Biochemistry (Moscow), Vol. 66, No. 8, 2001, pp. 821-831. Translated from Biokhimiya, Vol. 66, No. 8, 2001, pp. 1013-1025.

REVIEW

Na/K-ATPase as an Oligomeric Ensemble

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Received January 29, 2001
Revision received April 6, 2001

Abstract—Differences in the kinetic behavior and properties of monomeric and oligomeric forms of membrane-bound Na/K-ATPase are analyzed. It is concluded that enzyme molecules within oligomeric complexes are affected by extrinsic signals that result in change of enzyme activity, whereas the individual (protomeric) state is insensitive to these signals. Some of the major factors of such regulation are microviscosity of the lipid environment, reactive oxygen species, and intracellular protein kinases.

Key words: Na/K-ATPase, isoforms, oligomeric enzymes, reactive oxygen species, oxidative stress, protein kinases, ATP

About 50 years ago, R. D. Keynes showed that transport of sodium and potassium ions through the cell membrane against their electrochemical gradients is required for functional activities of cells [1]. Several years later, H. J. Schatzman showed that cardiac glycosides eliminate the asymmetric distribution of sodium and potassium ions across the erythrocyte membrane [2]. He suggested that these compounds inhibited a hypothetical enzyme that actively transports the cations through the cell membrane. In 1957, J. Ch. Skou discovered this enzyme activity in nerve tissue homogenates—an adenosine triphosphate phosphohydrolase that is specifically activated by sodium and potassium ions and requires magnesium ions. The enzyme was also inhibited by ouabain and other cardiac glycosides. Skou termed it (Na+K)-activated, Mg-dependent ATPase (current name, Na/K-ATPase, EC 3.6.1.37) [3]. Skou was a pioneer in studying this enzyme. He has provided important contributions to understanding of the mechanism of energy transformation by this enzyme system. Na/K-ATPase was the first example for which the molecular mechanisms of transformation of the chemical energy of ATP into the energy of electrochemical gradient were elucidated [4]. Forty years after the discovery of Na/K-ATPase, Skou together with G. Walker and P. Boyer were awarded the Nobel Prize for their studies in the field of molecular bioenergetics.

Since its discovery, Na/K-ATPase has constantly attracted the attention of researchers, and the directions of their interest have determined the process of accumulation of knowledge in modern biology. In the 60s, the membrane-bound Na/K-ATPase system was purified and used to reconstruct "partial reactions"; in the 70s, intensive kinetic studies were performed; in the 80s, the enzyme was expressed in *Xenopus* oocytes, and it was shown that Na/K-ATPase is an intracellular target for protein kinases. Later, the primary structure of the enzyme subunits and their topography in the membrane were described, the family of enzyme isoforms was described, and the participation of Na/K-ATPase in cell-to-cell and cell-to-cytoskeleton interactions was elucidated. These data on cellular functions of Na/K-ATPase have been analyzed in a number of recent reviews [5-9]. None of these, however, analyze the relationship between functional specificity and enzyme regulation and its quaternary structure. Therefore, analysis of the function of Na/K-ATPase as an oligomeric ensemble was chosen as the topic of the present article.

Na/K-ATPase consists of two polypeptide chains, the α chain (molecular mass ~112 kD) and the β chain, whose size varies in different tissues from 40 to 60 kD depending on the extent of its glycosylation (the molecular mass of the protein part is 35 kD). Thus, the molecular mass of the protomer is 150-170 kD. The protomer is the functional unit of Na/K-ATPase that possesses all the main functions of the enzyme. However, several approaches have demonstrated the ability of the enzyme to form supramolecular complexes within the membrane bilayer.

Maunsbach et al. [10] showed that Na/K-ATPase could form aggregates; the protein molecules form dimers and higher oligomers (up to octamers) in the membrane bilayer in the presence of potassium, while sodium prevents such oligomerization. The formation of enzyme oligomers has been demonstrated by several

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methods including cross-linking of the subunits by *o*-phenanthroline [11], gel filtration of the enzyme after detergent solubilization [12], and measurement of time-resolved phosphorescence decay of labeled membrane-bound enzyme [13]. However, these approaches cannot explain the functional significance of the oligomers that form.

A significant amount of data on the functional activity of oligomers has been obtained from analysis of kinetic cooperativity of the interaction of the enzyme with ATP [14, 15]. Disordering the inter-protomeric interactions by treatment of the enzyme samples with detergent or other chaotropic factors prevents positive cooperativity for the substrate [15]. Kinetic studies are very useful for making choices between different mechanisms. In this case, kinetic studies are a productive tool for comparison of enzyme properties when it operates within the oligomeric complex or as independent protomers. Calculation of Hill coefficient, n_H, for ATP and for activating ions, sodium and potassium, showed distinct positive cooperativity for these ligands under optimal conditions with n_H values of 3.4, 2.3, and 1.6 for ATP, Na^+, and K^+, respectively [16]. At low (micromolar) concentrations of ATP, where the substrate does not modify the reaction, the positive cooperativity of ATP becomes negative, i.e., $n_H < 1$. When the enzyme sample was treated with digitonin, which prevents protomer–protomer interaction, the Hill coefficient for activating ions did not change but that for ATP decreased to <1 in spite of high (saturating) ATP concentration. From these experiments, it was concluded that cooperativity for the ions transported by the enzyme occurs at the level of a single protomer, while positive cooperativity for ATP is based on the interaction between several protomers; negative cooperativity for ATP does not require protomer–protomer interactions and can be explained by "relaxation kinetics" [17]. This explanation is consistent with the reaction scheme suggested by L. C. Cantley [18].

