+-ATPase was incubated in media containing 1 mM $NaVO_3$, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.5). The samples were adhered to a Formvar-coated copper grid, negatively stained with 1% uranyl acetate, and viewed under a 80 kV beam at a magnification of 100000×. The above micrograph was obtained after incubation for 14 days, and similar results were obtained after incubation for 1 or 21 days at 4 °C. The bar is 0.1 μm long.

of the Na^+,K^+-ATPase. Furthermore, our data demonstrate that in a fully active preparation all $\alpha\beta$ diprotomers can be phosphorylated. These data do not support models which require half-of-the sites phosphorylation (*12*, *13*).

We have observed that the proteins form orderly aggregates under the proper conditions. Figure 14 shows an

electron micrograph of a membrane fragment from a purified Na^+,K^+-ATPase preparation that had been incubated in the presence of vanadate. There is a clear orderly patch of protein similar to that reported in other Na^+,K^+-ATPase preparations which had been incubated under similar conditions (*38*, *39*). A highly active and pure preparation may provide a sound basis for future structural determinations.

DISCUSSION

On the basis of a protein M_r of 147 000 for the $\alpha\beta$ protomer, and a molecular turnover number of 9690 min^{-1}, Na^+,K^+-ATPase should have a binding capacity of 6.8 nmol of phosphate/mg of protein and a specific activity of 66 EU under optimal conditions. Early attempts to purify the enzyme resulted in preparations with activities that were considerably smaller than the expected values (*8*, *40*). Over the next decade, attempts to purify the enzyme from a variety of sources—the mammalian kidney (*24*), rectal glands of dogfish (*41*−*44*), the electric organ of *Torpedo* (*42*, *43*, *45*), the salt gland of the duck (*23*), and brine shrimp (*46*)—resulted in preparations higher in activity than the original preparations, but still, with one exception (*24*), well below 50% of maximum theoretical activity. By 1980, the best preparations from renal medulla had a specific activity of 32−37 EU and a binding capacity of 3.5−3.7 nmol of phosphate/mg of protein (*47*). In most cases, as judged by either qualitative or quantitative comparison of the Coomassie staining of α and β chains of enzyme subjected to PAGE on SDS gels with the total amount of Coomassie staining of the enzyme preparation, α and β chains made up more than 90−95% of the most active preparations.

Moczydlowski and Fortes (*48*) attributed the discrepancy between the specific activity of highly purified membrane preparations and the apparent purity of the preparations as judged by Coomassie-stained SDS gels to overestimation of the protein concentration of the preparations by the Lowry method, which was almost universally used. When they measured the protein concentration by quantitative amino acid analysis, they concluded that the Lowry method overestimated the protein concentration by 7−18%. Using the values from the amino acid analysis, they estimated that the binding capacity of an eel electroplax preparation for the ATP analogue TNP-ATP [2′,3′-*O*-(2,4,6-trinitrophenyl-hexadenylidine) adenosine 5′-triphosphate] was 5.2−6.2 nmol/mg of protein and for ouabain 4.7−5.6 nmol/mg of protein, and the specific activity was 35−49 EU. Even greater corrections have been reported (*49*). Chetverin (*50*) compared simultaneous measurements of protein concentrations of purified Na^+,K^+-ATPase preparations by the Lowry method, the Kjeldahl procedure, and quantitative amino acid analysis and concluded that, while the Lowry and Kjeldahl procedures agreed, quantitative amino acid analysis significantly underestimated the protein concentration. Reynolds (*51*) and Jensen (*52*) came to a similar conclusion. In recent years, the best preparations of membrane-bound Na^+,K^+-ATPase have had specific activities of 30−40 EU if estimated by quantitative amino acid analysis (*20*). Jørgensen (*19*), who bases his protein determination on quantitative amino acid analysis, obtained specific activities approaching 50 EU and ligand binding capacities of 4.0−6.4 nmol/mg of protein. In our preparations, we evaluate the protein by the Peterson modification of the Lowry method (*26*).

