

S-Glutathionylation of the Na,K-ATPase Catalytic α Subunit Is a Determinant of the Enzyme Redox Sensitivity*

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Background: Na,K-ATPase activity is extremely sensitive to changes in the redox state.

Results: Binding of glutathione to the regulatory cysteine residues of the catalytic subunit completely inhibits the Na,K-ATPase by blocking the ATP-binding site.

Conclusion: S-Glutathionylation of the catalytic subunit is revealed as a mechanism controlling the Na,K-ATPase function.

Significance: Regulatory S-glutathionylation adjusts Na,K-ATPase activity to the changes in intracellular redox state and ATP levels.

Na,K-ATPase is highly sensitive to changes in the redox state, and yet the mechanisms of its redox sensitivity remain unclear. We have explored the possible involvement of S-glutathionylation of the catalytic α subunit in redox-induced responses. For the first time, the presence of S-glutathionylated cysteine residues was shown in the α subunit in duck salt glands, rabbit kidneys, and rat myocardium. Exposure of the Na,K-ATPase to oxidized glutathione (GSSG) resulted in an increase in the number of S-glutathionylated cysteine residues. Increase in S-glutathionylation was associated with dose- and time-dependent suppression of the enzyme function up to its complete inhibition. The enzyme inhibition concurred with S-glutathionylation of the Cys-454, -458, -459, and -244. Upon binding of glutathione to these cysteines, the enzyme was unable to interact with adenine nucleotides. Inhibition of the Na,K-ATPase by GSSG did not occur in the presence of ATP at concentrations above 0.5 mM. Deglutathionylation of the α subunit catalyzed by glutaredoxin or dithiothreitol resulted in restoration of the Na,K-ATPase activity. Oxidation of regulatory cysteines made them inaccessible for glutathionylation but had no profound effect on the enzyme activity. Regulatory S-glutathionylation of the α subunit was induced in rat myocardium in response to hypoxia and was associated with oxidative stress and ATP depletion. S-Glutathionylation was followed by suppression of the Na,K-

ATPase activity. The rat $\alpha 2$ isoform was more sensitive to GSSG than the $\alpha 1$ isoform. Our findings imply that regulatory S-glutathionylation of the catalytic subunit plays a key role in the redox-induced regulation of Na,K-ATPase activity.

Na,K-ATPase uses the energy of ATP to transport Na^+ and K^+ across the plasma membrane, thus mediating the transmembrane ion gradients responsible for the generation of the action potential in excitable tissues and for the secondary active transport of ions and metabolic substrates (1, 2). The enzyme is composed of α and β subunits. The catalytic α subunit is formed by the nucleotide binding, phosphorylation and actuator domains, and the ion transport pore. This subunit mediates ATP hydrolysis and ion transport. The β subunit is required for the enzyme translocation to the membrane and K^+ transport (1). In some tissues, the enzyme also contains a regulatory subunit belonging to the FXYD protein family. For each subunit, a number of isoforms showing tissue-specific expression patterns and functional diversity have been described (3, 4).

Na,K-ATPase is known to be redox- and oxygen-sensitive in a number of cell types (5, 6). H_2O_2 inhibits Na,K-ATPase in brain and kidneys (7). The tissue-specific $\alpha 2\beta$ isozyme is more susceptible to reduction in activity by H_2O_2 than ubiquitously expressed $\alpha 1\beta$ isozyme (8). Previously, the inhibitory action of oxidants on the Na,K-ATPase was attributed to irreversible oxidation of thiol groups. Whereas the α subunit possesses 23 reduced cysteine residues, the β subunit has only one of them (7). However, accumulating evidence suggests that redox-induced responses of the Na,K-ATPase cannot be explained by irreversible oxidation of -SH groups alone. Our earlier findings indicated that both loading of cerebellar granule cells with reduced glutathione (GSH) and GSH depletion were equally efficient in blocking Na,K-ATPase (9). Maximal activity of the enzyme in freshly isolated cerebellar neurons was only observed within a “physiological” range of pO_2 and redox state

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characteristic for neonatal rat cerebellum. Hypoxic and hyperoxic conditions and oxidative and reductive stress were associated with a decrease in Na,K-ATPase activity (9, 10). Alterations of NO production in response to hypoxia or ischemia have been shown to play a decisive role in oxygen-induced inhibition of Na,K-ATPase (10–13). Taken together, these findings suggest that Na,K-ATPase, similar to numerous other redox-sensitive enzymes (14, 15), may respond to shifts in the redox state and oxygen availability by S-nitrosylation and S-glutathionylation of the regulatory thiol groups. S-Glutathionylation of the Na,K-ATPase β 1 and FXYD subunits has been recently demonstrated (16, 17). Binding of glutathione to a single reduced cysteine residue in the regulatory β subunit caused a modest decrease in the Na,K-ATPase activity in cardiomyocytes (16). These minor effects of S-glutathionylation on the enzyme function did not explain robust redox-induced responses of the enzyme (9, 10, 12). S-Glutathionylation for any of the 23 evolutionarily conserved cysteine residues of the catalytic α subunit has never been reported. Fifteen of them are localized in the cytosolic loops of the subunit forming the ATP-binding site. These cysteine residues are potentially accessible for interaction with the cytosolic glutathione pool and for enzymes catalyzing deglutathionylation. Hence, we have hypothesized that S-glutathionylation of cysteine residues of the catalytic α subunit may be actively involved in redox-induced regulation of the Na,K-ATPase.

Using purified enzyme preparations from rabbit kidneys and duck salt glands (α 1 β 1 isozyme), and crude homogenate from isolated blood-perfused rat hearts (α 1 β and α 2 β isozymes), we have revealed the presence of basal and regulatory S-glutathionylation sites in the α subunit of the Na,K-ATPase. Binding of glutathione to the Cys-454, -458, -459, and -244 was associated with complete inhibition of the enzyme. The inhibitory action of S-glutathionylation was caused by occlusion of the adenine nucleotide-binding site by glutathione.

EXPERIMENTAL PROCEDURES

Animal handling and experimentation was approved by the Swiss Federal Veterinary Office and the Bioethic Committee of the Faculty of Biology, M.V. Lomonosov Moscow State University. Experiments were performed in accordance with the Swiss and Russian Federation animal protection laws and institutional guidelines that comply with the guidelines of the Institute for Laboratory Animal Research.

Myocardial Tissue Isolation and Handling—Wistar male rats (300–400 g) were anesthetized and heparinized, and 8–10 ml of blood and the heart were collected. The hearts were then perfused via aorta with autologous blood equilibrated with a humidified gas phase containing 20% (normoxia, hemoglobin oxygen saturation, $SO_2 = 98\%$) or 5% O_2 (hypoxia, $SO_2 = 35\%$), 5% CO_2 , and 75 or 90% N_2 , 37 °C, 1 h) (13). The hearts were then chilled and perfused with an ice-cold sodium/potassium-free isotonic buffer solution. Ventricular tissue was subsequently used to assess the Na^+ , K^+ , water content, GSH and GSSG content, and Na,K-ATPase activity (13).

Na,K-ATPase Purification and Activity Measurements—Na,K-ATPase (α 1 β 1 isozyme) was purified from duck salt glands and rabbit kidney medulla (for details see Refs. 18, 19) up

to the purity grade of 99 and 95% of total protein, respectively, as confirmed by electrophoresis. Na,K-ATPase-specific activity of the duck enzyme reached $\sim 2400 \mu\text{mol of } P_i \text{ (mg of protein} \times \text{h)}^{-1}$ at 37 °C, and in rabbit preparations was $800\text{--}1200 \mu\text{mol of } P_i \text{ (mg of protein} \times \text{h)}^{-1}$. The activity of Na,K-ATPase (α 1 β and α 2 β isozymes) from the rat heart was assessed either in the crude homogenate prepared from ventricular tissue (13) or in the sarcolemmal membrane fraction (20). Activity of the duck, rabbit, and rat Na,K-ATPase was measured as ouabain-sensitive (1 mM) ATP cleavage in the medium containing (in mM) 130 NaCl, 20 KCl, 3 $MgCl_2$, 3 ATP, and 30 imidazole, pH 7.4, 37 °C, when not stated otherwise (9, 13, 21).

