Two Isozymes of the Na,K-ATPase Have Distinct Antigenic Determinants

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Two isozymes of the Na,K-ATPase were purified from rat renal medulla and rat brainstem axolemma, and antisera were raised in rabbits. When antibody titers were measured, two sera showed specificity for either the kidney or axolemma Na,K-ATPases and had limited cross-reactivity which could be removed by cross-adsorption. In blots of polyacrylamide gels, these sera reacted with only the α or α(+) Na,K-ATPase catalytic subunits, while they cross-reacted with both types of β subunits. Two other sera each recognized both α and α(+), indicating that the catalytic subunit isoforms have additional shared antigenic determinants. A comparison of the Na,K-ATPases from the brains of different vertebrate species indicates that birds and fish differ from mammals and amphibians in the manifestation of Na,K-ATPase isoforms.

Neither neuraminidase nor endoglycosidase F treatment eliminated specific antibody reaction or affected the electrophoretic mobilities of the α and α(+) subunits, although endoglycosidase F increased the mobilities of the two types of β subunits to similar final apparent molecular weights. Blots of the peptide fragments produced by incomplete papain and trypsin digests of the α and α(+) subunits were stained with the specific sera, and the patterns of immunoreactive fragments were found to be markedly different. The results suggest that the antigenic differences reside in differences in the primary protein sequences of the two isozymes.

The (Na⁺ + K⁺)-stimulated adenosine triphosphatase (Na,K-ATPase) contains two types of protein subunits, a catalytic subunit of approximately M, 95,000 (α) and a glycoprotein subunit of approximately M, 50,000 (β) (reviewed in Ref. 1). There are two different molecular forms of the catalytic subunit of the Na,K-ATPase in mammals (2). The structure and kinetics of the form found in the kidney has been extensively studied, and its catalytic subunit is called α in accordance with accepted usage. The catalytic subunit of the form found in brain axolemma is called α(+) because of its apparently larger molecular weight after gel electrophoresis in SDS. The two catalytic subunit types can also be distinguished by their sensitivity to several biochemical probes, such as proteases, cross-linking agents, N-ethylmaleimide (2), and pyrithiamin (3).

In the rat and mouse, the two forms of the Na,K-ATPase have markedly different affinities for the cardiac glycosides (2, 4), which suggests at least one difference of potential functional significance. Both forms are present in the brain, and there is evidence that the form typical of the kidney is expressed in glia, while neurons may express either form or both (2, 5, 6). Both forms appear to be present in fat cells, where the α(+) form is selectively sensitive to stimulation by insulin (7, 8).

To look for more evidence for structural differences between the two Na,K-ATPases and to create useful immunological tools for immunocytochemistry and molecular biology, rabbit antisera were raised against the Na,K-ATPases purified from rat renal medulla and rat brain axolemma. If one form were derived from the other either by proteolytic processing or by the addition of something like carbohydrate, then one might expect to obtain antibodies that would be specific for the unique determinants of the larger form, but all determinants on the smaller form would be shared with the larger form. Here evidence was obtained for the existence of both shared and distinct antigenic determinants on both forms of the Na,K-ATPase.

MATERIALS AND METHODS

Purification of Antigen and Immunization of Rabbits—Na,K-ATPase was prepared from rat renal medulla by extraction with SDS by the method of Jorgensen (9). Specific activities obtained ranged from 600–1500 μmol of ATP hydrolyzed/h/mg protein. Axolemma with Na,K-ATPase specific activities of 125–250 μmol/h/mg protein was isolated from rat brainstems by the procedure of DeVries (10). The axolemma Na,K-ATPase was then further purified by extraction with SDS (9, 11), and specific activities of 790–1250 μmol/h/mg protein were obtained.