The supraorbital salt gland of the duck responds to increased intake of NaCl (and other salts such as KCl and $MgCl_2$) in two ways. The increase in plasma osmolality results in a rapid increase in the rate of NaCl secretion by the gland by means of a cholinergic mechanism, and a slower increase in the Na^+,K^+-ATPase content of the gland because of both an increase in its size and a 4-fold increase in its Na^+,K^+-ATPase specific activity (Na^+,K^+-ATPase activity per milligram of protein) (*53*). The increased Na^+,K^+-ATPase activity results from an increase in the rate of enzyme synthesis (*54*, *55*). Stewart et al. (*54*) prepared Na^+,K^+-ATPase from salt glands of salt-adapted ducks which had a specific activity of 17 EU; the preparation was not pure, and contamination of the Na^+,K^+-ATPase with the other proteins no doubt contributed to the low specific activity. Subsequently, Hopkins et al. (*23*) reported a careful attempt to purify the enzyme by a modification of the procedure of Jørgensen (*24*) which produced enzyme with a specific activity of 38 EU and a binding capacity of 3.6 nmol of ouabain/mg of protein which contained predominantly α and β chains. Since then, few investigators have used the salt gland enzyme except for Boldyrev and his colleagues (*34*), who used an enzyme with a specific activity of about 30 EU and a binding capacity of about 3 nmol of phosphate/mg of protein.

We selected the duck salt gland for our attempt to prepare pure fully active enzyme for two reasons. The preparation of microsomes from intact gland does not require a delicate and critical dissection, and the specific activity of the microsomes can be greatly increased by salt adaptation of the animals. Earlier work of Ernst et al. (*53*) suggested to us that young ducklings may have a more acute and sustained response to salt adaptation. The microsomes which we prepare have high specific activities, greater in some cases than the specific activity of the purified enzyme used in functional studies. In our first purification attempts, we readily obtained an enzyme with a specific activity of 40 EU, and with some modifications to the procedure, it became possible for us to regularly prepare an enzyme with a specific activity of >60 EU with a comparable binding capacity. While we believe the modification of the procedure accounted for the increase in the quality of the enzyme, we also believe there is a rather shallow learning curve. We now regularly prepare an enzyme with a specific activity and binding capacity near the theoretical maxima, but we still encounter occasional preparations with lower specific activities (40−55 EU). The procedure is not trivial. However, even our poorest preparations yield activities equal to or better than those of the best preparations that are currently used.

The enzyme preparation with near maximal phosphorylation capacity and specific activity clearly does not contain any denatured or partially denatured enzyme. Even when we obtain an enzyme with a considerably lower than maximal specific activity, we have found that the ratio of the specific activity we obtain to the theoretical maximum specific activity is closely correlated with the ratio of the amount of Coomassie stain bound to α and β chains in SDS gels to the total amount of protein-bound Coomassie stain in the gel. The decreased activity is therefore mainly due to contamination of the preparation with extraneous protein, and the activity of the enzyme remains constant and near the theoretical maximum throughout the purification procedure.

This is an important difference between our enzyme and enzyme preparations in which the specific activity and binding capacity are at best half of the theoretical maximum, yet nearly 95% of their protein is α and β chains on SDS gels. In those cases, nearly half of the enzyme must be denatured or partially denatured. This will be further discussed below.

The nasal gland enzyme is homogeneous. On the basis of immunoblot analysis with antibodies directed against the three α isoforms, we have concluded that our enzyme is nearly 100% α_1, with, at most, <1% α_2 and no α_3. Similar conclusions were drawn previously (*31*), but we found that the anti-α_3 antibody used in that study did not react with duck brain and therefore likely does not recognize duck α_3. By determining the N-terminal sequences of α and β chains from our duck gland preparation, we found that the enzyme contains only α_1 and β_1 isoforms. A similar conclusion was reached by Boldyrev et al. (*34*). The enzyme does not contain a γ chain (*35*). It recently has been shown that the γ chain is not present in all tissues; in the rat, it is limited to the Na^+,K^+-ATPase located in renal tubules (*36*).

The purity, functional competence, and homogeneity of the enzyme suggest that it may be suitable for structural studies.