Kinetics of the inhibitory action of GSSG on the rabbit Na,K-ATPase was monitored over 30 min in the presence of 25, 71.5, or 143 μM GSSG at room temperature. Samples containing 3–5 μg of Na,K-ATPase were collected every 5 min for activity measurements. An ~ 500 -fold excess of GSSG over the number of enzyme –SH groups made the Na,K-ATPase inhibition rate essentially independent of the inhibitor concentration. The interaction of GSSG with the enzyme was described by the pseudo-first order kinetics Equation 1,

$$A_t = A_0 \cdot e^{-k[GSSG]t} \quad (\text{Eq. 1})$$

where A_0 and A_t are the enzyme initial and current activity; k is the inhibition rate constant; $[GSSG]$ is the inhibitor concentration; and t is the time of exposure to GSSG. The product of k and $[GSSG]$ was then determined from the slope of the linear plot showing $\ln(A_t/A_0)$ as a function of time, and the inhibition rate constant was calculated.

Na,K-ATPase activity was assessed as a function of the GSSG concentration. The purified duck and rabbit enzyme as well as the sarcolemmal fraction isolated from the ventricular homogenate were exposed to 0.05–1 mM GSSG. Na,K-ATPase activity was then plotted against GSSG concentration and fitted using the logistic sigmoid function to obtain the values of apparent IC_{50} values (22) using Origin 7.0 (MicroCal).

Immunoblotting—S-Glutathionylation of the α 1 and β 1 subunits in purified Na,K-ATPase preparations and of the α 1 subunit in the crude ventricular homogenates and sarcolemmal fraction were assessed using immunoblotting. Proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane. After the blocking procedure, mouse monoclonal anti-glutathione antibody (Chemicon Millipore, MAB5310) was added. The membranes were then stripped, and mouse monoclonal anti-Na,K-ATPase α 1 antibody clone C464-6 (Upstate Millipore) and anti-Na,K-ATPase β 1 antibody clone C464-8 (Upstate Millipore) were applied to detect the total amount of α 1 and β 1 subunits, followed by horseradish peroxidase-conjugated secondary antibodies. Densitometric analysis was performed, and the results were expressed as α 1(β 1)-SSG/total α 1.

Isothermal Titration Calorimetry—The thermodynamic parameters of adenine nucleotide binding to rabbit Na,K-ATPase were measured using a MicroCal iTC200 instrument (MicroCal, Northampton, MA), as described elsewhere (23). Experiments with nonglutathionylated (dithiothreitol (DTT), 100 μM) and glutathionylated (GSSG, 1 mM) Na,K-ATPase were carried

out at 25 °C in imidazole buffer containing 25 mM imidazole, 1 mM EDTA, 250 mM sucrose, pH 7.5. Aliquots of the ligand (3.8 μ M, 20–30 μ M) were injected into the cell containing 2–3 μ M Na,K-ATPase to achieve a complete binding isotherm. To obtain the effective heat of binding, the heat of dilution was subtracted from the heat of the reaction. The resulting titration curves were fitted using the MicroCal Origin software, assuming one set of binding sites. Affinity constants (K_a) and enthalpy variations (ΔH) were determined, and the Gibbs energy (ΔG) and the entropy variations (ΔS) were calculated from Equation 2,

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad (\text{Eq. 2})$$

Mass Spectrometry—Cysteine residues undergoing S-glutathionylation in the duck Na,K-ATPase α 1 subunit were identified using MALDI-TOF MS. The enzyme was exposed to 1.7 mM GSH and 170 μ M GSSG for 30 min at room temperature and then incubated with SDS (5 min, 37 °C). α and β subunits were separated by SDS-PAGE in the absence of β -mercaptoethanol, and the band corresponding to the α 1 subunit was excised and subjected to an in-gel digestion by trypsin (24) or by α -chymotrypsin. For in-gel digestion, α -chymotrypsin or trypsin was dissolved in 50 mM ammonium bicarbonate buffer solution in a concentration of 30 or 13 ng/ μ l correspondingly just before use. MALDI-TOF MS analysis of the resulting peptide fragments was performed using Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany). Tryptic fragments in solution were transferred onto the MTP 384 target plate polished steel TF mass spectrometric target, dried on air, and then overlaid with a matrix solution consisting of 2,4-dihydroxybenzoic acid (201346, Bruker Daltonics) and α -cyano-4-hydroxycinnamic acid (201344, Bruker Daltonics) in concentrations of 2.4 and 3 mg/ml, respectively, in 50% acetonitrile in water, 0.1% TFA. Results of 4000 laser impulses (200 impulses from 20 different points of one spot) were summed up for every spectrum. The MS data were processed using Bruker Daltonics Flex Analysis 2.4 software, and the accuracy of mass determination of peptides was fixed to 100 ppm. Correlation of the MS data with the protein sequence was done using Bruker Daltonics BioTools 3.0 software.

Modeling—Comparison of the amino acid sequences of pig (P05024 in the UniProtKB database), duck (Q7ZYV1), rabbit (Q9N0Z6), and rat (P06685) indicates that the localization of cysteine residues is conserved in all species. This allowed us to use the existing crystallographic 3.5 Å structure of the porcine α 1 subunit of Na,K-ATPase (Protein Data Bank code 3b8e) to model the changes appearing in the enzyme after glutathionylation. Three-dimensional models of the S-glutathionylated Na,K-ATPase catalytic α 1 subunit were created on the basis of the previously published 3.5 Å structure of the porcine α 1 subunit (25). Cartesian coordinates were obtained from the Brookhaven Protein Data Bank (Protein Data Bank code 3b8e). Corresponding cysteine residues in duck, rabbit, or rat α 1 subunits are shifted upward by 2 compared with the numbering for cysteines in porcine α 1 sequence (denoted as cysteines^P). For the simulation of S-glutathionylation, GSH molecules were inserted into the protein via disulfide bridges with cysteines.

Two models have been built as follows: a model containing four glutathiones bound to the Cys-246^P, -452^P, -456^P, and -457^P residues, and a model with the ATP docked to the protein as described (26). Glutathione and ATP were inserted into protein with minimal geometric strain and no steric overlaps. Each model was subjected to energy minimization until convergence, using a combination of Steepest Descents, Conjugate Gradients, and Truncated Newton algorithms. The energy calculations were carried out under the MMFF94x force field using the MOE version 2009.10 modeling software (Molecular Operating Environment (MOE), 2011.10; Chemical Computing Group Inc., Montreal, Quebec, Canada). Then the models were superimposed using the structural alignment by MOE software.

Statistical Analysis—Values are shown as means \pm S.D. Statistical analysis was performed using GraphPad InStat 3 (GraphPad Software, Inc., La Jolla, CA). Either the Student's *t* test or one-way analysis of variance with Bonferroni post-test were applied depending on the type of experiments, and the difference was considered significant at *p* < 0.05.

