*Biochimica et Biophysica Acta*, 649 (1981) 691–700
Elsevier/North-Holland Biomedical Press



BBA 79505

# IMMUNOREACTIVITY OF SUBUNITS OF THE ($Na^+ + K^+$)-ATPase

## CROSS-REACTIVITY OF THE α, α+ AND β FORMS IN DIFFERENT ORGANS AND SPECIES

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(Received May 26th, 1981)

*Key words: ($Na^+ + K^+$)-ATPase subunit: Immunoreactivity; Phosphorylation; Protein A; (M. sexta brain)*

**The immunologic cross-reactivity of the α and α+ forms of the large subunit and the β subunit of the ($Na^+ + K^+$)-ATPase from brain and kidney preparations was examined using rabbit antiserum prepared against the purified holo lamb kidney enzyme. As previously reported by Sweadner ((1979) J. Biol. Chem. 254, 6060–6067) phosphorylation of the large subunit of the ($Na^+ + K^+$)-ATPase in the presence of $Na^+$, $Mg^{2+}$, and [γ-$^{32}$P]ATP revealed that dog and, very likely, rat brain contain two forms of the large subunit (designated α and α+) while dog, rat, and lamb kidney contain only one form (α). The cross-reactivity of the α and α+ forms in these preparations was investigated by resolving the subunits by SDS-polyacrylamide gel electrophoresis. The separated polypeptides were transferred to unmodified nitrocellulose paper, and reacted with rabbit anti-lamb kidney serum, followed by detection of the antigen-antibody complex with $^{125}$I-labeled protein A and autoradiography. By this method, the α and α+ forms of rat and dog brain, as well as the α form found in kidney, were shown to cross-react. In addition, membranes from human cerebral cortex were shown to contain two immunoreactive bands corresponding to the α and α+ forms of dog brain. In contrast, the brain of the insect *Manduca sexta* contains only one immunoreactive polypeptide with a molecular weight intermediate to the α and α+ forms of dog brain. The β subunit from lamb, dog and rat kidney and from dog and rat brain cross-reacts with anti-lamb kidney ($Na^+ + K^+$)-ATPase serum. The mobility of the β subunit from dog and rat brain on SDS-polyacrylamide electrophoresis gels is greater than the mobility of the β subunit from lamb, rat or dog kidney.**

## Introduction

The ($Na^+ + K^+$)-ATPase is a membrane-bound enzyme which catalyses the active transport of sodium and potassium. This enzyme is present in high concentrations in brain where it functions in neuronal transmission by maintaining the appropriate ion gradients. It may also be important for clearing extracellular potassium in brain [1]. Purification of the ($Na^+ + K^+$)-ATPase from many sources has consistently resulted in preparations composed of an α subunit of approx. 90 000 daltons and a β subunit of approx. 40 000–50 000 daltons. The α subunit contains the ATP-hydrolysis site as demonstrated by phosphorylation of this subunit in the presence of ATP, $Na^+$, and $Mg^{2+}$ or in the presence of $Mg^{2+}$ and $P_i$ [2–4]. The β subunit is a glycoprotein of unknown function. Recently, Sweadner [5] has demonstrated that under the conditions which result in the phosphorylation of the α subunit of the ($Na^+ + K^+$)-ATPase, two polypeptides in mammalian brain preparations (designated α and α+) are phosphorylated

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

while only one polypeptide (the $\alpha$ form) is observed by phosphorylation of the kidney enzyme. The $\alpha$+ polypeptide has been reported to be approx. 2000 daltons larger than the $\alpha$ polypeptide [5]. In addition, the $\alpha$ and $\alpha$+ forms of the intact enzyme may have different affinities for ouabain. Indications of multiple forms of the $\alpha$ subunit have also been reported in larval brine shrimp preparations [6] and also in grey matter from rat brain [7].

The following work was undertaken in order to examine the immunologic cross-reactivity of the $\alpha$ and $\alpha$+ subunits. Our laboratory is interested in using immunochemical techniques to study localization of the $(Na^+ + K^+)$-ATPase in the nervous system of various species. Thus, it is essential to define the specificity of the antisera used to localize the enzyme. The following data confirms the existence of distinct $\alpha$ and $\alpha$+ polypeptides and demonstrates that these forms contain common antigenic determinants.