Reaction Mechanism of the Na,K Pump in Light of a Fully Active Enzyme. A Na^+,K^+-ATPase preparation which can be phosphorylated simultaneously at all binding sites and which exhibits the theoretically maximal specific activity eliminates the possibility that half-of-the-sites or flip-flop mechanisms can be applied to this enzyme.

Half-of-the-sites mechanisms necessarily require that the enzyme exist as a functional dimer. In fact, much of the evidence advanced to support functional dimerization of this enzyme arises from experiments in which apparent half-of-the-sites behavior has been observed assuming that all αβ protomers are equivalent. The Albers-Post model for the Na^+,K^+-ATPase reaction mechanism accommodates interaction between αβ protomers only awkwardly, and the inability in the past to regularly prepare enzyme with an expected binding capacity and specific activity of much greater than 50% has frequently been used as evidence that the Albers-Post model cannot describe operation of the Na^+ pump. Such arguments must now be abandoned.

The data presented here do not address the question of whether the enzyme exists in the membrane as a monomeric αβ protomer or an $(\alpha\beta)_2$ diprotomer. In fact, we have found by measuring fluorescence resonance energy transfer between anthroylouabain and fluorescein ouabain attached to different αβ protomers that protomers are probably close enough in the purified preparation to form $(\alpha\beta)_2$ diprotomers (*56*). The question which must be answered is whether dimerization has any functional significance or is simply a result of the close packing of the enzyme in the membrane (*30*, *57*).

This is not to deny that dimerization, or the close proximity of αβ monomers in purified Na^+,K^+-ATPase preparations, may affect some property of the enzyme. Many such effects have been described, but often there is no independent evidence that protomer interaction is involved. However, in one case, an effect of protomer interaction has been convincingly shown (*58*). Scatchard plots of the level of ATP or ADP binding to the purified enzyme are curvilinear when K is present. Random inhibition of most of the protomers with ouabain converted the nonlinear Scatchard plot to a linear plot, strong evidence that nonlinearity is due to negative cooperativity between protomers in an enzyme unit. The conclusion was strengthened by the physical evidence that solubilization of the enzyme also converted the nonlinear plot to a linear one (*59*). The protomer interaction changes the dissociation constant for nucleotides, which is less than 1 μM, by a factor of ≤2, and it is not conceivable that this could have a significant effect on the reaction mechanism. On the other hand, other studies showing a biphasic relation between ATP activity and ATP concentration have long been cited as evidence for protomer interaction within a diprotomer. That has, however, been shown conclusively not to be the case because of two observations. (1) The biphasic activity curve persists when the enzyme is solubilized to form a monomeric preparation (*60*), and (2) the biphasic activity curve in red cell membranes in which αβ protomers are monomers (*28*) survives irreversible random inhibition of most of the αβ protomers (*61*) in an experiment similar to that of Ottolenghi and Jensen (*58*). Functional evidence for dimerization in the absence of physical evidence is, at best, unreliable.

In our fully active preparation, $(\alpha\beta)_2$ diprotomers, if they exist, must be made up of two functional αβ protomers. In partially active preparations, about 50% or more of the αβ protomers are nonfunctional since they can neither be phosphorylated nor hydrolyze ATP. It is often implicitly assumed that, in these preparations, $(\alpha\beta)_2$ diprotomers are made up of a functional αβ protomer and a nonfunctional protomer. Since the molecular turnover number of fully active nasal gland enzyme is the same as that of partially active preparations, the reaction mechanism of each αβ protomer must be the same whether its partner in a diprotomer is active or inactive, which is conceivable but less than credible. It is possible that in partially active preparations only functional protomers form diprotomers, in which case the similarity in the molecular turnover numbers of the two preparations cannot be used to eliminate an effect of protomer interaction on the reaction mechanism. There are, however, several observations in the literature which seem to rule out such an effect. When the specific activity of a detergent-solubilized enzyme, which has been shown to exist as enzymatically active αβ protomers (*60*), was compared with the specific activity of the purified enzyme from which it was prepared [in which the enzyme presumably exists as $(\alpha\beta)_2$ diprotomers], no difference was found (*62*). Hayashi et al. (*63*) separated αβ monomers from $(\alpha\beta)_2$ diprotomers in the same solubilized preparation and measured the specific Na^+,K^+-ATPase activity; the specific activity of the monomers was the same as that of the diprotomers. There is little doubt that αβ protomers hydrolyze ATP at the same rate whether they are monomers or diprotomers.