RESULTS

α Subunit of the Na,K-ATPase Is S-Glutathionylated—The presence of S-glutathionylated cysteine residues in ubiquitously expressed α 1 subunit of the Na,K-ATPase was assessed using immunoblotting. Basal S-glutathionylation was observed in the α 1 subunit of the duck (Fig. 1A), rabbit (Fig. 1C), and rat (Fig. 1D) enzyme. β 1 subunit was also S-glutathionylated as shown in Fig. 1B for the duck enzyme preparation. S-Glutathionylation of the α 1, but not of β 1, subunit was further enhanced upon exposure of the enzyme to GSSG (1 mM) (Fig. 1, A–C). Treatment of the rabbit enzyme with 100 μ M DTT reduced the basal S-glutathionylation level of the α 1 subunit (Fig. 1C). Exposure of rat myocardium to hypoxia was associated with an increase in the α 1 subunit S-glutathionylation in crude ventricular homogenates up to 1.5-fold over the values observed in normoxic heart (Fig. 1D). S-Glutathionylation of the α 1 subunit of the Na,K-ATPase in hypoxic myocardium occurred concurrently with an increase of GSSG levels in tissue from 86 ± 8 to 176 ± 10 μ mol/liter tissue water. Exposure of the purified duck Na,K-ATPase to a mixture of 1.7 mM GSH and 170 μ M GSSG imitating conditions occurring in hypoxic heart resulted in complete inactivation of the enzyme.

GSSG Treatment Causes Inhibition of the Na,K-ATPase—Exposure of rabbit Na,K-ATPase to GSSG resulted in time- and dose-dependent suppression of Na,K-ATPase activity (Fig. 2A). The inhibitory action of GSSG was biphasic. Fast interaction of GSSG with the enzyme accounting for \sim 80% inhibition of the Na,K-ATPase was followed by a slow interaction phase leading to complete inactivation of the enzyme (Fig. 2B). The corresponding rate constants were $1655 \text{ M}^{-1} \text{ min}^{-1}$ for the fast and $163 \text{ M}^{-1} \text{ min}^{-1}$ for the slow interaction phases (Fig. 2B).

The GSSG concentration at half-maximal inhibition (IC_{50}) was similar for the rabbit and duck α 1 β 1 isozymes (66 ± 3 and 59 ± 2 μ M, respectively) (Fig. 3, A and B). Complete inhibition of the Na,K-ATPase by 100 μ M GSSG was observed even at the presence of 10 mM GSH. The sensitivity of the duck Na,K-ATPase to GSSG was lost after 4 h of exposure of the enzyme to

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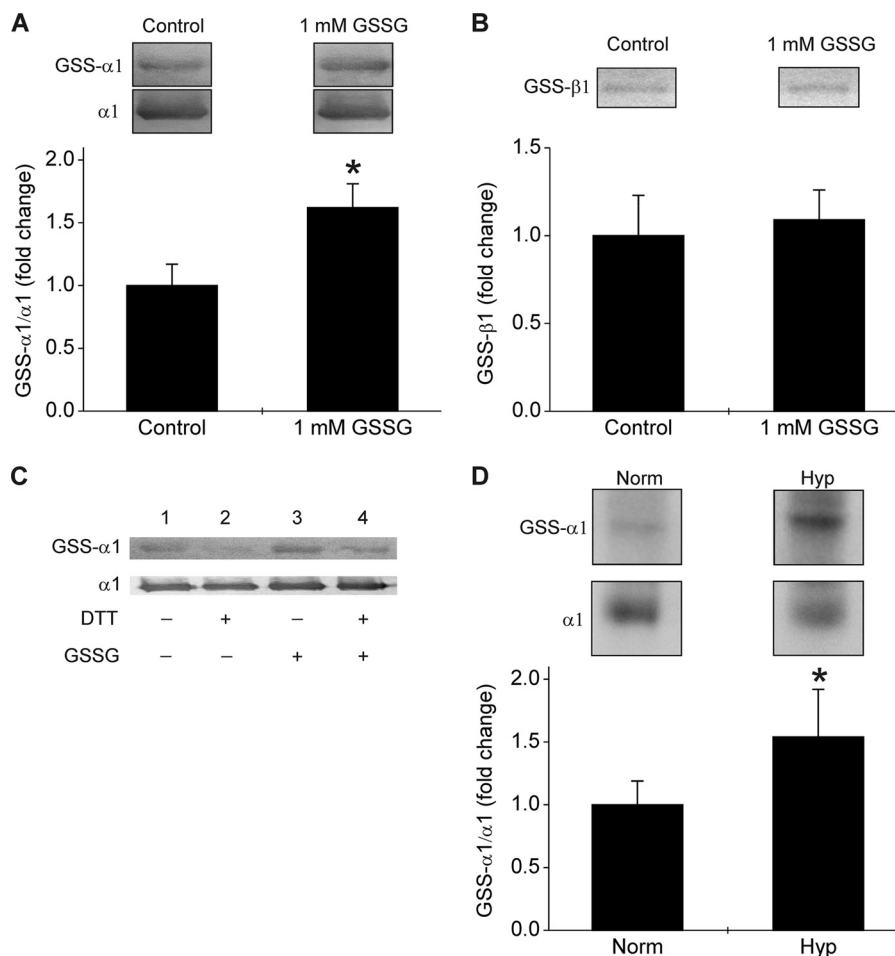


FIGURE 1. S-Glutathionylation of the α and β subunits of Na,K-ATPase. Basal S-glutathionylation and S-glutathionylation after 1 h of incubation with 1 mM GSSG for α 1 (A) and β 1 (B) subunits of the duck Na,K-ATPase. Bars represent the changes in the S-glutathionylated (GSS- α 1/ β) form of the protein normalized by its total amount. $n = 3$, mean \pm S.D. Presented above are the original immunoblotting readouts. Asterisk indicates significant differences ($p = 0.014$) relative to control as determined by the two-tailed t test. C, changes in S-glutathionylation of the α 1 subunit isolated from rabbit kidneys in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of 100 μ M dithiothreitol and thereafter exposed to 1 mM GSSG at 25 $^{\circ}$ C for 25 min (lanes 3 and 4). D, S-glutathionylation of the α 1 subunit in rat hearts perfused with normoxic (Norm, $n = 3$) or hypoxic (Hyp, $n = 5$) blood for 1 h. Bars represent the S-glutathionylated form of the protein (GSS- α 1) normalized to total amount of the α 1 protein (mean \pm S.D.). Asterisk indicates significant differences ($p = 0.0124$) relative to the normoxic heart sample as determined by one-way analysis of variance.

20% O_2 (air) at 4 $^{\circ}$ C prior to GSSG treatment (Fig. 3B). Similar desensitization to the inhibitory action of GSSG was confirmed for the rat sarcolemmal Na,K-ATPase pre-exposed to 100% O_2 for 30 min prior to GSSG treatment (Fig. 4A). The loss of sensitivity to GSSG was not associated with any profound effect on the Na,K-ATPase function (Fig. 4). Prolonged (6–9 months) storage of myocardial tissue at -80° C at 20% O_2 (air) was associated with the same loss of sensitivity of the rat Na,K-ATPase to GSSG along with preservation of its hydrolytic activity. This did not occur in tissue samples stored in liquid nitrogen (Fig. 4B).

α Subunit S-Glutathionylation Regulates Na,K-ATPase Activity in Hypoxic Rat Heart—S-Glutathionylation of the Na,K-ATPase α 1 subunit triggered by hypoxic exposure of rat heart (Fig. 1D) was associated with suppression of the enzyme function in crude ventricular tissue homogenate (Fig. 5A). Inhibition of the enzyme contributed to significant Na^+ accumulation in ventricular tissue (from 40.1 ± 4.3 to 55.0 ± 2.7 mmol kg^{-1} dry weight, $p = 0.043$) and K^+ loss (from 269 ± 9 to 222 ± 15 mmol kg^{-1} dry weight, $p = 0.031$) during hypoxic exposure.