## Experimental Procedures

*$(Na^+ + K^+)$-ATPase preparations.* $(Na^+ + K^+)$-ATPase used to produce antisera was prepared from lamb kidney medulla by the method of Lane et al. [8]. Specific activities of 1000–1500 $\mu$mol $P_i$ released/mg protein per h were routinely obtained with the purified enzyme. Dog brain $(Na^+ + K^+)$-ATPase was partially purified as described by Sweadner [9] and the specific activity ranged from 300 to 400 $\mu$mol $P_i$ released/mg protein per h. In several instances, microsomes and partially purified enzyme were isolated from dog brain in a medium saturated with PMSF. *Electrophorus electricus* electric organ $(Na^+ + K^+)$-ATPase was purified using the method of Cantley et al. [10] and this preparation had a specific activity of 1100. Microsomes from rat brain, rat kidney, human brain, dog kidney medulla, and lamb kidney medulla were prepared by the following procedure. Samples of tissue (10%, w/v) in 0.32 M sucrose (adjusted to pH 7.4 with Tris-HCl) were homogenized by ten strokes of a motor-driven Teflon-glass homogenizer at 0°C. This crude homogenate was centrifuged at 850 × *g* for 15 min. The pellet was resuspended in sucrose and centrifuged again at 8700 × *g* for 15 min; the supernatants from the two steps were pooled. The resulting material was centrifuged at 8700 × *g* for 20 min and the microsomes were pelleted from the resulting supernatant by centrifugation at 39 000 × *g* for 1 h. The microsomal pellet was resuspended in 0.32 M sucrose and stored at −20°C until needed. All preparative steps were performed at 5°C using a Sorvall SS-34 rotor. Frog retina membrane fragments were prepared by homogenizing retina (25 mg) in cold water (0.5 ml) followed by centrifugation at 760 × *g* for 10 min. The supernatant was recentrifuged for 30 min at 165 000 × *g* and the pellet was resuspended in water and stored at −20°C. Microsomes from *Manduca sexta* brain were prepared as previously described for ventral nerve cords [11]. Nal-treated rat brain microsomes were prepared as previously described [12].

*Antisera production.* Rabbit antiserum against the purified lamb kidney $(Na^+ + K^+)$-ATPase was raised by injecting rabbits subcutaneously with 0.5 mg protein in complete Freund's adjuvant followed by three injections of 0.5 mg protein in incomplete Freund's adjuvant at 7, 14, and 21 days after the initial challenge. The animals were subsequently injected every 3 weeks with 0.5 mg protein in incomplete Freund's adjuvant. The antisera used in this paper were collected 107 days after the initial injection. Control serum was obtained prior to injection of antigen.

*Electrophoresis.* SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [13] observing the conditions described by Sweadner [5] for resolving the $\alpha$ and $\alpha$+ subunits. Gels were prepared from a stock solution containing 30% acrylamide and 0.8% bisacrylamide. Electrophoresis was carried out in a jacketed apparatus (Hoefer Scientific Co.) maintained at room temperature by circulation of cooled water. For visualization of protein, the gels were stained overnight in 0.05% Coomassie brilliant blue in 25% isopropanol/10% acetic acid and were destained in the same solution without dye (destaining solution). Gels containing phosphorylated samples were immediately fixed in destaining solution for 1 h followed by a second change of destaining solution for another hour to remove $^{32}P_i$ and [$\gamma$-$^{32}$P]ATP. Gels were rinsed briefly, dried on filter paper, and placed in contact with Kodak X-Omat XR-2 X-ray film for autoradiography. Negatives were scanned using a Joyce, Loebl microdensitometer. Gels for immunologic characterization experiments were used immediately after electrophoresis without

fixing in destaining solution. The molecular weights of $(Na^+ + K^+)$-ATPase subunits, resolved on SDS gels, were determined as described by Weber and Osborn [14] using myosin (200 000 daltons), β-galactosidase (116 250 daltons), phosphorylase *b* (92 500 daltons), bovine serum albumin (66 200 daltons) and ovalbumin (45 000 daltons) as standards.

The purity of the lamb kidney $(Na^+ + K^+)$-ATPase was estimated by scanning Coomassie blue-stained SDS gels at 550 nm using a Gilford spectrophotometer and gel scanning attachment modified to accept slab gel lanes. The linear range of absorption by stained bands was determined by scanning several gel lanes loaded with different amounts of the enzyme. Integration of the areas of the α and β subunit peaks indicated that staining and absorption were linear up to 25 μg total protein loaded per gel lane. Purity of the $(Na^+ + K^+)$-ATPase was estimated as the percent of the total area under the Coomassie-stained bands accounted for by the α and β subunits.

*$(Na^+ + K^+)$-ATPase assay*. The $(Na^+ + K^+)$-ATPase was assayed essentially as described by Stahl [12] and the activity was defined as the portion of ATP-hydrolysis which was inhibitable by 0.1 mM ouabain. In assays where antisera were tested for ability to inhibit the $(Na^+ + K^+)$-ATPase, the enzyme and the antisera or control sera were preincubated at 37°C for 15 min prior to the initiation of the assay. In these experiments, the reaction was terminated by the addition of trichloroacetic acid (5% final) and the protein removed by centrifugation prior to the extraction of $^{32}P_i$.

*Phosphorylation*. The phosphorylated intermediate of the $(Na^+ + K^+)$-ATPase from different sources was obtained essentially as described by Jorgenson [15]. Reaction mixtures contained 3 mM $MgCl_2$/140 mM NaCl/30 mM Tris-HCl (pH 7.1)/50 μM [$\gamma$-$^{32}$P]-ATP, and enzyme or microsomes in a final volume of 200 μl. Where indicated, 20 mM KCl was also present. The reaction was terminated after 10 s at 4°C by the addition of 200 μl cold solution containing 10% trichloroacetic acid/1.5 mM $H_3PO_4$/1.6 mM ATP. Samples were washed by centrifugation at 4°C twice with 0.1% trichloroacetic acid/10 mM $H_3PO_4$/0.1 mM ATP and once with water. Samples for electrophoresis were resuspended in Laemmli sample buffer [13] containing 1 mM EDTA/1 mM PMSF and were used immediately.