Rabbits K2, A2, and K3 were injected intramuscularly at several sites in the hind leg with 100 μg of purified kidney (rabbits K2 and K3) or axolemma (rabbit A2) Na,K-ATPase in complete Freund’s adjuvant, and they were boosted with 100 μg of the appropriate Na,K-ATPase in incomplete Freund’s adjuvant at 2, 4, and 10 weeks. Highest titer bleeds were obtained at 12 and 14 weeks. Rabbits K1 and A1 were injected and boosted on the same schedule with approximately 50 μg of α or α(+) subunits, respectively, cut from unfixed SDS gels after visualizing the bands by soaking in 1 M KCl for 30 min.

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Solid-phase Antibody Titer Determination—Titers were determined essentially by the procedure of Gee and Langone (12) by adsorbing 1–2 μg of Na,K-ATPase to plastic wells (Immunon-2 Removawells, Dynatech Laboratories, Alexandria, VA) and then measuring the binding of serial dilutions of antibody, followed by 125I-goat-anti-rabbit IgG (New England Nuclear). Control experiments performed by varying the amount of antigen bound to the wells or the amount of antibody added confirmed that this technique measures the avidity of the antibodies, rather than the amount of either antibody or antigen.

Gel Electrophoresis—Gel electrophoresis employed the buffer system of Laemmli (13). Western blots were made by electrophoretic transfer to nitrocellulose sheets (14, 15). Blots were stained with the avidity of the antibodies, rather than the amount of either adsorbing 1–2 pg of Na,K-ATPase to plastic wells (Immunlon-2 ensuring the binding of serial dilutions of antibody, followed by '9-peroxidase-conjugated goat-anti-rabbit IgG (Bio-Rad). Autoradiograms of 125I were prepared with Kodak XAR-5 film, and chloronaphthol horseradish peroxidase color reaction was developed with the Bio-Rad Immun-Blot reagents.

Chromatographic Separation of Na,K-ATPase Subunits—Purified preparations of either kidney or axolemma Na,K-ATPase were dissolved in 2% SDS in 50 mM Tris-Cl, pH 8.0, and chromatographed on a 1 × 50-cm column of Sepharose CL-6B in 50 mM Tris-Cl, pH 8.0, with 0.1% SDS. Column fractions were analyzed by electrophoresis on SDS gels, and the fractions containing only α or α(+) or β subunits were pooled, dialyzed against phosphate-buffered saline, concentrated by incubating the dialysis tubing with dry Sephadex, and treated with polystyrene beads (Bio-Beads SM-2) to adsorb the SDS.

Membrane Preparations from Brain—Crude microsomal fractions from the brains of all species were prepared as described previously (11). Human specimens were obtained from postmortem brains at autopsy. Aliquots of 20 μg of membrane protein were electrophoresed and blotted for the analysis of antibody specificity.

Peptide Maps by Incomplete Proteolytic Digestion—Partial digests of the α and α (+) subunits were compared by the method of Cleveland et al. (16), using α and α (+) subunits cut from unfixed SDS gels. Solutions of protease dissolved in 0.1% SDS, 0.0625 M Tris-Cl, pH 6.8, 5–10% glycerol were layered on top of the gel slices, and digestion was allowed to proceed during gel electrophoresis. The peptide fragments and molecular weight markers were transferred to nitrocellulose and stained with antibodies as described above.

RESULTS

Purification of Antigen and Immunization of Rabbits—The Na,K-ATPases of rat renal medulla and brainstem axolemma were purified by SDS extraction and sucrose density centrifugation. Fig. 1A shows the SDS-gel electrophoretic pattern of the purified preparations, separated in the Laemmli buffer system on a gradient gel of 5–15% polyacrylamide and stained with Coomassie Blue. The position of molecular weight markers was marked by the open arrows. The catalytic subunits of the Na,K-ATPase have been previously identified by their specific Na+-dependent, K+-inhibitable phosphorylation by [γ-32P]ATP (2). The catalytic subunits, α and α (+), had apparent molecular weights of approximately 92,000 and 94,000, while the β subunits had apparent molecular weights of approximately 53,000 and 47,000.