Some of the enzyme remained inhibited by the α subunit S-glutathionylation even in normoxic myocardium because deglutathionylation (DTT exposure) was associated with only a modest increase in Na,K-ATPase activity (Fig. 5B). Induction of S-glutathionylation of the α subunit by supplementation of glutaredoxin 1 (GRX)⁴ and its substrates, NADPH and GSH, caused inhibition of the enzyme (Fig. 5B). The inhibitory effect of GRX treatment was directly proportional to the S-glutathionylation level (Fig. 5C). Depending on GSH/GSSG availability, GRX catalyzed either S-glutathionylation or deglutathionylation of the α subunit as described earlier for other GRX targets (27). Shifting the GSH levels by treating the tissue homogenate with glutathione reductase resulted in GRX-induced S-glutathionylation and the enzyme inhibition (Fig. 5C). GSH binding to cysteine residues affecting the Na,K-ATPase activity occurred only in the presence of GRX as a catalyst (Fig. 5, B and C). Deglutathionylation of the α subunit was catalyzed by GRX in a

⁴ The abbreviation used is: GRX, glutaredoxin 1.

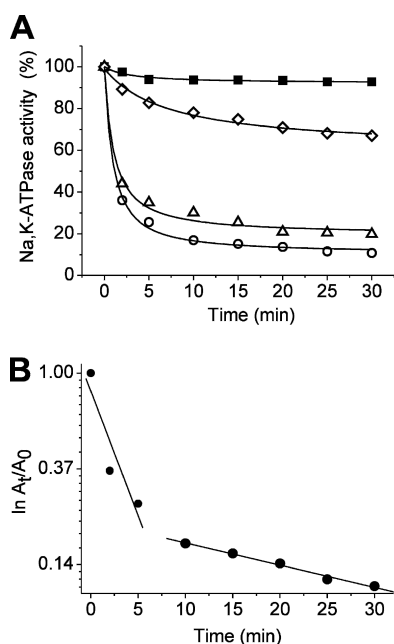


FIGURE 2. Kinetics of GSSG-induced inhibition of rabbit kidney Na,K-ATPase. A, changes in the activity of the Na,K-ATPase during the incubation in the absence (filled squares) or presence of 25 (open diamonds), 71.5 (open triangles), or 143 μ M (open circles) GSSG. Data are represented as the mean of three experiments \pm S.D. Errors are less than 2 (not shown). B, logarithm of the relative Na,K-ATPase activity A_t/A_0 was plotted against the time (t) of incubation with 143 μ M GSSG. A_0 denotes the activity of Na,K-ATPase without GSSG, and A_t denotes the activity at time (t) of incubation with GSSG. The inhibition constants (k) for the fast and slow phases of the reaction were obtained from the slope of the linear part of the curve by dividing it by the GSSG concentration in the medium.

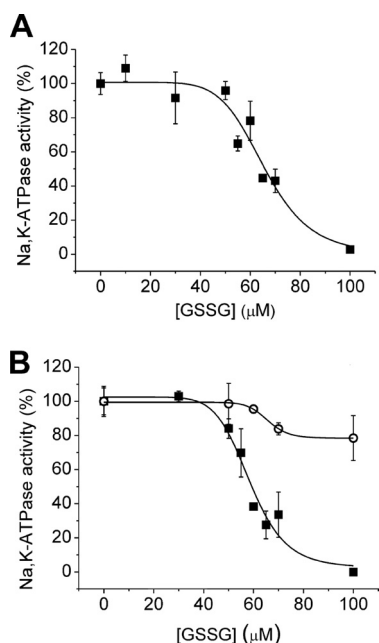


FIGURE 3. Dose response of the inhibitory effect of GSSG on the purified Na,K-ATPase preparations. A, inhibition of the Na,K-ATPase isolated from rabbit kidneys by GSSG as a function of GSSG concentration. The enzyme activity was assessed after incubation with GSSG (25 min, 25 $^{\circ}$ C) and normalized to activity of the nontreated enzyme. The apparent IC_{50} , obtained by fitting the data using the logistic sigmoid function, was $66 \pm 3 \mu$ M. B, inhibition of the Na,K-ATPase isolated from duck salt glands by GSSG as a function of GSSG concentration (filled squares), as described above. The effect of exposure to air (4 h, 4 $^{\circ}$ C) prior to treatment with GSSG was also determined (open circle). The apparent IC_{50} for the inhibition with GSSG of the duck enzyme before exposure to air was $59 \pm 2 \mu$ M. Data are represented as the mean of three experiments \pm S.D.

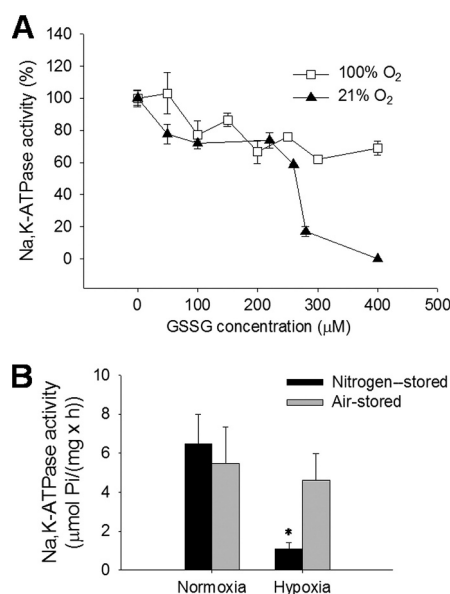


FIGURE 4. Effect of incubation of the SL fraction in the atmosphere of pure O_2 and tissue storage at -80° C in contact with air on the response of the Na,K-ATPase to hypoxia or GSSG treatment. A, effect of various concentrations of GSSG on the Na,K-ATPase function in freshly prepared sarcolemmal membranes (filled triangles) or sarcolemmal membranes pre-exposed to an atmosphere of 100% O_2 for 30 min before GSSG treatment (open squares) $n = 4$ per condition, means \pm S.D. B, Na,K-ATPase activity in crude ventricular tissue homogenates prepared from ventricular tissue and stored for 4–8 weeks either at -80° C in contact with air (gray bars) or in an atmosphere of liquid nitrogen (black bars). Data are presented as a mean of five independent experiments \pm S.D. Asterisk indicates significant differences ($p = 0.0078$) relative to the corresponding N_2 -stored normoxic control as determined by the two-tailed unpaired t test.

sarcolemmal fraction pretreated with GSSG to induce S-glutathionylation (Fig. 5D). As mentioned above, Na,K-ATPase in the myocardium is presented by $\alpha 1\beta$ and $\alpha 2\beta$ isoforms. The $\alpha 2\beta$ isoform was known to be more susceptible to oxidation than the $\alpha 1\beta$ isoform and may be blocked by 10 μ M ouabain, whereas the $\alpha 1\beta$ isoform in rat did not respond to this inhibitor concentration (28). We have used this difference in ouabain sensitivity to assess the responses of both isoforms in rat sarcolemmal membranes to GSSG treatment. Na,K-ATPase function was assessed in two sets of samples, one of which contained 10 μ M ouabain and the other was ouabain-free. As shown in Fig. 5E, the $\alpha 2\beta$ isoform was blocked by GSSG at concentrations 6-fold lower than the $\alpha 1\beta$ isoform with IC_{50} at 43.6 ± 9.2 and $265 \pm 13 \mu$ M for the $\alpha 2$ and $\alpha 1$ isoforms, respectively. Progressive inhibition of the $\alpha 1\beta$ isoform followed a dose-dependent increase in S-glutathionylation of the $\alpha 1$ subunit shown as bars in Fig. 5E.

S-Glutathionylation Prevents the Adenine Nucleotide Binding to Na,K-ATPase—The experiments presented above were performed in the absence of ATP because GSSG treatment of the Na,K-ATPase precluded enzyme activity measurements. Pretreatment of rabbit Na,K-ATPase with GSSG prior to exposing the enzyme to ATP completely inhibited the enzyme (Fig. 6A). However, when GSSG was added to the enzyme, in the presence of ATP at a concentration exceeding 0.5 mM, the inhibitory effect of GSSG was completely averted (Fig. 6B). Thus, the inhibitory action of GSSG on the Na,K-ATPase was caused by its interaction with the free enzyme and not with the enzyme-substrate complex.