Na/K-ATPase is well known to possess no absolute specificity to the substrate: in addition to ATP, it can hydrolyze other nucleoside triphosphates and several organic phosphoethers. When UTP or GTP was used instead of ATP, no cooperativity was found over a broad range of substrate concentrations, which indicates a specific role of ATP not only as the source of energy for ion translocation but also a modifier of the enzyme [4]. This feature focused on the shape of the substrate dependence curve during hydrolysis of ATP by Na/K-ATPase.

The dependence of the enzyme activity on ATP concentration is described by a curve with an intermediate plateau, and this feature was found for enzymes at different levels of purification and prepared from different tissues, including brain, heart, kidney, and salt gland [15, 19, 20]. Such substrate dependence might result from the summation of two components, one being close to hyperbolic (while precise analysis showed the presence of negative cooperativity [5, 21]) and another sigmoid component whose shape reflects the modifying action of high ATP concentrations (Fig. 1a). After enzyme solubilization, the sigmoid component disappears and the overall curve becomes nearly hyperbolic (Fig. 1b).

Analyzing conformational lability of the enzyme by measuring the transition between the potassium and sodium conformations (E2–E1), Skou found that the transition depends on pH in such a way that proton dissociation from a group conceivably located near the active center of the enzyme is able to accelerate the E2–E1 transition. Skou named this phenomenon the "Bohr effect" [22] in analogy with the pH effect on hemoglobin conformation and its affinity to oxygen noted by Bohr earlier. Differences in the structure of nucleotides inducing (ATP) and not inducing (UTP, GTP) non-Michaelis behavior of the ATPase consist in the ability (ATP) or inability (UTP, GTP) to facilitate proton dissociation from a specific group of the active center. This group was later identified by P. L. Jorgensen as the His-13 residue located in the N-terminal domain of the α-subunit (see [23]). Thus, the well-known ability of high ATP concentrations to facilitate the E2–E1 transition and modification of the substrate dependence of Na/K-ATPase connected with this property can be explained by the readiness of the substrate molecule to stimulate proton dissociation from His-13 because of its transient binding to the unprotonated N_1-atom of the pyrimidine ring of ATP. The corresponding nitrogen atoms in UTP and GTP molecules at physiological pH are fully protonated.

Use of synthetic analogs of ATP with different proton accepting ability of the nitrogen atom (N_1-methoxy-ATP having no such ability, N_1-carboxymethoxy-ATP characterized by partial ability to bind H^+, and N_1-oxy-ATP possessing proton accepting capacity comparable with that of ATP) strongly supported the above-mentioned point of view [24]. The ability of these substrate analogs to modify the so-called "K-phosphatase reaction" eliminating sodium-induced inhibition of *p*-nitrophenyl phosphate hydrolysis depended on their proton accepting capacity: activation of the phosphatase in the presence of potassium ions and the ligands tested changed in the following way: N_1-methoxy-ATP (no effect) < N_1-carboxymethoxy-ATP < N_1-oxy-ATP (the two latter ligands have similar efficiency). Moreover, CTP, whose N_3-atom also possesses proton-accepting capacity (the p*K* values for ATP and CTP are 3.5 and 4.2, respectively, whereas the value for UTP and GTP is 9.2) at physiological pH, can also modify the phosphatase reaction [24], accelerate the E2–E1 transition [25], and change the substrate dependence from Michaelis–Menton kinetics [26].

All these data suggest that ATP can modify Na/K-ATPase by transient facilitation of dissociation of a pro-

