A Novel Topology and Redox Regulation of the Rat Brain K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger, NCKX2*

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In this study we have examined the roles of endogenous cysteine residues in the rat brain K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger protein, NCKX2, by site-directed mutagenesis. We found that mutation of Cys-614 or Cys-666 to Ala inhibited expression of the exchanger protein in HEK-293 cells, but not in an \textit{in vitro} translation system. We speculated that Cys-614 and Cys-666 might form an extracellular disulfide bond that stabilized protein structure. Such an arrangement would place the C terminus of the exchanger outside the cell, contrary to the original topological model. This hypothesis was tested by adding a hemagglutinin A epitope to the C terminus of the protein. The hemagglutinin A epitope could be recognized with a specific antibody without permeabilization of the cell membrane, supporting an extracellular location for the C terminus. Additionally, the exchanger molecule could be labeled with biotin maleimide only following extracellular application of \textbeta-mercaptoethanol. Surprisingly, mutation of Cys-395, located in the large intracellular loop, to Ala, prevented reduction-dependent labeling of the protein. The activity of wild-type exchanger, but not the Cys-395→Ala mutant, was stimulated after application of \textbeta-mercaptoethanol. Co-immunoprecipitation experiments demonstrated self-association between wild-type and FLAG-tagged exchanger proteins that could not be inhibited by Cys-395→Ala mutation. These results suggest that NCKX2 associates as a dimer, an interaction that does not require, but may be stabilized by, a disulfide linkage through Cys-395. This linkage, perhaps by limiting protein mobility along the dimer interface, reduces the transport activity of NCKX2.

Cytosolic Ca\textsuperscript{2+} ions play key second messenger roles in numerous physiological processes in virtually all types of animal cells (1). Ca\textsuperscript{2+} entering the cell across the plasma membrane during calcium signaling must be quantitatively extruded to the extracellular environment to maintain long term cellular Ca\textsuperscript{2+} homeostasis. Plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers are a crucial component of the Ca\textsuperscript{2+} efflux process and have been extensively investigated in a wide range of tissues, particularly in the heart and brain (2, 3). Various functional and molecular studies have revealed the existence of two families of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger proteins that share sequence similarity in two intramolecular homologous domains known as \textalpha-repeats (4). One family, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCX),¹ are thought to catalyze the electroneutral exchange of either 3 or 4 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+} (2, 5, 6). The NCX family is made up of at least three distinct gene products: NCX1 (7), NCX2 (8), and NCX3 (9). NCX1 is expressed at high levels in cardiac muscle, brain, and kidney and is also present to a lesser extent in many other tissues (10, 11). NCX2 and NCX3, in contrast, are expressed primarily in only two tissues: brain and skeletal muscle (8, 9). All three exchangers share an overall amino acid identity of ~70% that rises to more than 80% within the predicted transmembrane segments (TMS) (9). The second family, K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCKX), are believed to catalyze the electroneutral countertransport of 4 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+} and 1 K\textsuperscript{+} (12–14). NCKX exchangers differ from NCX proteins in their absolute requirement for K\textsuperscript{+}, lower Ca\textsuperscript{2+} transport rates, and primary amino acid sequence outside the \textalpha-repeats (2). NCKX1 was initially cloned from bovine rod photoreceptors and was believed to play a central and unique role in the mammalian phototransduction pathway because its ionic stoichiometry represented an adaptation to the unusual ionic environment of the vertebrate eye (15, 16). However, evidence from functional measurements revealed some Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange processes that were dependent on K\textsuperscript{+} in tissues other than eye, for instance brain synaptic plasma membrane (17) and platelet (18). This result led to the search for other putative NCKX family members. Consequently, NCKX2 was first cloned from rat brain (19) and then from chick and human cone photoreceptors (20), and NCKX3 was recently cloned from brain and skeletal muscle (21). Expansion of the NCKX family suggests a wider role for K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in maintaining cellular Ca\textsuperscript{2+} homeostasis than previously anticipated. The tissue-specific expression patterns of these known NCKX members may reflect the different Ca\textsuperscript{2+} handling properties of different tissues or cells.