*Determination of immunologic cross-reactivity*. The immuno-cross-reactivity of the anti-lamb kidney $(Na^+ + K^+)$-ATPase sera with the α, α+ and β subunits from various sources was examined by the technique of Towbin et al. [16] as modified by Burnette [17]. Subunits, resolved by SDS-polyacrylamide gel electrophoresis, were electrophoretically transferred (100 mA for 18 h) to nitrocellulose filter paper in an apparatus similar to that described by Bittner et al. [18] using 25 mM $KH_2PO_4$ (pH 6.5)/0.05% $NaN_3$ as the electrode buffer. After the transfer was complete, the nitrocellulose sheets were incubated for 90 min at 40°C with agitation in Tris-saline (0.15 M NaCl/10 mM Tris-HCl, pH 7.4) containing 5% bovine serum albumin. The nitrocellulose sheet was then incubated with antiserum (1–200 dilution) in Tris-saline containing 5% bovine serum albumin for 90 min at room temperature with agitation. The antiserum was removed by successive washes in Tris-saline (10 min), Tris-saline containing 0.05% Nonidet P-40 (twice for 20 min each), and Tris-saline again (10 min). Each wash was performed at room temperature with agitation using 200 ml buffer per 8 × 13 cm sheet of nitrocellulose. The antibody-antigen complex bound to the nitrocellulose was detected with $^{125}$I-labeled protein A. Nitrocellulose sheets were gently agitated for 60 min in a minimal volume (approx. 10 ml/sheet) of Tris-saline containing 5% bovine serum albumin and 2–5 ($\cdot 10^5$) cpm $^{125}$I-labeled protein A/ml. Unbound $^{125}$I-labeled protein A was removed by washing as described above for the removal of unbound antiserum. The nitrocellulose sheets were dried on a hot plate and then placed in direct contact with X-ray film. Proper exposure required 1–14 days depending on the amount of protein loaded on the original gel.

*Protein determination*. Protein concentrations were determined by a modification of the method of Lowry et al. [19] using bovine serum albumin as a standard.

*Materials*. Nitrocellulose (0.45 μm) was obtained from Schleicher and Schuell and Nonidet P-40 was from Particle Data Laboratories, Inc., Elmhurst, IL. PMSF was from Sigma. The reagents for electrophoresis and the molecular weight standards were from Bio-Rad. [$\gamma$-$^{32}$P]ATP was prepared by the method of Buetler and Guinto [20]. $^{125}$I-labeled protein A was a gift from Dale Lindsley.

## Results

$(Na^+ + K^+)$-ATPase purified from the outer medulla of lamb kidney by the method of Lane et al. [8] was used to innoculate rabbits and generate the antisera used in this study. This source of antigen was selected since at least 200 mg of the purified enzyme can be obtained in routine preparations. In addition, the purified $(Na^+ + K^+)$-ATPase preparation has a relatively high specific activity (1000–1500 $\mu$mol $P_i$/mg protein per h) with only minor contamination by other polypeptides. Fig. 1 (lane A) and Fig. 2 (lane A) illustrate the $(Na^+ + K^+)$-ATPase resolved by SDS-polyacrylamide gel electrophoresis with the predominant bands being the $\alpha$ subunit (96 600 daltons, S.D. = 610; $n$ = 5) and the $\beta$ subunit (41 000–53 000 daltons). When polyacrylamide gels were heavily loaded with protein, other contaminating polypeptides became visible. The $\alpha$ and $\beta$ subunits were estimated to comprise at least 80% of the protein present as judged by spectrophotometric scanning of Coomassie blue-stained gels. The $\alpha$ subunit could be phosphorylated in the presence of 140 mM $Na^+$, 3 mM $Mg^{2+}$ and 50 $\mu$M [$\gamma$-$^{32}$P]ATP and the steady state level of phosphorylation was decreased by the addition of 20 mM KCl, (Fig. 1, lanes B and C) confirming that this polypeptide is the $\alpha$ subunit of the $(Na^+ + K^+)$-ATPase.

Antisera against the lamb kidney $(Na^+ + K^+)$-ATPase were characterized by two methods. Initially, antisera were checked for ability to inhibit the enzymatic activity of the $(Na^+ + K^+)$-ATPase. As shown in Table I, the sera used in this study were able to inhibit $(Na^+ + K^+)$-ATPase from a number of sources including lamb kidney, dog kidney, dog brain and rat brain but not insect brain. Lack of complete inhibition of lamb kidney $(Na^+ + K^+)$-ATPase activity by antiserum could be due either to an insufficient amount of specific antibodies in the antiserum or saturation of antigenic sites resulting in a reduction of the turnover rate of the enzyme without producing complete inhibition. Partial inhibition of enzymatic activities by specific antisera has been reported by others [21]. Inhibition of enzymatic activity does not reveal whether antibody is directed against the $\alpha$ or $\beta$ subunits. In addition, since inhibition of neither kidney nor brain enzymes was complete, one cannot establish whether $\alpha$+ reacts with the antiserum.

