ISSN 0026-8933, Molecular Biology, 2018, Vol. 52, No. 2, pp. 247–250. © Pleiades Publishing, Inc., 2018.
Original Russian Text © E.A. Dergousova, I.Yu. Petrushanko, E.A. Klimanova, V.A. Mitkevich, R.H. Ziganshin, O.D. Lopina, A.A. Makarov, 2018, published in Molekulyarnaya Biologiya, 2018, Vol. 52, No. 2, pp. 289–293.

MOLECULAR CELL BIOLOGY

UDC 577.1

Enhancement of Na,K-ATPase Activity as a Result of Removal of Redox Modifications from Cysteine Residues of the α1 Subunit: the Effect of Reducing Agents

E. A. Dergousova[a, b], I. Yu. Petrushanko[a], E. A. Klimanova[a, b], V. A. Mitkevich[a], R. H. Ziganshin[c], O. D. Lopina[a, b], and A. A. Makarov[a, *]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia*
[b] *Faculty of Biology, Moscow State University, Moscow, 119991 Russia*
[c] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997 Russia*
**e-mail: aamakarov@eimb.ru*

Received November 30, 2016; in final form, December 6, 2016

Abstract—Na,K-ATPase is a transmembrane enzyme that creates a gradient of sodium and potassium, which is necessary for the viability of animal cells. The activity of Na,K-ATPase depends on the redox status of the cell, decreasing with oxidative stress and hypoxia. Previously, we have shown that the key role in the redox sensitivity of Na,K-ATPase is played by the regulatory glutathionylation of cysteine residues of the catalytic alpha subunit, which leads to the inhibition of the enzyme. In this study, the effect of reducing agents (DTT, ME, TCEP) on the level of glutathionylation of the alpha subunit of Na,K-ATPase from rabbit kidneys and the enzyme activity has been evaluated. We have found that the reducing agents partially deglutathionylate the protein, which leads to its activation. It was impossible to completely remove glutathionylation from the native rabbit kidney protein. The treatment of a partially denatured protein on the PVDF membrane with reducing agents (TCEP, $NaBH_4$) also does not lead to the complete deglutathionylation of the protein. The obtained data indicate that Na,K-ATPase isolated from rabbit kidneys has both regulatory and basal glutathionylation, which appears to play an important role in the redox regulation of the function of Na, K-ATPase in mammalian tissues.

Keywords: Na,K-ATPase, glutathionylation, cysteine residues, reducing agents

DOI: 10.1134/S0026893318020024

INTRODUCTION

Na,K-ATPase is one of the transport proteins that creates the transmembrane gradient of sodium and potassium ions necessary for the viability and functioning of animal cells [1]. Functional monomer of Na,K-ATPase comprises catalytic (α) and regulatory (β) subunits. The activity of Na,K-ATPase is sensitive to changes in the redox status of the cell [2–5]. Earlier, we showed that the key role in redox sensitivity of Na,K-ATPase is played by glutathionylation of cysteine residues of its α subunit, consisting in the binding of the intracellular tripeptide glutathione to thiol group of protein cysteine to form a disulfide bond [6–9]. We found that increase in the level of glutathionylation of the α subunit led to inhibition of the enzyme caused by impaired ATP binding [6]. Particularly, this modification is observed under conditions of acute hypoxia [6, 7, 9]. In addition to regulatory glutathionylation, which leads to inhibition of Na,K-ATPase activity and can be reversed by reducing agents, we also discovered basal glutathionylation of the α subunit; in the latter case glutathione can be removed only from a denatured protein [7]. Basal glutathionylation and its increase upon prolonged hypoxia have been demonstrated in murine fibroblast cell line SC1 [7]. Furthermore, we found that, in purified samples of Na,K-ATPase isolated from duck salt gland tissue and rabbit kidneys, the α subunit is partially glutathionylated. Salt glands and kidneys contain only the α1 isoform of the enzyme; this is why these tissues are used to isolate purified Na,K-ATPase in order to study its functions. Nevertheless, the issue of the effect of glutathionylation on properties and activity of Na,K-ATPase remains open. In particular, it is not clear how the decrease in the level of glutathionylation in the presence of reducing agents influences activity of Na,K-ATPase of mammalian kidney and whether bound glutathione can be completely removed from the native protein molecule.

Abbreviations: ME, β-mercaptoethanol; DTT, dithiothreitol; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; EDTA, ethylenediaminetetraacetic acid; PBST, phosphate-buffered saline supplemented with Tween-20.