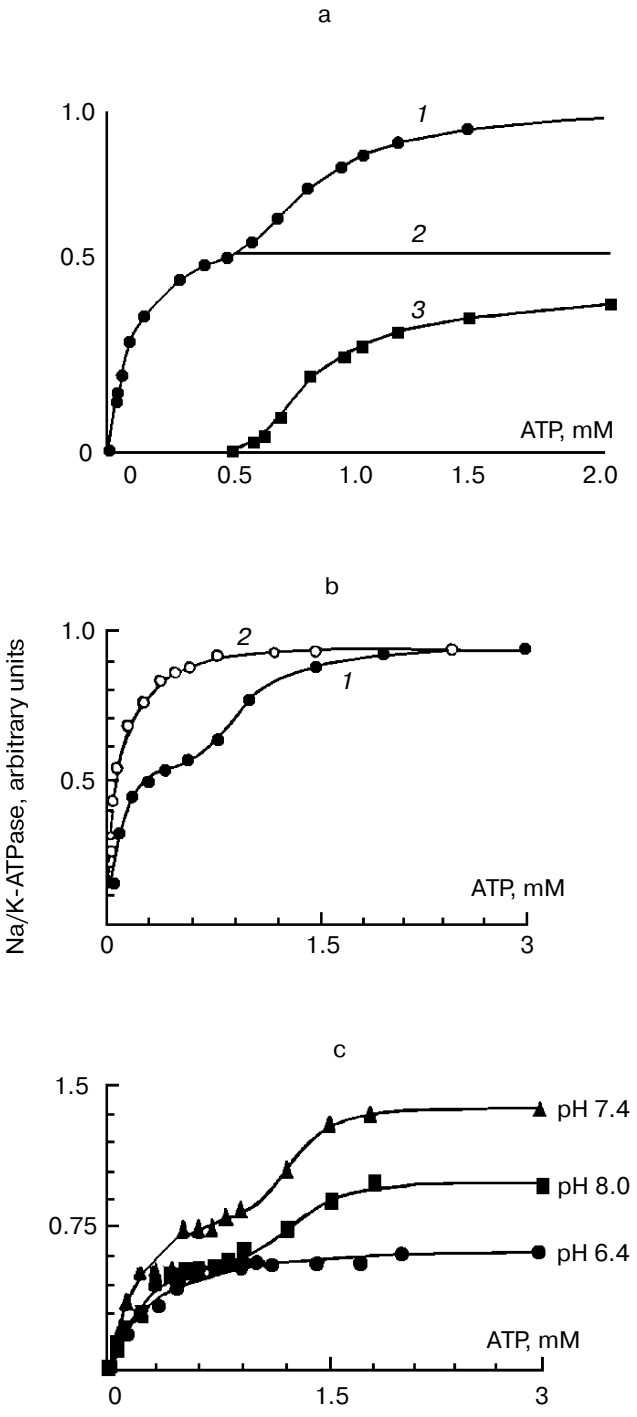

Fig. 1. Dependence of Na/K-ATPase activity on ATP concentration under different conditions: a) normal experimental curve with a marked intermediate plateau (*1*) consisting of sigmoid (*2*) and nearly hyperbolic (*3*) curves; b) normalized substrate dependence for the enzyme under normal conditions (*1*) and after solubilization of the enzyme with $C_{12}E_8$ (*2*); c) substrate dependence for native enzyme measured at different pH values.

ton from His-13, and this in turn initiates the conformational transition. Because the sodium and potassium conformer protomers have different tendency to oligomerize, ATP must influence the formation of oligomers during the reaction cycle. In other words, the substrate will be able to regulate the function of the enzyme, increasing the efficiency of the sodium pump. In fact, if the protomers in an oligomeric ensemble operate with a time shift in relation to their neighbors (thus working in a synphasic manner), the superposition of the endergonic step at one protomer with an exergonic step at another will result in more effective use of the energy of ATP [27].

An important argument in favor of proton dissociation from a particular protein group as a reason for the substrate dependence was found from the pH dependence of ATP hydrolysis presented in Fig. 1c. It was found [28] that alkalinization of the medium to pH 8.0 decreased the rate of ATP hydrolysis by the enzyme but did not change the shape of the curve, while acidification of the medium (to pH 6.4) removed the intermediate plateau, making the curve close to that obtained with UTP or GTP being hydrolyzed under optimal conditions.

Kinetic studies have revealed a number of illustrations of the functional importance of Na/K-ATPase oligomers; the final decision, however, required direct evidence in favor of this hypothesis. This evidence was obtained using time-resolved phosphorescence decay measured when Na/K-ATPase was labeled with eosin-5′-isothiocyanate [13]. After labeling the Na/K-ATPase prepared from duck salt glands, the label was found only in the electrophoretic band related to the α-subunit. The labeling caused a partial decrease in the enzyme activity. After labeling, short-term excitation of the sample with a laser beam was accompanied by a phosphorescence signal whose decay in time was measured thoroughly (256 measurements during 500 μsec). The experimental data were described well by a three-exponential curve [29]. A fast component (with rotation correlation time of about 10-14 μsec) corresponded to the behavior of particles with radii of ~2.4 nm, which correlated well with the calculated size of the (α + β)-protomer (approximated as a cylinder). This value was not affected by temperature, pH, or ligands. The second and third components reflected slower rotation and are related to the mobility in the membrane of larger complexes. The amplitude of the third component was small and varied from one enzyme preparation to another. This component was removed by addition of $C_{12}E_9$. This was suggested to belong to aggregates of large size and low mobility [29]. The second component of the experimental curve having rotational correlation time of several hundred microseconds corresponds to particles with radii varying between 7 and 13 nm depending on the presence of sodium or potassium (Table 1). These particles were thought to reflect the presence of rotating Na/K-ATPase oligomers within the membrane.

Table 1. Apparent radii (nm) of duck salt gland Na/K-ATPase calculated from phosphorescence anisotropy of the enzyme labeled by eosin-5′-isothiocyanate [45]. The measurements were performed at 20°C in the presence of 10 mM KCl (K-conformer) or 100 mM NaCl (Na-conformer)

pH	K-conformer		Na-conformer	
	"fast" component	"slow" component	"fast" component	"slow" component
6.0	2.4	13.2	2.2	6.9
6.5	2.5	12.2	2.4	7.9
7.0	2.4	11.0	2.4	7.7
7.5	2.4	9.8	2.3	8.4
8.0	2.4	10.3	2.4	9.4
8.5	2.3	9.3	2.4	9.7

Measurement of phosphorescence anisotropy of the labeled enzyme demonstrated that near the pH optimum for Na/K-ATPase (pH 7.5), the "slowly rotating" oligomers in the potassium (E2) or sodium (E1) forms are close to each other in their size (9.8 and 8.4 nm) but reacted on change in medium composition in different ways. For example, decrease in temperature did not change significantly the apparent size of the Na-conformer but significantly increased that of the K-conformer, which resulted in an increase in rotational correlation time. Acidification of the medium from 8.0 to 6.0 acted in the same manner, and the size of the Na-conformer decreased from 8.4 to 6.9 nm, while that of the K-conformer increased from 9.8 to 13.2 nm. Addition of ATP increased the mobility of oligomers approximately to the same value, thus having decreased the apparent size of the oligomers both in sodium and potassium medium, and this effect was more pronounced in the latter case because the starting size of the K-conformer was larger [29].

It is difficult to decide whether change in apparent size of oligomers is induced by variation of the number of protomers in the oligomers or by a change in the density of their packing. In terms of relatively small variations in particle radii, the latter is more probable. In other words, tightness of inter-protomer interactions varies through a single cycle: the oligomer is less compact when potassium binds and more compact after sodium and/or ATP bind. This property of Na/K-ATPase makes it sensitive to the microviscosity of the lipid environment, and the consistency of the lipid package may regulate the conformational lability of the enzyme.

The specificity of the interaction between protomers within the oligomeric enzyme ensemble was also demonstrated in studies on radiation inactivation of Na/K-ATPase. Frozen samples of the enzyme were exposed to a monoenergetic neutron beam from a cobalt source, which resulted in a dose-dependent decrease in enzyme activity measured after the exposure in the thawed samples. The curve obtained was characteristic of exponential dependence with irradiation and presentation of the activity versus dose in semilogarithmic coordinates was used to calculate the apparent molecular size of the irradiated target. This approach is known in the literature as the "molecular target method" [30, 31].