Cysteine accessibility studies have suggested that the initial hydropathy-based topological model of NCX1 needed to be re-

¹ The abbreviations used are: NCX, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; AMS, 4-acetamido-4-maleimidylstilbene-2,2'-disulfonic acid, disodium salt; BBS, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; FITC, fluorescein isothiocyanate; HA, hemagglutinin A; HEK, human embryonic kidney; IP, immunoprecipitation; \textbeta-ME, \textbeta-mercaptoethanol; MBP or biotin maleimide, 3-(4-n-maleimidylpropionyl)biocytin; NCKX, K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; PBS, phosphate-buffered saline; PCSCM, PBS supplemented with 0.1 mM CaCl\textsubscript{2} and 1 mM MgCl\textsubscript{2}; TMS, transmembrane segment.
vised so that mature NCX1 is now thought to contain nine TM domains with two re-entrance loops (22–24). The current topological model of NCX1, based solely on hydrophy analysis, is reminiscent of the original NCX model before modification. Recently, examination of the hydrophy profile for NCX3 gave rise to a new topological model in which the C-terminal hydrophobic domain contains only five TM5s, thus placing the C terminus of the exchanger outside the cell (21), in conflict with the initially proposed NCX3 model in which the C-terminal half contained six TM domains in a TMSN (21). Indeed, experimental determination of the topology of the *Escherichia coli* inner membrane protein YrbG, a putative bacterial Na+/Ca2+ exchanger, suggested the C-terminal half has five TM5s and placed the C terminus extracellularly (25).

Cardiac Na+/Ca2+ exchanger activity was observed to be enhanced dramatically after treatment with a combination of reducing and oxidizing reagents (26). Thiols-disulfide interchange was proposed to be the molecular mechanism underlying redox modification of exchange activity, although the precise amino acid(s) involved have not yet been identified (27). To date, experimental evidence for dynamic regulation of NCX-type exchangers is quite limited. In this study, we have used site-directed mutagenesis to investigate the role native cysteine residues play in NCX2 exchanger protein stability and in transport activity. A preliminary report describing some of these results was published previously in abstract form (28).

**EXPERIMENTAL PROCEDURES**

All molecular procedures were performed according to standard protocols (29, 30) or the directions of reagent manufacturers, unless noted otherwise. Common chemical reagents were obtained from Fisher, Sigma, or BD and were of analytical grade or better, unless indicated otherwise. 3-(N-Maleimidomethylpropionyl)biotinoyl (biotin maleimide, or MBP) was from Sigma or Molecular Probes. 4-Acetamido-4-maleimido-lisulfobine-2,2'-disulfonic acid (AMS) was from Molecular Probes.

**Construction of NCX2 Mutants**—The construction of the wild-type and the FLAG-tagged full-length rat brain NCX2 cDNA was described previously (19). Site-directed mutagenesis was performed with the polynucleotide chain reaction (PCR) overlap extension method using the Expand High Fidelity PCR system from Roche Molecular Biochemicals. Briefly, a pair of complementary primers in which cysteine-coding nucleotides were changed to those for alanine were synthesized. PCR fragments were generated using these mutagenic primers and external primers to match convenient unique restriction endonuclease sites in a two-step process. The purified fragment was digested and subcloned into the corresponding digested full-length exchanger clone in pBluescript II SK (+) (Stratagene). The cDNA clone plasmid was then digested with *Kpn*1 and *Bam*HI, and the ~2.5-kilobase pair fragment containing the full-length NCX2 clone was subcloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen). We made point mutations for each of the last four native cysteine residues of NCX2, Cys-395→Ala, Cys-614→Ala, Cys-633→Ala, and Cys-666→Ala. We also generated combined cysteine to alanine mutations named according to the linear order of cysteines in NCX2: C1–4 (Cys-16→Ala, Cys-24→Ala, Cys-154→Ala, and Cys-224→Ala), C1–5 (Cys-16→Ala, Cys-24→Ala, Cys-154→Ala, Cys-224→Ala, and C1–5,7 (Cys-16→Ala, Cys-24→Ala, Cys-154→Ala, Cys-224→Ala, Cys-395→Ala, and Cys-633→Ala). An additional cysteine residue was reintroduced back into the C1–5,7 construct to substitute Ser-105 in the N terminus (named C1–5,7–Ser-105→Cys), or one residue at a time at selected sites between the putative loops in the C-terminal half. An HA epitope was inserted at the C terminus of FLAG-tagged NCX2 as a 9-amino acid peptide extension (YPYDVPDYA) by a similar PCR overlap mutagenesis procedure as described above, and the HA-tagged construct was designated as FLAG-NCX2-HA671. All constructs were confirmed by sequencing to ensure that no polymerase errors were introduced into the amplified segments.