EXPERIMENTAL

Isolation of Na,K-ATPase from rabbit kidney. Na,K-ATPase from a rabbit kidney was isolated according to [10], while that of a duck salt gland was isolated according to the method described in [11]. A cocktail of protease inhibitors was added to the isolation buffer, along with 5 mM EDTA and 5 μM thiorphan. The purity of the sample was determined by polyacrylamide gel electrophoresis (PAGE) according to Laemmli [12] using 3.5% concentrating and 5–20% gradient separating gel. After PAGE, the proteins were stained with Coomassie brilliant blue. The protein concentration was measured according to Lowry and coauthors [13].

Na,K-ATPase activity was evaluated by concentration of inorganic phosphate (Pi) formed upon ATP hydrolysis according to [14] as we have previously reported [6]. Protein was added to the reaction mixture containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, and 30 mM imidazole (pH 7.5) and incubated at 37°C for 5–7 min. In the presence of 1 mM ouabain, a specific inhibitor of Na,K-ATPase, ATPase activity was absent, which showed the absence of other ATPases in the sample.

Determination of Na,K-ATPase glutathionylation level by immunoblotting. Samples for PAGE were prepared in a 4× buffer containing 250 mM Tris-HCl (pH 6.8), 8% sodium dodecyl sulfate (SDS), 40% sucrose, and bromophenol blue (without β-mercaptoethanol, ME). Samples were heated for 30 min at 37°C. PAGE according to Laemmli [12] was performed in 6% concentrating and 10% separating gels. After PAGE, proteins were transferred from the gel onto a nitrocellulose membrane, which was then blocked for 1 h in 5% casein solution in PBST (10 mM KH_2PO_4, pH 7.4, 150 mM NaCl, 0.1% Tween-20). After washing, the membrane was placed into solution of primary anti-glutathione MAB5310 (Chemicone Millipore, United States) antibody diluted 1 : 1000 or anti-α1 subunit of Na,K-ATPase C464-6 (Upstate Millipore, United States) diluted 1 : 10000 antibody as previously described [6, 7]. Membranes were incubated in solutions of primary antibodies for 15–17 h at 4°C under constant shaking. Then, they were washed and incubated for 2 h in a solution of secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1 : 3000). Protein complexes with primary and secondary antibodies were visualized by enhanced chemoluminescence (ECL) using a Chemi-Doc MP Bio-Rad molecular imager and the Western Lightning Plus-ECL (PerkinElmer, United States) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, United States) kits.

Deglutathionylation of microsome samples and enzyme purified from duck salt gland and rabbit kidney on a PVDF membrane. Samples of microsomes and Na,K-ATPase purified from duck salt gland and rabbit kidney were used. PAGE was performed according to Laemmli in 6% concentrating and 10% separating gels. Five micrograms of protein were applied to each well. After separation, proteins were transferred to a PVDF membrane and further incubated in 25 mM TCEP (Tris(2-carboxyethyl)-phosphine hydrochloride), 3% $NaBH_4$, or reducing agent-free buffer (25 mM imidazole, 1 mM EDTA, pH 7.5) for 30 min at room temperature.

Deglutathionylation using chemical reagents. Glutathionylated SH groups were reduced by adding 10 mM DTT, 30 mM ME, or 25 mM TCEP-HCl. The Na,K-ATPase sample was diluted with buffer (0.25 M sucrose, 1 EDTA, 25 mM Tris-HCl, pH 7.5) to a final concentration of 0.5 mg/mL and incubated with reducing agents for 30 min at 37°C and the activity was measured. The level of glutathionylation of Na,K-ATPase α subunit was analyzed by immunoblotting.

Statistical analysis. The average values ± the standard deviation (SD) are reported. Group data were compared using Student's *t*-test; differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Incubation with Reducing Agents Leads to Decrease of the Glutathionylation level of Rabbit Na,K-ATPase