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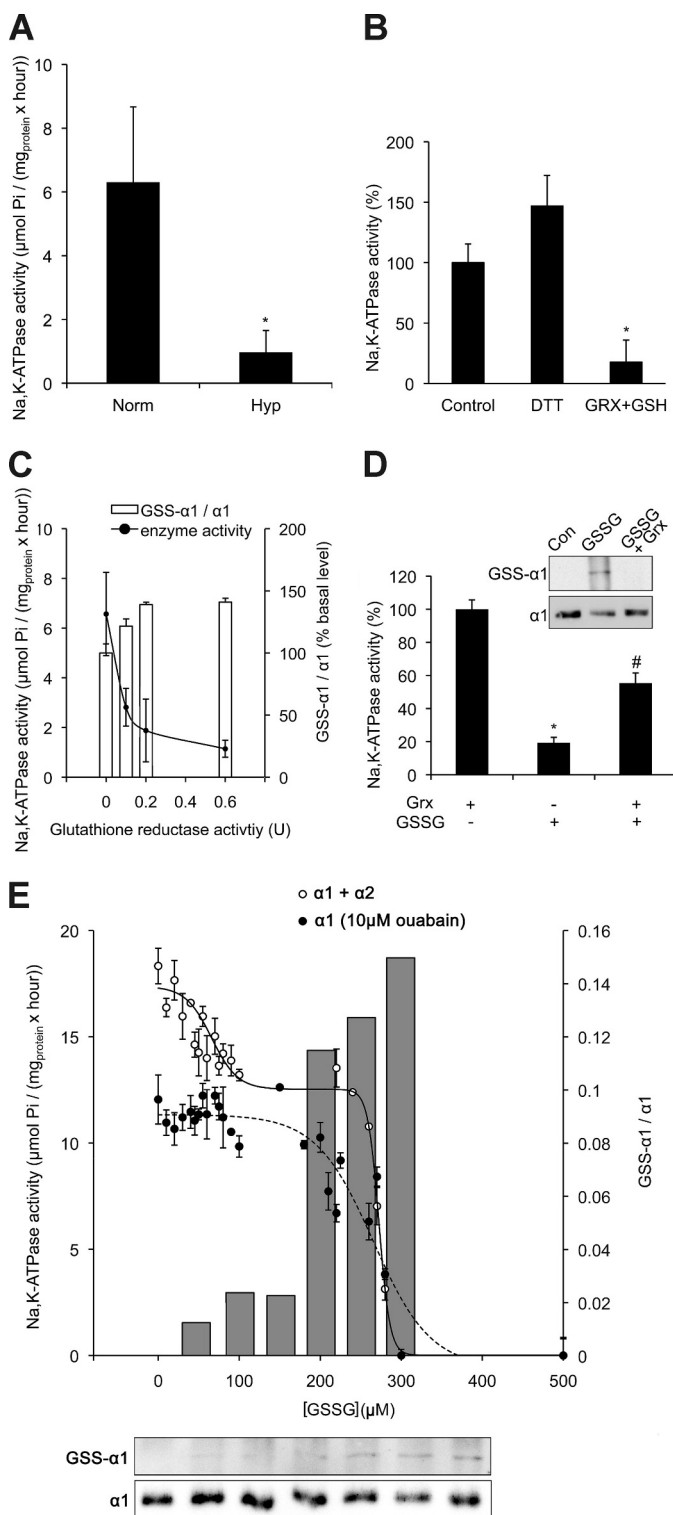


FIGURE 5. Regulation of the Na,K-ATPase in rat heart by S-glutathionylation. **A**, activity of the Na,K-ATPase in crude homogenate prepared from normoxic (Norm) and hypoxic (Hyp) hearts. $n = 5$ per condition. Asterisk denotes $p = 0.0039$. **B**, effects of 100 μM DTT and GRX/NADPH-catalyzed (0.6 units of GRX/200 μM NADPH) S-glutathionylation by 300 μM GSH on Na,K-ATPase activity in normoxic crude ventricular homogenates. $n = 4$ per group. Asterisk indicates $p = 0.0059$ relative to the nontreated crude homogenate sample as determined by the two-tailed paired t test. **C**, dose-dependent GSH-induced S-glutathionylation (open bars) and the corresponding changes in the activity of the enzyme (line) in crude homogenate treated with glutathione reductase and 0.6 units GRX/200 μM NADPH. $n = 4$. **D**, effect of GSSG-induced glutathionylation and GRX-catalyzed deglutathionylation on the enzyme function

Isothermal titration calorimetry was used for direct assessment of the thermodynamic parameters for nucleotide binding to rabbit Na,K-ATPase in nonglutathionylated and glutathionylated forms. Heat production associated with the interaction of ADP with the rabbit enzyme was measured in the presence of DTT or GSSG. A set of original data obtained in such experiments is shown in Fig. 5C. The ADP binding to Na,K-ATPase was enthalpy-driven ($\Delta H = -8.2 \pm 0.3 \text{ kcal mol}^{-1}$, $T\Delta S = 1.1 \text{ kcal mol}^{-1}$) with a binding constant K_a of $6.8 \pm 1.4 \times 10^6 \text{ M}^{-1}$. The stoichiometry of ADP binding to Na,K-ATPase was ~ 0.8 . S-Glutathionylation of the enzyme by GSSG completely abolished ADP binding to the Na,K-ATPase (Fig. 6C).

Glutathionylated SH Groups Are Localized in the Large and Small Cytosolic Loops of the α Subunit—Identification of the cysteine residues of the duck Na,K-ATPase that are S-glutathionylated in the native active enzyme and cysteines undergoing S-glutathionylation upon exposure to GSSG was performed using mass spectrometry. Duck Na,K-ATPase was exposed to a mixture of 1.7 mM GSH and 170 μM GSSG, and the concentrations were found to be present in hypoxic heart and to effectively inhibit the enzyme function. Enzyme activity measurements were performed in control and GSH/GSSG-treated protein samples. Thereafter, two enzyme samples were collected from control and treated enzyme, and each of them proteolyzed by either trypsin or chymotrypsin and MALDI-TOF MS was used to detect cysteine thiol modifications in resulting proteolytic fragments. The $\alpha 1$ sequence coverage reached 70–80% for chymotrypsin-digested fragments and was 50–60% for tryptic fragments. The list of cytosolic cysteine residues of the $\alpha 1$ subunit undergoing S-glutathionylation in the control and treated enzyme is summarized in Table 1. Listed there are the m/z ratios for each fragment and relative peak intensities. Localization of cysteines within the sequence is schematically shown in Fig. 6D. As shown in Table 1, treatment of the enzyme with GSH/GSSG was associated with an increase in S-glutathionylation of the Cys-454, -458, and -459 of the big cytosolic loop and the Cys-244 localized within the small cytosolic loop of the $\alpha 1$ subunit. Cysteine residue 423 has never been found S-glutathionylated.

