Biochemistry (Moscow), Vol. 68, No. 9, 2003, pp. 1040-1047. Translated from Biokhimiya, Vol. 68, No. 9, 2003, pp. 1271-1279.
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Revealing of Proteins Interacting with Na,K-ATPase

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Received December 11, 2002
Revision received March 12, 2003

Abstract—Proteins interacting with α1β1-type of Na,K-ATPase were revealed in pig kidney outer medulla and duck salt glands using three different methods (immunoprecipitation, protein overlay, and chemical cross-linking). Immunoprecipitation was performed after solubilization of protein homogenate with Triton X-100 so that both membrane and cytosol proteins bound to Na,K-ATPase could be revealed. Two other methods were used to study the interaction of cytosol proteins with purified Na,K-ATPase. The sets of proteins revealed by each method in outer medulla of pig kidney were different. Proteins interacting with Na,K-ATPase that have molecular masses 10, 15, 70, 75, 105, 120, and 190 kD were found using the immunoprecipitation method. The chemical cross-linking method revealed proteins with molecular masses 25, 35, 40, 58, 68-70, and 86-88 kD. The protein overlay method revealed in the same tissue proteins with molecular masses 38, 42, 43, 60, 62, 66, 70, and 94 kD.

Key words: Na,K-ATPase, protein–protein interactions, immunoprecipitation, chemical cross-linking, protein overlay

Na,K-ATPase (Na-pump) that catalyzes the active transport of Na^+ and K^+ across the plasma membrane of animal cells consists of two subunits: α-subunit with molecular mass of about 110 kD and β-subunit that is a glycoprotein. The molecular mass of the protein part of the β-subunit is about 35 kD, and after glycosylation it increases to 55-60 kD. Both subunits are integral proteins of the plasma membrane. The α-subunit is the catalytic one: it hydrolyses ATP, forms channel for Na^+ and K^+, and couples ATP hydrolysis to the transport of cations against the electrochemical gradient through the channel [1]. The β-subunit does not participate directly in the catalysis and in the translocation of the cations. It plays an important role in the transportation of newly synthesized subunits from endoplasmic reticulum to plasma membrane [2]. The β-subunit appears to have also another function: it was identified as a factor responsible for cell adhesion in nervous tissues [3].

In various tissues Na,K-ATPase is involved in different functions: maintenance of intracellular electrolyte homeostasis, creation of transmembrane potential, conduction of action potential in neurons and contraction of muscles, transepithelial transport of Na^+, and Na^+-dependent transport of different substances into the cells (Na^+/Ca^{2+} and Na^+/H^+-exchange, Na^+-dependent transport of sugars and amino acids) (for review see [4]). All these suggest complex mechanisms of Na-pump regulation, including the insertion of Na,K-ATPase molecules into the membrane and their removal from the membrane, concentration of pump molecules in certain parts of the plasma membrane (such as basolateral membrane of kidney epithelial cells), and interaction of Na-pump molecules with different protein regulators.

Na,K-ATPase is not only a pump but also a receptor for steroid compounds like ouabain and its derivatives. First ouabain and similar steroids (cardiac glycosides) were found in plants that do not have Na,K-ATPase, but later cardiac glycosides were revealed in animal tissues. Ouabain binding to Na,K-ATPase in animal cells results in its interaction with certain intracellular proteins that triggers different signal transduction pathways and results finally in the increase of intracellular concentration of some secondary messengers and in the expression of some genes [5].

Thus, the interaction of Na,K-ATPase with tissue specific sets of proteins is necessary for the normal function of the enzyme in different tissues. Four Na,K-ATPase α-subunit isoforms (referred to as α_1, α_2, α_3, and α_4) and three types of β-subunit isoforms (β_1, β_2, and β_3) have

Abbreviations: DTSP) 3,3′-dithio-bis(propionic acid N-hydroxysuccinimide ester); DTT) dithiothreitol; TBST) Tris-buffered saline containing Tween-20; PMSF) phenylmethylsulfonyl fluoride; EGS) ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester).

