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Anomalous mobilities of Na,K-ATPase α subunit isoforms in SDS-PAGE: identification by N-terminal sequencing

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Three isoforms of the α subunit of Na,K-ATPase, $\alpha 1$, $\alpha 2$, and $\alpha 3$ have been characterized at the DNA, mRNA and protein levels. In admixtures, isoforms migrate as doublets (i.e. $\alpha 1$ and another band originally designated $\alpha +$, comprising $\alpha 2 + \alpha 3$) when analyzed by SDS-PAGE. As deduced from cDNA sequences their masses range from 111.7 to 112.6 kDa. With conventional protein standards, however, SDS-PAGE yields nominal masses of 85–105 kDa. In this system, the presence of a doublet that reacted with a polyclonal anti-Na,K-ATPase antibody in the kidney was interpreted as indicating two molecular or conformational species of the kidney α sub-unit (Siegel, G.J. and Desmond, T.J. (1989) J. Biol. Chem. 264, 4751–4754). We report that Na,K-ATPase purified from dog, guinea pig and rat kidney medulla or from rat brain, can yield two distinct bands when analyzed by SDS-PAGE or STS-PAGE, migrating between 85 and 105 kDa. An additional band migrating at 117 and 120 kDa appears often in enzyme purified from rat and guinea pig kidney medulla. The apparent molecular weights and relative intensities of these bands vary with temperature and duration of incubation during sample preparation. N-terminal sequencing and monospecific antibody probes revealed that the two distinct bands obtained from the kidney enzyme consist only of the $\alpha 1$ isoform. The band appearing at 117–120 kDa also contains only the $\alpha 1$ N-terminal sequence. In contrast, as reported earlier (Sweadner, K.J. (1979) J. Biol. Chem. 254, 6060–6067), the doublet seen in brain preparations consists of $\alpha 1$ and $\alpha 2$ or $(\alpha 2 + \alpha 3)$. We conclude that monospecific antibody probes or N-terminal sequencing must be used to identify Na,K-ATPase isoforms by SDS- or STS-PAGE. In addition, gel conditions that may affect the mobilities of the isoforms are discussed.

1. Introduction

The existence of isoforms of the α subunit of Na,K-ATPase were first reported independently in brine shrimp [3] and in mammalian rat brain [2] on the basis of a closely spaced doublet on SDS PAGE. The bands in the brine shrimp and brain were identified as Na,K-ATPase subunits by K^+-sensitive phosphorylation [4]. Mammalian brain enzyme exhibited biphasic Strophanthidin and ouabain inhibition curves while those for mammalian kidney were monophasic [2,5,6]. The mammalian isoforms also differed in a number of kinetic parameters, sulfydryl crosslinking, sensitivity to tryptic cleavage and N-terminal sequences [2,7]. In rat brain, the isoforms $\alpha 1$, and $(\alpha 2 + \alpha 3)$ originally designated $\alpha +$ were isolated from separate subcellular fractions [2,8]. In the brine shrimp, the isoform-specific enzymes differed in their sensitivity to ouabain, Na^+ and K^+, in their relative abundance in zonal fractions, in osmolar tolerance and in ionic optima [4,9]. Na,K-ATPases of the mammalian kidney and eel electric organ yielded α subunits that migrated as single bands in SDS-PAGE, whereas the dog, mouse, rat and frog brain enzyme yielded α doublets [1,2]. In other studies, however, α doublets were also described in Na,K-ATPases isolated from the eel electric organ and from various kidney preparations [1,10,11]. Na,K-ATPase α subunit isolated from dog kidney, split into two bands when heat-treated with 1% SDS for 5 min at 70 °C, 80 °C or

Abbreviations: SDS, sodium dodecyl sulfate; STS, sodium tetradecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Na,K-ATPase, $(Na^+ + K^+)$-dependent adenosine triphosphatase; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

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100 °C [11]. Siegel and Desmond [1] obtained two α bands by STS-PAGE, with Na,K-ATPase purified from mouse, lamb and beef kidneys, even though the samples were not heated with the detergent. The bands were considered to represent the α1 and α2 isoforms of renal Na,K-ATPase, but the designated renal α2 was not considered identical to brain α2. The existence of an α doublet in SDS- or STS-PAGE, however, may not necessarily indicate the presence of more than one α isoform as discussed previously [1]. Previous analyses of the kinetics of ouabain inhibition, and for Na- and K-activation of the renal enzyme failed to reveal the kinds of heterogeneities exhibited in brain or other multiple isoform containing enzymes [2]. Moreover, only $mRNA_{\alpha 1}$ was detected by Northern blot analysis in extracts of rat kidney [12].