The catalytic subunit of Na,K-ATPase of rabbit kidney contains glutathionylated cysteine residues (Fig. 1). To study the effect of reducing agents on glutathionylation level, Na,K-ATPase sample was incubated for 30 min with reducing agents of various redox potential: standard redox potential of ME, DTT, and TCEP is −0.26, −0.33, and −0.29 V, respectively. According to the data obtained here, the compounds, despite the difference in redox potential value, remove bound glutathione from Na,K-ATPase α subunit in a similar manner (Fig. 1). Apparently, failure to remove residual glutathionylation is due, not to reducing agents, which are not powerful enough to reduce the disulfide bond between glutathione and protein, but some glutathionylated cysteine residues in native protein being out of reach of the reducing agents. We have previously observed a similar effect in lysates of murine SC1 fibroblasts, when after treatment with TCEP some residual (basal) glutathionylation was retained and could be removed only upon protein denaturing [7]. Isolated cavities in the Na,K-ATPase structure that contain cysteine residues found upon the exploration of its structure allowed for the proposal that these cysteine residues are subjected to basal glutathionylation [7]. Therefore, purified protein from the rabbit kidney also has basally glutathionylated cysteine residues that cannot be reduced in a native protein since they apparently bind glutathione in the course of folding.

Deglutathionylation Leads to Increase Activity of Na,K-ATPase from Rabbit Kidney

In contrast to cell lysates, purified protein samples allow for the direct evaluation of the effect of glutathionylation on the enzyme activity. To determine how deglutathionylation affects the activity of Na,K-ATPase of rabbit kidney, we removed bound glutathione residues nonenzymatically using chemical reducing agents DTT, ME, and TCEP-HCl. Protein-containing samples were incubated with 10 mM DTT, 30 mM ME, or 25 mM TCEP for 30 min at 37°C in a buffer (25 mM Tris-HCl, 1 mM EDTA, 250 mM sucrose, pH 7.5). The change of Na,K-ATPase activity over time was controlled in samples not treated with any reducing agent. As such, incubation for the given time was not found to influence the hydrolytic activity of the enzyme. Incubation in the presence of ME, DTT, and TCEP led to increase in hydrolytic activity of the enzyme by 45, 53, and 62%, respectively (Fig. 2). The highest increase in the activity of the enzyme was caused by the strongest reducing agent (TCEP). Therefore, the activity of Na,K-ATPase isolated from rabbit kidney was decreased due to the partial glutathionylation of regulatory cysteine residues. It should be noted that, according to previously published data neither site-specific substitution of individual cysteine residues [15], nor the replacement of all cysteine residues of α subunit with alanine [16] changed considerably the enzyme activity, therefore function of cysteine residues is not quite clear yet. At the same time, redox-dependent modification of cysteine residues in α subunit can considerably influence the enzyme activity; in particular, the glutathionylation of regulatory cysteine residues can cause the complete inactivation of the enzyme [6]. We assume that one of the major functions of cysteine residues in α subunit is redox-dependent regulation of the enzyme activity.

Treatment with Reducing Agents Does Not Lead to Complete Deglutathionylation of Na,K-ATPase from Rabbit Kidneys

To compare the deglutathionylation of Na,K-ATPase in rabbit kidney and duck salt gland under the conditions of partial denaturation, the deglutathionylation of purified and microsomal Na,K-ATPase from rabbit kidney and duck salt gland transferred onto a PVDF membrane was performed (Fig. 3). PVDF membranes with proteins were incubated for 30 min with 25 mM TCEP or a stronger reducing agent, 3% sodium borohydride ($NaBH_4$, standard redox potential: −1.24 V). Control membrane was not treated with reducing agents. Similar to rabbit kidney Na,K-ATPase, neither purified nor microsomal Na,K-ATPase from the duck salt gland was deglutathionylated completely with these reducing agents (Fig. 3). TCEP and $NaBH_4$ deglutathionylated purified Na,K-ATPase to the same level. No differences between deglutathionylation of purified enzyme of the salt gland or rabbit kidney was revealed (Fig. 3). At the same time, microsomal protein is less easily available for deglutathionylation by TCEP than by a stronger