The α and α (+) bands could be resolved on gels of lower polyacrylamide concentration. Fig. 1B shows such resolution in the Laemmli buffer system on a gel of 5% polyacrylamide: lane 1 contained the purified kidney enzyme, lane 3 the purified axolemma enzyme, and lane 2 a mixture of the two. In Fig. 1C the experiment was repeated with electrophoresis in the Amory gel system (17). This electrophoresis system employs buffers in the acidic pH range and uses tetradecylammonium bromide as a detergent instead of SDS, and electrophoresis is in the direction of the cathode instead of the anode. Nonetheless, the α and α (+) subunits were resolved at the same apparent molecular weights as in the Laemmli system, indicating that their separation is not an artificial.

Two rabbits, A1 and K1, were immunized with axolemma α(+) and kidney α subunits, respectively, cut from SDS gels. Rabbit A2 was immunized with purified axolemma Na,K-ATPase containing both α(+) and β subunits, and rabbits K2 and K3 were immunized with purified kidney Na,K-ATPase containing both α and β subunits. Rabbit A1 did not produce detectable antibodies, but rabbits A2, K1, K2, and K3 each produced antisera with different specificities. Antiserum A2 and K2 have been characterized most extensively.

Antibody titer was measured by the binding of serial dilutions of A2 or K2 antisera to axolemma or kidney Na,K-ATPase preparations adsorbed to microtiter wells, followed by the binding of 125I-goat-anti-rabbit IgG. Fig. 2 shows that A2 and K2 each had a much higher titer when binding the homologous Na,K-ATPase type than the heterologous type. If the antisera were cross-adsorbed prior to determining the titer, i.e., if A2 was preadsorbed with membranes from the kidney and K2 was preadsorbed with axolemma, the binding to the heterologous Na,K-ATPase type was reduced to the background level seen when control rabbit serum was used instead of K2 or A2, while the binding to the homologous Na,K-ATPase type was affected very little. Cross-adsorption thus enhances antibody specificity. Final titers (50% of maximum binding) were approximately 1:1000 for K2 and 1:2500 for A2 in the bleeds shown.

The ability of the antisera to inhibit Na,K-ATPase activity was assayed by preincubating the antibodies with preparations of the Na,K-ATPase from either axolemma or kidney for 30 min at 0°C and then measuring ATPase activity in a reaction mixture containing the same amount of antibody and saturating amounts of Na+, K+, and ATP. None of the antisera had any detectable effect on ATPase activity at dilutions as low as 1:20.

Antibody Specificity by Western Blot—Electrophoretic blots of membrane preparations from kidney, axolemma, and whole brain were used to assess antibody specificity. Unpurified membrane fractions were used so that antibodies against components other than the Na,K-ATPase could be detected. In Fig. 3, the lanes marked C (control) show the binding of untreated A2 and K2 antisera to the homologous membrane preparations. Antiserum A2 stained two bands in axolemma whose molecular weights corresponded to the α(+) and β subunits of the Na,K-ATPase. Antiserum K2 stained bands
Fig. 2. Titers of the A2 and K2 antisera. The binding of antibodies to kidney and axolemma Na,K-ATPases adsorbed to plastic microtiter wells was measured. Dilutions of the A2 and K2 antisera are shown along the abscissa, and counts/min of the iodinated second antibody along the ordinate. In the upper panel: ■, binding of untreated A2 antiserum to purified axolemma Na,K-ATPase; □, binding of A2 to axolemma Na,K-ATPase after preadsorption with 100 μg of renal medulla membranes/10 μl of antiserum; △, binding of untreated A2 to purified renal medulla Na,K-ATPase; ◼, binding of A2 preadsorbed with renal medulla membranes to renal medulla Na,K-ATPase. Control rabbit serum gave the same level of background binding as seen with the open triangles. In the lower panel: ●, binding of untreated K2 to purified renal medulla Na,K-ATPase; ○, binding of K2 to renal medulla Na,K-ATPase after preadsorption with 100 μg of axolemma membranes/10 μl of antiserum; ◼, binding of K2 to purified axolemma Na,K-ATPase; ○, binding of K2 preadsorbed with axolemma membranes to axolemma Na,K-ATPase.