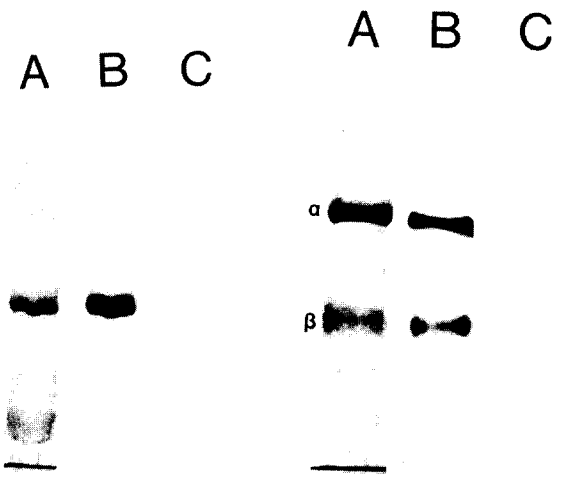


Fig. 1. SDS-polyacrylamide gel electrophoresis of phosphorylated lamb kidney $(Na^+ + K^+)$-ATPase. The purified lamb kidney enzyme was phosphorylated and subjected to SDS-polyacrylamide gel electrophoresis (10 $\mu$g/gel lane) as described in Experimental Procedures. The resolving gel was formed using 5% acrylamide. After electrophoresis, the gel was stained with Coomassie blue, destained, and prepared for autoradiography. A, $(Na^+ + K^+)$-ATPase phosphorylated in absence of KCl and visualized by Coomassie blue staining; B and C, autoradiographs of the enzyme phosphorylated in the absence (B) or presence (C) of 20 mM KCl. The autoradiograph in B was obtained by exposing the gel lane in A to X-ray film.

Fig. 2. Resolution of the $\alpha$ and $\beta$ subunits of the purified lamb kidney $(Na^+ + K^+)$-ATPase by SDS-polyacrylamide gel electrophoresis. Polypeptides visualized by Coomassie blue staining or autoradiography after binding of $^{125}$I-labeled protein A and antibodies. Samples (5 $\mu$g/lane) were separated by SDS-polyacrylamide gel electrophoresis using 7% acrylamide resolving gels. The sample in lane A was stained with Coomassie blue. Samples in lanes B and C were transferred to nitrocellulose paper, reacted with primary antiserum against the $(Na^+ + K^+)$-ATPase (lane B) or preimmune serum (lane C). The bound antibody was detected using $^{125}$I-labeled protein A followed by autoradiography as described in Experimental Procedures. '$\alpha$' and '$\beta$' indicate the position of the large and small subunits of the enzyme.

A more direct method of examining the reactivity of the subunits was utilized. The $\alpha$, $\alpha$+ and $\beta$ subunits were first resolved by SDS-polyacrylamide gel electrophoresis, then electrophoretically transferred to nitrocellulose filter paper. The proteins examined in this study appeared to bind tightly to the nitrocellulose

TABLE I

INHIBITION OF $(Na^+ + K^+)$-ATPase ACTIVITY BY ANTISERUM TO THE LAMB KIDNEY $(Na^+ + K^+)$ATPase

$(Na^+ + K^+)$-ATPase activity was assayed at 37°C for 15 min as described under Experimental Procedures. Controls were run with preimmune serum which caused less than 5% inhibition of enzyme activity.

| Enzyme | Final dilution of antiserum in assay | Inhibition (%) |
|---|---|---|
| Purified lamb kidney $(Na^+ + K^+)$-ATPase | 22 | 42 |
| | 220 | 19 |
| Dog kidney microsomes | 10 | 68 |
| | 22 | 67 |
| | 220 | 35 |
| Partially purified dog brain $(Na^+ + K^+)$-ATPase | 10 | 79 |
| | 22 | 50 |
| | 220 | 0 |
| Rat brain microscomes | 10 | 49 |
| | 22 | 46 |
| | 220 | 0 |
| *M. sexta* brain microsomes | 10 | 0 |
| | 22 | 0 |

without the necessity of using derivatized paper [17]. Transfer was highly reproducible and no loss of resolution was observed when the protein patterns of fixed polyacrylamide gels were compared to the pattern of proteins on the nitrocellulose filter paper. The resolved subunits bound to the nitrocellulose paper were reacted with rabbit anti-$(Na^+ + K^+)$-ATPase serum and the antibody-antigen complex was detected using $^{125}$I-labeled protein A. Fig. 2, lanes B and C, illustrate the reactivity of the purified lamb kidney $(Na^+ + K^+)$-ATPase with immune serum and control serum, respectively. The predominant immuno-reactive bands correspond to the $\alpha$ and $\beta$ subunits and no reactivity or background binding was observed with preimmunization serum (lane C). The antibody preparation appeared to be highly specific for the $\alpha$ and $\beta$ subunits. When the reactivity of unfractionated kidney membranes was examined, the predominant reactive polypeptides again corresponded to the $\alpha$ and $\beta$ subunits even though the crude membranes exhibited a highly complex polypeptide pattern when examined by SDS-polyacrylamide gel electrophoresis using Coomassie blue staining (compare Fig. 3, lane B and Fig. 6, lane F).