**In Vitro Translation**—In vitro translation of wild-type or mutated NCX2 was performed essentially as described previously (21). In brief, cDNA constructs in the pcDNA3.1 (+) vector were transcribed and translated in *vitro* using the TNT-7-T system (Promega) together with [35S]-methionine (Amersham Biosciences), in the presence of 0.1% Triton X-100. Following an incubation of 90 min at 30 °C, the products were resolved on an SDS-polyacrylamide gel, dried, and detected by autoradiography using Biomax MR film (Eastman Kodak Co.).

**Indirect Immunofluorescence**—Location of the HA epitope was determined using immunofluorescence essentially as described previously (19) with some modifications. In brief, HEK-293 cells transfected with different constructs were washed twice, the reaction was quenched by application of 3 ml of 2% ethanol in PBS for 15 min at room temperature. Cells were then fixed in 4% paraformaldehyde in PBS and blocked with 0.2% gelatin/PBS for 30 min. A rhodamine-conjugated anti-rabbit secondary antibody (1:500) was then incubated with the rabbit anti-HA polyclonal antibody (1:500) in PBS containing 0.2% gelatin for 1 h at room temperature. The cells were then fixed in 4% paraformaldehyde in PBS and blocked with 0.2% gelatin/PBS for 30 min. A rhodamine-conjugated anti-rabbit secondary antibody (1:500) was employed in 0.2% gelatin/PBS for 30 min. After extensive washing with PBS, the cells were then treated with 1 ml of 2% anti-FLAG monoclonal antibody (1:500) followed by a FITC-conjugated mouse anti-FLAG antibody. In permeabilization experiments, the cells were first fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 before consecutive application of first and second antibodies as described above. Immunofluorescence microscopy was performed using standard epifluorescence optics on a Zeiss Axiovert II through a Fluor 63× objective. Images were captured using a Spot digital camera and processed with Photoshop.
incubating the membranes with PBS plus 0.1% Tween 20 containing 0.1 μg/ml horseradish peroxidase-conjugated streptavidin for 1 h after blocking with 2% bovine serum albumin for 1 h. After washing, the membranes were then developed using SuperSignal Plus ECL reagents (Pierce). To assess the level of protein present in each lane, the membranes were stripped with 0.2 M NaOH for 15 min and reprobed with rabbit anti-NCKX2 polyclonal antibody F, followed by application of horseradish peroxidase-conjugated anti-rabbit IgG antibody. The membranes were developed using ECL reagents.

Calcium Imaging and Data Analysis—Calcium transport into transfected HEK-293 cells was measured by fura-2 fluorescent ratio digital imaging essentially as described previously (19, 21) with modification. In brief, 2 days after transfection, HEK-293 cells grown on poly-L-lysine-coated coverslips were loaded with 5 μM fura-2 AM (Molecular Probes) and mounted in a perfusion chamber on a microscope stage. The ratio of fura-2 fluorescence was captured with excitation at 340 or 380 nm using the ImageMaster system (Phonton Technology International). Several perfusion solutions were used: solution I (145 mM NaCl, 10 mM HEPES-trimethylamine, pH 7.4), solution II (in which the NaCl of solution I was replaced with 140 mM LiCl and 5 mM KC1), and solution III (in which the NaCl of solution I was substituted with 140 mM NaCl and 5 mM KC1). For testing activity of mutants, cells were initially perfused with solution I for 5 min, followed by alternating changes to solution II for 2 min. For investigating redox-dependent regulation of NCKX2, cells were first perfused with solution III for 17 min without collecting ratio imaging data. Upon changing to perfusion solution I for 2 min, fura-2 fluorescence measurements were started. Perfusion was changed successively to solution II for 2 min and solution I for 2 min. Then, the cells were incubated with either 2% β-ME or 2% ethanol in solution III for 15 min, followed by perfusion with solution III for 2 min. Finally, the cells were subjected consecutively to perfusion solutions I, II, and I for 2 min each.