Antibody Dilution

Fig. 3. Western blot analysis of the specificity of the A2 and K2 antisera. Samples of unpurified axolemma (upper panel) and kidney membranes (lower panel) were loaded into 10-cm-wide wells on slab gels of 7.5% polyacrylamide and were electrophoresed and blotted onto sheets of nitrocellulose. Strips of the blots were then cut and incubated individually with the Na,K-ATPase antisera, and the bound antibodies were detected with horseradish peroxidase-conjugated second antibody and chloronaphthol color development. 

Evidence has been obtained that the α and β subunits of the kidney Na,K-ATPase were the subunits of the Na,K-ATPase (18); we infer that it might have been a highly antigenic contaminant present in the original antigen. It was not detected in blots of membranes from the brain, which are prepared in such a way that blood vessels are removed in the first centrifugation step (see Fig. 4).

To test whether the bands stained by the K2 and A2 antisera were the subunits of the Na,K-ATPase, the antisera were preadsorbed with purified Na,K-ATPase prior to staining the blots. Preadsorptions were done in two ways: with undenatured purified Na,K-ATPase and with α or α(+) and β subunits separated in SDS on a column of Sepharose CL-6B. In both panels of Fig. 3, lanes 1–3 show the effects of a single preadsorption of 100-μl aliquots of 1:10 dilutions of the antisera with 25-, 50-, and 100-μg aliquots of undenatured...
preparations of the homologous Na,K-ATPases. Lanes 4 and 5 show the effect of preadsorption with 20 and 40 μg, respectively, of the homologous separated denatured α or α(+) subunits from the Sepharose column, while lanes 6 and 7 show the effect of preadsorption with 20 or 40 μg of the separated denatured β subunits. The reduction of β stain by purified subunit seen in lane 5 of the top panel was not a reproducible observation. In aggregate, the identity of the stained bands as the α, α(+), and β subunits is supported by the data if one accepts that the antibody preparation is likely to be heterogeneous, with populations that recognize native enzyme and others that recognize denatured enzyme, or both.

Fig. 4 shows blots of 5% polyacrylamide gels, where the α and α(+) subunits can be resolved (arrows); the β subunits migrate at the dye front and are not shown. Lane 1 in each panel contains kidney microsomes, with the α catalytic subunit; lane 2 contains whole brain microsomes, with both α and α(+) catalytic subunits; and lane 3 contains axolemma, with only the α(+) catalytic subunit as judged by Coomassie Blue stain (not shown). Antiserum A2 stained only the α(+) subunit in brain and axolemma preparations, and it did not stain the α catalytic subunits in either the kidney or brain preparations. Antiserum K2 stained only the α subunit in kidney and brain preparations, and it detected a small amount of the α subunit contaminating the axolemma preparation. Thus the specificity of A2 and K2 antiserum implicit in the titer determinations of Fig. 2 can be accounted for by their selective recognition of the α(+) and α subunits, respectively. Later bleeds of both K2 and A2 rabbits contained more cross-reactive antibodies than shown here (see Fig. 7 for an example), indicating that time and multiple boosts changed the composition of the antisera. The A2 and K2 antisera contained antibodies which cross-reacted with the β subunits of both the kidney and axolemma Na,K-ATPases (see Figs. 6 and 7); these antibodies were diminished in later bleeds.

Antiserum K1 stained both α and α(+) subunits approximately equally well. Antiserum K3 stained both α and α(+) subunits but stained the α subunit more intensely than the α(+) subunit. Thus each antiserum showed a characteristic pattern of specificity with respect to the catalytic subunits of the Na,K-ATPase (Fig. 4). Antiserum K3 also normally stained the same M, 135,000 protein stained by the K2 antiserum, but the preparation of kidney microsomes used for the K3 stain shown in Fig. 4 apparently had little of it.