The objective of this study was to identify α-isoforms by N-terminal sequencing and monospecific antibody probes, in Na,K-ATPases derived from kidneys of various species, compared to rat brain. The results indicate that depending on the conditions used to prepare the enzyme for analysis, the α subunit often migrates anomalously, and that the existence of α isoforms, different in sequence, may not be reliably inferred from SDS- or STS-PAGE alone. The importance of N-terminal sequencing in isoform detection is emphasized.

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Materials and Methods

Na,K-ATPase preparations. Purification of Na,K-ATPase from dog, guinea pig and rat kidney medulla was performed as described by Jørgensen [13]. Briefly, the procedure consisted of dissection of the outer medulla of dog, guinea pig and rat kidneys, as well as rat brain. Each was then homogenized in 10 ml of 0.25 M sucrose, 0.03 M histidine (pH 7.4), per g of tissue by 20 strokes in a teflon-glass homogenizer. Sodium deoxycholate was added to the brain homogenate to a final concentration of 0.1%. The homogenates were centrifuged at 6000 × *g* for 15 min and the microsomes were pelleted by centrifugation of the supernatant at 48000 × *g* for 30 min. The microsomes from kidney preparations were incubated with 0.57 mg SDS/1.4 mg protein and from the brain with 0.4 mg SDS/1.4 mg protein at 20 °C for 30 min in the presence of 5 mM ATP. The purified enzyme was then pelleted in a 50% glycerol gradient. Protein content was determined by the method of Lowry et al. [14], as modified by Peterson [15].

Polyacrylamide gel electrophoresis (PAGE). Analysis of protein composition was determined by SDS-PAGE by the Hokin et al. [16] modification of the method of Laemlii [17]. Best resolution of subunits was obtained using 7.5% acrylamide, 0.23% bisacrylamide as the separating gel, and a 4% acrylamide, 0.10% bisacrylamide stacking overlay. The samples were made up to 2% (w/v) SDS + 5% (w/v) β-mercaptoethanol and incubated at room temperature for 30 min or as indicated in the various experiments. After addition of Bromophenol blue tracking dye to a final concentration of 0.01% (v/v) and adjusting the solution to 10% glycerol, the samples were electrophoresed at room temperature with a constant current of 40 mA until the tracking dye reached the bottom (usually in 3–4 h). The gels were fixed and stained with Coomassie blue [18]. Analysis by sodium tetradecyl sulfate (STS)-PAGE was done according to Siegel and Desmond [1]. STS replaced SDS in all steps and buffers, and the gel was run at 4 °C.

FITC labeling. Fluorescien isothiocyanate (FITC) labeling was performed as described by Salon et al. [18]. Purified enzyme at 1 mg protein per ml of 100 mM Tris, 2 mM EDTA (pH 9.2) was incubated with and without 5 mM ATP for 5 min at 4 °C. FITC was then added to a final concentration of 10 μM and the incubation continued for 30 min at room temperature. The reaction mixture was diluted 300 × with 50 mM Tris pH 7.2 (with HCl) and the membranes were then pelleted at 48000 × *g* for 30 min. The pellet was analyzed by SDS-PAGE. The fluorescent bands were photographed under 345 nm ultraviolet light and the gel was then stained with Coomassie blue.

Western blots. Electroblotting and immunoblotting was performed on PVDF membranes (Immobilon, Millipore), as described by Matsudaira [19]. The bands of interest were excised and N-terminal sequencing was accomplished at the Protein Core Facility of Columbia University, using an Applied Biosystems model 470A Sequencer equipped with model 120A PTH analyzer. The duplicate lanes on the PVDF were then probed with a monospecific anti-α1 antibody (McK1) or anti-α2 antibody (McB2) as in Urayama et al. [20] and Felsenfeld and Sweadner [21].