Using this approach, the apparent size of the Na/K-ATPase molecule during its operation can be calculated (Table 2). The data from these experiments showed that the apparent size of the enzyme depended on the conditions of enzyme functioning. As noted above, the molecular mass of the Na/K-ATPase protomer is close to 150-170 kD, and significant deviation from this value toward larger size can be considered as evidence in favor of inter-protomer interaction during enzyme operation. As seen from Table 2, during the hydrolysis of GTP the calculated molecular mass of the enzyme is 140 kD, which is close to the calculated size of the protomer (in other words, when enzyme protomers hydrolyze GTP they do not interact with each other). The same value was obtained when 30 μM ATP was used, a substrate concentration which induces negative rather than positive cooperativity. This fact confirms that inter-protomer interaction is not required for negative cooperativity, which can occur in isolated protomers following "relaxation kinetics" [17]. At the same time, increasing the ATP concentration to the level inducing positive cooperativity (3 mM) significantly elevates the apparent molecular mass of the enzyme, involving single protomers into oligomeric ensembles (Table 2).

The actual size of such enzyme ensembles is difficult to clearly determine because in different studies various methods of measurements and various properties (and sources) of enzyme were used; this may account for apparent inconsistencies in the data and conclusions. For kidney enzyme containing α1 and β1 subunits, stud-

Table 2. Apparent size of the duck salt gland Na/K-ATPase under different conditions calculated by the molecular target method (measurements were made at 37°C except where indicated otherwise)

Substrate, mM	Reaction performed	Molecular target size, kD
ATP, 3 mM	Overall reaction	240 ± 15
ATP, 3 mM	Overall reaction (10°C)	360 ± 10
ATP, 30 μM	Overall reaction	138 ± 10
GTP, 3 mM	Overall reaction	140 ± 20
p-NPP, 10 mM	K-phosphatase	90 ± 10
ATP, 3 mM (no K^+ added)	Na-ATPase	95 ± 10

ies on both ATP binding [14] and kinetic cooperativity for the substrate [32] suggest that each oligomer consists of two protomers. For brain ATPase, which is characterized by a number of different isoforms, n_H for ATP suggested four protomers in the oligomer [15]. For Na/K-ATPase prepared from duck salt gland ($\alpha_1 + \beta_1$), the maximal number of protomers in an oligomer calculated from the sigmoid component of the substrate curve is between 5 and 8 [19]; the latter coincides well with the maximal number of protomers in two-dimensional crystals of the enzyme from mammalian kidney [10]. For the duck salt gland enzyme, the molecular target method gives data corresponding to dimeric (diprotomeric) enzyme structure under optimal conditions. Decreasing the temperature from 37 to 10°C increased the apparent size of the target (Table 2). The diversity of results obtained by several authors might be explained by differences in methods, experimental conditions, sources of the enzyme, and methods for its preparation. Nevertheless, the functional significance of oligomeric structure of Na/K-ATPase is clear, especially in terms of feature of the molecular target method, which estimates the actual molecular size of the protein during its functioning.

This approach clearly showed small differences in the size of the functional oligomer under various experimental conditions, giving additional information on the molecular properties of Na/K-ATPase. As seen from Table 2, the apparent molecular mass of the protein performing the so-called partial reactions "Na-ATPase" or "K-phosphatase" is smaller than that of the protomer and close to the size of the α-subunit. However, the overall cycle of ATP or GTP hydrolysis is performed by a protein with apparent size close to that of the (α + β)-protomer. This suggests that the β-subunit takes part in the overall hydrolytic cycle, but it is not essential for the partial reactions. Moreover, from the generally accepted reaction scheme [18], a step is predicted where the participation of the β-subunit is essential, and this step is the E2–E1 conformational transition because it is the only step not participating in the partial reactions measured in the absence of activating ions: sodium for K-phosphatase and potassium for Na-ATPase.

Careful analysis of the diversity in molecular size of the enzyme performing the partial reactions or the overall hydrolytic cycle under optimal conditions (in the presence of a modulating concentration of ATP, 3 mM) suggests that the partial reactions are carried out with no protomer–protomer interactions, but that the overall cycle includes a step where the protomers are interacting. This step is the E2–E1conformational transition. It should be noted that this step is influenced by ATP, resulting in acceleration of the overall ATPase cycle. Consistent with this idea, the high apparent molecular mass of the native enzyme is decreased to the size of the protomer (130 kD) after disrupting protein–protein interaction by the detergent $C_{12}E_8$, even when ATP is used as the substrate [19].

Based on these data, it was suggested that during the regular Na/K-ATPase cycle (under optimal conditions), potassium ions induce the formation of short-lived oligomers at the step of transition of the K-conformer into the Na-conformer, and that ATP accelerates their dissociation. This explains the molecular mechanism by which ATP decreases the affinity of the enzyme for potassium and increases it for sodium [19, 33]. Thus, the loosening of the oligomeric ensemble by potassium ions described above is a step making the oligomers ready for independence and resulting in the substitution of potassium by sodium. It is quite possible that such loosening of the oligomer facilitates the conformational transition, which in turn provides for dissociation of the ions from sites where they were occluded. Such a step can be accelerated by ATP due to transient dissociation of the proton from the His-13 residue of the α-subunit inducing the E2–E1 transition.

All these processes are accompanied by changes of molecular size and packing (density) of both the oligomer and the protomer itself that are reflected in variation of apparent size of the molecular target depending on different conditions (see Table 2). This scheme is in good agreement with many known facts that also explain the molecular mechanism of activation of Na/K-ATPase by high concentrations of ATP, resulting in deviation of the shape of the substrate curve (see Fig. 1a). Another explanation is also possible: if two interconvertible forms of the enzyme are present in the membrane—protomeric and oligomeric—and increase in ATP concentration results in transformation of less active protomers into more active oligomers [22].