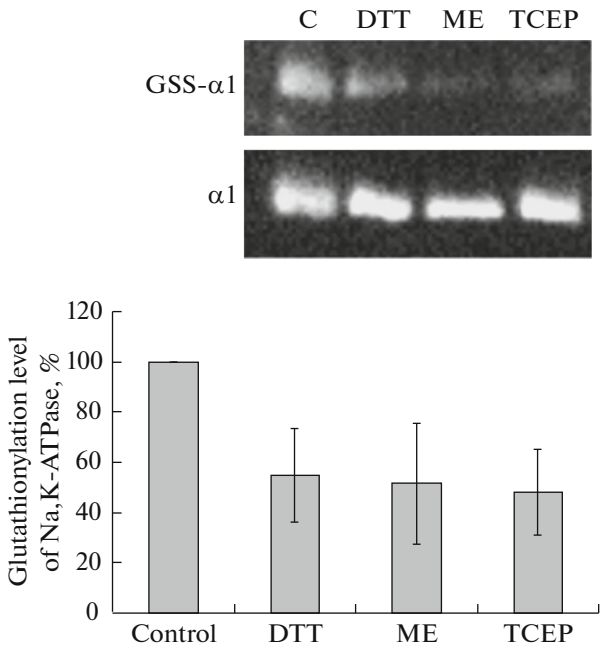

Fig. 1. Level of glutathionylation Na,K-ATPase α1 subunit upon incubation with reducing agents. Immunoblotting with antibodies against glutathionylated protein (GSS-α1) and α1-isoform of Na,K-ATPase samples pretreated with 10 mM DTT, 30 mM mercaptoethanol (ME), or 25 mM TCEP for 30 min. Upper panel: immunoblotting results visualized with chemoluminescence. Lower panel: digital processing of immunoblotting results. Glutathionylation level of untreated Na,K-ATPase (control) was taken as 100%. The results were normalized over α1 subunit content. Average values of three independent measurements ± SD are reported.

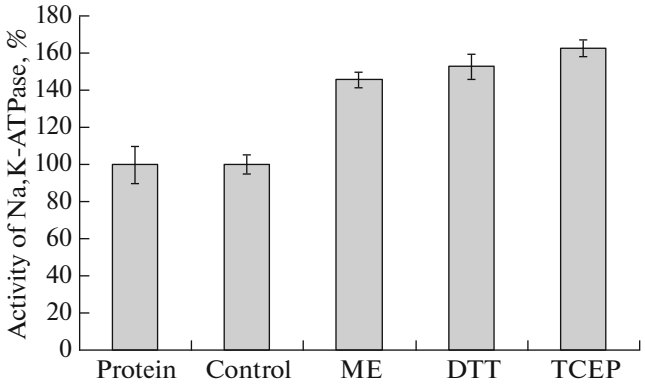

Fig. 2. Changes in hydrolytic activity of Na,K-ATPase from rabbit kidney upon incubation with reducing agents. Protein activity prior to incubation (protein) and after incubation in the absence of reducing agents (control) or in the presence of 10 mM DTT, 30 mM ME, or 25 mM TCEP is expressed in percent to control. Activity in control sample upon 30 min incubation is considered to be 100%.

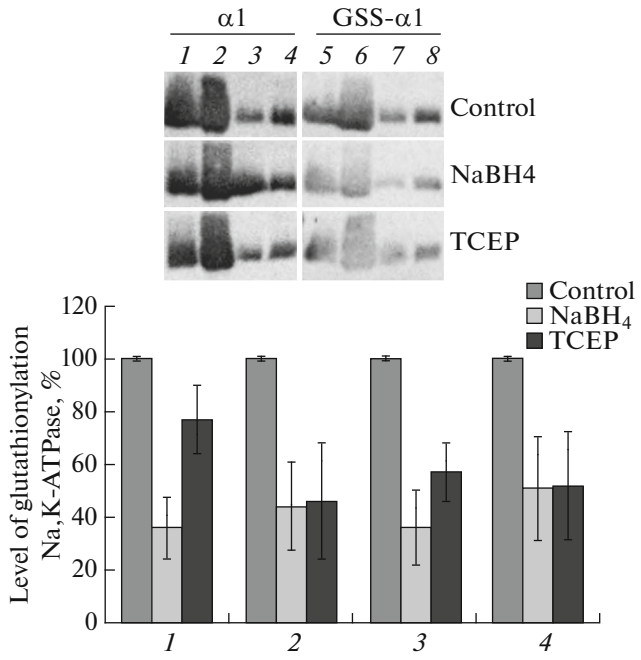

Fig. 3. Level of glutathionylation Na,K-ATPase α subunit in samples of (*1*) duck salt gland microsomes, (*2*) duck salt gland purified enzyme, (*3*) rabbit kidney microsomes, and (*4*) rabbit kidney purified enzyme upon treatment with 3% $NaBH_4$ or 25 mM TCEP on a PVDF membrane at room temperature for 30 min. Upper panel: immunoblotting result visualized with chemoluminescence. Lower panel: immunoblotting analysis result. Level of glutathionylation was calculated as a ratio of signal intensity of glutathionylated α1 subunit (GSS-α1) to signal intensity of α1-isoform of Na,K-ATPase. Level of glutathionylation of Na,K-ATPase in untreated cells (control) was taken to be 100%. Values obtained in three independent experiments ± SD are reported.

low molecular weight reducing agent $NaBH_4$. The difference is more pronounced in the case of the duck salt gland protein (Fig. 3). The lower efficiency of enzyme deglutathionylation within microsomes may be due to thiol groups being less readily available for reducing agents.

