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Anomalies in the electrophoretic resolution of Na^+/K^+-ATPase catalytic subunit isoforms reveal unusual protein–detergent interactions

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Three different isozymes of the Na^+/K^+-ATPase α-subunit have slightly different electrophoretic mobilities in sodium dodecyl sulfate (SDS). Certain procedures (reduction and alkylation, heating, and the use of sodium tetradecyl sulfate) have been reported either to improve the electrophoretic separation of isoforms or to reveal the presence of new isoforms. The variables affecting gel electrophoretic mobility were investigated here. Reduction and alkylation decreased the mobility of all three isozymes, and slightly improved the separation of α1 from α2 and α3 without causing a qualitative change in the α isoforms detected. Heating the enzyme in SDS caused splitting into two bands. Both bands were intact polypeptides but migrated differently in 5% and 15% polyacrylamide, disclosing an anomalous conformation in detergent. The use of sodium tetradecyl or decyl sulfate instead of dodecyl sulfate altered the relative mobilities of the isozymes, revealing differences in detergent affinity, but no new isoforms were found. In conclusion, Na^+/K^+-ATPase α-subunit mobility reflects complex detergent–protein interactions that can be affected by experimental conditions. The existence of more than one band on gels may reflect different conformations in detergent, but should not be accepted alone as evidence for subunit structural heterogeneity.

Introduction

The Na^+/K^+-ATPase is a membrane protein with two different kinds of subunit, an α-subunit of M_r 112000 and a glycosylated β-subunit with a protein component of M_r 34000. The existence of more than one form of the catalytic subunit of the Na^+/K^+-ATPase in membrane preparations from the brain was first noticed because of the presence of a doublet after gel electrophoresis in sodium dodecyl sulfate [1]. Evidence that the two bands were structurally different included different sensitivities to proteolysis by trypsin; different sensitivities to crosslinking by a reagent that oxidizes sulfhydryl groups; and different numbers of exposed sulfhydryl groups reactive with *N*-ethylmaleimide. More compellingly, the two forms were physically separated by fractionating brain tissue prior to isolating the enzyme: an axolemma fraction had only one form, while cultured non-neuronal cells had only the other. The electrophoretic mobilities of the separated forms ran true. Furthermore, it was shown that they had different sensitivities to inhibition by cardiac glycosides. The basic kinetic properties of the separated forms were examined [2], and the two forms were found to be antigenically different [3].

Molecular genetics later revealed that the resolution of isoforms based on gel electrophoretic mobility had not told the full story. Not two, but three different cDNA's were found in rat brain libraries [4] Similarly, three cDNA's were found in chicken brain libraries [5], although only one band had been resolved on gels of chicken brain samples [1] The molecular weights calculated from the translation of the three rat brain cDNA's were slightly different [4], but the rank order of gel electrophoretic mobility was the opposite of that expected: the form with the highest calculated M_r (α1) migrated fastest, and the form with the lowest M_r (α3) migrated slowest [6–8]. The band originally called "$\alpha(+)$" is now known to contain both α2 and α3 [7–9].

Abbreviations: Na^+/K^+-ATPase, $(Na^+ + K^+)$-stimulated adenosine triphosphatase. SDS, sodium dodecyl sulfate. STS, sodium tetradecyl sulfate. S10S, sodium decyl sulfate.

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The Na^+/K^+-ATPase α-subunit had long been suspected of migrating anomalously fast in SDS [10–12]. The reason is not known, but is likely to be due to excess detergent binding to hydrophobic regions of the protein [13,14]. Several recent reports have detailed ways in which the gel electrophoretic mobility of this enzyme can be changed. The reduction and alkylation of sulfhydryl groups has permitted the apparent resolution of isoforms in Na^+/K^+-ATPase preparations from rat heart [15]. Heating of SDS-denatured enzyme causes an extra band of smaller apparent M_r to appear on gels [16,17]. The use of alkyl sulfate detergents of different chain lengths has been reported to reveal two bands in kidney Na^+/K^+-ATPase preparations [18,19]. In each instance, the hypothesis was put forward that the newly-resolved bands represented additional isoforms of the Na^+/K^+-ATPase: not necessarily derived from different genes, but sufficiently different structurally to permit their reproducible separation. In none of these instances, however, was evidence produced to document that the newly resolved bands were different in sequence, covalent structure, or even immunological reactivity.

In view of the controversy engendered by reports of new Na^+/K^+-ATPase isoforms or structural variants, experiments have been undertaken to compare and contrast the effects on the mobility of Na^+/K^+-ATPase α-subunits of reduction and alkylation, heating in SDS, and substitution of alkyl sulfates of different chain lengths. To identify the bands, a panel of monoclonal and peptide-directed antibodies has been used, all of which bind to the Na^+/K^+-ATPase on Western blots, and most of which bind to regions which have isoform (α1, α2 and α3) specificity.

Materials and Methods

Enzyme preparation

Rat renal medulla microsomes [20], rat brain cerebrum microsomes [21], and a sarcolemma-enriched fraction from newborn or adult rat heart [22] were prepared as described previously. All enzyme preparations were stored in 320 mM sucrose/20 mM Tris/1 mM EDTA (pH 7.2) at −70°C.

Gel electrophoresis

All gel electrophoresis was performed with the Laemmli stacking buffer system [23]. The pH of separating gel buffer (8.8) and stacking gel buffer (6.8) was adjusted at room temperature in 4-times concentrated stock solutions. No Na^+ was added. Electrode buffer was prepared by mixing Tris base and free glycine exactly as in the original Laemmli recipe [23]; no pH adjustment was made, and no Cl^- was added. Over many years, poor gel resolution has invariably been attributable to use of a pH meter that required maintenance and recalibration or a new electrode. Attempts to use commercially available preset-pH Tris buffers were not very successful because of lot-to-lot variation. Acrylamide and bisacrylamide (used at a ratio of 1 : 0.0265) were from Bio-Rad.

Because Tris buffers are temperature-sensitive as well as concentration-dependent, the final pH reached in the separating gel during electrophoresis was not known precisely. The geometry of the gel affects its resistance and therefore how hot it gets, and thus can contribute to different results obtained in different laboratories. Electrophoresis was performed here at room temperature in slabs of polyacrylamide, 15 cm wide, 9–10 cm high plus a 1–2 cm stacking gel, and 0.75 mm thick. The gels were run at constant current at 15–20 mA; voltage was typically 75–90 V at the beginning (depending on the % polyacrylamide) and 200–250 V by the end. The time required for the tracking dye to reach the bottom was 2.5–3 h. Gels made with 15% polyacrylamide were run at 10 mA to avoid excessive heating. The temperature of the gels generally reached 30°C measured at the surface of the glass with a flat thermoprobe.