The K1 antiserum, which was raised against denatured α subunit cut from SDS gels, stained only the α and α(+) subunits. Unlike all of the other antisera, it showed little or no binding (titer approximately 1:150 or less) when measured by binding to antigen in plastic wells. Since it was known to bind to the Na,K-ATPase on Western blots, dilutions of the K1 antiserum were bound to strips of blots, followed by 125I-goat-anti-rabbit IgG. The resulting autoradiographs were scanned with a densitometer, and a titer of 1:800 was obtained. This result suggested that the K1 antiserum contains antibodies that recognize SDS-denatured Na,K-ATPase but which do not recognize native enzyme bound to microtiter wells. The other antisera apparently contain antibodies that recognize either or both. The K3 antiserum stained all of the proteins stained by K2 in Western blots and two or three other unidentified bands as well (not shown).

Na,K-ATPase Heterogeneity in Other Species—The A2 and K2 antisera were used to screen crude membranes from the whole brains of a variety of vertebrate species for the presence of α(+) and α (Fig. 5). Replicate blots of 5% polyacrylamide gels were stained with A2 or K2 alone, or with a mixture of the two antisera. The A2 antiserum stained a component with the same mobility as the α(+) subunit from rat brain in samples from all mammals tested and from the frog. It did not detectably cross-react with the α subunit in any species. The K2 antiserum was specific for the α subunit only in the rat, mouse, and hamster. In the calf, sheep, guinea pig, dog, cat, human, rabbit and frog, it cross-reacted with both α and α(+) and stained them relatively weakly. The results obtained with the chicken and three species of

**Fig. 5.** A2 and K2 stain of the brain Na,K-ATPases from a variety of species. Electrophoretic blots of 5% polyacrylamide gels of unpurified membrane preparations from whole brain are shown, stained with the indicated antiseras at 1:200 dilutions. 1, rat; 2, calf; 3, cat; 4, chicken; 5, dog; 6, guinea pig; 7, hamster; 8, human; 9, mouse; 10, rabbit; 11, sheep; 12, rat; 13, carp; 14, perch; 15, goldfish; 16, bullfrog. The chicken and the three species of fish show only one band of intermediate molecular weight at the position of the catalytic subunit of the Na,K-ATPase.
Antibodies to Isozymes of the Na,K-ATPase

bony fishes were surprising. Membranes from the brains of the chicken, carp, goldfish, and perch had only one band at the position of the catalytic subunit of the Na,K-ATPase. This band had a mobility roughly halfway between those of \( a \) and \( a(+) \), and in all four cases, it was stained by both the A2 and the K2 antiserum.

The Effect of Removing Carbohydrate on Antibody Specificity—Neuraminidase and endoglycosidase F were used to determine whether the observed electrophoretic mobility differences and antibody specificity of rat kidney and rat axolemma Na,K-ATPases could be ascribed to differences in glycosylation. Fig. 6 illustrates the results obtained when purified preparations of kidney and axolemma Na,K-ATPase were stained with the K2 antiserum before and after treatment with neuraminidase. The panel on the left (lanes 1–7) shows a Coomassie Blue-stained Laemmli gel of 7.5% polyacrylamide. Lanes 1 and 7 are molecular weight markers, while lane 2 is Na,K-ATPase from the rat renal medulla, and lane 3 is Na,K-ATPase from axolemma from the rat brainstem. The darkest bands are the catalytic subunits, and that from the axolemma preparation has a characteristiclly slightly higher apparent molecular weight. The positions of the \( \beta \) subunits are marked with circles. The axolemma preparation had several contaminating proteins which migrated as sharp bands in the vicinity of the \( \beta \) subunit; the \( \beta \) subunit, however, migrated as a broader band, though not so broad as that of the kidney Na,K-ATPase. Lanes 4 and 5 show kidney and axolemma Na,K-ATPase preparations that were treated with Clostridium perfringens neuraminidase (Worthington/Cooper Biochemicals, Malvern, PA) at 50 \( \mu \)g/ml for 30 min at 37°C prior to electrophoresis. Lane 6 shows the faint bands at approximately \( M, 80,000 \) that were contributed by the neuraminidase. The neuraminidase digestion had no effect on the mobility of the \( a \) and \( a(+) \) subunits. Neuraminidase treatment caused a shift in the mobility of the \( \beta \) subunits of both preparations, although it did not cause them to display the same mobility. Before digestion, their apparent molecular weights were 53,000 and 47,000, respectively, while after the digestion they were 49,000 and 45,000.