Imaging data were analyzed as described previously (19, 21) using the ImageMaster program and Excel (Microsoft). For redox experiments, all the ratio data were normalized to the height of the first peak ratio. The height of peaks (with base line subtracted) following treatments, all the ratio data were normalized to the height of the first peak ratio. Data were then tested for statistical significance using one-way analysis of variance with Newman-Keuls multiple comparison.

RESULTS
Effects of Mutating Native Cysteine Residues—There are eight native cysteine residues in the NCKX2 molecule (Fig. 1A). Three constructs with combined cysteine-to-alanine mutations were made: C1–4 (Cys-14 → Ala, Cys-24 → Ala, Cys-154 → Ala, and Cys-224 → Ala), C5–8 (Cys-395 → Ala, Cys-614 → Ala, Cys-633 → Ala, and Cys-666 → Ala) and a Cys-less exchanger, C1–8. Immunoblotting showed C1–4 was expressed well in HEK-293 cells, whereas C5–8 and C1–8 expression was too low to be detected (Fig. 1B). In vitro translation demonstrated that the C5–8 mutant could be translated (Fig. 1C), suggesting mutation of the last four cysteine residues might affect protein stability in transfected HEK-293 cells.

To identify which cysteine residue(s) was(were) involved in the stability of NCKX2, single cysteine mutants Cys-395 → Ala, Cys-614 → Ala, Cys-633 → Ala, and Cys-666 → Ala were made in FLAG-tagged NCKX2. Cys-614 → Ala and Cys-666 → Ala mutants were observed to cause a reduction in protein expression level. A mutant named C1–5,7, in which all cysteines except Cys-614 and Cys-666 were mutated to Ala, was well expressed in HEK-293 cells (Fig. 1B). C1–4 and C1–5,7 mutants were functionally active when tested by calcium imaging (Fig. 1D), as were the NCKX2 mutants Cys-395 → Ala, Cys-633 → Ala, and C1–5. Thus, the integrity of both Cys-614 and Cys-666 is essential for the functional expression of NCKX2.

Native disulfide bonds are believed to play an important role

![Fig. 1. Initial topological model of NCKX2 and effects of native cysteine mutagenesis](image-url)
in developing proper protein conformational folding (34). Thus, we speculated that Cys-614 and Cys-666 might form a structurally and functionally important cystine disulfide bond. Disulfide bonds are usually found extracellularly, as the cytoplasm is a reducing environment. This speculation regarding Cys-614 and Cys-666 would place the C terminus of NCKX2 outside the cell, in conflict with the original topological model for NCKX2 based on hydropathy analysis, where the C terminus as well as Cys-614 and Cys-666 were located intracellularly (19), as illustrated in Fig. 1A.

Location of the C Terminus of NCKX2—We previously used the epitope insertion approach to prove that the N terminus of mature NCKX2 was on the outside of the plasma membrane (19). To explore the location of the C terminus, an HA epitope was added at the C terminus of an NCKX2 construct that was also tagged with a FLAG epitope in the N-terminal extracellular loop (see Fig. 2C). The double-tagged construct named FLAG-NCKX2-HA671 was shown to be functional, as tested by calcium imaging (data not shown) and thus preserved the structural integrity of the NCKX2 protein. Cells transfected with vector alone revealed no remarkable fluorescent staining with FLAG and HA antibodies after permeabilization (Fig. 2A, Negative Control). Control experiments used FLAG-NCKX2 (without the HA tag) double-stained using both sets of antibodies, and FLAG-NCKX2-HA671 stained with only one or other of the primary antibodies. The images shown are representative of 12 microscopic fields analyzed from four different experiments. C, a new putative topological model for NCKX2 is proposed. In this model the C terminus is extracellular and putative transmembrane segment M6 is placed inside the cytoplasm to form part of the large intracellular loop. Cys-614 and Cys-666 may form a cystine disulfide bond. Sites for the FLAG and HA epitopes are shown, as well as the site of glycosylation (CHO). Other details are similar to those of Fig. 1A.