Results and Discussion

Na,K-ATPase was purified to varying extents from dog, guinea pig and rat kidney medulla. The specific activities of these preparations were 530 u, 960 u, and 1670 u (u = μmol P_i/mg protein per h) for the guinea pig, rat and dog kidney enzymes, respectively. The highest purities obtainable with the Jørgensen method yields specific activities of approx. 2000 u [22]. When preheated to 80 °C for 10 min these preparations yielded a doublet at 85 kDa and 92.5 kDa (Fig. 1). The rat and guinea pig enzyme preparations also exhibited an additional doublet at 117–120 kDa and 130 kDa. As shown in Fig. 2, FITC labeling followed by SDS-PAGE

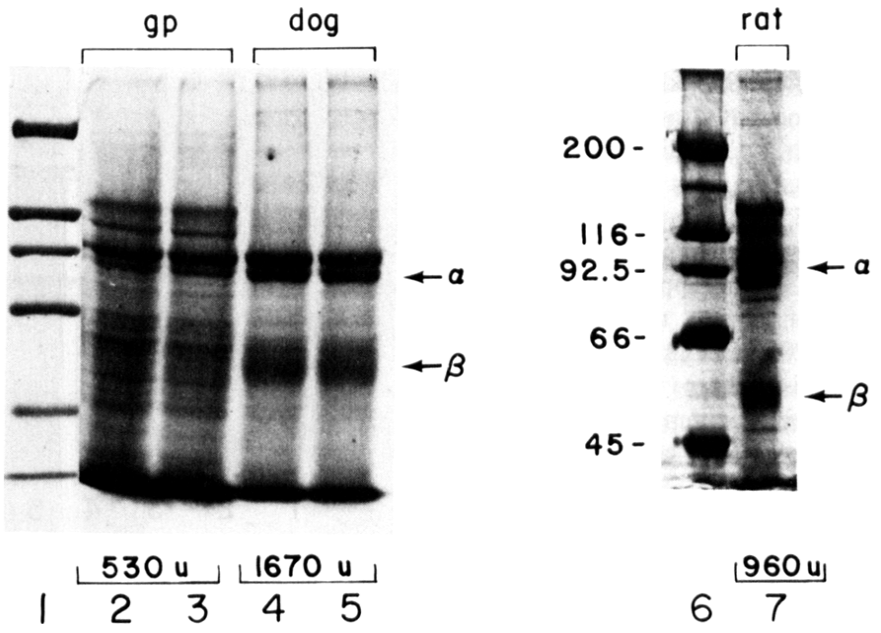

Fig. 1. SDS-PAGE of Na,K-ATPase partially purified from renal outer medulla. All of the preparations were pre-incubated at 80 °C for 10 min in 2% SDS/5% β-mercaptoethanol. The gels were stained with Coomassie blue. Lane 1, molecular weight standards (Bio-Rad) consisting of (all in kDa): Myosin 200, β-galactosidase 116, phosphorylase *b* 92, bovine serum albumin 66, ovalbumin 45. Lane 2, guinea pig (gp) Na,K-ATPase incubated with FITC (10 μM) and ATP (5 mM) prior to denaturation. Lane 3, guinea pig Na,K-ATPase incubated with FITC (10 μM) alone prior to denaturation. Lane 4, dog Na,K-ATPase incubated with FITC (10 μM) and ATP (5 mM), prior to denaturation. Lane 5, dog Na,K-ATPase incubated with FITC (10 μM) alone, prior to denaturation. Lane 6, molecular weight standards (Bio-Rad) (see above). Lane 7, rat Na,K-ATPase. The enzyme specific activities for each of the preparations are listed at the bottom of each column (u = μmol P_i /mg protein per h).

revealed that only the doublet at 85 kDa and 92.5 kDa (cf. Fig. 1) were labelled in an ATP-sensitive manner, implying that they are α subunits of Na,K-ATPase [18].