A replica of lanes 2–4 was electrophoresed on the same slab gel, transferred to nitrocellulose, and stained with K2 antiserum followed by 125I-goat anti-rabbit IgG. The autoradiograph is shown in the panel on the right (Fig. 6, lanes 2–4'). As in Fig. 4, the K2 antiserum stained the \( a \) subunit of the kidney Na,K-ATPase well, and it detected a minor contaminant in the axolemma Na,K-ATPase preparation which was not visible by Coomassie Blue stain. The antiserum detected the \( \beta \) subunits of both preparations, before and after treatment with neuraminidase. Similar results were obtained with the A2 antiserum, although it stained the \( \beta \) subunits much less intensely (not shown). Neuraminidase treatment had no effect on the specific antibody reactivity of the K2 and A2 antisera toward the \( a \) and \( a(+) \) subunits.

Endoglycosidase F was used to remove N-linked carbohydrate side chains (19). Fig. 7 shows blots of kidney and axolemma Na,K-ATPases before and after digestion with 0.043 unit of endoglycosidase F (New England Nuclear) per \( \mu \)g of purified Na,K-ATPase for 75 min at 37°C. Lanes 1–4 show kidney and axolemma Na,K-ATPases before and after endoglycosidase F digestion and stained with K2, while lanes 5–8 show the same samples stained with A2. No alteration of the mobility or immunoreactivity of the \( \alpha \) and \( a(+) \) subunits was seen (even after overnight incubation with endoglycosidase F and separation on gels of 5% acrylamide (not shown)), while the \( \beta \) subunits were partially digested to lower molecular weight products. Cross-reactivity of the K2 antiserum with the \( a(+) \) subunit in the axolemma Na,K-ATPase is seen in this experiment, because a later bleed of the K2 rabbit was used. Both antisera stained the same \( \beta \) subunit products, but the stain with the A2 antiserum was so faint that only the smaller arrows.
most prominent bands can be detected in Fig. 7.

The apparent molecular weights of the kidney and axolemma β subunits before digestion were 54,500 and 47,500, respectively. After digestion, the kidney β subunit showed products at 41,500, 40,000, and 35,500 molecular weights, while the axolemma β subunit showed products at 45,500, 40,000, and 35,500 molecular weights. In other experiments it was observed that the 35,500 molecular weight product was the smallest one obtained, when detected by either antibody stain or by Coomassie Blue stain, even after overnight electrophoresis in the presence of 0.064 unit of endoglycosidase Flpg of protein. The data suggest that 35,500 is the molecular weight of the β subunit apoprotein of both the rat kidney and the rat axolemma Na,K-ATPases.

Immunoreactive Peptide Fragments of α and α(+)—One-dimensional peptide maps of partial proteolytic digests of the α and α(+) subunits were performed by the Cleveland method (16). In this procedure, the subunits cut from SDS gels were layered onto new gels, protease was added to the gel sample well, and proteolysis was allowed to occur during electrophoresis. The extent of digestion was most easily controlled by varying the concentration of protease added to each well. Heavy digestion, in which most of the fragments were reduced to molecular masses of 10,000–20,000 daltons, was used to maximize the possibility of detecting small fragments bearing discrete antigenic differences between the two isoforms of the Na,K-ATPase. Papain and trypsin were the proteases used, at concentrations of 0.25 and 5.0 µg/well, respectively; it is estimated that the gel slices containing the α and α(+) subunits contained 2–3 µg of protein each. The resulting fragments, electrophoresed on a gradient of 10–20% polyacrylamide, were transferred to nitrocellulose and stained with the antisera. “Prestained” (dyecoupled) molecular weight markers (Bethesda Research Laboratories) were electrophoresed on the same gels and transferred in parallel. In the experiment illustrated in Fig. 8, the kidney and axolemma catalytic subunits were electrophoresed in adjacent wells to make their comparison most convincing, and the blot was stained with a mixture of K2 and A2 antisera. In other cases the kidney and axolemma Na,K-ATPases were digested, electrophoresed, blotted, and stained separately to verify that the patterns of fragments shown in Fig. 8 were reproducible and ascribable to specific reaction with the K2 or A2 antisera. The result was that the immunoreactive peptide fragments obtained from digestion of α and α(+) were unambiguously different. Papain and trypsin gave different characteristic fragment maps with each isozyme.