Redox-modulated Accessibility of Cysteine Residues to Biotin Maleimide and Effects of Cys-395→Ala Mutation—The accessibility of the cysteine residues in wild-type and functional cysteine mutants was examined by covalently modifying the protein expressed in transfected HEK-293 cells with the membrane-permeant cysteine-selective reagent MPB. Cysteine res-
which the endogenous Cys-633 remained intact, were expressed in HEK-293 cells and analyzed by immunoblot and Ca\(^{2+}\) imaging. C1–5 and the cysteine mutants at Ser-471, Ala-503, and His-531 were all expressed and functional. Lys-567 and Leu-569 cysteine mutants were expressed but were not functional. However, none of these expressed proteins could be labeling with the MPB reagent, and they were thus uninformative for our topological studies.

To investigate whether Cys-614 and Cys-666 form a disulfide bond, transfected HEK-293 cells were treated with the reducing agent β-ME, or ethanol as a control for β-ME treatment, for 15 min before incubation with the MPB reagent. The results demonstrated that endogenous cysteine residue(s) in wild-type NCKX2 and C1–4 became accessible for MPB labeling only following extracellular application of β-ME (Fig. 3B). Interestingly, C1–5,7 could not be labeled even after β-ME treatment (Fig. 3B). Thus we concluded that Cys-614 and Cys-666 were not accessible for labeling by the hydrophilic MPB even after treatment with β-ME, and so might be concealed within the hydrophobic portion of the membrane. Because the NCKX2 construct, C1–4, was labeled with MPB, but C1–5,7 was not, we reasoned that Cys-395, Cys-633, or both, might be involved in the β-ME-dependent labeling of the NCKX2 exchanger. By using the C1–5 and the Cys-395 → Ala (C5) mutants we demonstrated that mutation of Cys-395 to Ala, the only cysteine residue located in the large intracellular loop, completely abolished the β-ME-dependent labeling of NCKX2 by MPB (Fig. 3B). We also found that after β-ME treatment of transfected HEK-293 cells, application of AMS could not block the subsequent MPB labeling of wild-type NCKX2 (Fig. 3C), confirming that Cys-395, the single cysteine underlying reduction-dependent labeling of NCKX2, was located inside the cell as predicted by hydrophathy analysis (19).

**Redox Regulation of Wild-type NCKX2 Activity and Involvement of Cys-395**—The observation that MPB labeling of NCKX2 was β-ME-dependent led to the hypothesis that reducing agent might also play a role in regulating exchanger activity, as reported for cardiac NCX1 (26, 27). Therefore, cells were subjected to two sequential switches to Na\(^{+}\)-free solution II (140 mM Li\(^{+}\), 5 mM K\(^{+}\)) to measure NCKX2 activity, separated by a 15-min incubation with either 2% β-ME or ethanol in physiological solution III (140 mM Na\(^{+}\), 5 mM K\(^{+}\)) followed by a 2-min wash with solution III. The magnitude of the difference between peak and base line for each perfusion-induced fluctuation in fura-2 ratio was compared before and after the application of reagents. Fig. 4A shows the effects of 2% β-ME or ethanol on the increase in fura-2 ratio for transfected HEK-293 cells. Cells transfected with vector alone (traces a and b) had no significant change in fura-2 ratio. After a 15-min application of 2% β-ME, the fura-2 ratio peak of wild-type NCKX2 (trace c) was enhanced to an average of 113.2 ± 3.2%, and the peak of the Cys-395 → Ala mutant (trace d) was decreased to 86.33 ± 7.3%. In comparison, the control treatment with 2% ethanol for 15 min caused the fura-2 ratio peak after treatment in cells expressing wild-type (trace e) and Cys-395 → Ala (trace f) to decrease to 81.4 ± 8.4 and 71.6 ± 19.8%, respectively. Similar small inhibitory effects of ethanol have been observed for a number of neurotransmitter receptors and ion channels, such as voltage-gated Ca\(^{2+}\) channels and the glutamate receptor (38). One-way analysis of variance revealed that Ca\(^{2+}\) transport activity of wild-type NCKX2 treated with β-ME differed significantly from that of the other three groups (p < 0.01). No significant difference was observed among results for the Cys-395 → Ala mutant treated with β-ME, the wild-type treated with ethanol, and the Cys-395 → Ala mutant treated with ethanol (p > 0.05).
The peak ratios for the Cys-395 Ala mutation were significantly different from the peak ratio for wild-type NCKX2 incubated with 2% -mercaptoethanol (left column) and with PA1–926 anti-NCKX2 antibody (right column), detected by anti-FLAG antibody (top) and with PA1–926 anti-NCKX2 antibody (bottom), respectively.