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0006-2979/03/6809-1040$25.00 ©2003 MAIK "Nauka/Interperiodica"

been identified. All isoforms of α- and β-subunits are encoded by different genes, and α- and β-forms can interact each other in different combinations forming various αβ-isoenzymes of Na,K-ATPase that are differently expressed in various tissues [6, 7]. In a tissue Na,K-ATPase may be present as different isoenzymes, and it seems that various sets of proteins can interact with different Na,K-ATPase isoenzymes. Some proteins that interact with Na,K-ATPase (mainly with $\alpha_1\beta_1$-isoenzyme) have been identified. Among them are members of FXYD-family [8]. The first representative of the family was found in preparations of Na,K-ATPase from mammalian kidney in the 1970s; it was called γ-subunit. Other identified proteins interacting with Na,K-ATPase are ankyrin [9], adducin [10], 3-phosphoinositide kinase [11], proteins providing Na,K-ATPase insertion into vesicles (SNAPAP [12] and AP-2 [11]), catalytic subunit of protein phosphatase 2 [12], and Src-kinase [5]. One of the identified proteins, β_3-tubulin, was shown to bind covalently to $\alpha_3\beta_1$-isoenzyme of Na,K-ATPase in brainstem [13]. Although many proteins interacting with Na,K-ATPase have been identified, it is clear that list of proteins which are partners of Na,K-ATPase will be increased. Thus, it is necessary to develop new methods for revealing proteins interacting with Na,K-ATPase that will be suitable for further identification of proteins bound to different isoenzymes of Na,K-ATPase in various tissues.

The goal of the present work was to develop methods that can be used for revealing proteins that interact with Na,K-ATPase with subsequent identification of the proteins. Three different approaches were used in the study: 1) immunoprecipitation under mild conditions that do not destroy the interaction of Na,K-ATPase with protein partners; 2) protein overlay, a method that reveals proteins interacting with the pump if the mixture of proteins was subjected to separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); 3) chemical cross-linking of the proteins with Na,K-ATPase. Experiments were done with Na,K-ATPase from two tissues: outer medulla of pig kidney and duck salt glands in which $\alpha_1\beta_1$-isoenzyme is presented.

MATERIALS AND METHODS

ATP, sodium deoxycholate, EDTA, EGS, DTSP, leupeptin, pepstatin A, and protein A-agarose were obtained from Sigma (USA); sucrose, glycine, Tris, and acrylamide were from Khelikon (Russia); SDS was purchased from ICN (USA); Coomassie Brilliant Blue R-250 was from Serva (Germany); methylene-bis-acrylamide and nitrocellulose membranes were from Bio-Rad (USA); other reagents of the highest purity available were produced in Russia.

Na,K-ATPase was purified in membrane-bound form from duck salt glands and outer medulla of pig kidney in accordance with methods described by Smith [14] and Jorgensen [15], respectively. Homogenate was obtained from each tissue as described in the original papers and was centrifuged at 5000*g* for 15 min. The supernatant was kept at 4°C, the pellet was suspended in 10 ml of buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM sucrose, and was centrifuged under the same conditions. The supernatants were combined and centrifuged at 48,000*g* for 1 h. The supernatant was used in experiments as cytosol fraction. Purification of Na,K-ATPase was continued using the pellet in accordance with the original methods. Protein concentration was measured according to the method of Lowry et al. [16].

Two rabbits were immunized using as antigen purified preparation of Na,K-ATPase from duck salt gland according to the following scheme. The first injection was made intradermally using 500 μg of purified Na,K-ATPase that was injected together with complete Freund's adjuvant. Then 300 μg of purified Na,K-ATPase was injected 3 times through each two weeks with incomplete Freund's adjuvant. Immune serum obtained from two rabbits was combined, and antibodies were partially purified by treating the serum with ammonium sulfate. Then the antibodies were purified using affinity chromatography with immobilized α-subunit of Na,K-ATPase from duck salt glands [17]. The titer of the polyclonal antibodies obtained was determined using the DOT-ELISA method and consisted of 70,000-90,000.

Immunoprecipitation. Immunoprecipitation was carried out using the following procedures. Duck salt gland weighing about 0.3 g or an equal amount of medullar layer of pig kidney were cut by scissors into small pieces and mixed with 10 volumes of buffer containing 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM DTT, 2 mM EDTA, and protease inhibitors (2 mM PMSF, 100 μM leupeptin, 1 μM pepstatin A). Tissues were homogenized in a Potter homogenizer and then protein concentration was measured. For immunoprecipitation under mild conditions, buffer A was added to homogenate (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, protease inhibitors at concentrations noted above, and 1% Triton X-100) to the final protein concentration 4 mg/ml. Then it was homogenized and kept at room temperature with constant agitation for 10 min. The homogenate was centrifuged at 100,000*g* for 1 h at 4°C. Aliquots of supernatant (1 ml) containing 3-4 mg of protein were added to preimmune serum and the mixture was agitated for 30 min at room temperature. Then 25-50 μl of protein A-agarose was added, which was previously washed 5 times with buffer A. The mixture was kept for 30 min under similar conditions and then centrifuged at 5000*g* for 5 min at 4°C. Purified polyclonal rabbit antibodies against Na,K-ATPase α-subunit (50-100 μl) were added to the supernatant. The mixture was incubated at room temperature with constant agitation for 1 h, then