Independently from the molecular mechanisms discussed, the most significant feature is the inconstancy of inter-protomer interactions. This is clearly demonstrated by comparison of the apparent target size measured under varying conditions of enzyme functioning. As seen from Table 2, decrease in temperature increases the size of the molecular target, thereby indicating that protomers are undergoing oligomerization. This again suggests the importance of the lipid environment whose microviscosity (if it increases) can fix the enzyme molecules in the oligomeric state. It is quite possible that other factors affecting membrane properties (clustering of acidic phospholipids by calcium ions, effects of membranotropic agents, etc.) will affect the properties of the enzyme like the change in temperature does.

Moreover, the dependence of enzyme activity on inter-protomer interactions can be used to estimate the energetic status of the cell. In a living cell, a gradient of ATP exists directed from the energetic center (where mitochondria are located) to the periphery of the cell; this gradient changes depending on oxygen supply and other conditions. For this reason, enzymes located in the outer cell membrane, like Na/K-ATPase will be especially sensitive to energy deficit. For Na/K-ATPase, it will cause reversible suppression of the activity because of the "sliding" of substrate level along the substrate curve toward decreased activity (Fig. 1a). In terms of kinetic parameters measured earlier, the K_m of the protomers for ATP is 70 μM, and K_H, substrate concentration providing semi-maximal activation, is 0.7 mM [19]. Thus, the high affinity of protomers to ATP makes Na/K-pump operation possible even under sharp decrease in the cellular ATP level when inter-protomer interactions are weakened. Such partial switching the Na/K-pump off is reversible, and restoration of the cellular pool of ATP quickly restores the normal efficiency of the pump.

Another mode of regulation of the enzyme is its phosphorylation by protein kinases. It has been demonstrated in several laboratories that Na/K-ATPase is a target for at least two cellular protein kinases, cAMP-dependent protein kinase A and (Ca+phospholipid)-dependent protein kinase C [34]. In pig kidney Na/K-ATPase, protein kinase A phosphorylates the Ser-938 residue which is localized in the loop between 8th and 9th transmembrane domains which is exposed to the cytoplasm. This phosphorylation occurs *in vitro* only in the presence of detergents. Depending on experimental conditions, such modification can inhibit or stimulate Na/K-ATPase activity or even have no effect on it [5]. It is quite possible that this depends on enzyme conformation and/or on the accessibility of Ser-938 for protein kinase A.

Protein kinase C phosphorylates serine residues located in the rat kidney enzyme in the N-terminus of the α-subunit (Ser-11 and Ser-18, numbering corresponding to the α1-subunit of rat kidney), the intensity of labeling of the enzyme from several sources being significantly different [9, 34]. In duck salt gland Na/K-ATPase, besides serine, a threonine residue is also phosphorylated by this kinase [5]. In all cases, the effect of protein kinase C results in inhibition of the enzyme activity [34]. No publications have discussed whether the kinetic properties of Na/K-ATPase phosphorylated by protein kinases were modified, but one intriguing fact was noted. In the presence of the Na/K-ATPase α-subunit, protein kinase C gained the ability to be self-phosphorylated [34]. This illustrates that Na/K-ATPase can affect other proteins resulting in modification of their properties and conformation.

A recent publication [35] showed that suppression of Na/K-ATPase activity by protein kinases is proportional to the incorporation of radioactive phosphate into the α-subunit. This suggests that protein kinases (at least protein kinase A, which was carefully studied by the authors) switch off the ATPase molecules after their phosphorylation and subsequent reactivation is possible only by the action of protein phosphatases.

It is a well-known fact that various isoforms of Na/K-ATPase are present in different tissues. There are at least four isoforms of catalytic α-subunit and clearly three β-subunits of the enzyme. The isoforms of the catalytic subunit slightly differ from each other by the pattern of their posttranslational modification, molecular mass, and affinity to sodium and potassium [5, 8]. They show distinct differences in relation to their affinities to cardiac glycosides. Na/K-ATPase prepared from kidney (predominantly the α_1-subunit) is resistant to ouabain, while the heart enzyme (containing also α_2- and α_3-subunits) demonstrates high affinity to the inhibitor [36]. Another important difference is the response to hormonal signal. As shown for adipose tissue and skeletal muscles, activation of Na/K-ATPase by insulin is explained by increased enzyme affinity to sodium, and this property is characteristic only of the α_2- (but not the α_1-) subunit [37].

It is important to note that during ontogeny and on variation of conditions of functioning, changes in the isoform spectrum of Na/K-ATPase was often demonstrated. For example, during differentiation of skeletal muscle the α1-subunit appears first; later, its amount decreases and α2 appears, the latter progressively substituting the former as differentiation progresses [38]. In human heart after ischemic injury, a change of isoform pattern was also demonstrated, resulting in an increased share of α1, which is more resistant to ouabain [39].