The reactivity of the $\alpha$+ subunit of the $(Na^+ + K^+)$-ATPase was examined using crude rat brain membranes and enzyme purified from dog brain. The latter had a specific activity of 400 $\mu$mol $P_i$/mg protein per h. As reported by Sweadner [5], two forms of the $\alpha$ subunit are apparent when the purified dog brain enzyme is subjected to SDS-polyacrylamide gel electrophoresis and the polypeptides detected by Coomassie blue staining (Fig. 3, lane E). The two forms of the large subunit were also observed in dog brain $(Na^+ + K^+)$-ATPase preparations prepared in the presence of the protease inhibitor PMSF (data not shown). The dog and rat brain samples were also phosphorylated in order to demonstrate that the doublet in the 96 000 molecular weight region actually represented $(Na^+ + K^+)$-ATPase subunits and not contaminating bands. Samples were phosphorylated in the presence of $Na^+$, ATP and $Mg^{2+}$. The phosphorylation was reduced by the addition of $K^+$. This is consistent with the properties of phosphorylation and dephosphorylation of the purified $(Na^+ + K^+)$-ATPase [2,3]. The kidney preparations had a single phosphorylated band. The most heavily phosphorylated band in the dog brain preparation had a slower mobility than the kidney samples and corresponded to the $\alpha$+ subunit (Fig. 4, lane C). We consistently had difficulty demonstrating the presence of a discrete phosphorylated $\alpha$ band in brain although occasionally we did observe a $^{32}$P-labeled $\alpha$ subunit (Fig. 4, lanes B and C). More typically, a smear of labeled material beneath the $\alpha$+ form was seen in the region of the $\alpha$ polypeptide. Densitometric scanning of a number of autoradiography negatives (Fig. 5) revealed a slight increase in $^{32}$P-labeling where the $\alpha$ subunit migrates in brain preparations (scans A and B). Our difficulty in observing the $\alpha$ form in brain does not appear to stem from poor resolution since the $\alpha$ form of kidney is clearly resolved from the $\alpha$+ form of brain (Fig. 3, lane E and Fig. 4). Both forms appear to be present in similar concentrations in the dog brain preparation, based on Coomassie staining and antibody reactivity. However, it may be possible that the brain $^{32}$P-$\alpha$ subunit is more unstable under

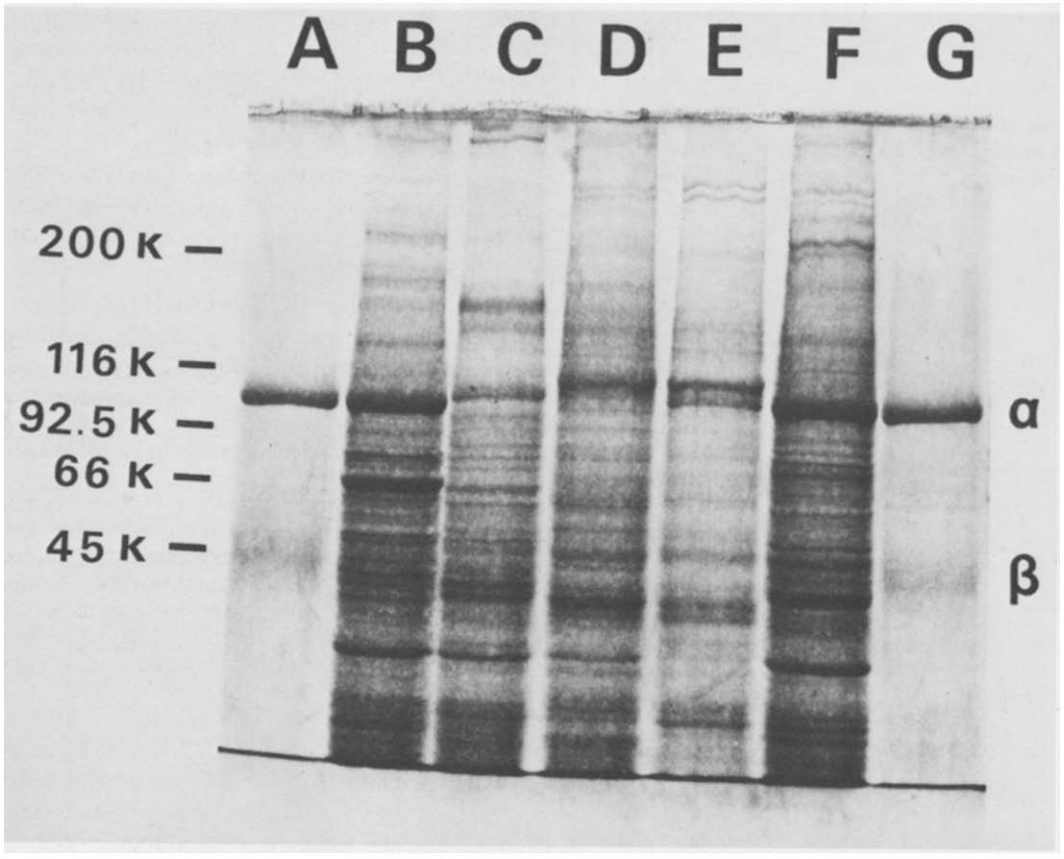


Fig. 3. SDS-polyacrylamide gel electrophoresis of crude and purified preparations of the $(Na^+ + K^+)$-ATPase from lamb, dog and rat kidney and dog and rat brain. SDS-polyacrylamide gel electrophoresis was performed as described in Experimental Procedures using a 7% acrylamide resolving gel. A, purified lamb kidney $(Na^+ + K^+)$-ATPase (5 μg); B, lamb kidney microsomes (20 μg); C, rat kidney microsomes (20 μg); D, rat brain microsomes (20 μg); E, partially purified dog brain $(Na^+ + K^+)$-ATPase (6 μg); F, dog kidney microsomes (20 μg), and G, purified lamb kidney $(Na^+ + K^+)$-ATPase (5 μg). The positions and molecular weights of standard proteins are given in the margin. 'α' and 'β' indicate the position of the large and small subunits of the purified lamb kidney $(Na^+ + K^+)$-ATPase.