Glutathionylation of Residues Cys-452^P, -456^P, and -457^P Disrupts the ATP Binding by $\alpha 1$ Subunit of Na,K-ATPase—The structural alignment of the model containing three glutathionyl residues bound to the Cys-452^P, -456^P, and -457^P residues (cor-

in sarcolemmal membranes prepared from normoxic crude homogenate. S-Glutathionylation was induced by treating the sarcolemmal membranes with 300 μM GSSG and reversed with 0.6 units of GRX/200 μM NADPH. Data are represented as the mean of four hearts per condition \pm S.D. Shown in the upper panel is a representative Western blot for the total and S-glutathionylated $\alpha 1$ subunit. * denotes $p = 0.0001$ compared with the GRX-treated control (Con) and # stands for $p = 0.002$ compared with the sample treated with GSSG alone. **E**, differential sensitivity of the $\alpha 1$ and $\alpha 2$ isozymes to the inhibitory action of GSSG. Activity of the Na,K-ATPase ($\alpha 1 + \alpha 2$) or the $\alpha 1$ isozyme alone was assessed in sarcolemmal membranes prepared from the normoxic heart treated with various GSSG concentrations. Activity of the $\alpha 2$ isozyme was calculated by subtracting the activity of the $\alpha 1$ isoform from the total Na,K-ATPase activity. Fitting of the plots with double ($\alpha 1 + \alpha 2$) or single ($\alpha 1$ or $\alpha 2$ alone) logistic sigmoidal functions was performed giving apparent IC_{50} values for $\alpha 1$ as $271.1 \pm 1.7 \mu\text{M}$ and for $\alpha 2$ as $43.6 \pm 9.2 \mu\text{M}$. Gray bars and the lower panel show the changes in S-glutathionylation of the $\alpha 1$ subunit followed by the corresponding changes in the enzyme activity. $n = 5$ per group. All plotted data are represented as mean values \pm S.D.

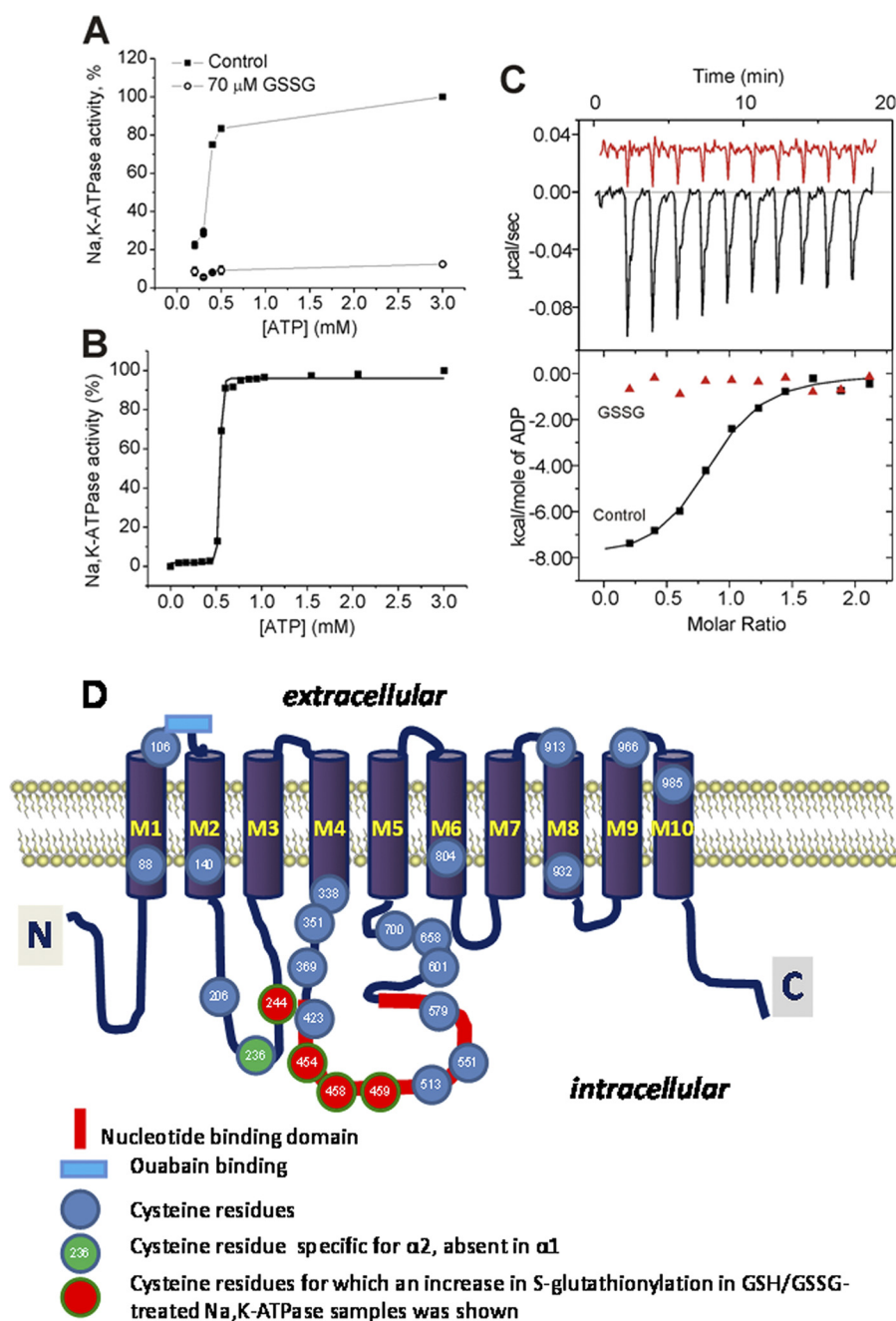


FIGURE 6. Competition between nucleotides and GSSG for the Na,K-ATPase nucleotide-binding site. *A*, pretreatment of the Na,K-ATPase prevents the dose-dependent activation of the Na,K-ATPase with ATP. Na,K-ATPase purified from rabbit kidney was preincubated both in the presence and absence 70 μ M GSSG for 25 min and then its activity was measured as a function of ATP availability. The data are represented as mean values \pm S.D. $n = 3$. *B*, ATP causes dose-dependent prevention of the inhibitory action of 1 mM GSSG when ATP and GSSG are simultaneously present in the incubation medium. The data are represented as mean values \pm S.D. $n = 3$. *C*, inhibitory effect of S-glutathionylation on the ADP binding to Na,K-ATPase. An original isothermal titration calorimetry recording (*upper panel*) and binding isotherms (*lower panel*) of the Na,K-ATPase interaction with ADP in the presence of DTT (100 μ M, *black*) or GSSG (1 mM, *red*) at 25 $^{\circ}$ C. *D*, localization of S-glutathionylation sites on the $\alpha 1$ subunit. Membrane domains of the $\alpha 1$ subunit are shown as barrels numbered as M1–M10. Cytosolic or extracellular domains are shown as lines, where the ouabain-binding site is shown in blue and nucleotide binding domain is in red. “C” and “N” indicate the C and N terminus. Cysteine residues are presented as filled circles with numbers corresponding to the duck/rabbit/rat $\alpha 1$ sequence. Cysteines, which undergo S-glutathionylation upon GSH/GSSG treatment, are shown in red. The Cys-236 absent in the $\alpha 1$ but present in the $\alpha 2$ isozyme is shown in green.

responding to the Cys-454, -458, and -459 residues in the duck, rabbit, and rat sequences), and the model with the ATP molecule docked to the protein, has been done by the MOE software (Fig. 7A). According to the model, the distance between the terminal negatively charged phosphate of the ATP molecule and the carboxyl group of glutathione bound to the Cys 452^P carrying the same negative charge is less than 8 Å (Fig. 7A).