SDS for most experiments was obtained from Bio-Rad, but was recrystallized from hot 95% ethanol before use. SDS from Kodak was crystalline, and was not further purified. STS and S10S, also from Kodak, were used without further purification, at the same concentration by weight as SDS. Tris and glycine were from Sigma. All samples were treated with β-mercaptoethanol (2%) or dithiothreitol (10 mM).

Electroblotting onto nitrocellulose (Bio-Rad) or Immobilon-P (Millipore) membranes was performed according to Towbin et al. [24], except that Immobilon was prewetted with 100% methanol. The blots were quenched with 0.5% Tween-20 (Sigma) in Tris- or phosphate-buffered saline (pH 7.2). The blots were incubated with antibody for 1 h, washed, incubated with alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Sigma) for 1 h, and washed again, all in Tween 20-containing buffer. The alkaline phosphatase-conjugated secondary antibody was detected by the indoxy phosphate/nitro blue tetrazolium method of Blake et al. [25]. Molecular weight markers 7B and 6H were obtained from Sigma. High and low-range molecular weight markers were obtained from Bio-Rad.

Antibodies

Monoclonal antibodies were as follows: McK1 is specific for α1, and was described in Felsenfeld and Sweadner [26]. McB2 is specific for α2; McB-X3 recognizes α3 but also weakly cross-reacts with α1 and the H^+/K^+-ATPase of gastric mucosa, described in Urayama et al. [8]. AMOG-7C9 (the gift of H. Antonicek and M. Schachner, U. Heidelberg) reacts with α1

and α2, and possibly α3 [27]. Other antibodies used to stain heated kidney α-subunit included AMOG-2F12, which reacts with all three isozymes [27], and McB7, which reacts with α1 and α3; the data obtained with these antibodies are not shown. All monoclonal antibodies were used as cell culture supernatants, in most cases diluted 1 : 20.

Polyclonal antibodies were as follows: Anti-peptide 1 (GRDKYEPAAVSE, near the N-terminus) and anti-peptide 9 (RRRPGGWVEKE, near the C-terminus) antisera [28] were the gift of William J. Ball Jr., U. Cincinnati, Cincinnati, OH. Both peptides were derived from the sequence of sheep α1 but react with rat α1 as well. Anti-peptide 1 antiserum was used at 1 : 1000, and anti-peptide 9 antiserum was used at 1 : 250. An antiserum against a peptide representing the N-terminus of rat α3 (GDKKDDKSSPKKS) was the gift of Robert W. Mercer, Washington U. School of Medicine, St. Louis, MO, and was used at 1 : 1500.

Reduction and alkylation

Reduction and alkylation was performed essentially as described by Charlemagne et al. [15]. Dithiothreitol was from Bio-Rad; urea ('molecular biology reagent grade') and iodoacetamide were from Sigma. Reduction was performed for a minimum of 30 min in 10 mM dithiothreitol, a final concentration of urea of from 3.5–7 M, 50–130 mM Tris buffer, and 0.1 mM EDTA (pH 8.8). Iodoacetamide (in 100 mM Tris buffer (pH 8.0)) was added to a final concentration of 120 mM, and allowed to react at 37°C for 1 h. After reaction, the mixture was diluted 50 : 50 with 2 × Laemmli sample buffer (pH 6.7) containing 20% glycerol, and applied directly to the gels.

Results

Anomalous mobility of Na^+/K^+-ATPase α-subunits

The rat Na^+/K^+-ATPase α1, α2 and α3 subunits should be M_r 112 573, 111 736 and 111 727, respectively, as calculated from the cDNA sequences [4]. Five amino acids are removed from the N-terminus of both α1 and α2 when mature [29]. The protein is otherwise thought to be intact, however, because antisera to peptides terminating just 3–8 amino acids from the C-terminus do bind to it [28,30]. The molecular weights estimated from gel electrophoretic mobility vary considerably from laboratory to laboratory, but usually average 95 000–98 000.

The M_r for the rat kidney α1 subunit was estimated by comparison with molecular weight standards from several commercial sources. An excellent correspondence between standards was obtained with four different mixtures from two suppliers when electrophoresed on the same gel (some batches of the Sigma 7B prestained markers used did not migrate anomalously, while others did). If one compares heavily-loaded M_r standards to lightly-loaded Na^+/K^+-ATPase bands and uses the mid-point or leading edge of the bands to calculate the relative mobilities, the resulting M_r calculated for the Na^+/K^+-ATPase is erroneously high, because of the tendency of heavily-loaded bands to bulge downwards. The most consistent values for α1, obtained by comparing the trailing edges of bands, were M_r 97 000–98 000, which is 13% lower than would be predicted from the amino-acid sequence.

Reduction and alkylation

The state of oxidation of sulfhydryl groups has been suspected for some time to affect the mobility of the Na^+/K^+-ATPase α-subunit (an example can be seen in Ref. 1). Reduction with β-mercaptoethanol or dithiothreitol sometimes increases the gel electrophoretic mobility (decreases the apparent M_r). Since this effect is not always observed, it may be related to how much air-oxidation the enzyme has experienced during preparation. Charlemagne et al. [15], however, observed the appearance of a new band of higher apparent M_r in preparations from adult rat heart when they were first reduced and then alkylated with iodoacetamide. Reduction alone did not produce the extra band. Although both bands reacted equally well with the available antibodies, the conclusion was made that the reduction and alkylation revealed the existence of a form of the Na^+/K^+-ATPase that could account for the high-affinity ouabain binding site observed in this tissue.

The effect of reduction and alkylation on rat brain α1, α2, and α3 (detected by isozyme-specific monoclonal antibodies) is illustrated in Fig. 1. Reduction and alkylation (asterisks) was observed to increase the apparent M_r of all three of the isozymes. The separation of α1 from α2 and α1 from α3 was improved over untreated enzyme, although the bands were also broader. After reduction and alkylation, α2 and α3 ran together.

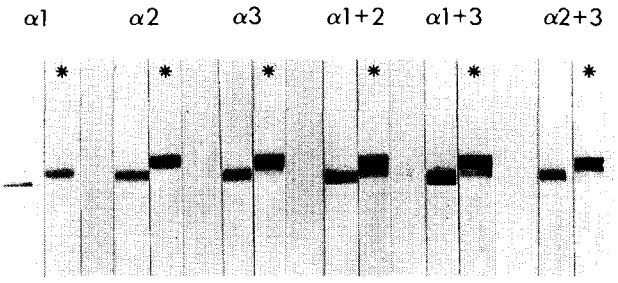

Fig. 1. Reduction and alkylation of rat brain Na^+/K^+-ATPase isozymes. Rat brain microsomes were prepared for gel electrophoresis after reduction with dithiothreitol alone (unmarked lanes) or after reduction and alkylation with iodoacetamide (asterisk-marked lanes). Reduction and alkylation were performed prior to dissolving the samples in SDS, using the procedure of Charlemagne [15]. Samples were electrophoresed in wide lanes (25 μg protein per 3 cm lane); Strips were cut from the resulting blots and stained with isoform-specific antibodies individually or in combination, as indicated. The antibodies were McK1 (α1), McB2 (α2), and McB-X3 (α3).