We have demonstrated that the Na^+,K^+ pump in the human red cell, the source of much of our knowledge about how the enzyme functions, is a monomeric αβ protomer (*28*). This means that monomeric αβ protomers are capable of all functions of the Na^+ pump, including cation transport.

The conclusion that subunit interaction has no significant role in the reaction mechanism of the Na^+,K^+-ATPase is inescapable.

Characteristics of Partially Active Protein in Light of the Existence of Fully Active Nasal Gland Enzyme. The prepara-

tion of fully active nasal gland enzyme demonstrates that there is no intrinsic functional reason other preparations have phosphorylation capacities and specific activities that are ≤50% of the theoretically expected maxima even though α and β chains make up all of the protein. It must be concluded that in almost all membrane-bound ATPase preparations ≥50% of the αβ protomers are nonfunctional in that they can neither be phosphorylated nor hydrolyze ATP under standard conditions.

When Na^+,K^+-ATPase preparations with approximately half-maximal binding capacity are digested with trypsin, the time course of the loss of activity and the size of the proteolytic fragments produced depend on whether trypsinization takes place in a Na^+ solution or a K^+ solution (*64*). If digestion occurs in K^+, the loss of activity is described by a single exponential and the α chain is cleaved into 58 and 48 kDa fragments. On the other hand, if trypsinization occurs in a Na solution, the loss of activity is biphasic and a 78 kDa fragment appears. Ottolenghi and Jensen (*65*) pointed out that, in these experiments, although only half of the enzyme is capable of phosphorylation, all of the enzyme is capable of undergoing the conformational changes induced by Na^+ and K^+. The nonfunctional protomers must be only partially inactive or partially denatured.

There are several reports that inactive α chains, which cannot be phosphorylated under standard conditions, can be phosphorylated under extraordinary circumstances. When enzyme is solubilized in the detergent $C_{12}E_8$, the solubilized enzyme (*66*) or the solubilized enzyme reconstituted in phospholipid vesicles (*67*) exhibits near maximal ouabain binding or phosphorylation capacity. ATPase molecules are very closely crowded in renal membranes (*30*, *68*), and the major effect of detergent solubilization followed by reconstitution at low protein/lipid ratios is a great reduction of their crowding. Comparison of different crystal structures of T_4 lysozyme showed that crystal contacts perturb the backbone structure of the protein by 0.2−0.5 Å (*69*). In one case, two-dimensional crystals of Na^+,K^+-ATPase seemed to contain both E_1 and E_2 forms even though, under similar ligand conditions, less tightly packed molecules are either one conformation or the other (*70*). Na^+,K^+-ATPase molecules in renal membranes are probably not much farther apart than they are in two-dimensional crystals, and it may be that intermolecular contacts are responsible for inactivation. On the other hand, Na^+ pumps of nasal gland membranes seem to be packed equally close, but inactivation does not occur.

Two other surprising studies have been published which suggest that inactive protomers can be reactivated under extraordinary circumstances. When phosphorylation of partially inactive enzyme was carried out at 50 μM ATP rather than at 10 μM, an extent of transient phosphorylation 2−4 times greater than the extent of stable phosphorylation at 10 μM ATP was found (superphosphorylation) (*71*). Phosphorylation capacity at 10 μM ATP was very low (<1 nmol of phosphate/mg of protein), although some specimens had a higher capacity (2.4 nmol of phosphate/mg of protein). The amount of superphosphorylation seemed to vary inversely with the amount of stable phosphorylation. Transient phosphorylation at the nearly theoretically maximum phosphorylation capacity was achieved when phosphorylation was carried out at 500 μM ATP and 40 mM imidazole rather than Na^+. Transient phosphorylation does not occur when the enzyme is turning over, and the Na-ATPase activity is the same at 10 and 500 μM ATP so that the enzyme which is superphosphorylated is not an intermediate in the reaction pathway. The authors believed their findings showed that, under unusual circumstances, the functional α chain can be phosphorylated at up to four extra sites. However, in view of the low proportion of functional αβ protomers in the partially active enzyme preparations used in this study and the reciprocal relation between the amount of superphosphorylation and the amount of stable phosphorylation, a more compelling explanation is that there are phosphorylation sites on the inactive protomers with characteristics different from those of the sites on the active protomers. At high ATP concentrations, these anomalous sites can be transiently phosphorylated; i.e., the inactive protomers are only partially denatured.