Electrostatic repulsion forces between these two negative charges are sufficient to hinder attachment of the ATP to the S-glutathionylated binding site moiety. The same is true for glutathione binding to the Cys-452^P in the presence of ATP in docked position. This electrostatic repulsion will become even more pronounced as an additional two cysteines in the vicinity of the ATP-binding site, Cys 456^P and 457^P (Fig. 7A), are S-glu-

Catalytic α Subunit S-Glutathionylation Blocks Na,K-ATPase

TABLE 1

MALDI-TOF-MS analysis of the glutathionylated Cys-containing peptides of $\alpha 1$ subunit of the Na,K-ATPase

Na,K-ATPase isolated from duck salt glands was treated with a mixture of GSH (1.7 mM) and GSSG (170 μ M), and the $\alpha 1$ subunit of the enzyme before (control, active enzyme) and after (GSH/GSSG, inactivated enzyme) treatment was subjected to either tryptic or chymotryptic digestion. The resulting fragments were analyzed by MALDI-TOF MS. Shown in the table are only the tryptic and chymotryptic fragments containing S-glutathionylated cysteine residues. Listed in the table are the experimental and calculated m/z ratio values (monoisotopic mass-to-charge ratio of the singly charged peptide ions) of the fragments. The calculated m/z values are for the fragments in which all cysteine residues are present in nonmodified (reduced) form. Cysteine modification was assessed by comparison of the experimental m/z values with the calculated ones. Binding of glutathione ($-SG$) increases mass of the fragments by 305 Da. Peaks with maximal intensity were chosen as referent ones for analysis of tryptic and chymotryptic fragments. These peaks differed for tryptic and chymotryptic fragments. The same referent peaks were used for analysis of fragments obtained from control and GSH/GSSG-treated samples. The peak intensity of the fragment normalized to intensity of the corresponding referent peak is presented in the table as relative intensity. Peak detection was made by the SNAP algorithm of the program Flex Analysis (Bruker Daltonics, Germany). Experiment has been repeated three times.

Cysteine no.	Fragment/ type of fragment	Sequence	Experimental m/z		Calculated m/z for nonmodified fragment	Calculated m/z for modified fragment	Cysteine modification	Relative intensity	
			Control	GSH/GSSG				Control	GSH/GSSG
206	194–207 tryptic	IPADLRISAHGCK	1798.82	1798.89	1493.82	1798.89	SG	0.72	0.77
244	236–250 tryptic	NIAFFSTNCVEGTAR	1934.95	1934.86	1629.76	1934.84	SG	0.08	0.47
338, 351	336–353 chymotryptic	TVCLTLTAKRMARKNCLV	2326.19	2326.19	2021.14	2326.21	SG, SH	0.14	0.21
351, 369	350–372 tryptic	NCLVKNLEAVETLGSTSTICSDK	3035.49	3035.48	2425.19	3035.33	SG, SG	0.03	0.03
454, 458, 459	454–468 tryptic	CIELCCGSVKEMRER	2671.20	2671.19	1755.79	2671.01	SG, SG, SG	0.05	0.13
458, 459	456–462 chymotryptic	ELCCGSV	1015.34	1015.34	710.28	1015.35	SG, SH	0.28	0.49
513	502–516 chymotryptic	MKGAPERILDRCSSI	1981.0	1981.01	1675.86	1980.93	SG	2.98	2.62
551	549–564 chymotryptic	GFCHLALPDDQPEGF	2097.96	2097.95	1792.79	2097.87	SG	1.88	2.13
658	640–660 chymotryptic	AARLNIPVSQVNPRDAKACVV	2526.24	2526.36	2221.22	2526.29	SG	0.19	0.13
700	694–702 tryptic	LIIVEGCQR	1335.65	1335.65	1030.56	1335.64	SG	0.58	0.78

tathionylated. The results of modeling comply with the observation that interaction of ATP with its binding site and binding of glutathione to the regulatory cysteine residues are mutually exclusive (Fig. 6, A–C). Modeling based on the crystal structure of the enzyme in E2P conformation did not show any significant interaction of Cys-246^P with the ATP-binding site. However, this may not hold true for the enzyme in E1 conformation (25).

DISCUSSION

The obtained data indicate that S-glutathionylation of the catalytic α subunit may result in complete inactivation of the enzyme by making its adenine nucleotide-binding site inaccessible for ATP. Regulatory S-glutathionylation does not occur spontaneously, only when ATP depletion reaches a threshold of ~ 500 μ M (Fig. 6B). Therefore, inactivation of the enzyme prevents irreversible ATP deprivation under conditions of limited ATP supply. S-Glutathionylation of regulatory cysteines is promoted under oxidative stress when GSSG concentration increases in the cytosol. However, it may also be mediated by GSH in the presence of GRX (Fig. 5C) indicating that oxidative stress is not necessarily required to trigger S-glutathionylation. ATP depletion on the contrary is absolutely required to induce regulatory S-glutathionylation. The ability of ATP to protect Na,K-ATPase from HO[•]-induced inactivation has been shown previously (27).

Basal S-Glutathionylation of α Subunit—In contrast to regulatory S-glutathionylation, endogenous basal S-glutathionylation is an intrinsic feature of the Na,K-ATPase catalytic subunit and is independent of the ATP availability. Removal of basal glutathionylation by DTT was not followed by an alteration of the Na,K-ATPase activity. Physiological relevance of basal S-glutathionylation remains unclear. However, its high abundance suggests that basal S-glutathionylation is required for the maintenance of optimal protein function. Similar to the α subunit of Na,K-ATPase, basal S-glutathionylation was described for its structural homologue, SERCA-2A (28), as well as for ryanodine receptors (29).

S-Glutathionylation of α Subunit Leads to Complete Inhibition of the Na,K-ATPase—Interaction of glutathione with regulatory cysteine residues resulted in complete inhibition of the enzyme (Figs. 3 and 5). S-Glutathionylation of the catalytic subunit associated with the changes in enzyme activity represents typical regulatory glutathionylation as described earlier (30). Hypoxia is a physiological stimulus that induces regulatory S-glutathionylation in rat heart. Oxygen consumption rate in the myocardium exceeds that in the brain, and reduction of the O₂ supply of this tissue is followed by rapid reduction in ATP levels (13) along with GSSG accumulation (see above). As we have demonstrated in this study, these conditions promote regulatory S-glutathionylation. Na,K-ATPase may be S-glutathionylated in a reaction of thiol-disulfide exchange within the physiological concentration range of GSH and GSSG of 1–10 mM and 50–500 μ M, respectively (Figs. 3 and 5E) (9, 31, 32). Thiol-disulfide exchange reaction with glutathione is comparatively rare, and the few other proteins where it is also physiologically relevant are c-Jun (33) and aldose reductase (34). For the vast majority of other proteins, including the β subunit of the Na,K-ATPase, intermediate S-nitrosylation step or other thiol modifications preclude the formation of S-glutathionylated adducts (16).

Regulation of the Na,K-ATPase function by S-glutathionylation is fast (Fig. 2) and completely reversible (Fig. 5D). GRX actively participates in deglutathionylation or induction of S-glutathionylation depending on the changes in GSH and NADPH levels. Therefore, GRX coordinates the activity of numerous redox-sensitive proteins adjusting to the changes in the redox microenvironment (Fig. 5, C and D) (35–37). Spontaneous deglutathionylation catalyzed by GRX and thioredoxins is most likely to be the cause of the gradual loss of the inhibitory effect of ischemia on the Na,K-ATPase in heart tissue homogenate with time, as reported by Fuller *et al.* (12).