Because oxidative stress is one of the factors of oxygen metabolism disorders accompanied by a rise in reactive oxygen species (ROS), it was important to study the effect of free radicals on different isoforms of the enzyme. It was shown that dog kidney enzyme (α1) provides a lower rate of ATP hydrolysis, decreased affinity to potassium, and slower rate of E1–E2 conformation tran-

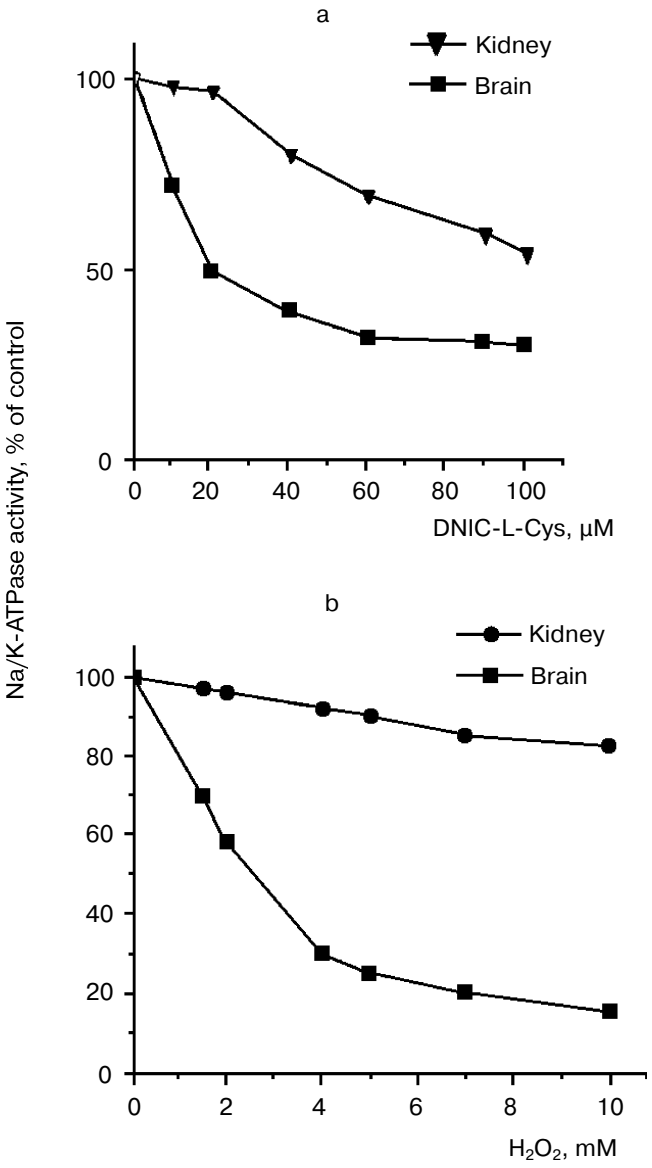

Fig. 2. Comparison of oxidative resistance of Na/K-ATPase from kidney and brain. Enzyme activity was measured after 30-min incubation of the samples with dinitrosyl-Fe-cysteine complex (DNIC-L-Cys) (a) or with hydrogen peroxide (b).

sition after its oxidative modification [40]. Comparison of sensitivity to oxidation of Na/K-ATPase from kidney preferably containing the α_1-subunit and from brain having also α_2- and α_3-subunits showed that the α_1-isoform is much more resistant to free radical oxidation, whereas the α_2- and α_3-subunits easily lose their activity after oxidation (Fig. 2). Sulfhydryl groups of the protein are oxidized and decrease in enzyme activity correlates well with their disappearance [41], while addition of ascorbate [41], cysteine, or DTT [42] after oxidative modification of the enzyme results in nearly total restoration of both parameters. Because different isoforms of Na/K-ATPase do not significantly differ in the number of cysteine residues (23 in α_1, 24 in α_2, and 24-25 in α_3), higher sensitivity of the brain enzyme compared to that of the kidney enzyme can be related rather to their location along the polypeptide chain rather than their number. It is reasonable to suggest that SH-groups of the α_2- and α_3-subunits are more readily oxidized. In fact, in the primary structure of α1 there are fewer sulfhydryls exposed to the cytoplasm [43].

It is of particular interest that after oxidative modification the substrate dependence of the enzyme is drastically changed. The curve with an intermediate plateau is transformed into a nearly hyperbolic curve, as was found after acidification of the medium or solubilization of the enzyme preparation. In all cases inter-protomer interactions became disordered, suggesting that S-S bridge formation induces changes in protein structures hindering protomer–protomer interactions. These areas responsible for the oligomer stability are located in the loop between the M4 and M5 columns [44] and between the M1 and M2 and between the M8 and M10 columns [46]. These areas contain easily oxidized cysteine SH-groups.

Both acidosis and increase in ROS production are typical features of brain metabolism when the blood supply is impaired and brain ischemia occurs. To determine whether these conditions modify Na/K-ATPase, a comparative study was carried out using a model of experimental ischemia in rat brain (Table 3). It was found that the enzyme prepared from normal rat brain showed the usual dependence on ATP concentration expressed as a curve with an intermediary plateau. From the "Michaelis part" of this curve, the maximal rate of ATP hydrolysis ($V_{maxMich}$) and K_m for ATP were calculated, whereas from

Table 3. Kinetic parameters of Na/K-ATPase prepared from rat brain before and after 15-min occlusion of the *arteria carotis communis* with subsequent 60-min reperfusion [47]

Parameter	Control	After experimental ischemia
$V_{maxMich}$, nmol/min per mg	1315	1520
$V_{maxHill}$, nmol/min per mg	325	—
K_m, μM	260	235
K_H, μM	155	—
Hill coefficient for ATP, n_H	8	1
Correlation coefficient, n	0.986	0.995

the sigmoid component of the curve $V_{maxHill}$ and $K_{0.5}$ characteristic of the modulating action of ATP were determined. In contrast, enzyme prepared from brain after ischemic stroke was characterized by a nearly hyperbolic substrate dependence and no cooperativity for ATP (the Hill coefficient was $n_H = 1$). This curve was similar to the "hyperbolic component" of the curve obtained for control brain, demonstrating that oxidative modification of the enzyme affects inter-protomer interactions rather than the catalytic properties of the protomers themselves (Table 3). This suggests that under *in vivo* conditions the oxidative modification of the enzyme suppresses its activity because of weakening of inter-protomer interactions.