Based on the data, one can conclude that the α subunit of Na,K-ATPase isolated from rabbit kidney contains glutathionylated cysteine residues. The level of glutathionylation of the residues decreases upon treatment with reducing agents, leading to an increase in protein activity. Bound glutathione cannot be completely removed from native proteins, which indicates that there is a basal glutathionylation; presumably [7] occurs in the course of protein folding; and unavailable for reducing agents.

ACKNOWLEDGMENTS

The work was supported by the Russian Science Foundation (project no. 14-14-01152).

REFERENCES

1. Kaplan J.H. 2002. Biochemistry of Na,K-ATPase. *Annu. Rev. Biochem.* **71**, 511–535.
2. Bogdanova A., Petrushanko I.Yu., Hernansanz-Agustín P., Martínez-Ruiz A. 2016. "Oxygen Sensing" by Na,K-ATPase: These miraculous thiols. *Front. Physiol.* **7**, 1–21.
3. Bogdanova A., Petrushanko I., Boldyrev A., Gassmann M. 2006. Oxygen- and redox-induced regulation of the Na/K-ATPase. *Curr. Enzyme Inhib.* **2**, 37–59.
4. Petrushanko I.Yu., Bogdanov N.B., Lapina N., et al. 2007. Oxygen-induced regulation of Na/K-ATPase in cerebellar granule cells. *J. Gen. Physiol.* **130**, 389–398.
5. Petrushanko I., Bogdanov N., Bulygina E., et al. 2006. Na-K-ATPase in rat cerebellar granule cells is redox sensitive. *Am. J. Physiol.: Regul. Integr.Comp. Physiol.* **290**, R916–R925.
6. Petrushanko I.Y., Yakushev S., Mitkevich V.A., et al. 2012. S-glutathionylation of the Na,K-ATPase catalytic alpha -subunit is a determinant of the enzyme redox sensitivity. *J. Biol. Chem.* **287**, 32195–32205.
7. Mitkevich V.A., Petrushanko I.Y., Poluektov Y.M., et al. 2016. Basal glutathionylation of Na,K-ATPase alpha-subunit depends on redox status of cells during the enzyme biosynthesis. *Oxid. Med. Cell. Longevity.* **2016**, ID 9092328.
8. Meng Xianyu, Petrushanko I.Yu., Klimanova E.A., et al. 2014. Glutathionylation of the alpha-subunit of Na,K-ATPase from rat heart by oxidized glutathione inhibits the enzyme. *Biochemistry* (Moscow). **79** (2), 158–164.
9. Petrushanko I.Yu., Simonenko O.V., Burnysheva K.M., et al. 2015. The ability of cells to adapt to low-oxygen conditions is associated with glutathionylation of Na,K-ATPase. *Mol. Biol.* (Moscow). **49** (1), 153–160.
10. Jorgensen P.L. 1988. Purification of Na,K-ATPase: Enzyme sources, preparative problems, and preparation from mammalian kidney. *Methods Enzymol.* **156**, 29–43
11. Smith T.W. 1988. Purification of Na^+,K^+-ATPase from the supraorbital salt gland of the duck. *Methods Enzymol.* **156**, 46–48.
12. Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**, 680–685.
13. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
14. Rathbun W.B., Betlach M.V. 1969. Estimation of enzymically produced orthophosphate in the presence of cysteine and adenosine triphosphate. *Anal. Biochem.* **28**, 436–445.
15. Shi H.G., Mikhaylova L., Zichittella A.E., Arguello J.M. 2000. Functional role of cysteine residues in the (Na,K)-ATPase alpha-subunit. *Biochim. Biophys. Acta.* **1464**, 177–187.
16. Hu Y.K., Eisses J.F., Kaplan J.H. 2000. Expression of an active Na,K-ATPase with an alpha-subunit lacking all twenty-three native cysteine residues. *J. Biol. Chem.* **275**, 30734–30739.

Translated by N. Onishchenko