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Immunoaffinity isolation of Na,K-ATPase α3 isoform from pig kidney

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The Na,K-ATPase α3 isoform of the catalytic subunit has been isolated from pig kidney microsomes. The procedure employs immunoaffinity chromatography on Sepharose 4B covalently coupled with monospecific antibodies a-II against the synthetic peptide including the putative α3 N-terminus. The structural analysis provides unambiguous proof that the isolated protein corresponds to the third transcript for the α3 isoform. The N-terminal amino acid sequence determined, Met-Gly-Asp-Lys-Lys-Asp-Asp, shows that unlike the α1 and α2 proteins, the mature Na,K-ATPase α3 isoform lacks post-translational proteolytic processing.

ATPase isozyme, $(Na^+ + K^+)$-; Immunoaffinity chromatography; N-terminal sequence analysis

1. INTRODUCTION

Na,K-activated adenosine triphosphatase is a widely distributed ion pump which maintains monovalent cation (Na^+ and K^+) gradients across the cell plasma membrane. The enzyme molecule consists of two types of polypeptides – catalytic α-subunit (112 kDa) and glycoprotein β, the function of which is still unknown [1]. Nowadays the existence of the gene family encoding the enzyme catalytic subunit is generally accepted [2]. At least three different transcripts, namely α1, α2 and α3 isoforms, are expressed in various animal tissues. If the α1 protein is the most abundant in kidney cells, the α2 chain is shown to be the predominant form in the nervous system [3]. The tissue distribution of the α3 protein seems, at present, to be controversial. Using monospecific antibodies, we have recently identified this form of Na,K-ATPase catalytic subunit in human and animal brain and kidney [4]. However, it is in disagreement with [5] where the α3 protein is supposed to be transcribed predominantly in brain tissues and is completely undetectable in rat kidney. To tackle the problem we have tried to isolate the α3 protein from pig kidney microsomes by immunoaffinity chromatography and performed its direct sequence analysis. The results demonstrate unambiguously that the Na,K-ATPase α3 protein is expressed in renal tissues.

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2. MATERIALS AND METHODS

Microsomes were isolated from pig kidney as in [6]. A freshly prepared solution of dodecyloctaethylene glycol monoether (Calbiochem) was dropped into the microsome suspension at a final concentration of 1 mg/ml in a buffer containing 10% sucrose, 30 mM histdine, pH 7.2, 3 mM EDTA, in the presence of 0.1 M KCl up to a detergent/protein ratio of 3:1 (5 min, 20°C). Solubilized proteins were pelleted (20000 × *g*, 20 min, 4°C), then chromatographed on an Ultro Pak TSK G-3000 SWG column (LKB) (21.5 × 600 mm). Production, purification of antibodies a-II and indirect solid-surface adsorption binding assay (ELISA type) were described in [4]. The affinity purified antibodies a-II (6 mg) were covalently linked to BrCN-activated Sepharose 4B (1.5 ml) as in [7]. The coupling efficiency was about 80%. The fractions after TSK-chromatography were dialyzed against Dowex AG 1 × 4 for two days to remove the detergent then applied to the affinity column. For elution from the immunosorbent use was made of 1 M acetic acid with 0.1% $C_{12}E_8$. The fractions were collected, dialyzed against 0.05% (w/v) SDS for 16 h at 4°C and then analyzed by SDS-PAGE (7.5%) [8] in the presence of 0.1% SDS. After electrophoresis, the gels (1.5 mm thickness) were transferred to a Bio-Rad tank apparatus and electroblotted in a lateral direction onto PVDF membranes [9]. After staining (0.2% Coomassie R-250/50% methanol), the protein bands were cut into thin strips and applied directly to a microsequencer. The sequencing was performed by a model 470 A gas-phase sequencer with an on-line model 120 A PTH analyzer (Applied Biosystems) [9]. The results were calculated with a C-R3A integrator (Shimadzu).

3. RESULTS AND DISCUSSION

At present the protein product of the Na,K-ATPase α3 gene is believed to be expressed in brain tissues. In rat [5,10] and human [4] brain the α3 isozyme was detected through its interaction with specific antibodies. Rat brainstem axolemma was also shown to contain α3 by determining the N-terminal sequence of

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a specific fragment which resulted from the Asp-Pro cleavage [11]. Slightly different isozyme electrophoretic mobilities and trypsin resistance [5,10] favour their discrimination in brain preparations. However, in agreement with our previous data [4], the $\alpha 3$ content in mammalian kidney is very low. (It is noteworthy that this isoform could be identified by immunoblotting only if the avidin-biotin system amplifies the signal.) So, to procure independent proof for the expression of the $\alpha 3$ gene in animal kidneys, we attempted to isolate an individual protein and to analyse its structure.