TABLE I

Apparent molecular weights of brain Na^+/K^+-ATPase isoforms with and without reduction and alkylation

	α1	α2	α3
Reduction only	98000	99000	102500
Reduction and alkylation	102000	105000	106000

Table I lists the apparent molecular weights seen with all three isoforms with and without reduction and alkylation.

When the technique was applied to samples from either adult or newborn rat heart, similar mobility shifts were seen (Fig. 2). The slightly larger shift of α2 and α3 relative to α1 may well explain the separation of a higher M_r band reported by Charlemagne et al. [15]. No new isoforms were detected that could not already be seen by the isoform-specific antibodies without alkylation of the samples, however.

Heating in SDS causes α1 to split into two bands

Ohta et al. [16] first reported that when kidney Na^+/K^+-ATPase is dissolved in SDS and then heated,

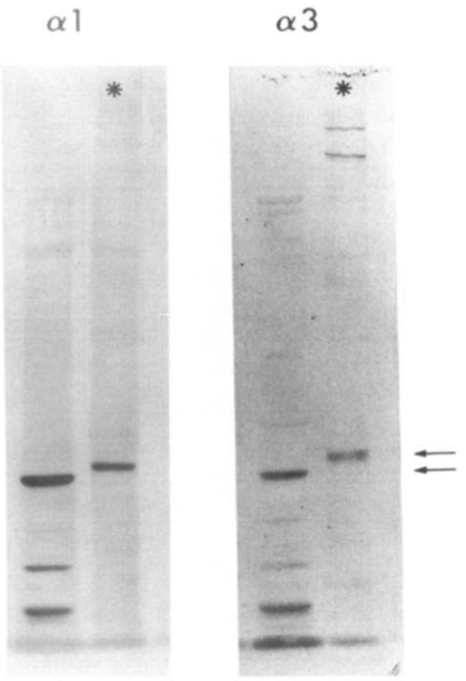

Fig. 2. Reduction and alkylation of newborn rat heart Na^+/K^+-ATPase isozymes. Samples of sarcolemma preparation from newborn rat hearts (50 μg protein per lane) were electrophoresed after reduction alone (unmarked lanes) or after reduction and alkylation as in Fig. 1 (asterisk-marked lanes) Samples were run in adjacent lanes and blots were stained as indicated with either McK1 (α1-specific monoclonal antibody) or a peptide-directed polyclonal antibody specific for α3, obtained from R.W. Mercer. The mobilities of both α1 and α3 were decreased by the alkylation, but no new bands appeared. Similar results have been obtained with adult rat heart samples where the isoforms are α1 and α2.

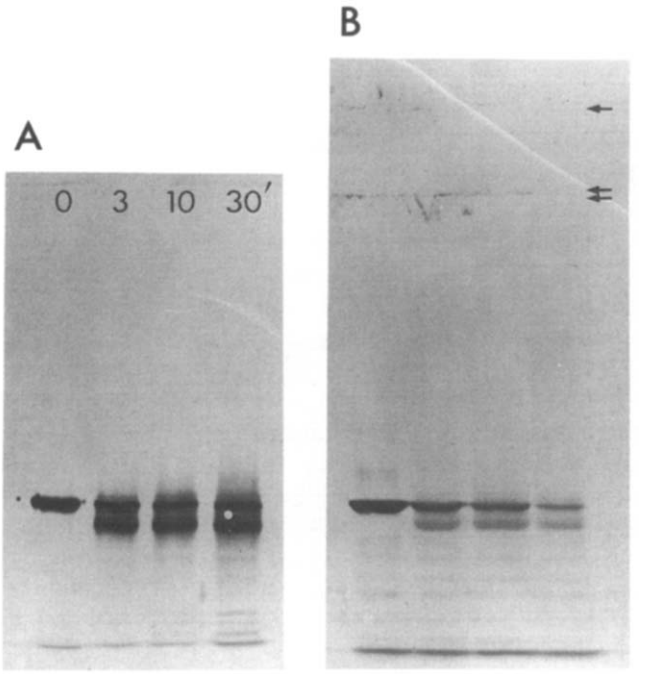

Fig. 3. Heating in SDS causes splitting of the kidney α1 band. Rat kidney microsomes were dissolved in SDS-containing Laemmli sample buffer with 10% glycerol at room temperature. Aliquots (40 μg protein per lane) were heated at 80°C for 3, 10, or 30 min as indicated and cooled to room temperature prior to applying to the gel. Panels A and B represent two similar experiments. In panel A, the blot was stained with McK1, a monoclonal antibody specific for α1. In panel B, the same time course of heating was performed except that the blot was stained with AMOG-7C9 (obtained from H. Antonicek and M. Schachner, U. Heidelberg), a monoclonal antibody that cross-reacts with the Na^+/K^+-ATPase α isoforms. In panel B, care was taken to ensure that the stacking gel and top of the separating gel were included on the blot; the single arrow points to the bottoms of the sample wells, while the double arrow points to the top of the separating gel. There was little or no aggregated material at these positions. The different staining intensities in the two panels reflects the intrinsic sensitivities of the antibodies.

the α-subunit splits into two bands with distinctly different electrophoretic mobilities. Both bands labeled with [^{3}H]NAP-ouabain, a covalent label of the cardiac glycoside binding site. The phenomenon was characterized with respect to the temperature required and the length of incubation. Cortas reproduced this with Na^+/K^+-ATPases from the kidneys of several different species (Cortas, N. and Edelman, I.S., Columbia University College of Physicians and Surgeons, personal communication), demonstrating that it was a general characteristic of kidney Na^+/K^+-ATPases. Cortas et al. [17] determined that the N-terminal amino-acid sequences of both bands produced from rat kidney Na^+/K^+-ATPase were identical and characteristic of α1.

The results shown in Fig. 3 confirm the observations of both of these groups of investigators: rat kidney Na^+/K^+-ATPase α subunit, when heated in SDS at

80°C for any length of time from 3 to 30 min, split into two bands. The slower-migrating band had the same apparent M_r as that of unheated enzyme (97 000–101 000), while the new band migrated faster, at (90 000–93 500). In seven different experiments, the difference in apparent M_r averaged 7600 on gels of 5% polyacrylamide. This difference is of much greater magnitude than the typical separation of isozymes.