100-150 μl of protein A-agarose was added. The suspension was kept under similar conditions for 1 h and then was centrifuged (5000*g* for 5 min at 4°C). The pellet was washed 5 times with buffer A, kept 5 min at 95°C, and treated with buffer containing 0.25 M Tris-HCl, 8% SDS, 2% 2-mercaptoethanol, and 40% sucrose. Then the protein A-agarose beads were pelleted by centrifugation as previously described, and the supernatant was subjected to SDS-PAGE. Electrophoresis was performed in accordance with the Laemmli method [18] using 3.5% stacking and 5-20% running gels. After electrophoresis the gels were washed with a solution of 10% acetic acid and 20% isopropanol, and protein bands were stained with Coomassie Brilliant Blue R-250 (0.25% solution prepared using 10% acetic acid and 20% isopropanol).

Immunoprecipitation under severe conditions was performed as described above except that buffer A contained additionally 3.7% SDS and the homogenate with antibodies was incubated for 2 h.

Protein overlay. This method is often used to study protein–protein interactions [19]. Cytosol fractions obtained from the homogenate of duck salt glands and outer medulla of pig kidney were subjected to SDS-PAGE according to the Laemmli method with 5% stacking and 8-20% or 10-20% running gels. All wells of the gel were joined and 400 μg of protein of the cytosolic fraction were added into this well. After electrophoresis, part of the gel was cut out and proteins on this part of the gel were stained with Coomassie Brilliant Blue. The remaining part of the gel was used for transfer of proteins to nitrocellulose membrane. The transfer was made by blotting in a Mini Trans-Blot device (Bio-Rad) during 1.5 h at 80 V in buffer containing 25 mM Tris/192 mM glycine (pH 8.3), 20% methanol, and 0.1% SDS. A strip of the nitrocellulose was cut out and stained with Amido Black to check the protein transfer. Then the nitrocellulose was treated twice with TBST (50 mM Tris, 0.15 M NaCl, pH 7.9, 0.05% Tween-20) and was blocked for 1 h in solution of 10% nonfat dry milk prepared using TBST. After that the nitrocellulose was washed twice for 10 min with TBST and placed into solution containing purified Na,K-ATPase (500 nM) obtained from the tissue that was used for purification of cytosol fraction. The nitrocellulose was kept overnight at 4°C with constant agitation, washed twice for 10 min using TBST solution, and then agitated for 1 h at room temperature in solution containing antibodies against Na,K-ATPase α-subunit (20 ml of TBST, 0.02 ml antibodies, and 0.2 g nonfat dry milk). Then the nitrocellulose was washed twice with TBST for 10 min and incubated for 1 h in solution of secondary antibodies (20 ml of TBST and 20 μl of anti-rabbit antibodies conjugated with horseradish peroxidase, titer 1 : 1000). After washing the nitrocellulose was stained in the dark using 3,3′-diaminobenzidine (0.55 mg/ml) dissolved in 50 mM solution of Tris-HCl, pH 8.3, and 0.1% hydrogen peroxide.

Cross-linking of proteins from cytosol fraction with purified Na,K-ATPase. EGS and DTSP were used as cross-linking agents in these experiments. Both compounds interact with protein amino groups located at the distance of 16 and 12 Å, respectively. Purified Na,K-ATPase, cytosol fraction or mixture of purified Na,K-ATPase and cytosol fraction (protein ratio was 1 : 2 (mg/mg), respectively) were incubated for 1 h at room temperature. Then either DTSP prepared using a solution of NaH_2PO_4 (50 mM, pH 7.4), or EGS dissolved in dimethylformamide to final concentration 20 mg/ml were added. Proteins were cross-linked for 15 min at room temperature and various ratios of protein/cross-linking agent. The reaction was stopped by the addition of glycine (50 mM) and after that proteins were subjected to SDS-PAGE. Samples treated with DTSP were prepared for electrophoresis both in the presence and in the absence of 2-mercaptoethanol that destroys internal S–S bond in the molecule of DTSP that results in the separation of cross-linked proteins. After SDS-PAGE the gels were stained with Coomassie R-250, destained, and scanned using an UltroScan XL laser densitometer (LKB, Sweden). The content of protein in protein bands was calculated using the GelScanXL program (LKB).

RESULTS

To obtain antibodies we used as the antigen purified Na,K-ATPase from duck salt glands. According to SDS-PAGE more than 98% of the protein in this preparation is represented by two proteins with molecular masses of about 100 and 55 kD (Na,K-ATPase α- and β-subunits, respectively). It was shown using an immunoblotting procedure that purified polyclonal antibodies against Na,K-ATPase α-subunit interact with α-subunit of these enzyme from both tissues: duck salt glands and outer medulla of pig kidney (data not shown). The antibodies were used for immunoprecipitation of Na,K-ATPase from homogenate of outer medulla of pig kidney and for the identification of proteins interacting with Na,K-ATPase in both tissues using the protein overlay method.