It seems reasonable to apply biospecific chromatography to the antibodies raised against a synthetic peptide derived from the $\alpha 3$ sequence used as a ligand. This procedure could facilitate the goal-directed selective isolation of the $\alpha 3$ isoform from the mixture of highly homologous proteins. (The structural homology between the $\alpha 1$ and $\alpha 3$ isoforms is about 85% [3].) To prepare the immunoaffinity sorbent we employed polyclonal antibodies a-II to the synthetic peptide GDKKDDKSSP corresponding to the putative $\alpha 3$ N-terminus [4]. The antibodies were preliminarily purified on thiol-Sepharose 4B coupled with the peptide – CGDKKDDKSSP. Pig kidney microsomes, the most abundant membrane fraction, were chosen for isolating the desired protein.

Initial strategy for the $\alpha 3$ isolation included kidney microsomes titration in nonionic detergent $C_{12}E_8$ followed by immunoaffinity chromatography. Analytical experiments established that the optimal level of soluble $\alpha 3$ protein according to ELISA, is observed at a detergent/protein ratio of 3 : 1. However, under these conditions the antigen was shown to fail immunosorbent binding. Partial aggregation was observed due to dilution of the starting material in order to decrease the detergent concentration. Later it was discovered that better binding could be obtained

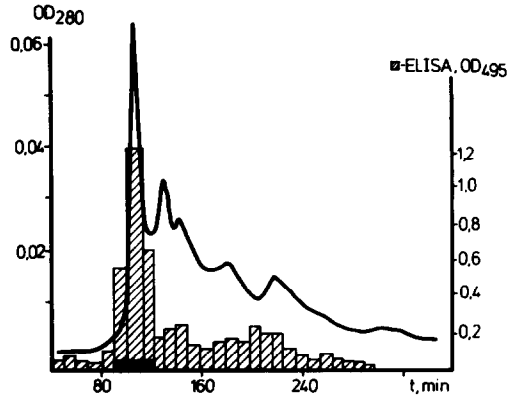

Fig.1. Separation of pig kidney microsomes preliminarily solubilized in $C_{12}E_8$ on an Ultro Pak TSK G-3000 SWG (21.5 × 600 mm). Running buffer is 0.1 M sodium acetate, pH 4.5, 0.2% SDS. Elution rate 1 ml/min. The sample was resolubilized in 5% SDS, 2% 2-mercaptoethanol. The fractions applied onto immunosorbent are marked.

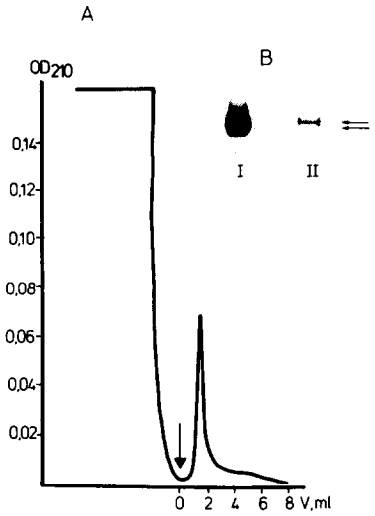

Fig.2. (A) Affinity chromatography of TSK fractions (fig.1) on immunosorbent (1.5 ml) with affinity purified antibodies a-II (6 mg). The arrow indicates the elution start with 1 M acetic acid in the presence of 0.1% $C_{12}E_8$. Elution rate 8 ml/h. (B) Electrophoretic mobilities of the $\alpha 1$ subunit (I) and the protein eluted from the immunosorbent (II) in 7.5% SDS-PAGE [8].

with more inclusive $\alpha 3$ fractions. The microsomes solubilized in $C_{12}E_8$ were first chromatographed on TSK-gel (fig.1). The fraction of 100 kDa proteins was applied directly to the affinity column after SDS removal. It should be stressed that $C_{12}E_8$ added to the 'wash buffer' induced the partial protein nonspecific desorption. Consequently, we used only NaCl concentrations (up to 0.5 M) to remove unbound material. The antigen-antibody complexes were destroyed by decreasing the pH to 2.5 (1 M acetic acid with 0.1% $C_{12}E_8$) (fig.2A). The choice of acidic elution is supported by high antigenic activity of the fractions obtained in ELISA.