Both of the bands reacted strongly with a monoclonal antibody (McK1) that is completely specific for α1 as opposed to α2 and α3, and which is sequence-specific to the degree that it reacts only with α1 from certain species and not others (Fig. 3A). Its deduced binding site is very near the N-terminus [26]. Antibodies specific for α2 and α3 did not detect anything in kidney preparations, with or without heating (Ref. 17; Fig. 4 of Ref. 32). Thus, no evidence exists to suggest either that the new band is due to one of the known isoforms, or that it results from cleavage at the N-terminal end of the molecule. Three other monoclonal antibodies specific for Na^+/K^+-ATPase α-subunits (AMOG-7C9, AMOG-2F12 and McB7) were also used to stain blots of heated rat kidney microsome samples. Each of these antibodies reacts with α1 at a different site, as well as with at least one other isozyme, and all three stained both bands of the doublet (one example is shown in Fig. 3B). The amount of staining declined with increasing heating time in some experiments but not in others (Fig. 3A and B). Immunoreactive material was not found at the top of the gel or at the top of the stacking gel (Fig. 3B).

The faster-migrating band might conceivably be the product of a chemical cleavage occurring about 70 amino-acids from the C-terminal end of the protein. To test this hypothesis, blots were stained with antibodies raised against synthetic peptides corresponding to the N- and C-termini of α1 [28]. Fig. 4 shows the result: the faster-migrating band contained the full length of the Na^+/K^+-ATPase sequence, since it was stained by both antibodies.

The observations above suggest that the new band produced by heating in SDS contains intact α1 Na^+/K^+-ATPase subunit. If so, it must be migrating even more anomalously than unheated enzyme. Anomalous gel mobility could in principle be produced by excess binding of SDS (to raise the net charge-to-size ratio), or by forming a more globular particle (smaller Stokes radius), or both. A more globular particle would be expected to migrate differently with respect to molecular weight markers in higher % polyacrylamide gels, and so heated and unheated rat kidney Na^+/K^+-ATPase samples were compared on 15% polyacrylamide gels (Fig. 5). The unheated α1 subunit gave the same calculated M_r seen on 5% gels: 98 000. The striking result was that the new band produced by heating migrated as if it were M_r 155 000, rather than M_r

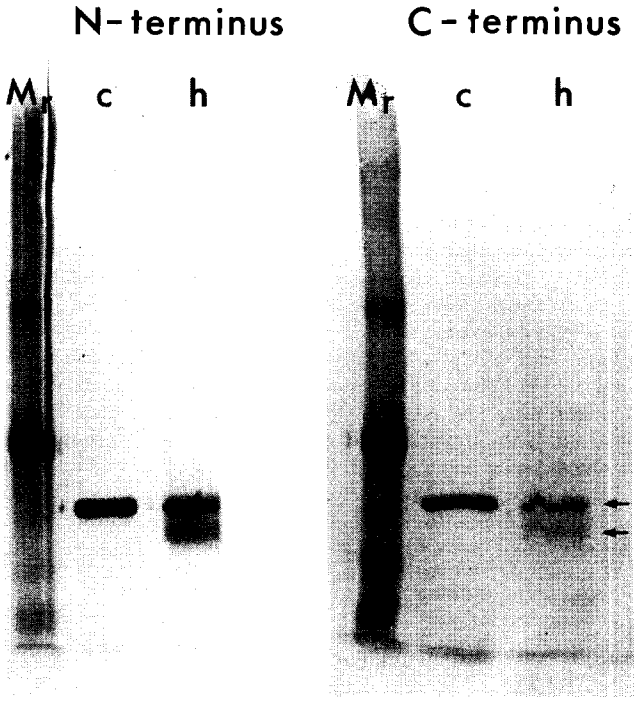

Fig. 4. N- and C-termini of the α subunit after heating. Two identical blots are shown, each of which had M_r markers (Sigma prestained 7B), unheated rat kidney microsomes (lanes marked c), and rat kidney microsomes heated in SDS for 10 min at 80°C (lanes marked h). On the left, each lane contained 10 μg of protein, and the blot was stained with an antibody against the N-terminus of α1 and on the right, each lane contained 20 μg of protein, and the blot was stained with an antibody against the C-terminus. The difference in protein per lane was necessitated by the much lower sensitivity of the C-terminus-directed antibody.

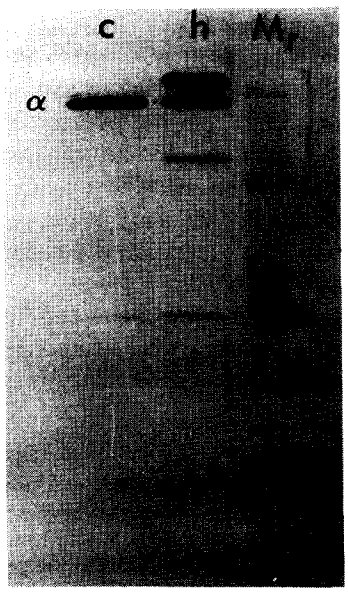

Fig. 5. Anomalous mobility of the heated α1 band. Samples of rat kidney microsomes (10 μg protein per lane), unheated (c) and heated (h) in SDS, were electrophoresed on a 15% polyacrylamide gel. The resulting blot was stained with McK1, a monoclonal antibody specific for α1. The M_r markers were Sigma 7B.

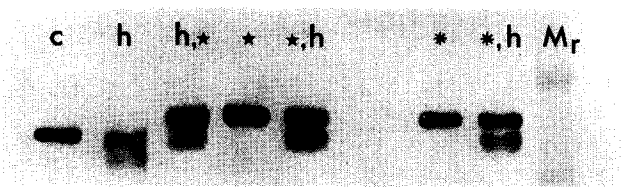

Fig. 6. Reduction and alkylation do not prevent or reverse the effect of heating. Reduction and alkylation, before and after dissolving the Na^+/K^+-ATPase in SDS and before and after heating, was tested for its ability to prevent or reverse the formation of a second α1 band. The lanes are marked as follows: (c) is control rat kidney microsomes, reduced with dithiothreitol, but not further treated. (h) stands for heating at 80°C for 10 min. (★) stands for reduction and alkylation after dissolving the sample in SDS. (*) stands for reduction and alkylation of the sample prior to dissolving it in SDS. When samples were both heated and alkylated, the order of the symbols (i.e., h, ★) indicates the order in which the procedures were carried out. The M_r markers were Sigma 7B. Each lane had 10 μg of kidney microsomes. The blot was stained with McK1, a monoclonal antibody specific for α1.