Cleavage of a membrane-bound enzyme preparation by chymotrypsin in a Na^+ solution produced an 83 kDa fragment of the α chain (*22*). The phosphorylation capacity of the enzyme was not given, but based on the reported specific activity of 29 EU, more than 50% of the αβ protomers in the preparation were nonfunctional. The phosphorylation capacity of the α chain fragments in the proteolyzed preparation was nearly twice that of intact α chains in the original enzyme preparation, although Na^+,K^+-ATPase activity was lost. The authors concluded that "dormant" phosphorylation sites were uncovered as a result of proteolysis (according to J. G. Nørby,[2] "Dormant partner is the dream of the sleeping scientist"). A less optimistic interpretation (which the authors considered, but declared "irrational") might conclude that dormant sites are phosphorylation sites on partially denatured α chains.

Although αβ protomers in nasal gland membranes are all active and homogeneous, at least half of the protomers in other preparations are either denatured or partially denatured. Denaturation might occur during detergent purification of the microsomal preparations, but we believe the protomers in the microsomal membranes are probably already denatured; protomers in nasal gland microsome membranes seem to be fully active. We do not know if all denatured protomers are denatured in the same way, nor do we know if denaturation serves some physiological function, although no such function has been identified. But it is clear that denaturation of half of the α chains can only impair pump function since our enzyme has twice the specific activity of other preparations.

Over the past two decades, many reports of experiments with purified ATPase preparations which are inconsistent with the standard Albers-Post mechanism have appeared. It is possible that some of these findings can be attributed to the use of enzyme which is not fully active. Two of these reports may be mentioned.

(1) Partially active Na^+,K^+-ATPase preparations simultaneously bind 1 mol of the ATP analogue $Cr(H_2O)_4$ ATP and 1 mol of the analogue $Co(NH_3)_4$ ATP per phosphorylation site (*72*). Simultaneous binding of the two analogues was taken as evidence that there are two independent ATP binding sites per αβ protomer. But in view of the fact that

[2] Nørby, J. G. (1998) Discussion at symposium honoring J. C. Skou, Aarhus, Denmark, October 10, 1998.

half of the $\alpha\beta$ protomers in the preparation are inactive, and in view of the likelihood that inactive protomers are partially denatured, a more likely explanation is that one of the analogues binds to functional protomers and the other to partially denatured protomers and there is a single ATP binding site per protomer. The situation could be resolved with the help of the nasal gland enzyme.

(2) Binding stoichometrics of pump ligands have been measured for many years. A consensus has developed that the number of binding sites for phosphate, vanadate, nucleotides, and ouabain are equal in any given preparation, and equal to about half the number of $\alpha\beta$ protomers in the preparation. However, Yamazaki et al. (*73*), using a pig renal enzyme with a maximal specific activity of 20 EU, determined a ligand binding stoichiometry of 1:0.5:1:1 for phosphate from PNPP, phosphate from ATP, ouabain, and ATP. In addition, under certain circumstances, quarter-site binding of phosphate from PNPP was found, from which the authors concluded that the enzyme is an $(\alpha\beta)_4$ tetramer. Although full site phosphorylation was found in some experiments, half of the protomers were unable to hydrolyze ATP (the specific activities of the two enzyme preparations were about half or less of the theoretic maximum). It would be of interest to perform the same experiments with the nasal gland enzyme in which all the protomers can be phosphorylated and hydrolyze ATP.

It has long been known that biochemical mechanisms can be unambiguously elucidated only with pure, fully active enzyme preparations such as the one described here.

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