**DISCUSSION**

**Multiple Forms of the Na,K-ATPase**—The significance of the detection of antigenic determinants unique to the α and α(+) subunits is that it provides evidence for structural differences between the Na,K-ATPase isoforms. Previous analysis of Cleveland proteolytic maps of the α and α(+) subunits from canine brain (less heavily digested than in the present experiments) indicated that the two forms have considerable homology (2). Two of the antisera described here, K1 and K3, and an antiserum described by Schellenberg et al. (20) all contained antibodies that recognized both α(+) and α, which is further support for the presence of structural homology. Although antigenic differences between the Na,K-ATPases of different tissues have been found in several laboratories (21–25), the present report is the first case in which tissue-specific differences in Na,K-ATPase antigenic determinants can be assigned to the α and α(+) catalytic subunits.

The A2 antiserum recognizes determinants unique to the α(+) form of the Na,K-ATPase in all mammals and the one amphibian tested, and it recognizes the only catalytic subunit detected in the chicken and bony fishes. In no case did it cross-react with the α subunit in mammals or the frog, and thus it must recognize a domain that is uniquely characteristic of α(+). The K2 antiserum recognized determinants that were specific for α in only three closely related species, the rat, mouse, and hamster. These species are among those with the lowest affinities for the cardiac glycoside ouabain. The K2 antiserum detected more weakly a determinant that was shared by α and α(+) in all of the other mammals and the frog and which may be present but harder to detect in the rat, mouse, and hamster. It is likely that there are at least two antibody populations present: one, of high avidity, that is specific for α, and another, of lower avidity or less abundant, that recognizes a determinant that is shared by α and α(+).

The observation that the chicken, like the teleost fishes, shows only one band on SDS gels at the position of the catalytic subunit was surprising in view of the prior demonstration of Na,K-ATPase heterogeneity with monoclonal antibodies in the same species (24, 25). There is more than one possible explanation; the heterogeneity detected by the monoclonal antibodies may reside in the β subunit, or there may be multiple forms of the α subunit in the chicken with identical electrophoretic mobilities. If the one catalytic subunit detected in the chicken and bony fishes is a single protein species recognized by both A2 and K2 antisera, that form of the enzyme must have both types of antigenic determinants.

The Na,K-ATPase of invertebrates also shows heterogeneity. Multiple bands at the position of the catalytic subunit were observed in gels of SDS-extracted membranes from the nervous system of the lobster (2) although no other criteria were applied to determine whether the bands really represented the Na,K-ATPase. In contrast, Schellenberg et al. (20) found a single band with mobility between mammalian α and α(+) in the nervous system of an insect, Manduca sexta, and in this case the band was recognized by antibodies raised against sheep kidney Na,K-ATPase. Two forms of the catalytic subunit in the brine shrimp Artemia salina, named α1 and α2, have been studied more extensively (26–29). In electrophoretic pattern they resemble the α and α(+) forms of