The only prominent band corresponding to the protein with a molecular mass of about 100 kDa was revealed upon eluate analysis in 7.5% SDS-PAGE. Its electrophoretic mobility is slightly lower as compared to that of the $\alpha 1$ isoform (fig.2B). This is similar to [5]

Table 1

Microsequence analysis of the protein eluted from the immunosorbent (fig.2) electrotransferred onto PVDF membranes[a]

Sequence cycle no.	Amino acid	pmol
1	Met	8.32
2	Gly	2.08
3	Asp	3.64
4	Lys	2.42
5	Lys	3.08
6	Asp	2.04
7	Asp	2.50

[a] The transfer yield was about 70–80%. The sequence yields at each cycle of Edman degradation are shown

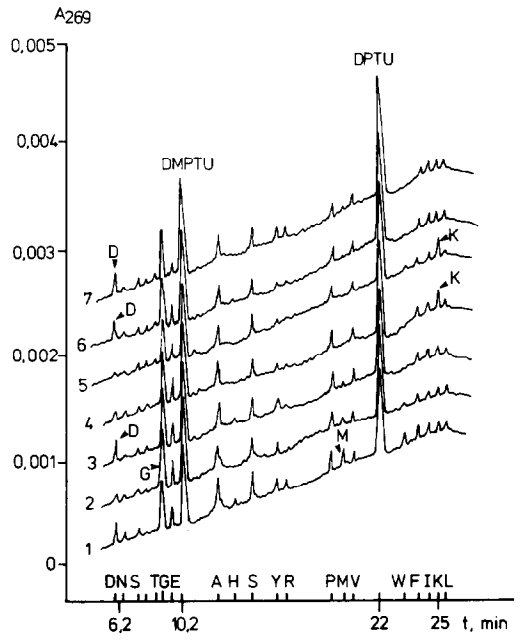

Fig.3. Sequence analysis of the protein eluted from the immunosorbent (fig.2) after electrotransfer to PVDF membranes. About one-third of the sample was analyzed for each cycle. Quantitation is shown in table 1.

where a lower electrophoretic mobility of the rat brain $\alpha 3$ isoform was assumed as compared with that of a1 and a2 proteins.

To determine whether the eluted protein is the $\alpha 3$ polypeptide, it was transferred to the hydrophobic membrane just after electrophoresis and subjected to amino acid analysis. Results summarized in table 1 and fig.3 illustrate the chromatogram of seven cycles of Edman degradation. The integrator application allowed the unambiguous sequence identification: Met-Gly-Asp-Lys-Lys-Asp-Asp that really belongs to the $\alpha 3$ isoform. The $\alpha 1$ subunit, the so called renal-like form, lacks this sequence. So, this is evidence for the $\alpha 3$ isoform expression in pig kidney. As the antipeptide antibodies, a-II recognized equally well the $\alpha 3$ protein upon immunoblotting in pig and human kidney preparations [4], this Na,K-ATPase isoform is supposed to be in human kidney tissue.

With regard to our data, the failure to detect $\alpha 3$ using monoclonal antibodies in rat kidney preparations [5,11] could be due to species specificity. The very low $\alpha 3$ content in this tissue seems to be a more plausible explanation.

The protein yield after immunoaffinity chromatography was about 0.05% relative to the starting material (20 mg of microsomes give about 10 mg of the $\alpha 3$ protein). Thus the $\alpha 3/\alpha 1$ ratio in kidney is approximately 1:100 if the protein loss at various enrichment stages is taken into account. However, since the $\alpha 3$ isoform was shown to be more sensitive to the proteolytic enzyme action, e.g. trypsin [10], partial proteolysis can occur upon isolation and, consequently, the $\alpha 3$ protein content in kidney tissues can be higher.

The Na,K-ATPase $\alpha 3$ isozyme has an N-terminal amino acid structure coinciding with that deduced from the $\alpha 3$ gene nucleotide sequence [2]. It means that no proteolytic processing occurs in the mature $\alpha 3$ isoform, or, at least, demonstrates the existence of the form that does not undergo processing in animal organisms. This distinguishes the $\alpha 3$ isoform of the Na,K-ATPase catalytic subunit from $\alpha 1$ and $\alpha 2$ subunits missing their five amino acids due to post-translational changes [3].

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