



Structural and functional analysis of extracellular loop 4 of the Nhe1 isoform of the Na⁺/H⁺ exchanger

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ABSTRACT

The mammalian Na⁺/H⁺ exchanger isoform 1 (NHE1) is a ubiquitously expressed plasma membrane protein. It regulates intracellular pH by removing a single intracellular H⁺ in exchange for one extracellular Na⁺. The membrane domain of NHE1 comprises the 500 N-terminal amino acids and is made of 12 transmembrane segments. The extracellular loops of the transmembrane segments are thought to be involved in cation coordination and inhibitor sensitivity. We have characterized the structure and function of amino acids 278–291 representing extracellular loop 4. When mutated to Cys, residues F277, F280, N282 and E284 of EL4 were sensitive to mutation and reaction with MTSET inhibiting NHE1 activity. In addition they were found to be accessible to extracellular applied MTSET. A peptide of the amino acids of EL4 was mostly unstructured suggesting that it does not provide a rigid structured link between TM VII and TM VIII. Our results suggest that EL4 makes an extension upward from TM VII to make up part of the mouth of the NHE1 protein and is involved in cation selectivity or coordination. EL4 provides a flexible link to TM VIII which may either allow movement of TM VII or allow TM VIII to not be adjacent to TM VII.

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1. Introduction

A key pH regulatory protein in mammalian cells is the Na⁺/H⁺ exchanger, type I isoform (NHE1). NHE1 is a ubiquitous plasma membrane protein that catalyzes removal of a single intracellular proton in exchange for a single extracellular sodium ion [1–3]. Aside from regulation of intracellular pH, NHE1 has numerous other indirect physiological roles. It promotes cell growth and differentiation [4], facilitates inward sodium flux in response to osmotic shrinkage [5], and enhances cell motility [6]. Additionally, NHE1 has several pathological roles. Studies in culture have suggested that NHE1 may be important in breast cancer cell invasiveness and have demonstrated that in NHE1 activity enhances invasion by breast cancer cells not only by raising intracellular pH, but also by acidification of the extracellular microenvironment of tumor cells [7–10]. The extracellular acidification may be necessary for protease activation which facilitates the digestion and remodeling of the extracellular matrix [8], critical in metastasis.

In the myocardium it promotes heart hypertrophy and exacerbates the damage that occurs during myocardial ischemia and reperfusion.

Abbreviations: CHO, Chinese hamster ovary; EL, extracellular loop; HA, hemagglutinin; NHE1, Na⁺/H⁺ exchanger type 1 isoform; MTSES, ([2-Sulfonatoethyl] methanethiosulfonate); MTSET, ([2-(Trimethylammonium)ethyl] methanethiosulfonate); TM, transmembrane segment

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Inhibitors of NHE1 have proven useful in preclinical trials, for protecting the myocardium in various disease states [11–14]. However clinical trials with NHE1 inhibitors have been disappointing, possibly due to a lack of specificity of the inhibitors [15]. Further knowledge of NHE1 structure and function may facilitate the development of more specific inhibitors for clinical use.

The mammalian NHE1 protein is believed to consist of 12 transmembrane (TM) segments that comprise the N-terminal 500 amino acids. These are connected by a series of extra membrane segments which form intracellular and extracellular loops (EL). Loops between α -helices can influence their arrangements and packing [16] and can modulate protein function [17]. For NHE1, EL5 may be involved in drug sensitivity and cation binding [18,19]. EL2 links TM segments I and II and mutation of residues of EL2 affects both the drug sensitivity and the activity of NHE1 [20,21]. Two models of the topology of NHE1 are in question with two different models, one model is based on cysteine accessibility studies [22] and a second being based on computational comparison with the deduced structure of the *Escherichia coli* Na⁺/H⁺ exchanger NhaA suggested [23]. Although significant variation in the predictions occurs after amino acid 315, prior to this region the models are essentially identical with the exception of the latter model predicting that the first two transmembrane segments are cleaved free of the protein. Amino acids 278–290 are predicted to form an EL in both models. They form EL4 connecting TM VII and VIII in the model of Wakabayshi et al. [22] and are predicted to form the same loop in the model of Landau et al. [23] connecting the same two transmembrane segments that are numbered V and VI in that model. Little is known about this particular loop connecting

TM VII (amino acids 251–273) and VIII (amino acids 291–311) in terms of its functional role and contribution by the amino acids present in it. We have earlier examined the structure and function of TM VII. We found that TM VII is a predominantly α -helical segment, that is critical to NHE1 function and that it contained pore lining residues [24,25].