ROS generation increases in neurons in response to the action of various unfavorable factors resulting in their death. Na/K-ATPase supports the stability of ionic homeostasis, thus preventing neuronal death. From this point of view, it seems strange that this enzyme is a target for ROS and its neuronal isoforms ($\alpha 2$ and $\alpha 3$) possess higher sensitivity to oxidative modification (Fig. 2). The only reasonable explanation is that modification of Na/K-ATPase by ROS has regulatory (adaptive) sense and appears not only under extreme conditions, but also during normal neuronal function.

It is well known that activation of the glutamatergic system by specific ligands in cerebellar neurons is accompanied by elevation of intracellular ROS level because of stimulation of some metabolic reactions [48, 49]. It is important to know whether Na/K-ATPase takes part in realization of these signal mechanisms, in other words, if the enzyme is sufficiently sensitive to the changes of intracellular ROS level when glutamate receptors of neuronal membrane are activated. This problem was studied in our laboratory by E. Bulygina, M. Yuneva, and L. Lyapina. The neurons carefully prepared from cerebella of young (12-14 days old) rats or mice surviving as homogenous suspensions were used for this study [49, 50]. Generation of intracellular ROS induced by exposing the cells to agonists and antagonists of glutamate receptors of different types was measured using the flow cytometry with a fluorescent label for ROS, 2′,7′-dichlorofluorescein, DCF, or 1,2,3-dehydrorhodamine. It was found that ROS elevation within the neuronal space after neuronal activation is characterized by a direct dependence on ligand concentration; moreover, it can be specifically prevented by antagonists. For example, the fluorescent signal induced by kainate was averted by the kainic receptor antagonist DNQX but not by MK-801, a specific antagonist of NMDA receptors. Activation of metabotropic glutamate receptors stimulated ROS production by NMDA receptors and suppressed that by kainate receptors [51]. These data demonstrated that ROS in neurons fulfilled a signal role and the reaction of Na/K-ATPase to these signals was of special interest.

To clarify this problem, we measured the enzyme activity in neuronal suspension after their incubation with glutamate or its analogs specified for different types of glutamate receptors and subsequent disruption of the neuronal cells by freezing–thawing in order to reveal latent Na/K-ATPase. Nonspecific effects of ligands were measured after their addition to the suspension of the cells after their disruption, and the enzyme activity was tested with no preincubation (when subsequent membrane receptors were not occupied with the ligands); the nonspecific action was subtracted from the overall effect. Specific inhibition on Na/K-ATPase of the activation of neuronal cells by both glutamate and its agonists was compared and the sensitivity of the enzyme to synthetic analogs of glutamate was found to be higher than that to glutamate itself [52]. It is seen from Fig. 3a that $K_{0.5}$ for the specific action of glutamate on Na/K-ATPase is 175 μM, whereas that of ACPD, an agonist which activates metabotropic receptors, is only 50 μM. Maximal inhibition of the enzyme by glutamate resulted in 35% decrease in Na/K-ATPase activity, which is undoubtedly significant for survival of neurons. It was shown that under experimental brain ischemia inhibition of Na/K-ATPase in the ischemic area amounted to 24% in 30 min, which resulted in significant damage to tissue; a similar effect was achieved by the application of ouabain [53].

Further research showed that specific inhibition of neuronal Na/K-ATPase takes place after activation of both ionotropic and metabotropic receptors. Different classes of glutamate receptors are used in cells for a diversity of functions: ionotropic receptors are connected with ionic channels and provide excitation potential after opening of these channels and ionic fluxes (Na^+ and K^+ in the case of kainate or AMPA-receptors and Na^+, Ca^{2+}, and K^+ in the case of NMDA-receptors). The function of metabotropic receptors associated in the membrane with G-proteins is regulation of the above-mentioned ionic channels via metabolic reactions including protein kinases, phospholipases, and other regulatory proteins controlled by G-proteins (see [51, 54]). From this point of view, it was reasonable to suggest that mechanisms of inhibition of Na/K-ATPase mediated by metabotropic or ionotropic receptors differ from each other. In fact, Fig. 3b shows the inhibiting effect of NMDA is fully prevented by its antagonist, MK-801, but only partially by the protein kinase C inhibitor chelerythrine. However, the inhibitory effect of the metabotropic receptor agonist ACPD is totally prevented by both its antagonist MCPG and chelerythrine. Thus, activation of metabotropic receptors involving G-proteins and protein kinase C results in enzyme inhibition because of its regulatory phosphorylation, and this effect is prevented by switching off protein kinase C. The inhibiting effect of NMDA occurs only partially in this way and to an extent that is determined by calcium