Immunoprecipitation. Results of the immunoprecipitation of Na,K-ATPase from homogenate of pig kidney outer medulla are presented in Fig. 1. The homogenate was treated with the detergents (1% Triton X-100 or mixture of 1% Triton X-100 and 3.7% SDS) and after that nonsolubilized membrane fragments were pelleted by centrifugation at 100,000*g* for 1 h. It can be seen that more than 10 proteins with molecular masses between 10 and 190 kD are pelleted together with beads of protein A-agarose if proteins solubilized from the homogenate by Triton X-100 were used for immunoprecipitation (Fig. 1, lane *1*). Only three proteins were found in the precipitate if the procedure of immunoprecipitation was performed after the solubilization of homogenate proteins by a mix-

ture of 1% Triton X-100 and 3.7% SDS (Fig. 1, lane *2*). One of them is a 100-kD protein. The two others (25- and 55-kD proteins) were bound with protein A-agarose when the procedure of immunoprecipitation was performed without homogenate. After transfer of the 25- and 55-kD proteins to nitrocellulose, they were stained with the secondary antibodies (data not shown). These proteins appear to be heavy and light chains of immunoglobulins (antibodies). When proteins of the immunoprecipitate obtained in the presence of 1% Triton X-100 (Fig. 1, lane *1*) were transferred from the gel to nitrocellulose and stained with antibodies against Na,K-ATPase α-subunit, only 100-kD protein was revealed (Fig. 1, lane *3*). Thus 7 of 10 proteins found in the immunoprecipitate (Fig. 1, lane *1*) coprecipitate together with the Na,K-ATPase α-subunit–antibody complex. Na,K-ATPase β-subunit seems to coprecipitate with the complex too, but its electrophoretic mobility is similar to the mobility of the immunoglobulin heavy chains and we cannot identify this subunit.

Hence, using the immunoprecipitation method we were able to precipitate together with Na,K-ATPase at least seven proteins which appear to form sufficiently stable complexes with Na,K-ATPase that withstand treatment with 1% Triton X-100.

Protein overlay. Results obtained using the protein overlay method that revealed cytosolic proteins that form complexes with Na,K-ATPase in duck salt glands and outer medulla of pig kidney are presented in Fig. 2. Gels stained with Coomassie Brilliant Blue after separation of proteins of cytosol fractions from these tissues by SDS-PAGE are presented in Figs. 2a and 2b (left parts). The right parts of Figs. 2a and 2b represent nitrocellulose membranes after the transfer to them of proteins separated by electrophoresis and stained with purified Na,K-ATPase and antibodies against Na,K-ATPase. It can be seen that six proteins in the cytosolic fraction from duck salt glands (Fig. 2a, right) and nine proteins from cytosolic fraction of pig kidney outer medulla (Fig. 2b, right) interact with Na,K-ATPase. Proteins of the cytosolic fraction from duck salt glands forming complexes with Na,K-ATPase have molecular masses between 31 and 90 kD, and proteins of cytosolic fraction from outer medulla of pig kidney—between 38 and 94 kD. Among cytosol proteins that interact with Na,K-ATPase in these two tissues were found proteins with close values of molecular masses: 43-49, 54-58, 70, and 90-94 kD.

Cross-linking of cytosolic proteins with purified Na,K-ATPase. Treatment of purified Na,K-ATPase from outer medulla of pig kidney with EGS or DTSP and subsequent separation of protein using SDS-PAGE shows the decrease in the amount of 100-kD protein corresponding to Na,K-ATPase α-subunit and in the increase in the amount of protein that does not enter into the gel (data not shown). It can be seen in Fig. 3b (top) that the