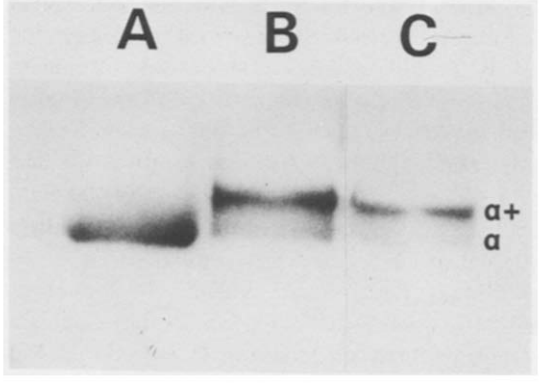


Fig. 4. Resolution of the phosphorylated α and α+ subunits by SDS-polyacrylamide gel electrophoresis. Purified lamb kidney $(Na^+ + K^+)$-ATPase (12 μg, lane A), rat brain microsomes (38 μg, lane B) and partially purified dog brain $(Na^+ + K^+)$-ATPase (12 μg, lane C) were phosphorylated, subjected to electrophoresis using a 7% acrylamide resolving gel, and prepared for autoradiography as described under Experimental Procedures.

the conditions of electrophoresis than the $^{32}$P-α+ form.

*Immunoreactivity of subunits*

The immuno-cross-reactivity of the α and α+ subunits of the $(Na^+ + K^+)$-ATPase was examined

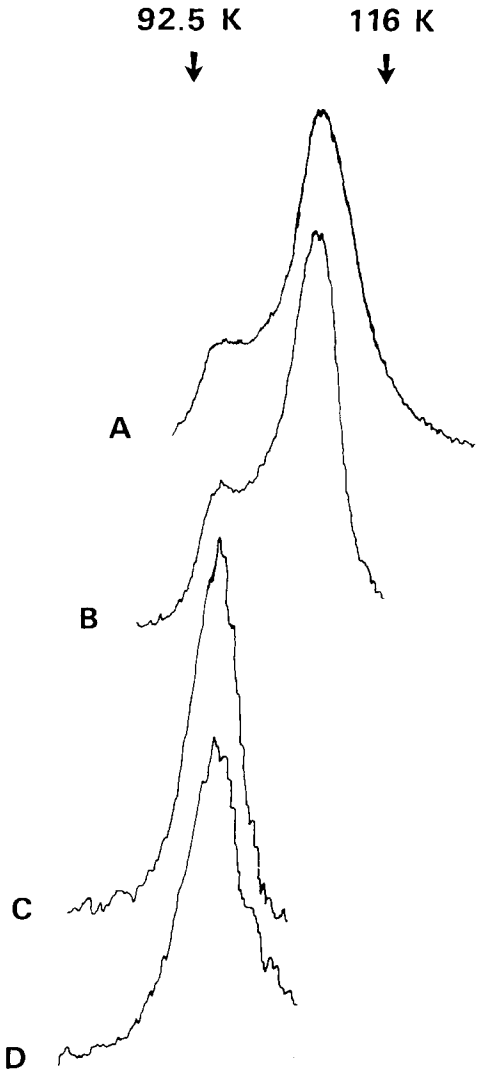


Fig. 5. Densitometric scans of autoradiograms of phosphorylated $(Na^+ + K^+)$-ATPase preparations. Samples were phosphorylated and resolved by SDS-polyacrylamide gel electrophoresis (5% acrylamide resolving gel) as described in Experimental Procedures. Autoradiograms from the gels were scanned using a Joyce, Loebl scanning microdensitometer. Samples are: A, rat brain microsomes (38 μg); B, partially purified dog brain $(Na^+ + K^+)$-ATPase (12 μg); C, dog kidney microsomes (10 μg); and D, purified lamb kidney $(Na^+ + K^+)$-ATPase (12 μg). Arrows indicate the position of molecular weight standards phosphorylase *b* (92 500 daltons) and β-galactosidase (116 000 daltons).

using the nitrocellulose transfer method. In lamb, dog and rat kidney, which have been shown to contain only the α form, a single immunoreactive band was