Biphasic kinetics of the inhibitory action of GSSG on Na,K-ATPase activity may reflect the existence of two dis-

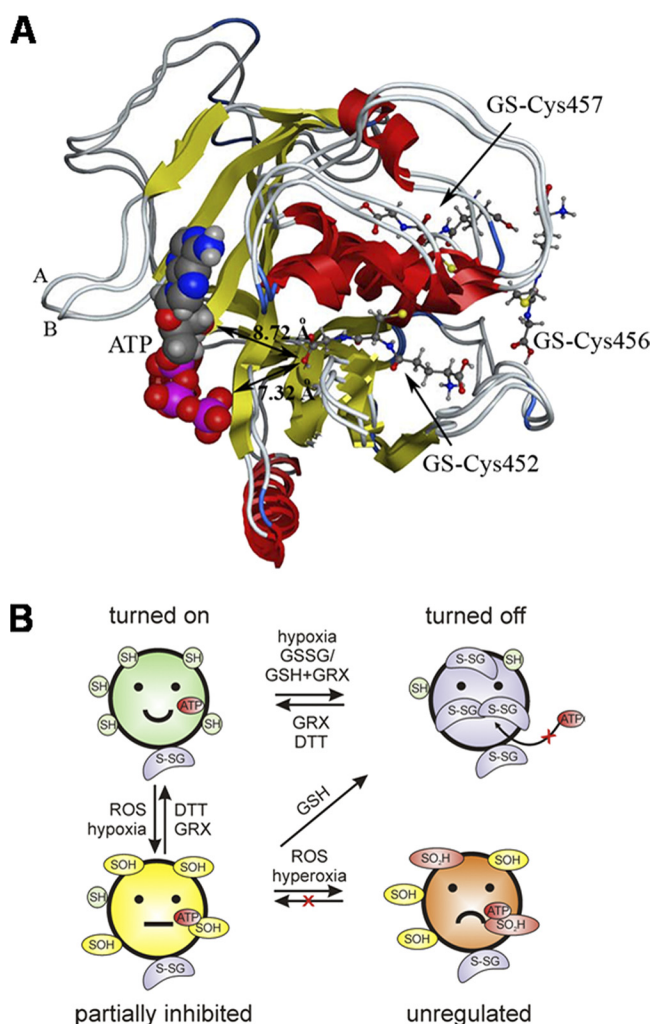


FIGURE 7. S-Glutathionylation of the α subunit as a mechanism of regulation of the Na,K-ATPase. A, superimposition of the three-dimensional structures of the nucleotide binding domain (Arg-378 to Arg-589) with ATP A and glutathione B bound to Cys-452^P, -456^P, and -457^P. Shown as a ribbon diagram is a model created on the basis of 3.5-Å structure of the porcine α 1 subunit (Protein Data Bank code 3b8e). The structural alignment was simulated with the program MOE. Glutathione is shown as a ball-and-stick representation, and ATP is presented as a space-filling van der Waals representation with atoms in standard colors for atom type (carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow; phosphorus, pink). The distance between the negatively charged phosphate ATP tail and the negatively charged carboxyl group of the glutathione bound to the Cys-452^P is shown in green and is less than 8 Å. B, schematic representation of the regulatory S-glutathionylation of the Na,K-ATPase. The enzyme is shown in dynamic equilibrium between three distinct states as follows: “turned on” (active, regulated), “turned off” (reversibly inhibited), and “partially inhibited.” Transformation to the “unregulated” state is irreversible. Maximal activity of the enzyme may be achieved under the conditions supporting the optimal redox environment. Mild oxidation (GSSG accumulation coupled to ATP depletion or -SH to -SOH transformation of the cysteine residues in the partially inhibited mode) or GSH overload in the presence of GRX transfers the Na,K-ATPase from turned-on to the turned-off state in which the regulatory cysteines are S-glutathionylated. Severe oxidative stress causes oxidation of the -SH groups of the regulatory cysteines to -SO₂H or -SO₃H and thus turns the enzyme into the unregulated state, in which it is unable to respond to the changes in redox state and remains constantly active.

tinct classes of regulatory thiol groups, as shown before for the glycogen debranching enzyme in rabbit skeletal muscle (38) or alternatively two conformational states (e.g. E₁ and E₂) of the Na,K-ATPase in which the same thiol groups become more or less accessible to interactions with GSSG.

Greater susceptibility of the α 2 β isozyme to inhibitory S-glutathionylation by GSSG in rat sarcolemmal membranes is of physiological importance (Fig. 5E). The α 2 isoform of the catalytic subunit is mainly localized in the T-tubular zones, where it is associated with the Na⁺/Ca²⁺ exchanger (36). Thus, S-glutathionylation and the following inhibition of the α 2 β isozyme may have a profound effect on the intracellular Ca²⁺ handling in cardiomyocytes (36, 37, 39). As the α 2 isoform has been reported to be particularly prone to oxidation (8), more favorable binding of glutathione to this isoform will protect it from oxidation.

Severe oxidative stress is associated with irreversible oxidation of the regulatory thiols to sulfinic or sulfonic acid. When oxidized to -SO₂⁻ or -SO₃⁻, the thiol groups cannot be S-glutathionylated, and the Na,K-ATPase loses its redox sensitivity. In our study, oxidation has been induced by exposure of the enzyme to 20–100% O₂ (Figs. 3B and 4). Isolation of cardiomyocytes from adult myocardium also inevitably results in oxidative stress and most likely leads to the inaccessibility of the α subunit cysteines for regulatory glutathionylation (16). Cys-46 of the β subunit is localized almost within the lipid bilayer, which is less exposed to the cytosol and hence is more resistant to oxidation. It may still undergo S-glutathionylation even in isolated cardiomyocytes as reported earlier (16).

Localization of the Regulatory Cysteines—The obtained results indicate that inhibition of the Na,K-ATPase activity may be induced by S-glutathionylation of at least three regulatory cysteine residues localized within the big cytosolic loop of its α subunit, Cys-454, -458, and -459. The inability of the enzyme to bind ATP in S-glutathionylated form results from an increase in negative charge within the ATP binding pocket. The role of S-glutathionylation of the Cys-244 remains to be clarified. Along with the data of mass spectrometry, clear differences in sensitivity to the inhibitory action of GSSG on the α 1 β and α 2 β isozymes (Fig. 5E) suggest that this cysteine residue may play a regulatory role as well. The more sensitive α 2 isoform of the catalytic subunit possesses one additional cysteine is at position 236, which may be a target for S-glutathionylation along with Cys-244. It is tempting to suggest that this alteration in the α 2 sequence compared with that of α 1 is a cause of amplification of the inhibitory effect of GSSG observed for the α 2 β isozyme in rat heart (Fig. 5E).

Ca²⁺-transporting ATPase SERCA2A, a homologue of the Na,K-ATPase, has also been reported to possess a site of regulatory S-glutathionylation (28). But in contrast to Na,K-ATPase, SERCA2A is activated upon S-glutathionylation of a single cysteine residue, Cys-674. This cysteine is absent in all α subunit isoforms of the Na,K-ATPase. The conserved localization of cysteine residues within the sequence of SERCA2A and the α subunit of the Na,K-ATPase is necessitated by the regulatory function of these amino acids. Although mutations of the Cys residues to Ser or Ala in the α subunit were not associated with significant changes in the enzyme activity (40), reversible thiol modifications of some of them may have a striking effect on the Na,K-ATPase function. Our data indicate that the resulting mutants will render the enzyme largely redox-insensitive due to the lack of sites of regulatory S-glutathionylation.

Schematic representation of the role of regulatory S-glutathionylation in the control of Na,K-ATPase is shown in Fig. 7B. Induction of regulatory S-glutathionylation allows us to quickly inactivate the enzyme under conditions of limited ATP supply. Mild oxidative stress (e.g. associated with hypoxic exposure) results in an increase in GSSG and oxidation of regulatory thiols to sulfenic acid ($-SOH$). Taken together, these changes promote S-glutathionylation of cysteine residues thereby inhibiting the Na,K-ATPase and protecting thiols from irreversible oxidation. When not protected, the regulatory thiols get further oxidized to sulfinic and sulfonic acid making the enzyme insensitive to the changes in ATP levels and the redox state.

Our findings reveal the importance of S-glutathionylation of cysteine residues of the Na,K-ATPase catalytic subunit in redox-induced responses of the enzyme. S-Glutathionylation of the regulatory cysteine(s) acts as a switch turning off the Na,K-ATPase at the low ATP level to prevent irreversible ATP depletion.

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