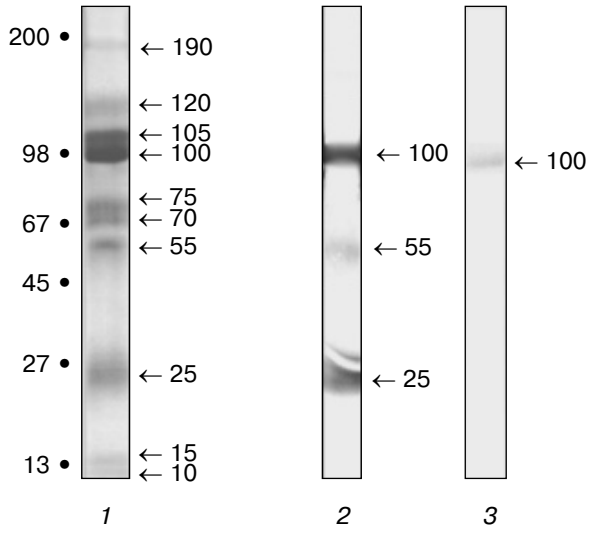

Fig. 1. Immunoprecipitation of Na,K-ATPase from the homogenate of outer medulla of kidney. *1*, *2*) Results of electrophoretic separation of precipitate obtained by immunoprecipitation of the complex of proteins interacting with Na,K-ATPase in the presence of 1% Triton X-100 (lane *1*) and in the presence of mixture of 1% Triton X-100 and 3.7% SDS (proteins are stained by Coomassie Brilliant Blue R-250) (lane *2*); *3*) results of immunoblotting procedure performed with the proteins that are shown on lane *1* and stained with antibodies against the Na,K-ATPase α-subunits. Dots show molecular masses of marker proteins (kD), arrows show molecular masses of precipitated proteins (kD).

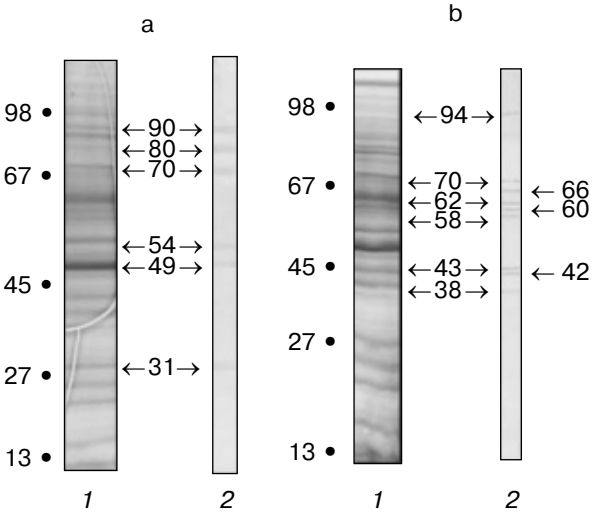

Fig. 2. Proteins of cytosolic fraction from duck salt glands (a) and cytosolic fractions from outer medulla of pig kidney (b) after their separation by SDS-PAGE. Staining of proteins with Coomassie Brilliant Blue (lanes *1*) and staining after incubation with purified Na,K-ATPase and antibodies against the α-subunit (lanes *2*). Dots show molecular masses of marker proteins (kD), arrows show molecular masses of cytosol proteins interacting with Na,K-ATPase (kD).

amount of α-subunit in purified Na,K-ATPase is decreased after its incubation with raising concentrations of DTSP. Because we used gradient running gel with minimal concentration 5%, the cross-linked protein complexes with molecular mass more than 500 kD did not enter the gel. We suggested that cross-linking results in the formation of high molecular mass aggregates (with molecular mass more than 500 kD) consisting of Na,K-ATPase α-subunits. Unfortunately, we did not observe significant formation of aggregates with molecular masses lower than 500 kD even when we decreased time of incubation with cross-linking agent or molar ratio cross-linking agent/Na,K-ATPase. The DTSP molecule is known to contain an S–S bond that can be destroyed when reducing agents are added. Incubation of purified Na,K-ATPase previously treated by DTSP with 2-mercaptoethanol (50 mM) for 30 min led to an increase in the amount of protein in the 100-kD band to the initial level (data not shown). These results confirm the suggestion that the decrease in the amount of 100 kD protein after incubation of purified Na,K-ATPase with DTSP can be explained by the cross-linking of enzyme α-subunits with the formation of high molecular mass aggregates.

Treatment of cytosolic fraction by cross-linking agents EGS and DTSP also results in a decrease in the amount of some proteins (different when DTSP or EGS were used) with parallel increase in the share of protein that does not enter into the gel. It is shown in Fig. 3c (bottom) how the amount of cytosolic 68-kD protein is decreased after the incubation of cytosolic fraction with increasing concentrations of DTSP. Incubation of cytosolic fraction previously treated with DTSP with 2-mercaptoethanol (50 mM) for 30 min also led to an increase in the amount of proteins in the bands to the initial level. Thus, the loss of some proteins as a result of the treatment of cytosol fraction with DTSP also is due to the cross-linking of these cytosolic proteins with the formation of large aggregates.