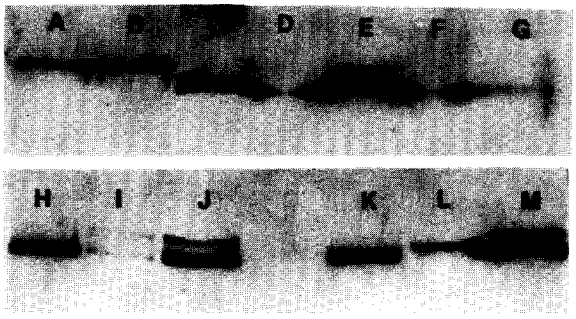


Fig. 6. Immunologic cross-reactivity of the α and α+ subunits of the $(Na^+ + K^+)$-ATPase. Samples were subjected to electrophoresis (5% acrylamide resolving gels), transferred to nitrocellulose paper and reacted with rabbit-anti-lamb kidney $(Na^+ + K^+)$-ATPase serum. Bound antibodies were detected with $^{125}I$-labeled protein A followed by autoradiography. Only the α subunit regions of the gels are shown. Samples are: A, rat brain microsomes (5 μg); B, NaI-treated rat brain microsomes (7 μg); C, rat kidney microsomes (10 μg); D, dog kidney microsomes (1 μg); E, J, and M, partially purified dog brain $(Na^+ + K^+)$-ATPase (2 μg); F, lamb kidney microsomes (1 μg); G, H, and K, purified lamb kidney $(Na^+ + K^+)$-ATPase (0.4 μg); I, human cerebral cortex microsomes (5 μg); and L, *M. sexta* brain microsomes (5 μg).

observed (Fig. 6, lanes C, D and G). In contrast, the dog brain sample (Fig. 6, lane E) showed a band identical in mobility to the α band of kidney as well as a second band with a higher molecular weight of 106 000 (S.D. 558, $n = 5$) corresponding to the α+ form.

Rat brain preparations consistently showed an α+ immunoreactive band and generally a very faint α band (Fig. 6, lanes A and B). It has been reported that both forms are present in rat brain [5]. In addition, human brain (Fig. 6, lane I) and frog retina (data not shown) had both α and α+ forms of the large subunit, while the insect *M. sexta* brain (Fig. 6, lane L) had only a single band with a mobility intermediate to the α and α+ forms of dog brain (Fig. 6, lane M). The α subunit of purified *E. electricus* electric organ $(Na^+ + K^+)$-ATPase also cross-reacts with antibody to the lamb kidney $(Na^+ + K^+)$-ATPase (data not shown).

*Cross-reactivity of the glycopeptide*

The glycopeptide subunit of the $(Na^+ + K^+)$-ATPase prepared from brain or kidney of lamb, dog and rat cross-reacted with anti-lamb kidney $(Na^+ +$

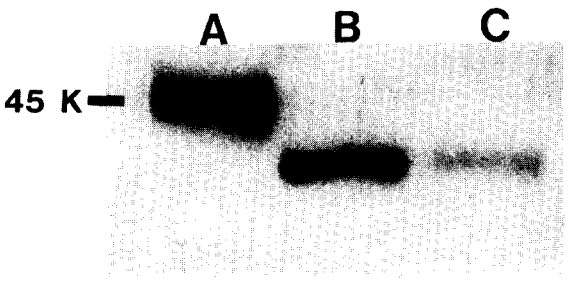


Fig. 7. Immunologic cross-reactivity of $\beta$ subunit of the $(Na^+ + K^+)$-ATPase. Samples were subjected to SDS-polyacrylamide gel electrophoresis (7% acrylamide resolving gels), transferred to nitrocellulose paper, and reacted with rabbit-anti-$(Na^+ + K^+)$-ATPase serum. Bound antiserum was detected with $^{125}I$-labeled protein A. Samples are: A, lamb kidney $(Na^+ + K^+)$-ATPase (1 $\mu$g); B, rat brain microsomes (23 $\mu$g); C, partially purified dog brain $(Na^+ + K^+)$-ATPase (4 $\mu$g).

$K^+)$-ATPase serum. The kidney $\beta$-subunit from these three species had identical mobilities (data not shown). However, the $\beta$ subunit from dog and rat brain (Fig. 7) had a faster mobility on SDS-polyacrylamide electrophoresis gels. Spector et al. [22] have reported that, when the $(Na^+ + K^+)$-ATPase was purified from mouse brain or Ehrlich ascites tumor cells in the presence of PMSF, the presumed $\beta$ subunit had a molecular weight of 53 000. In contrast, enzyme purified in the absence of PMSF from these sources contained polypeptides of 44 000 and 29 000 daltons in place of the 53 000 dalton subunit. These authors suggested that the 44 000 and 29 000 dalton polypeptides are proteolytically derived fragments of the 53 000 dalton polypeptide. Our data (Fig. 7) is consistent with the findings of Spector et al. [22]. Further experiments are presently being conducted to determined whether the immunoreactive $\beta$ subunit of rat and dog brain is a breakdown product of a larger polypeptide.

## Discussion

The results presented in this study substantiate the presence of two molecular forms of the $\alpha$ polypeptide of the $(Na^+ + K^+)$-ATPase. As in previous work by Sweadner [5], the identity of the $\alpha$ and $\alpha$+ polypeptides was demonstrated by sodium-stimulated phosphorylation of the polypeptides in the presence of $[\gamma\text{-}^{32}P]ATP$ which was decreased by the addition of $K^+$. In this way, Sweadner demonstrated a clear-cut distinction between a higher molecular weight $\alpha$+ and the $\alpha$ species. Although the $\alpha$+ form was present in brain and absent in kidney, not all nervous tissue studied contained $\alpha$+. In particular, membranes prepared from primary cultures of glial and neuronal cells as well as membranes from neuroblastoma and glioma cell cultures contain only the $\alpha$ form. Similarly, the electric organ of electric eel contains only the $\alpha$ form of the large subunit. On the other hand axolemma prepared from rat brain contain only the $\alpha$+ form [5]. Highly purified enzyme from brine shrimp contains both $\alpha$ and $\alpha$+ large subunits [6].