**DISCUSSION**

In this study, we have prepared cDNA constructs that express the plasma membrane NCKX2 exchanger protein with various single or combined mutations of cysteine residues, to investigate the role of native sulfhydryls in the expression and function of NCKX2. We demonstrated that, of eight endogenous cysteine residues, both Cys-614 and Cys-666 were critical for functional expression of NCKX2 in HEK-293 cells. In mammalian cells, the cytosol is a reducing environment, which prevents the formation of inter- or intrachain disulfide bonds between intracellularly exposed cysteine residues. Therefore, disulfide bonds of an integral membrane protein most likely exist either extracellularly, embedded internally in the protein structure, or within the lipid bilayer. Thus, we speculated that Cys-614 and Cys-666 might form a structurally and functionally important cystine disulfide bond, exposed on the extracellular side of the plasma membrane.

To test this hypothesis, we examined the location of the nearby C terminus of NCKX2 with carefully controlled immunofluorescence experiments. Our data confirmed an extracellular location of the C terminus. On the basis of these data, we propose a new topology model for NCKX2 (Fig. 2C) that is consistent with both the prediction for NCKX3 (21) and the data on the putative bacterial Na\(^{+}/\)Ca\(^{2+}\) exchanger protein, YrbG (25). These findings give rise to the possibility that NCKX-type exchangers may have a different topology than NCX-type exchangers in which the C terminus is believed to be outside the cell (3).

NCKX-type exchangers and NCX-type exchangers share no significant similarity in their amino acid sequences outside the α-repeat regions. However, both new models for NCKX- and NCX-type exchangers place the α-repeat regions on the opposite face of the membrane (22). Thus, it is possible that NCX and NCKX exchangers have similar conserved structural elements formed by the α-repeat regions, surrounded by a different overall transmembrane structure. It remains an intriguing possibility that such structural differences between NCKX- and NCX-type exchangers may underlie their distinctive ion stoichiometry. The accuracy of this new NCKX-type exchanger topology will need more supportive proof from further experimental studies.

Studies using cysteine-scanning mutagenesis of NCX1 have revealed a novel C-terminal structure that differs remarkably from the previous putative topological model based on hydropathy analysis (3). A cysteine-labeling experiment using biotin precipitation, and thus Cys-395 was not required for the NCKX2 oligomeric complex.
maleimide demonstrated that the endogenous sulphydryls of NCKX2 could not be detected under normal conditions. Therefore, cysteine residues were reintroduced, one at a time, at sites in the putative C-terminal loops. None of these reintroduced cysteine residues reacted with biotin maleimide, even after β-ME treatment, suggesting they may be buried in the membrane and hence inaccessible for labeling. Furthermore, and discussed below, β-ME-dependent labeling of NCKX2 did not involve the endogenous Cys-614 and Cys-666 proposed to have an extracellular disposition. These results may indicate that the current model for threading of the C terminus of protein of NCKX2 through the membrane needs substantial revision, as demonstrated by the topological studies of NCX1 (22, 23). Furthermore, treatment of NCKX2 with reducing agent stimulated its activity, an effect that was abolished by mutation of Cys-395 to Ala. Redox signaling has been shown to participate in modulating the activity of several ion channels and transporters (39), such as the N-methyl-D-aspartate receptor NR1 subunit (40), the cystic fibrosis transmembrane conductance regulator (41), the ryanodine receptor 1 (42, 43), and the G protein-coupled inwardly rectifying K+ channel (44).