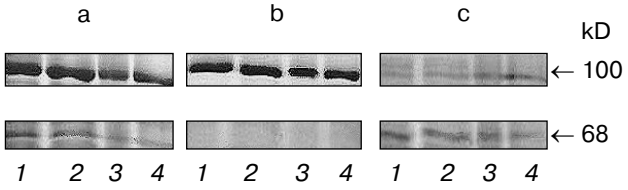

Fig. 3. Estimation of the amount of Na,K-ATPase α-subunit (top) and 68-kD cytosolic protein (bottom) after incubation with DTSP and subsequent separation by SDS-PAGE. Results were obtained after incubation of the mixture of purified Na,K-ATPase from outer medulla of pig kidney and cytosolic fraction from the same tissue (a), only Na,K-ATPase (b), and only cytosol fraction (c). *1*) Incubation was carried out without DTSP; *2*, *3*, *4*) incubation with DTSP at molar ratio Na,K-ATPase/DTSP equal to 1 : 50, 1 : 100, and 1 : 200, respectively; incubation time was 15 min.

Incubation of mixture of purified Na,K-ATPase and cytosol fraction with DTSP and EGS results in more significant decrease in the amount of Na,K-ATPase α-subunit and some cytosolic proteins than incubation of purified Na,K-ATPase and cytosolic fraction separately with the cross-linking agents (compare Fig. 3, a and c, top, and Fig. 3, a and c, bottom). These differences are more obvious when the molar ratio cross-linking agent/Na,K-ATPase is increased. The amount of high molecular mass aggregates with molecular mass more than 400 kD that are seen in the upper part of the gel also is increased as well as the amount of protein that does not enter into the gel (data not shown). The data demonstrating the decrease in the amount of some proteins of cytosolic fraction and Na,K-ATPase α-subunit as results of their incubation with cross-linking agents separately and in the mixture are presented in Fig. 4a (DTSP) and Fig. 4b (EGS). It can be seen that incubation of mixture of cytosolic fraction and purified Na,K-ATPase with DTSP significantly increases the loss of Na,K-ATPase α-subunit (100-kD protein). A decrease in the amount of cytosol proteins with molecular masses of about 25, 35, 40, 58, 68, 77, and 86-kD was observed simultaneously. The amount of α-subunit after incubation of Na,K-ATPase with DTSP for 15 min at molar ratio cross-linking agent/Na,K-ATPase 1 : 200 is decreased to 88% comparing with a control. If Na,K-ATPase was incubated under the same conditions together with cytosolic proteins the amount of α-subunit was decreased to 44% (Fig. 4a). Thus, the total loss of the amount of α-subunit that is due to its cross-linking with cytosolic proteins consists of 44%. Difference in the loss of cytosolic proteins during their incubation in the mixture with DTSP and purified Na,K-ATPase changes between 15-68%. The biggest loss (68 and 67%) was noted for the proteins with molecular masses of about 40 and 35 kD, respectively. These proteins are cross-linked only with purified Na,K-ATPase because their amounts do not decrease when cytosolic fraction is incubated with DTSP without Na,K-ATPase.

Incubation of the mixture of purified Na,K-ATPase and cytosolic fraction with EGS results in the loss of α-subunit (100-kD protein) and the only cytosolic protein with molecular mass of about 88 kD (Fig. 4b). The loss of the α-subunit in the control consists of 34%, and in the presence of cytosolic fraction, 47%. Thus, about 13% from the total amount of α-subunit is cross-linked with 88-kD cytosolic protein. The amount of this protein during the incubation of cytosolic fraction together with purified Na,K-ATPase decreases in comparison with the control by 17%.