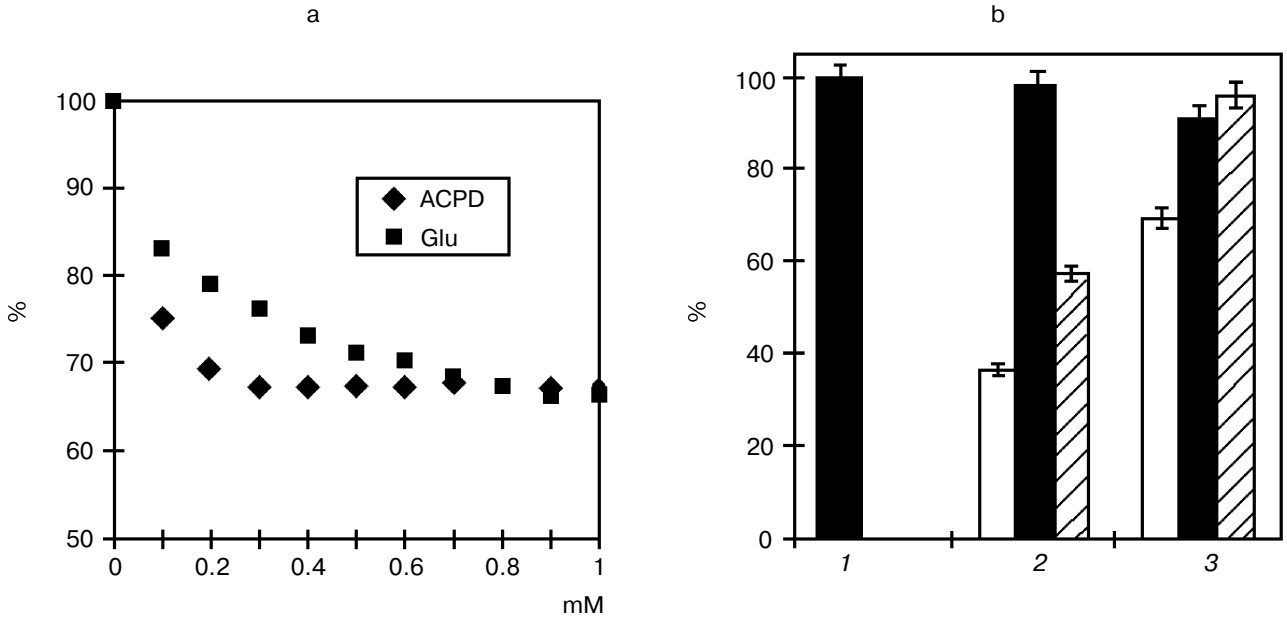

Fig. 3. Na/K-ATPase activity measured under optimal conditions after 30-min incubation of neuronal suspension with different ligands and subsequent disruption of the cell membrane [47]. a) Dependence of enzyme activity on concentration of glutamate (Glu) or activator of metabotropic receptors (ACPD); b) comparison of Na/K-ATPase activity under control conditions (100%, *1*) or in the presence of activator of ionotropic (NMDA, *2*) or metabotropic (ACPD, *3*) receptors: first bar, in the presence of agonist only; second bar, in the simultaneous presence of agonist and followed by antagonist (*2*, MK-801; *3*, MCPG); third bar, in the presence of agonists and the protein kinase C inhibitor chelerythrine.

ions entering through the NMDA-dependent ion channels. Most of the inhibiting effect of NMDA is presumably connected with intracellular ROS, which oxidize SH-groups of proteins, this suggestion being supported by the ability of cysteine to restore Na/K-ATPase activity after incubation of the neurons with NMDA [52].

Thus, we can conclude that in living cells there are several mechanisms of Na/K-ATPase suppression: ROS remove the activating effect of ATP by disturbing protomer–protomer interactions, whereas protein kinases remove from the active state that part of ATPase molecules which are subjected to regulatory phosphorylation. Both inhibiting effects are reversible, and the reversibility depends on the current situation within the cell—presence of reducing equivalents or antioxidants in the former case and active protein phosphatases in the latter.

Consequently, the activation of several types of glutamate receptors results in the generation of different signals informing Na/K-ATPase about the current state of the cell membrane, and the pattern of these signals depends on the concentration of glutamate released into the synaptic cleft. Moreover, sensitivity of different types of receptors to glutamate is varied and also depends on the excitability of the synaptic membrane. As seen for enzyme preparations isolated from adult brain, glutamate can demonstrate a dual effect: inhibition of Na/K-ATPase at high (300-750 μM) and activation of the enzyme at lower (<100 μM) concentrations [55-57]. Under normal functioning of glutamatergic synapses, the concentration of the neuromediator in the synaptic cleft varies from 10 μM to several mM [54]. This suggests that the described mechanisms of Na/K-ATPase regulation form gradually during ontogeny and operate not only under extreme conditions (regulating neuronal viability), but also during normal functioning of neuronal cells.

Recently Amir Askari and his coworkers obtained convincing evidence demonstrating the participation of ROS in the interaction of Na/K-ATPase with other cell proteins [58, 59]. It was found that inactivation of Na/K-ATPase of cultured myocytes by ouabain immediately increases intracellular Ca^{2+} level, results in activation of MAP kinase and tyrosine kinase, the phosphorylation of a number of intracellular proteins with molecular mass up to 200 kD, and selective gene expression. Decrease in outer potassium or addition of ionomycin, both factors leading to increase in ionized calcium in the cytoplasm, do not substitute for ouabain addition. Moreover, removal of calcium from the outer medium did not prevent the effect of ouabain on the ROS level. From these experiments it was concluded that inhibition of Na/K-ATPase not only affects ionic homeostasis inside cells, but it also prevents its inhibiting action on specific transducing protein whose activation switch on a chain of metabolic reactions regulating protein synthesis in the cells [59]. This

means that regulation of cellular metabolism involving Na/K-ATPase is taking place during both formation of Na/K-ATPase homo-oligomers and the interaction of Na/K-ATPase protomers with the other cellular proteins, for example protein kinases, which can explain the effect of Na/K-ATPase on protein kinase C resulting in its self-phosphorylation [30].

The data presented here demonstrate that Na/K-ATPase is not only the target for oxidative stress but also the sensor providing cellular sensitivity to the signal function of ROS, and the oligomeric organization of the enzyme in the membrane is involved in the realization of such signal effects.

This work was supported by the Russian Foundation for Basic Research (grant Nos. 99-04-4999430 and 00-04-48767). I give cordial thanks to O. D. Lopina, E. R. Bulygina, M. O. Yuneva, L. A. Lyapina (Lomonosov Moscow State University, Moscow), N. M. Vladimirova (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow), Natalia Fedosova (Aarhus University, Aarhus, Denmark), David Carpenter (SUNY, Albany, NY, USA), Amir Askari (Toledo Medical College, OH, USA), and Peter Johnson (Ohio University, Athens, OH, USA) for useful discussions.

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