The activity of cardiac Na+/Ca2+ exchanger was also shown to be markedly stimulated after incubation with a combination of both reducing and oxidizing agents (26). This observation was verified using cloned canine NCX1.1 expressed in Xenopus oocytes (27). Interconversion of thiol and disulfide groups was initially speculated to be the molecular mechanism involved in redox modulation of exchange activity (26). Analysis of redox stimulation using mutated NCX1.1 constructs, although ruling out the involvement of individual cysteine residues, did not identify which amino acids were responsible. Indeed, redox-dependent stimulation of wild-type NCX1.1 was suggested to be primarily an elimination of Na+-dependent inactivation (27), a process that involves several regions of the intracellular loop (45, 46).

Our data, on the other hand, clearly demonstrate the necessity of Cys-395 in the reduction-dependent modification and stimulation of NCKX2. The remaining question is what molecular component might have been associated with Cys-395 before the reducing reagent abolished the interaction, hence rendering Cys-395 available for MPB labeling? An intracellular cysteine may be subject to reducible modifications, such as S-nitrosylation (47), palmitoylation (48), and, if protected from the reducing environment of the cytoplasm, a disulfide bond. The level of nitric-oxide synthase in HEK-293 cells is too low to be detected (49), so it is very unlikely that Cys-395 is S-nitrosylated. The common protein palmitoylation motif requires the palmitoylated cysteine residue to be located either within a TMS or at the cytoplasmic side of the membrane near a TMS, for instance 12 amino acids away as found in the α2δ-adrenergic receptor or β2-adrenergic receptor (50, 51). These considerations indicate that Cys-395, found in the middle of the large cytoplasmic loop, is unlikely to undergo palmitoylation. Thus, formation of a disulfide bond is left as the most likely explanation.

We have demonstrated that NCKX2 monomers associate in a homo-oligomeric complex, based on the observation of co-immunoprecipitation of wild-type and FLAG-tagged NCKX2 proteins. However, our data show that the Cys-395 → Ala mutation in neither wild-type nor FLAG-tagged NCKX2 inhibited self-association of NCKX2 monomers. This implies that oligomerization of NCKX2 monomers is primarily based on non-covalent, possibly hydrophobic, interactions. A similar situation has been documented for the extracellular Ca2+-sensing receptor, where dimerization still occurs without covalent interactions through intermolecular disulfide bonds (52). Once formed, however, such oligomeric complexes are then often “locked” in place, giving rise to a structural constraint conferred by the formation of the covalent disulfide bond (52).

Self-association of ion transporters can play an important role in function, regulation, or possibly cellular location (53), as found in the oligomerization of serotonin transporters (54) and glutamate transporters (55). The stimulation of NCKX2 activity induced by β-ME may be mediated through disruption of the putative Cys-395 disulfide bond, but appears not to require elimination of NCKX2 oligomerization, because NCKX2 mutants lacking Cys-395 can still form oligomers. Recently, NCKX1 has been reported to exist as a homodimer in bovine rod photoreceptors (56) and an inhibitory protein domain was proposed to be present at the contact site of the dimerized NCKX1 exchanger (57). Thus, even though Cys-395 of NCKX2 is not required for oligomerization, this covalent linkage may constrain conformational changes required for NCKX2 transport function, in a manner analogous to NCX1. Therefore, reducing this bond, although not destroying oligomerization, may relieve a structural constraint, allowing increased exchange activity. This speculation is consistent with the requirement that Cys-395 is not accessible to the glutathione-mediated reducing environment of the cytoplasm, but once reduced by the small β-ME molecule, becomes accessible to MPB labeling.

The redox-dependent modification of Cys-395 in NCKX2 and its subsequent functional consequence is a novel aspect of dynamic regulation of K+-dependent Na+/Ca2+ exchangers, although the detailed molecular mechanisms underlying this modulation still need to be resolved. It seems likely that redox regulation of NCKX2 serves a potential protective mechanism by increasing efflux of Ca2+2 in response to hypoxic and ischemic conditions.

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