**Fig. 8.** Proteolytic digest maps of the α and α(+) isozymes of the Na,K-ATPase. Incomplete digests of the two catalytic subunit types were carried out during electrophoresis. Pieces of polyacrylamide gel containing α or α(+) were pushed to the bottom of individual wells, and solutions of protease in 0.0625 M Tris-Cl, 0.1% SDS, pH 6.8, were layered on top. The length of the stacking gel was 2.5 cm, the separating gel was 9 cm, the thickness of the gel was 0.75 mm, and electrophoresis was carried out at constant power at 2 watts for 5 h. Lanes 1 and 4 contained prestained molecular weight markers; the positions of β-lactoglobulin (M, 18,400) and cytochrome c (M, 12,300) are shown with the arrows. Lane 2, α digested with papain; lane 3, α(+) digested with papain; lane 5, α digested with trypsin; lane 6, α(+) digested with trypsin. The blot was shown to be stained with a mixture of K2 and A2 antisera at 1:200 dilutions, followed by horse-radish peroxidase-conjugated second antibody.
mammals, but their relative mobilities have been reported to be reversed when electrophoresis is performed at acidic pH (27). The most recent studies of in vitro protein synthesis of the Na,K-ATPase with Artemia mRNA indicated that the product of translation was two bands of higher molecular weight, suggesting the presence of precursors synthesized from different gene transcripts (28). Morohashi and Kawamura (29) have determined the N-terminal sequence of the α1 and α2 bands of the Artemia Na,K-ATPase and reported amino acid heterogeneity at the 3rd position, supporting the existence of different gene products.

The Structural Basis of the Differences between Kidney and Axolemma Na,K-ATPases—There are several reports of the presence of carbohydrate in the α subunit of the Na,K-ATPase of some species, and the β subunit is known to be heavily glycosylated (1). Since glycosylation differences could in principle give rise to both molecular weight and antigenic differences, it was imperative to attempt to determine whether the removal of carbohydrate would affect the mobility and recognition of α and α(+). Neither neuraminidase nor endoglycosidase F, the endoglycosidase of broadest specificity now available (19), affected α and α(+) under conditions where the mobility of the β subunits was clearly increased. The result argues against glycosylation as the molecular basis of the difference between α and α(+). We cannot rule out, however, that α or α(+) bears a form of glycosylation or other post-translational modification that is not sensitive to endoglycosidase F.

Previous investigators have noticed the difference in electrophoretic mobility of the β subunits from brain and kidney (1). The results of endoglycosidase F digestion suggest that the difference can be ascribed to two or more complex carbohydrate side chains. Kidney and axolemma Na,K-ATPases have similar digestion products at apparent molecular weights of 40,000 and 35,500 and different digestion products at molecular weights of 41,500 (kidney) and 45,500 (axolemma). The appearance of such discrete steps in endoglycosidase digestion is usually interpreted as the removal of individual complex carbohydrate chains, although they may represent the removal of multiples of chains (19). The smallest product, M, 35,500, is much like that described by Fambrough at M, 32,000 after biosynthesis of chicken skeletal muscle Na,K-ATPase in the presence of tunicamycin (25) and by Fisher et al. at M, 36,000 after in vitro synthesis of the Na,K-ATPase from brine shrimp mRNA (28). The simplest interpretation is that the β subunits of the kidney and axolemma Na,K-ATPases have very similar apoproteins, one or more similar carbohydrate side chains, and two or more dissimilar ones. Our antibodies against the β subunits cross-reacted with both axolemma and kidney β subunits, but in view of the diversity of the rabbits’ antigenic responses, the failure to detect specific antibodies does not rule out the existence of antigenic differences.

The most compelling evidence that the antigenic differences between α and α(+) are likely to lie in primary protein structure comes from the analysis of one-dimensional proteolytic peptide maps. After digesting heavily and using the specific antisera to detect only the immunoreactive peptide fragments, almost no correspondence was seen between the fragments derived from α and α(+) (+). It should be possible to purify peptide fragments bearing the antigenic differences by immunoaffinity chromatography and sequence them to determine the differences in primary protein structure. It should also be possible to use these specific antisera to clone the gene or genes for the Na,K-ATPase isozymes and to localize the two forms in cells and tissues by immunocytochemistry.

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