90000. A band of similar mobility has also been seen on gels of 10% polyacrylamide. This strongly suggests that it has an altered conformation in detergent; the possibility that it is a globular dimer cannot be ruled out. A minor band stained by the monoclonal antibody also appeared at M_r 57000. Such a low M_r band would not have been detected on the 5% polyacrylamide gels used in the other experiments because it would have been at the dye front.

Reduction and alkylation of the α subunit was tested for its ability to either reverse or prevent the formation of the anomalously-migrating band (Fig. 6). The rationale was that it would prevent the formation of either inter- or intrachain disulfide bonds, and that the introduction of alkyl groups might affect the detergent-protein interaction. The result was that reduction and alkylation, whether performed before addition of SDS or in the presence of SDS, did not prevent the subsequent splitting of the α1 band by heating, and also that reduction and alkylation after heating in SDS did not reverse the splitting *. The shift in the mobility of the α-bands indicates that alkylation did occur in all of the conditions.

Samples of rat brain microsomes containing all three Na^+/K^+-ATPase isoforms were heated (Fig. 7). α1, α2 and α2 all showed a similar tendency to split into two

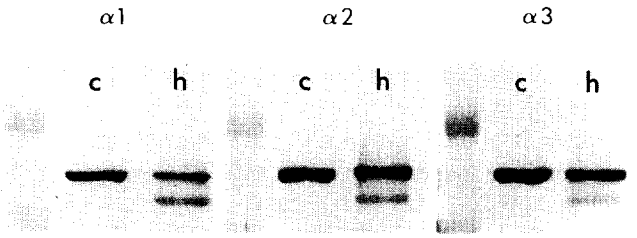

Fig. 7. Heating of α2 and α3 in SDS. Rat brain microsomes (20 μg protein per lane) containing all three Na^+/K^+-ATPase isoforms were prepared for electrophoresis in SDS without (c) and with (h) heating. Each gel also contained the Sigma 7B prestained M_r markers (lanes on the left of each blot). Three identical blots were prepared and stained with McK1 (α1), McB2 (α2) and McB-X3 (α3). Splitting of all three brain isoforms was similar to that seen above with α1 from the kidney.

bands, one of unaltered mobility and the other migrating faster in 5% polyacrylamide. This makes it very unlikely that the phenomenon reveals either a new, undiscovered isoform or an interesting post-translational modification.

The formation of a detergent-protein complex with unusual shape could in principle be affected by the structure of the detergent. The effect of heating the enzyme was found to be a function of the detergent used, as illustrated in Fig. 8. Splitting into two bands has been observed reproducibly every time heating in sodium dodecyl sulfate has been attempted (16 different experiments; over 50 separate samples). If sodium tetradecyl sulfate (the 14-mer, STS) was substituted for sodium dodecyl sulfate (the 12-mer, SDS) in the sample buffer, but the samples were then applied to gels made with SDS, much less splitting of rat kidney α1 was observed under identical conditions of heating (three different experiments). During electrophoresis, the STS present in the sample is diluted out and replaced by SDS migrating from the electrode buffer toward the anode. The extent of doublet formation in Fig. 8 thus represents events that occurred during heating, but

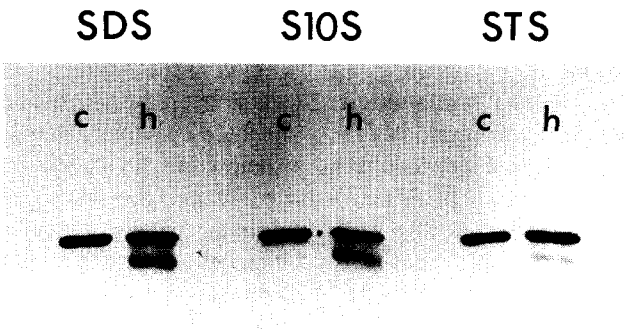

Fig. 8. Heating in different length alkyl sulfate detergents. In this experiment, samples of rat kidney microsomes (10 μg protein per lane) were dissolved in SDS, STS, or S10S as indicated, and half of them were incubated at 80°C for 10 min (lanes marked h). All of the samples were then applied to a gel made with SDS. The resulting blot was stained with McK1, a monoclonal antibody specific for α1.

* In the same experiment, a small amount of a stained band of M_r 200000 was seen in all of the unheated samples, with or without reduction and alkylation (not shown). Similar bands, thought to be dimers, have been reported occasionally in the past. Whatever its true structure, the M_r 200000 band disappeared from the gel in all samples that were heated. Cortas et al. observed the presence of bands at M_r 150000 [17], 200000, 120000, 66000 and 43000 in heated samples. Such bands were not seen in heated samples in any of the experiments in this laboratory, with the exception, of course, in the 15% polyacrylamide gel of Fig. 5.

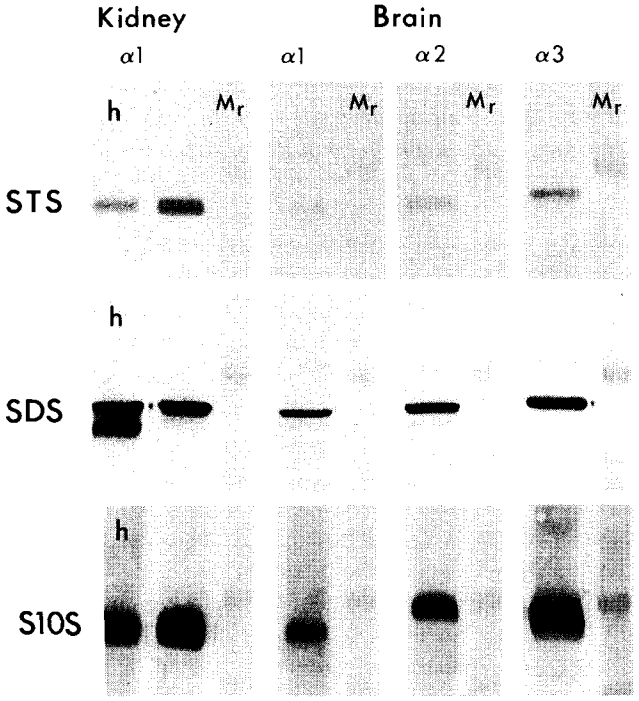

Fig. 9. Na^+/K^+-ATPase isoforms in STS, SDS, and S10S. In each panel, all of the blots were made from a gel run with STS, SDS, or S10S, as indicated; samples were prepared in the corresponding detergents. Sigma 7B prestained M_r markers were in the right-most lane of each blot. Each blot was stained with an isoform-specific monoclonal antibody: McK1 (α1), McB2 (α2) and McB-X3 (α3), as indicated. The first two lanes contained rat kidney microsomes (9 μg protein per lane), prepared with (h) or without heating at 80°C for 10 min. The remaining lanes contained rat brain microsomes (20 μg protein per lane) prepared without heating and stained for each of the α isoforms. The current passed through SDS gels has been varied to produce a range of gel temperatures during electrophoresis from 6°C (in the cold room) to 35°C (measured at the surface of the glass with a flat disk thermoprobe) without producing doublets. The pH of the separating gel buffer has been varied from 8.7 to 9.1 (at room temperature) without producing doublets. Siegel et al. [18,19] added chloride ions to the electrode buffer by making it with Tris (pH 8.8) preset crystals (Sigma) instead of Tris base, but the only effect of this modification in our hands was an odd splitting of the tracking dye front.

which were stable to subsequent replacement of the detergent. In three other experiments, samples were both heated and electrophoresed in STS. In two of those experiments, no splitting was observed in STS (an example is seen in Fig. 9); in the other, a small amount of splitting was seen in STS.