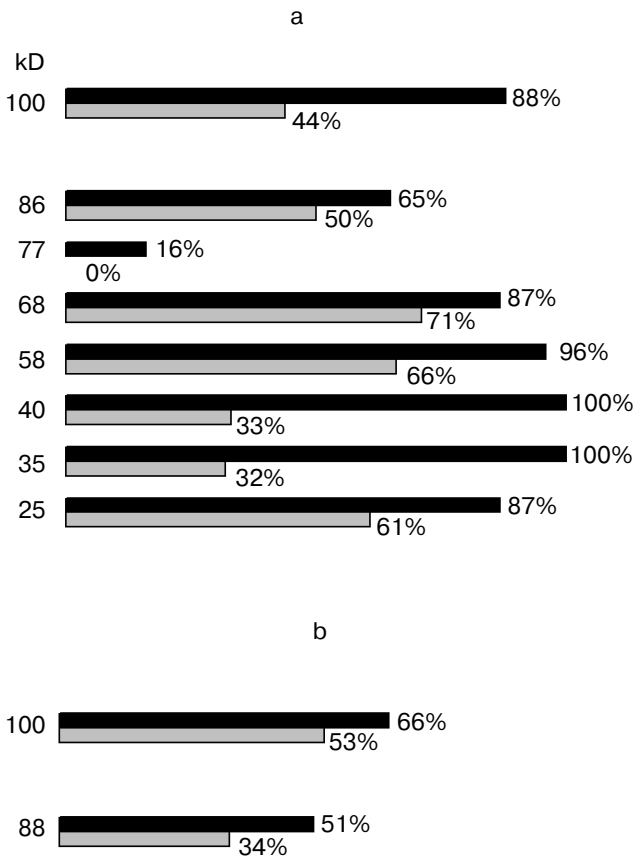

Fig. 4. Decrease in the amount of some proteins in cytosol fraction and Na,K-ATPase α-subunit (100-kD protein) from outer medulla of pig kidney during their incubation with cross-linking agents. Incubation of cytosol fraction and purified Na,K-ATPase with DTSP (a) or EGS (b) for 15 min at molar ratio 1 : 200 separately (black) or in the mixture with each other (gray).

One can suggest that mixing the cytosol proteins with Na,K-ATPase results in more intensive cross-linking of Na,K-ATPase α-subunits with cytosol proteins that form complexes with this subunit. Thus, among the proteins that interact with Na,K-ATPase may be cytosolic proteins from outer medulla of kidney with molecular masses 25, 35, 40, 58, 68-70, 77, and 86-88 kD.

DISCUSSION

The contemporary stage of development in biology is characterized by the intensive collection of data about the structures of genes and proteins that are encoded by these genes. Reading of genomes of some species, in particular the human genome, shows that until now a significant part of the proteins are not being studied using biochemical methods. This led to the appearance of proteomics, a branch of biochemistry that engages in the separation and identification of proteins. However, besides the identification of proteins it is necessary to know what cellular proteins interact with each other and what the results of these interactions are.

Recently, molecular biological approaches such as the yeast two-hybrid system began to be used for study of protein–protein interactions and for identification of proteins interacting with each other. This method is based on the unique properties of transcriptional factors [20]. It was shown recently using the yeast two-hybrid system that Na,K-ATPase interacts with proteins SNAPAP, phosphoprotein phosphatase 2 [12], and cofilin [21]. However, this approach also has some disadvantages, in particular, it is impossible to use the whole membrane protein to study protein–protein interaction with the two-hybrid system but only its cytoplasmic or extracellular domains. Besides this, yeasts do not have some post-translational modifications, which in higher eucaryotes can affect the interaction of proteins with each other.

To reveal proteins forming complexes with Na,K-ATPase, we used three approaches: immunoprecipitation in the presence of Triton X-100, protein overlay, and cross-linking of cytosolic proteins with purified Na,K-ATPase using two cross-linking agents.

Because two methods used are immunochemical methods, at first we produced antibodies against Na,K-ATPase. Purified Na,K-ATPase from duck salt glands was used as the antigen. This preparation is inhomogeneous, but more than 98% of the protein in this preparation according to the data of electrophoresis are represented by proteins with molecular masses of about 100 and 55 kD corresponding to α- and β-subunits of Na,K-ATPase. The obtained polyclonal antibodies were additionally purified using affinity chromatography with immobilized α-subunit. It was confirmed also that the purified antibodies interact with Na,K-ATPase α-subunit from pig kidney.

At least 7 proteins with molecular masses of about 10, 15, 70, 75, 105, 120, and 190 kD were coprecipitated as a complex with Na,K-ATPase from homogenate of outer medulla of kidney solubilized by Triton X-100 (Fig. 1, lane *1*). Using the protein overlay method 9 proteins (molecular masses of about 38, 42, 43, 58, 60, 62, 66, 70, and 94 kD) interacting with Na,K-ATPase were revealed in cytosolic fraction from that tissue (Fig. 2a). Among proteins of these two sets, there is only one 70-kD protein that was found by both methods. However, this does not mean that in both cases the same protein was revealed: this conclusion may be made only after identification of the protein (using mass-spectrometry or by reading of the N-terminal amino acid sequence). Thus, two immunochemical methods based on the use of the same antibodies revealed different sets of proteins forming complexes with Na,K-ATPase.

This result is not surprising. First, both cytosolic and membrane proteins are precipitated during the procedure of immunoprecipitation from the homogenate but among precipitated membrane proteins should be only proteins that are solubilized by Triton X-100. Besides this, both cytosolic and membrane proteins should form complexes with Na,K-ATPase that are not destroyed by the treatment with nonionic detergent Triton X-100. Only these proteins can coprecipitate with Na,K-ATPase. Using the protein overlay method we reveal only cytosolic proteins and that retain the ability to interact with Na,K-ATPase after their treatment by a mixture of ionic detergents and 2-mercaptoethanol. It is necessary to note that incubation of cytosolic proteins with Na,K-ATPase takes place after their transfer to a nitrocellulose membrane. Proteins with different molecular masses are transferred to the nitrocellulose with different velocity. We used time of transfer that was enough for transfer of all proteins with low molecular mass but during this time high molecular mass proteins were transferred to nitrocellulose only partially. Thus, we cannot reveal all proteins interacting with Na,K-ATPase using this method. However, we can compare proteins forming complexes with this enzyme in different tissues.