In the present study, our phosphorylation experiments (Fig. 5) clearly confirm the existence of the $\alpha$+ form of the $(Na^+ + K^+)$-ATPase; however, the presence of the $\alpha$-phosphorylated species in brain preparations is less clearly demonstrated than in experiments presented by Sweadner (cf. Ref. 5, Fig. 1). In her work, relatively similar levels of phosphorylation of $\alpha$ and $\alpha$+ were shown, especially in dog brain. The reason for this discrepancy is not immediately evident. The Coomassie-stained $\alpha$ and $\alpha$+ polypeptides found in dog brain (Fig. 3, lane E) certainly suggests rather similar amount of protein associated with each band. Perhaps the conditions used in our experiments favored higher phosphorylation or turnover of one form but not the other in brain. These experiments are particularly difficult to interpret in detail since after phosphorylation, the individual polypeptides must be separated by SDS-polyacrylamide gel electrophoresis under pH conditions where most of the acyl-$^{32}P$ is hydrolyzed. Our attempts to resolve the $\alpha$ and $\alpha$+ subunits by electrophoresis under acidic conditions [23], where the phosphorylated species would be more stable, have been unsuccessful.

The main thrust of the present work was to study the immunoreactivity of the $\alpha$ and $\alpha$+ forms of the $(Na^+ + K^+)$-ATPase. Sweadner found a 2000 dalton difference between these polypeptides in dog brain. After partial proteolytic cleavage, polypeptide maps of the two forms of the two subunits were essentially the same [5], suggesting close homology between the two forms. Our work shows a greater molecular weight difference, approx. 10 000, between the $\alpha$ and $\alpha$+ forms. However, antiserum obtained against the

purified lamb kidney ($Na^+ + K^+$)-ATPase clearly contained specific antibodies which reacted with both forms of the enzyme. This is perhaps most clearly shown with dog brain in the autoradiogram appearing in Fig. 6, lane E, where both α and α+ forms show impressive reactivity. The presence of an α form in rat brain (Fig. 6, lanes A and B) is less clear; however, much longer exposure of the autoradiograms often indicated faint reactivity in the α region. We have not seen convincing evidence for the α form of the enzyme from rat brain microsomes in Coomassie-stained gels (Fig. 3, lane D). Work by Petrali and Sulakhe [7] suggested that brain ($Na^+ + K^+$)-ATPase from young rats (28-days-old) contain only one high molecular weight polypeptide, whereas in adults (48-days-old) a doublet, perhaps corresponding to the α and α+ subunits, was found. However, rats used in our study were all adults. Work is continuing in order to establish whether both α and α+ forms can be demonstrated in rat brain.

In the insect brain ($Na^+ + K^+$)-ATPase, only one immunoreactive species was observed and this migrated between the α and α+ forms. Antiserum did not inhibit enzymatic activity (Table I) in *M. sexta* but clear immunoreactivity (Fig. 6, lane L) was observed. This suggests that in this case antibodies recognize antigenic sites outside the catalytic center of the *M. sexta* preparation and that sites involving substrate or ligand binding must not be affected by the antiserum in the insect. Thus, common antigenic determinants outside the active center must be present in insect as well as in a variety of species.

The antiserum used in this study clearly recognizes antigenic determinants on the β subunit of both the kidney and brain ($Na^+ + K^+$)-ATPase (Fig. 7). The differing mobilities shown in Fig. 7 may be due to proteolytic cleavage of the β subunit in brain or to microheterogeneity of the β subunit [24]. Spector et al. [22] showed that the β subunit from mouse brain, as well as from Erlich ascites tumor cells, is cleaved to yield 44 000 and 29 000 fragments unless PMSF was present during preparation of the enzyme. Further studies are in progress to further delineate these findings.

**Note added in proof** (Received November 5th, 1981)

Results identical to those shown in Fig. 7 were obtained when rat brain and kidney microsomes were prepared and electrophoresed in the presence of the protease inhibitors iodoacetamide, PMSF, pepstatin A, EDTA, phenanthroline and diisopropylfluorophosphate. Also the mobilities of the α and α+ forms of the ($Na^+ + K^+$)-ATPase were not altered in these tissues in the presence of the inhibitors.

## Acknowledgements

This work was supported in part by NIH grant NS-05424 and by the Veterans Administration. G. Schellenberg was supported by a National Research Service Fellowship from the NIH (NS 06388). Irene Pech received a stipend from the Graduate School Research Fund of the University of Washington. We thank Thomas Loomis for preparations of [$\gamma$-$^{32}$P]-ATP and eel ($Na^+ + K^+$)-ATPase, Dale Lindsley for $^{125}$I-labeled protein A, and Elaine Loomis for excellent technical assistance.

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