Even more striking results were obtained with a shorter alkyl sulfate detergent. In two experiments, samples heated and electrophoresed in sodium decyl sulfate (the 10-mer, S10S) failed to show any evidence of splitting, although the α1 band was very broad and fuzzy (Fig. 9). In two other experiments, samples heated in S10S but then electrophoresed in SDS were observed to split into two bands (Fig. 8). This result suggests that the effect of heating is to produce a change in the protein stable enough for SDS to recognize it and bind to it during electrophoresis, long after the sample has cooled.

Detergents of different chain lengths

Siegel et al. [18] reported finding doublets of α-subunits in kidney Na^+/K^+-ATPase preparations even without heating, and later concluded that the phenomenon was related to the purity of the SDS used for gel electrophoresis [19]. When pure SDS was used, a single band was observed, but when mixtures of STS and SDS were used (30% or more STS), splitting into two bands was observed. Unlike the effects of heating (in which case the new band had a faster electrophoretic mobility), the new band resolved in STS-containing gels had a slower electrophoretic mobility in gels of 6% polyacrylamide, indistinguishable from that of the brain "α(+)" band. The effect was reversible, in that samples prepared in one detergent adopted the electrophoretic pattern of the detergent used in the gel. Fig. 9 illustrates the fact that such detergent-dependent splitting has not been seen in this laboratory. Whether in 100% STS, SDS or S10S, rat kidney α1 subunits migrated as a single band. The principal differences in methodology were that the gels in this laboratory were run at room temperature at constant current, whereas those of Siegel's group were run in a cold room at constant power.

The substitution of detergents of different chain length had a significant effect on gel electrophoretic mobility, however. This was immediately obvious in the mobility of the molecular weight standard proteins, which migrated significantly faster in S10S and slower in STS, relative to the Bromphenol blue dye front. In fact, an S10S gel in 7.5% polyacrylamide resembled an SDS gel in 5% polyacrylamide, while an STS gel in 7.5% polyacrylamide resembled an SDS gel in 10% polyacryl-

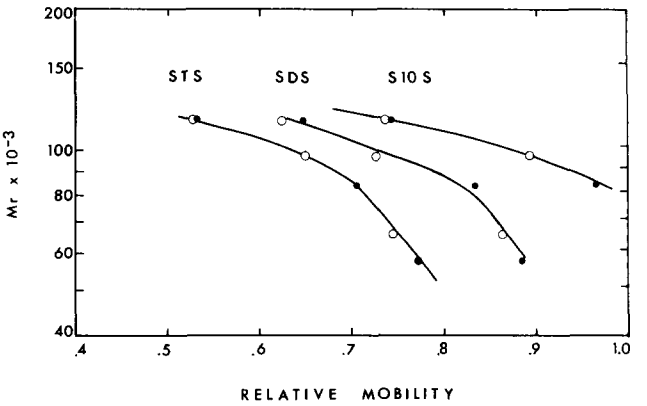

Fig. 10. Electrophoretic mobility of standards in STS, SDS and S10S. Two different mixtures of M_r markers (Sigma 6H, open circles; Sigma 7B, closed circles) were used to calibrate 5% polyacrylamide gels made with STS, SDS, or S10S. The graph shows the plot of relative mobility (measured against the tracking dye) vs. M_r for each of the alkyl sulfate detergents.

TABLE II

Apparent molecular weights of α isoforms in STS, SDS and S10S

	α1 (kidney)	α1 (brain)	α2 (brain)	α3 (brain)
STS	100000	102000	101500	106500
SDS	102000	98000	100000	102000
S10S	103500	101500	110500	112000

amide, with a corresponding shift in the plot of mobility vs. molecular weight. Identical shifts were seen whether normal molecular weight markers or 'prestained' molecular weight markers were used. Similar shifts were seen whether gels of 5% or 7.5% polyacrylamide were used; Fig. 10 shows data obtained with 5% gels. These results are different from those reported recently by Siegel and Desmond [19], who stated that the protein standards were not altered in their migration in STS or (STS + SDS) gels.

Siegel and Desmond [19] reported that the calculated molecular weights of the kidney enzyme subunits were lowest in SDS (90200–93800) and highest in STS (100000–101700 for the faster band, and 106900–107500 for the slower band that was not seen in the experiments presented here). Table II shows the calculated M_r in representative experiments for rat kidney α1 and rat brain α1 relative to markers in S10S, SDS and STS. The differences are slight and are likely to be due to gel-to-gel variation.

The extra band seen by Siegel and collaborators in kidney Na^+/K^+-ATPase preparations had the same mobility as the 'α(+)' band of mammalian brain preparations, which contains α2 and α3. As shown in Fig. 9, samples of rat brain microsomes as well as kidney microsomes were electrophoresed on gels made with the different alkyl sulfate detergents to see how the relative mobilities of the isoforms would be affected. The detergent chain length had different effects on the mobilities of the three Na^+/K^+-ATPase isozymes, detected on replicate blots with isozyme-specific monoclonal antibodies. In STS, α1 and α2 migrated closer together and α3 separated somewhat better. In S10S, α1 and α2 were better resolved from each other, but α3 appeared as an exceptionally large and fuzzy spot. The calculated molecular weights are shown in Table II.

While the calculation of apparent molecular weight was inexact, the differences in isoform mobility in the different detergents was reproducible. Fig. 11 shows the result of attempts to exploit the differences in a two-dimensional gel. Rat brain samples were first electrophoresed in STS, and pieces of the gel were cut out and laid lengthwise on the top of an S10S gel. Three identical blots were made, and each was stained by a combination of two isozyme-specific monoclonal antibodies, permitting unambiguous identification of the spots. As predicted, the three α subunit types did migrate differently. The α1 and α2 were quite well-separated, but the size and fuzziness of the α3 band prevented its complete separation from α2.