Fig. 2. FITC labelling of α subunits of guinea pig and dog Na,K-ATPase. The gels shown in lanes 2–5 of Fig. 1 were photographed through an orange filter, under UV light prior to staining with Coomassie blue. Lanes 1 and 2, guinea pig Na,K-ATPase (±5 mM ATP). Lanes 3 and 4, dog Na,K-ATPase (±5 mm ATP). The fluorescent bands migrated at 92.5 kDa and 85 kDa, respectively.

We confirmed that the doublet at 85 kDa and 92.5 kDa was enhanced by varying the temperature during sample preparation (Fig. 3), as reported by Ohta et al. [11]. Rat kidney Na,K-ATPase (specific act. 820 u) was analyzed by SDS-PAGE after pre-incubation in 2% SDS and 5% β-mercaptoethanol for 30 min at room

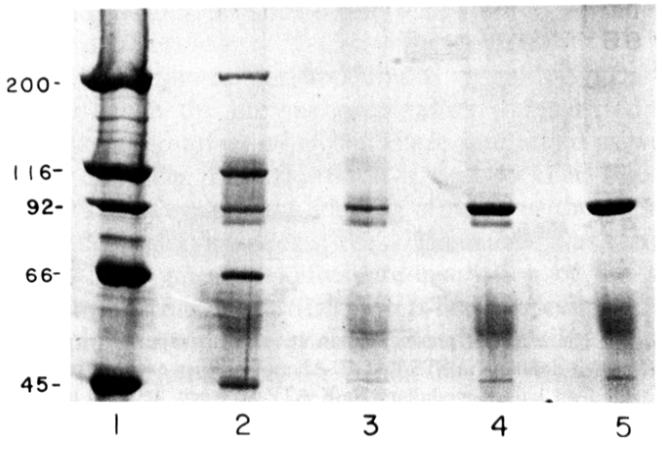

Fig. 3. The effect of pre-incubation at various temperatures on the yield of α doublets in SDS-PAGE. Aliquots (20 μg each) of partially purified rat kidney medullary Na,K-ATPase (spec. act. 960 u) were pre-incubated in 3% SDS/5% β-mercaptoethanol and then analyzed by SDS-PAGE post-stained with Coomassie blue. Lane 1, molecular weight standards (Bio-Rad), see legend of Fig. 1 for listing. Lane 2, after 3 min at 100 °C. Lane 3, after 10min at 80 °C. Lane 4, after 20 min at 55 °C. Lane 5, after 30 min at room temperature.

temperature, 20 min at 55 °C, 10 min at 80 °C, or 3 min at 100 °C. A single band at 92.5 kDa was obtained when the sample was pre-incubated at room temperature (Fig. 3). A doublet with a dense band at 92.5 kDa and a lighter band at 85 kDa was seen when the sample was incubated at 55 °C. The intensity of the upper band was reduced further when the sample was incubated at 80 °C or 100 °C; the apparent molecular weights remained constant. When the sample was boiled for 3 min, bands at 205 kDa, 117.5 kDa, 66 kDa and 45 kDa were also intensified.

Even when pre-incubated at room temperature in 2.3% STS/5% β-mercaptoethanol, analysis in STS-PAGE yielded a doublet (105 kDa and 98.5 kDa), as described by Siegel and Desmond [1] (Fig. 4). A triplet appeared with pre-incubation at higher temperature; the additional band appeared at 91 kDa. The intensity of this band increased with higher temperatures. At all pre-incubation temperatures, an unidentified band was evident at 130 kDa. Loading the same amount of protein on an SDS-gel under similar conditions as those for STS-PAGE, did not yield triplets in the 85–105 kDa range.

Ouabain inhibition curves and studies with monoclonal antibody probes as well as α-specific mRNA abundances indicate that α1 is the predominant isoform in the kidney [2,20,21,23]. mRNAs α2 and α3, if they are expressed in the kidney, are in extremely low abundance [24,25]. To verify the identity of the doublets seen with both kidney and brain Na,K-ATPase, at 85–92.5 kDa, Western blots of SDS-PAGE were probed with monospecific anti-α1 (McK1) and anti-α2 (McB2) antibodies. When the samples were incubated for 30 min at room temperature prior to electrophoresis the brain enzyme α subunits reacted with both anti-α1 and anti-α2 antibodies (Fig. 5). In contrast, the kidney α doublet reacted only with anti-α1 as reported by Urayama et al. [20]. To examine the possibility that α2 abundance in the kidney preparation was below the sensitivity of detection with McB2, an SDS gel was overloaded with kidney enzyme. Once again, significant binding was obtained only with the anti-α1 antibody (Fig. 6). These results imply either an extremely low abundance or absence of α2 polypeptide in kidney medulla.