The set of cytosolic proteins interacting with Na,K-ATPase and revealed by the protein overlay method is tissue specific: it can be seen that different cytosol proteins interact with Na,K-ATPase in duck salt glands and outer medulla of pig kidney (Fig. 2). Among them are proteins with similar molecular mass (94 and 90, 70, 54, and 58 kD); they might be the same or related proteins. However, a conclusion about their similarity or difference can be made only after identification of these proteins.

Starting the cross-linking study we supposed that this method will be most promising because in contrast to the first two methods it can reveal native proteins forming complexes with Na,K-ATPase. We suggested that it is possible to find conditions for cross-linking or cross-linking agents that will not make cross-linking between cytosolic proteins and Na,K-ATPase subunits itself but will covalently attach Na,K-ATPase subunits only to cytosol proteins that are partners of Na,K-ATPase. Unfortunately, both cross-linking agents used formed covalent bonds between molecules of Na,K-ATPase α-subunit and between numerous cytosolic proteins (Fig. 4). Furthermore, changing the time of cross-linking and molar ratio cross-linking agent/Na,K-ATPase and cross-linking agent/cytosolic proteins we cannot achieve good separation of the obtained aggregates during electrophoresis: they either do not enter into the gel, or enter into the gel but were formed in small amount. Thus, it was difficult to detect such aggregates. We have shown that the loss of proteins in corresponding bands after treatment of purified Na,K-ATPase, cytosol fraction and their mixture at least by one of the cross-linking agent (DTSP) is due to their cross-linking with each other: the addition of reducing agent destroys the internal S–S bond in the molecule of DTSP and increases the amount of protein in corresponding bands to the initial level. Besides this, we found that incubation of the mixture of cytosolic fraction and purified Na,K-ATPase with cross-linking agents led to more significant decrease in the amount of some cytosolic proteins and α-subunit than incubation of cytosolic fraction and Na,K-ATPase separately with the cross-linking agents. This can be considered as evidence of the formation of complexes of Na,K-ATPase α-subunit and cytosolic proteins. EGS cross-linked with α-subunit the only cytosolic protein with molecular mass of about 88 kD, and DTSP cross-linked with the α-subunit at least seven cytosolic proteins (25-, 35-, 40-, 58-, 68-, 77-, and 86-kD proteins). We cannot exclude the possibility that the proteins with molecular masses of about 86 and 88 kD are the same protein. The most interesting in this list of proteins are proteins with molecular masses of about 35 and 40 kD that are cross-linked only to Na,K-ATPase.

We should also note that proteins with similar molecular masses (38, 42, 58, and 70 kD) were revealed also by the protein overlay method. However, it is still impossible to elucidate whether the same or different proteins are revealed by the two methods because we cannot separate complexes and identified proteins that form these complexes.

Thus, at least two methods (immunoprecipitation under mild conditions and protein overlay) of the three methods used in this work can be useful for revealing and further identification of proteins forming complexes with Na,K-ATPase in different tissues. These methods revealed various proteins: both membrane and cytosolic proteins can be found using the method of immunoprecipitation: the conditions for their coprecipitation with Na,K-ATPase are the solubilization of membrane proteins by Triton X-100 (another nonionic detergent can be used) and the formation of stable complexes with Na,K-ATPase in the presence of this detergent. During the use of the protein overlay method, cytosolic proteins were revealed that can form a complex with the enzyme even after separation of cytosolic proteins by SDS-PAGE. However, it is necessary to use different times for transfer of proteins with different molecular masses from gel to the nitrocellulose. The cross-linking method allows cross-linking with Na,K-ATPase the proteins that form with the enzyme complex in the absence of the detergent, but further studies are necessary for separation of these complexes and for the identification of proteins of these complexes. We suggest that the combination of different methods will reveal in the future most of the proteins interacting with Na,K-ATPase. Proteins with molecular masses of about 38, 42, 58, and 70 kD that were revealed by different methods are the first candidates for identification of the possible protein partners of $\alpha_1\beta_1$-isoenzyme of Na,K-ATPase.

This study was supported by the Russian Foundation for Basic Research (grant No. 01-04-48237) and by INTAS (grant No. 01-0224).

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