It was curious that the α3 subunit also gave rise to higher-M_r aggregates in the second (S10S) dimension, including a spot at approx. M_r 170000, presumed to be a dimer. Both this and the fuzziness of the bands in S10S have a simple explanation. Because of its shorter alkyl group length, S10S must have a higher critical

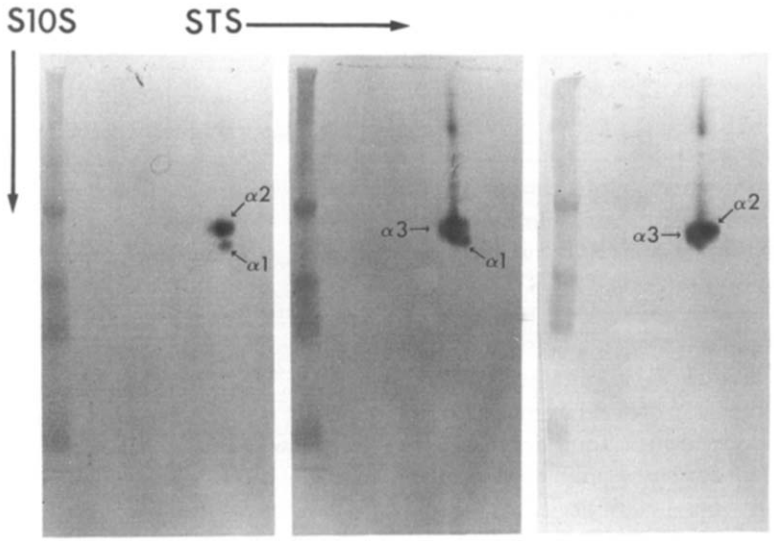

Fig. 11. Two-dimensional electrophoretic separation of the Na^+/K^+-ATPase isoforms. In the first dimension, samples of rat brain microsomes (8.5 μg of protein per sample) were electrophoresed in STS in slab gels. Using Sigma 7B M_r markers as a guide for where to cut, vertical portions of lanes containing the α-subunits were excised and laid lengthwise on the top of a slab gel made with S10S. The second dimension of electrophoresis resulted in the separation of the three isoforms, although the large and diffuse nature of the α3 spot limited the resolution. Electrophoresis in STS first and S10S second was chosen because of the propensity of STS to interfere with protein blotting.

micelle concentration than SDS, and thus higher concentrations may be required to achieve the same detergent properties. This hypothesis was confirmed in experiments in which the concentration of S10S in the separating gel was doubled, while the concentration in the electrode buffer was increased 5-fold. Not only were all Na^+/K^+-ATPase α-subunit bands much sharper, but they all migrated (relative to markers and each other) essentially as they do in SDS gels (data not shown). This suggests that the fuzziness and altered mobility are due to the binding of limiting amounts of the S10S; this property appears to differ between the isoforms. When SDS was omitted from the electrode buffer of an otherwise normal SDS gel, a similar phenomenon was observed: $\alpha 1$, $\alpha 2$, and $\alpha 3$ all migrated (relative to M_r markers and each other) just as they did in S10S gels, with additional smearing of antibody stain in the range of M_r 150 000–200 000 (data not shown). In this experiment, the SDS originally in the sample, stacking gel, and separating gel was migrating toward the anode and was not being replaced with SDS from the electrode buffer, and so the mobility differences reflect the affinity of the proteins for their bound SDS.

An incidental observation was that electrophoretic transfer of proteins from the gels to nitrocellulose was also affected by the detergent used: proteins in S10S gels transferred better than those in SDS gels, and those in STS gels transferred very poorly. One hypothesis is that STS blocks protein binding sites on the nitrocellulose more effectively than SDS or S10S. An observation that confirms this hypothesis was that transfer was poorer in the gels made with an elevated amount of S10S. Transfer to Immobilon (PVDF) membranes was better than transfer to nitrocellulose for gels with all three detergents in some experiments, although results with Immobilon were less consistent than those with nitrocellulose (data not shown). Oddly enough, the prestained molecular weight markers transferred more poorly to Immobilon than to nitrocellulose, in contrast to the other proteins.

Discussion

Anomalous gel mobility of unmodified Na^+/K^+-ATPase

Reported molecular weights for the Na^+/K^+-ATPase catalytic subunit, based on gel electrophoretic mobility in comparison with standard proteins, have varied from a low of 89 000 to a high of 113 000, with most reports falling between 95 000 and 98 000. Some of the variation may be due to genuine differences between the enzymes isolated from different species; lengths have been found to be as short as 1013 amino-acids for rat $\alpha 3$ [4] and as long as 1038 amino-acids for *Drosophila* α [33]. Some of the variation may be due to different degrees of protein oxidation during enzyme purification. Most of the variation is probably due to the interpretation of mobilities relative to M_r standards, however. There may be differences in the electrophoretic mobilities of the M_r standard proteins (obtained from different suppliers, or deteriorated with age), and there are almost certainly differences in the interpretation of the mobilities of overloaded bands.

A more fundamental question is why does the Na^+/K^+-ATPase generally migrate faster than its true molecular weight would predict. We previously compared the mobilities of Na^+/K^+-ATPase subunits purified from rat brain with $\alpha 1$, $\alpha 2$ and $\alpha 3$ polypeptides translated in vitro from synthetic mRNA's [8]. It was clear that the mobilities of native and synthesized polypeptides were similar and, as shown by Schneider et al. [6], it did not matter whether the synthetic α-subunits had been made in the presence or absence of rough microsomes. This implies that the final protein-detergent complex formed is not dependent on interaction with lipid or even on whether the protein has been folded correctly previously.

Under constant conditions, many proteins tend to bind an amount of SDS proportional to their weight [35,36], close to 1.4 g/g of protein. Two different possibilities could account for the fast migration of the Na^+/K^+-ATPase α subunits. First, an excess of SDS may be bound to hydrophobic regions, which would cause the protein to move faster relative to the M_r markers in the electric field. This may reflect a tendency for hydrophobic regions of the protein to not fully unfold (or to arrive at a more condensed conformation) in the presence of the detergent. The more compact shape of the particle might result in less of a sieving effect during migration through the gel. This hypothesis is attractive in view of the propensity of the Na^+/K^+-ATPase α-subunit to aggregate and become insoluble. Second, it has been observed that disulfide-bonded soluble proteins bind less SDS than proteins with no disulfide bonds and that reduction and alkylation increases SDS binding up to the 1.4 g/g level [35]. The theory advanced in that study was that reduction and alkylation permitted more complete unfolding and that only the fully unfolded protein was capable of accommodating the maximum amount of SDS. Reduced and alkylated Na^+/K^+-ATPase does migrate more slowly (Ref. 15, and Fig. 1 above). If it could be assumed that it, too, is more unfolded and that it binds more SDS, the suggestion would be that conformation, rather than the ratio of SDS to protein, plays more of a role in causing the faster-than-theoretical gel electrophoretic mobility. Neither hypothesis is directly supported by studies of gel sieving, however. Peterson and Hokin [34] performed an exhaustive analysis of the gel electrophoretic mobility of α-subunits of dog, eel, dogfish and brine shrimp Na^+/K^+-ATPases at different concentrations of polyacrylamide. With the exception of the brine shrimp enzyme (discussed below), they did not find any

significant differences in the values obtained at different polyacrylamide concentrations, indicating that the SDS-protein complex behaved like a conventional rod-shaped particle.