To explore the identity of the α isoforms further, each of the putative α bands from the doublets were excised separately and N-terminal sequencing was performed. The α isoform N-terminal sequences deduced from cDNAs are shown in Fig. 7. Both the 85 kDa and 92.5 kDa doublet of renal origin yielded the α1 sequence, whereas those from the brain gave both sequences with α2 being in higher abundance than α1

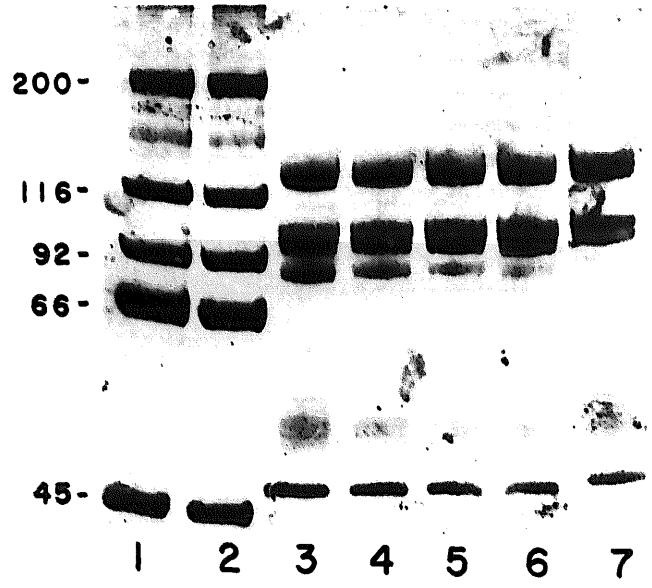

Fig. 4. The effect of pre-incubation at various temperatures on the yield of α doublets in STS-PAGE. Aliquots (20 μg each) of partially purified rat kidney medullary Na,K-ATPase (spec. act. 960 u) were pre-incubated in 3% STS/5% β-mercaptoethanol and then analyzed by STS-PAGE post-stained with Coomassie blue. Lanes 1 and 2, molecular weight standards (Bio-Rad). See legend of Fig. 1 for listing. To evaluate the effects of pre-incubation temperature on the standards the sample shown in lane 1 was boiled in STS/β-mercaptoethanol for 3 min and that in lane 2 was pre-incubated for 30 min at room temperature. Lane 3, Na,K-ATPase after 3 min at 100 °C. Lane 4, after 10 min at 80 °C. Lane 5, after 20 min at 55 °C. Lane 6, after 30 min at 37 °C. Lane 7, after 15 min at room temperature.

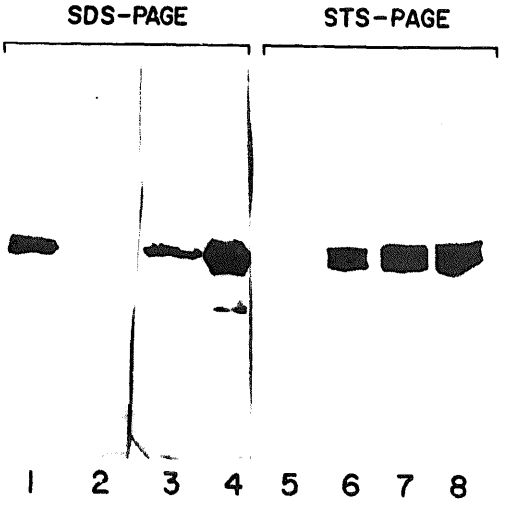

Fig. 5. Western blots of Na,K-ATPase purified from rat brain and kidney medulla. Aliquots of partially purified brain and renal medullary Na,K-ATPases (spec. act. 530 u and 870 u, respectively) were pre-incubated in 2% SDS/5% β-mercaptoethanol for 30 min at room temperature (lanes 1–4) or in 2% STS/5% β-mercaptoethanol for 15 min at room temperature (lanes 5–8). The respective samples were resolved by SDS-PAGE or STS-PAGE, electroblotted on PVDF and probed with anti-x1 (McK1) and anti-α2 (McB2) monoclonal antibodies. Lane 1, brain enzyme probed with McB2. Lane 2, kidney enzyme probed with McB2. Lane 3, brain enzyme probed with McK1. Lane 4, kidney enzyme probed with McK1. Lane 5, kidney enzyme probed with McB2. Lane 6, brain enzyme probed with McB2. Lane 7, kidney enzyme probed with McK1. Lane 8, brain enzyme probed with McK1.

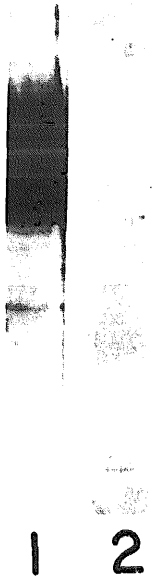

Fig. 6. Western blots of rat kidney medulla Na,K-ATPase (overloaded gel). Partially purified medullary enzyme (spec. act. 870 u) was pre-incubated for 30 min at 37 °C in 2% SDS/5% β-mercaptoethanol and resolved by SDS-PAGE; each lane was loaded with 100 μg of enzyme. The subunits were electroblotted on PVDF. Lane 1, probed with McK1. Lane 2, probed with McB2.

(approximately 5:1). The α3 sequence was not detected in either the kidney or the brain α aliquots. These results confirm that the rat kidney expresses the

Sequence derived from cDNAs.

Rat α3								M	G	D	K	K	D	D	K	S	S	P			
Rat α2	M	G	R	G	A	G	R	E	E	Y	S	P	A	A	T	T	A	E	N	G	G	G
Rat α1	M	G	K	G	V	G	R	D	K	Y	E	P	A	A	V	S	E	H	G	D		

Sequence derived from blotted bands.

Rat kidney medulla Na,K-ATPase												
1. 92.5 Kd	X	X	X	K	Y	E	P	A	A	V	S	
2. 92.5 Kd	X	X	D	K	Y	E	P	A	A	V		
3. 105 Kd	X	X	D	K	Y	E	P	A	A	V	S	E H
4. 85 Kd	X	X	X	K	Y	E	P	A	A	V	S	
5. 85 Kd	X	X	D	K	Y	E	P	A	A	V	S	
6. 98.5 Kd	X	R	D	K	Y	E	P	A	A	V	S	
7. 117.5 Kd	X	R	D	K	Y	E	P	A	A	V		
Rat brain Na,K-ATPase												
8. 97 & 87 Kd	X	X	X	E	Y	E	P	A	A	T	T	
	X	X	X	K	Y	E	P	A	A	V	S	

Fig. 7. N-terminal sequences of α subunits of Na,K-ATPase derived from rat kidney medulla and brain. The deduced sequences of rat α3, α2 and α1 were obtained from Shull et al. [29]. The N-terminal sequences of the bands obtained from rat kidney and brain preparations are listed in order, 1–8. X stands for unidentified amino acids. Sequences are shown from cycle 1. Note that the first five amino acids in the deduced sequences do not appear by N-terminal sequencing. This has been previously reported by Lytton [7]. Amino acid yield per cycle ranged from 0.9 to 12.6 pmoles. Of the 58 identified amino acids, all but eleven gave yields in excess of 3 pmoles. Rows 1–7, rat kidney Na,K-ATPase (spec. act. 870 u) and incubated as follows: Rows 1, 2, 4, 5 and 7; pre-incubated in SDS for 20 min at 55 °C. Rows 3 and 6; pre-incubated in STS for 15 min at room temperature. Row 8; pre-incubated in SDS for 30 min at room temperature.

α1 isoform and that the brain expresses α1 and α2. The reason why no sequence for α3 was detected in the brain aliquots is unresolved. The abundance of α3 in rat brain membranes may be less than the estimate derived using a monospecific anti α3 antibody which cross reacts slightly with α1 (Sweadner, personal communication). Specific mRNA α1, α2 and α3 abundances are equivalent in the rate brain [25]. These results, however, do not imply equivalent abundances at the polypeptide level.