The $\alpha 2$ isoform of the Na^+/K^+-ATPase sometimes migrates as a non-uniform band, with a small proportion of its mass at a larger apparent M_r, although the phenomenon did not occur in the experiments shown in Figs. 1 and 7. That this is most likely to be due to an anomaly of protein-detergent interaction and not to a structural heterogeneity is most persuasively indicated by the fact that we have seen it in gels of $\alpha 2$ synthesized in vitro by translation of synthetic mRNA in a reticulocyte lysate without the addition of rough microsomes [8]. Very occasionally we have also seen doublets of $\alpha 1$ in gels without regard to their detergent content. Whenever this occurred, both members of the doublet invariably stained with the same isoform-specific antibodies. Since the phenomenon has never been reproducible, it has not been possible to investigate it systematically.

Detergent-protein complexes of unusual shape

The new band produced by heating kidney Na^+/K^+-ATPase in SDS migrated very differently in gels of 5% and 15% polyacrylamide. Ohta et al. [16] had originally reported that they were not able to detect both bands when the concentration of polyacrylamide in the gel was 7% or higher (it is not clear how high they went). A similar phenomenon was reported by Liang and Winter [37], who saw the formation of a second α band in digitonin-solubilized canine kidney Na^+/K^+-ATPase that was oxidized with copper-phenanthroline complex. Since copper-phenanthroline crosslinks subunits through the formation of disulfide bonds, they inferred that the second band had an intramolecular disulfide that prevented it from completely unfolding. The altered form migrated faster than the unmodified form in 3.5% polyacrylamide, but slower in 7% polyacrylamide.

Peterson and Hokin [34] observed a different phenomenon in Na^+/K^+-ATPase purified from the brine shrimp. In this case, the α-subunit migrated as a doublet. The '$\alpha 1$' subunit migrated at the same apparent M_r regardless of the concentration of polyacrylamide, but the brine shrimp '$\alpha 2$' form (the faster-migrating component, not to be confused with mammalian $\alpha 2$) migrated relatively more rapidly (at a smaller apparent M_r) at higher % polyacrylamide. The brine shrimp '$\alpha 1$' and '$\alpha 2$' subunits were also reported to reverse their relative gel mobility in gels of acidic pH [38]. Both of these observations suggest that the brine shrimp doublet might reflect structural modifications of one protein affecting its mobility, rather than two separate gene products. It is notable that in another arthropod (*Drosophila*), only one α gene has been detected in 58 cDNA isolates examined by restriction mapping [33].

The evidence presented here appears compelling that the new band produced by heating the rat kidney Na^+/K^+-ATPase in SDS is a relatively stable conformational form. Its production is time and temperature-dependent and is influenced by the chain-length of the detergent used. It is unaffected by reduction and alkylation before or after formation. The $\alpha 2$ and $\alpha 3$ isoforms of the Na^+/K^+-ATPase have a similar tendency to form anomalously-folded forms. What remains puzzling is why only part of each α subunit converts to the anomalously-folded form. It is possible that there is an underlying heterogeneity of structure that leads to its unusual reaction with detergent at elevated temperatures. It remains possible, however, that the proportion of the misfolded form is merely the result of stochastic conformational events.

Detergents of different chain length

Siegel and collaborators [18,19] saw doublets after performing electrophoresis in STS in the cold room. All of the STS gels in this laboratory were run at room temperature and no doublets were seen. The critical factor may indeed be temperature, since doublets in STS have been seen by Cortas and collaborators in gels run in a cold room (personal communication). Yamaguchi and Post [39] observed an effect of temperature on the reversible splitting of pig kidney Na^+/K^+-ATPase in isoelectric focusing gels. The polypeptide appeared to equilibrate between two states, and it is of interest that the Ca^{+2}-dependent ATPase of sarcoplasmic reticulum behaved similarly.

Schmidt and McDonough [40] reported obtaining a better separation of 'α' and '$\alpha(+)$' subunits in brain preparations when impure SDS from Pierce was used, containing up to 30% STS. Pierce SDS was also tried here, but the resolution of kidney $\alpha 1$ and axolemma $\alpha 2 + \alpha 3$ was not markedly improved over that in recrystallized SDS gels and only a single band was seen in kidney preparations (data not shown).

The two bands seen by Siegel et al. in kidney preparations [18,19] were approximately equally abundant and the slower-migrating band appeared much like the '$\alpha(+)$' of brain samples, except that STS was required to reveal it. The possibility that renal medulla Na^+/K^+-ATPase preparations may contain $\alpha 2$ or $\alpha 3$ isoforms of the Na^+/K^+-ATPase at levels approaching the levels of $\alpha 1$ is extremely unlikely. All studies of isoform mRNA levels with the exception of one have indicated the overwhelming preponderance of $\alpha 1$ mRNA in the kidney (reviewed in Ref. 32). In addition, 16 tryptic fragments of kidney α-subunit were purified and sequenced by Collins et al. [41]; 11 of the peptides were from regions where the isoforms differ in sequence and yet all of the sequences detected were later shown to be

those of α1, not α2 or α3. Finally, isoform-specific antibodies have detected either only α1 in mammalian kidney preparations [7,8,32,42], or only very small amounts of α3 (approx. 1%) [43,44]; if α2 and α3 are present, their concentrations must usually be below the threshold for detection. Cortas and collaborators (personal communication) have observed that both bands seen in STS gels have the same α1 N-terminal sequence. The most conservative conclusion is that the extra band is α1, showing altered detergent-protein interaction. Like the effect of heating, however, the phenomenon may ultimately be explained by an underlying structural heterogeneity.

Conclusions

It is clear that the Na^+/K^+-ATPase α-subunits interact with SDS in ways that are not typical of most other proteins. Each of the manipulations tested had some effect on their electrophoretic mobility. The three identified isoforms in many cases reacted differently from each other, despite their 90% sequence identity. Given these practical problems, it seems imperative that gel electrophoretic mobility differences alone should not be accepted as rigorous evidence that Na^+/K^+-ATPases from two sources are different. In particular, differences in gel electrophoretic behavior cannot be taken alone as a way to identify the presence of α2 and α3.

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