The 85 kDa band generated by pre-heating renal Na,K-ATPase in SDS has been identified as an α1 isoform both by immuno-detection and N-terminal sequencing. This raises the possibility that the 92.5 kDa band is full-length and is subject to a proteolytic C-terminal clip during pre-incubation in SDS at an elevated temperature.

To investigate this possibility, a mixture of six proteinase inhibitors (pepstatin A, 10 μM, PMSF, 0.1 mM; leupeptin, 2 μM; iodoacetamide, 10 μM; antipain, 50 μg/ml and EDTA 5 mM) were included in the homogenization buffer and at every step during purification of rat kidney enzyme. In other experiments, microsomes from rat kidney medulla were incubated for 2 h at 37 °C prior to treatment with SDS in the purification scheme. In both of these experiments, the relative abundance of the two bands, at 85 and 92.5 kDa after heating in SDS remained the same, with the upper band always being more intense than the lower (data not shown).

Sweadner [26] investigated also the possibility of C-terminal proteolysis, as the basis for the 85 kDa band in SDS, by probing with an anti-α1 antibody raised against synthetic peptide that corresponds to C-terminal residues. She found that this antibody bound to both α bands of the temperature-dependent SDS doublet.

In addition to the appearance of the 85 kDa α band, when the enzyme preparation is incubated at high temperatures, other bands are intensified as well (e.g. 205 kDa, 120 kDa, 66 kDa, and 45 kDa; Fig. 3, lane 2). To explore the identify of some of the higher molecular weight species, the 97 kDa form was recovered from gel slices, after pre-incubation of the rat kidney enzyme in 2% SDS/5% β-mercaptoethanel for 30 min at room temperature. The recovered polypeptide was then boiled for 3 min in 2% SDS/5% β-mercaptoethanol and reanalyzed by SDS-PAGE. This manuever produced bands at 117.5 kDa, 92.5 kDa and 66 kDa (data not shown). The 117.5 kDa form obtained from the holoenzyme, was electroblotted onto PVDF membranes and subjected to N-terminal sequencing. Only the rat α1 sequence was obtained with this preparation (Fig. 7). Since the computed molecular weight of α1 is 112 kDa, the 117.5 kDa form may be the completely denatured intact α1. The 92.5 kDa and 85 kDa

forms may contain sufficient secondary structure to yield anomalously high R_f values.

Whereas pre-incubation of rat medullary Na,K-ATPase with SDS resulted in a temperature-dependent doublet formation (85 and 92.5 kD), pre-incubation with STS produced a doublet (98.5 and 105 kDa) even at room temperature. Antibody probing of these 98.5 and 105 kDa bands with McB2 and McK1 showed that both bands reacted as α1. No α2 was detected (Fig. 5). This was confirmed by N-terminal sequencing which revealed that both bands had only the α1 sequence (Fig. 7).

The results obtained with the α isoform specific antibodies and N-terminal sequencing indicate that the appearance of an α doublet in the 85–105 kDa range obtained with either SDS- or STS-PAGE is not sufficient to indicate the presence of more than one α isoform. In the case of the rat kidney medulla, α1 migrates at various R_f values, with apparent molecular masses of 117–120 kDa, 92.5 kDa or 85 kDa in SDS-PAGE depending on the pre-incubation conditions. Similarly, each of the 97 and 87 kDa bands from the brain contained sequences of α2 and α1. Hence, the α1 and α2 isoforms of Na,K-ATPase produce conformational 'pseudoisozymes', defined as multiple, thermodynamically metastable conformational states of the same molecular species [27,28], which migrate on SDS or STS-PAGE with anomalously low R_f values. The formation of conformational 'pseudoisozymes' may be enhanced by the long central hydrophobic stretches in both α1 and α2, which can form variable hydrophobic-hydrophobic and hydrophobic-detergent interactions. The bands that migrate with lower R_f values, especially after the sample is boiled, may result from molecular aggregation. The occurrence of 'pseudoisozymes' renders quantitation by immunoblot analysis difficult to interpret. We conclude that definitive identification of α isoforms requires isoform-specific antibody assays or N-terminal sequencing (or both).

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