ELs of transporters are often critical to their function. For example in the serotonin transporter, the glutamate transporter and the glycine transporter [26–28] ELs play key roles in function. For Na^+, K^+ ATPase the second EL is a major determinant of extracellular cation affinity [29,30]. Amino acids of the acetylcholine receptor [31,32] and NhaA [33] ELs are also involved in the attraction of cation to the extracellular surface. In this study we examined the role of EL4 (amino acids 274–290) of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. We also used NMR to characterize the structural properties of synthetic peptides representing EL4.

2. Materials and methods

2.1. Materials

Streptavidin–agarose resin was from Sigma-Aldrich (St. Louis, MO, USA). Toronto Research Chemicals, (Toronto, Ontario, Canada) supplied MTSET and MTSES. Lipofectamine™ 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA) and PWO DNA polymerase was obtained from Roche Applied Science (Roche Molecular Biochemicals, Mannheim, Germany). Sulfo-NHS-SS-biotin was from Pierce Chemical Company (Rockford, IL, USA).

2.2. Site-directed mutagenesis

For expression and mutagenesis of the human NHE1 protein we used the expression plasmid pYN4+ that contains a fully functional hemagglutinin (HA) tagged human NHE1 isoform of the Na^+/H^+ exchanger with all native cysteines mutated to serine [34]. Mutations to the NHE1 cDNA in pYN4+ changing the indicated amino acids to cysteine (Supplementary Table 1), where indicated, were made using the functional cysteineless NHE1 protein that we described earlier [35]. We have characterized amino acids 271–273 as part of our earlier work on TM VII [25]. Site-directed mutagenesis was as described earlier [35]. Mutations were designed to create or delete a restriction enzyme site. DNA sequencing confirmed the fidelity of DNA amplification and mutations.

2.3. Cell culture and transfections

Stable cell lines were made using AP-1 cells transformed with control and mutant pYN4+ Cysless DNA [19]. AP-1 cells lack their endogenous Na^+/H^+ exchanger as described earlier. Transfection was with Lipofectamine™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) [34]. Stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 5 and 9. At least two independently made clones of each mutant were made and results are shown from one mutant. Independently made clones all had very similar functional characteristics.

2.3.1. SDS-PAGE and immunoblotting

Cell lysates were made as described earlier [34] and were used for Western blot analysis on 10% SDS/polyacrylamide gels [36]. Identification of the NHE1 protein was via the hemagglutinin (HA) tag on the Na^+/H^+ exchanger using an anti-HA monoclonal antibody. The second antibody was peroxidase-conjugated goat anti-mouse antibody. Chemiluminescence was used to visualize immunoreactive proteins. Quantification was using ImageJ 1.35 software (National Institutes of Health, Bethesda, MD, USA) examining protein expression on X-ray films.

2.4. Cell surface expression

The amount of NHE1 protein that was targeted to the cell surface was measured essentially as described earlier [34]. Briefly, cell surfaces were labeled with sulfo-NHS-SS-biotin and immobilized streptavidin was used to remove plasma membrane labeled NHE1 protein. Equivalent amounts of total and unbound proteins were analyzed by blotting for HA-tagged NHE1 protein. It was not possible to efficiently and reproducibly elute proteins bound to immobilized streptavidin resin. The relative amount of NHE1 on the cell surface was calculated for both the 110 kDa and the 95 kDa (partial or de-glycosylated) HA-immunoreactive species in Western blots of the fractions as indicated in the figures and legends.

2.5. Accessibility of EL4 residues

The accessibility of residues of EL4 was determined based on a modified procedure of [37]. Stable cell lines with mutants of EL4 were grown to confluence and after washing with phosphate buffer saline (PBS), were treated with or without 10 mM MTSET for 20 min at 37 °C in a buffer consisting of 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1.0 mM MgSO_4 , 5.5 mM glucose and 10 mM HEPES, and pH 7.3 (normal buffer). Cells were washed with PBS three times and then 2 ml of a lysis buffer was added, consisting of 25 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol and 37.5 μM ALLN and a protease inhibitor cocktail described earlier [38]. Cells were scraped off and sonicated two times for 15 s. The solution was spun at 35,000 rpm (100,000 $\times g$) for 1 h and the supernatant was collected. The supernatant was used to immunoprecipitate the HA-tagged NHE1 protein. For this purpose we used the Pierce Crosslink IP kit in which the primary antibody was crosslinked to Protein A/G agarose enabling immunoprecipitation without contamination from the primary antibody. The primary antibody used for immunoprecipitation was a commercially obtained rabbit polyclonal (Santa Cruz, sc-805). After immunoprecipitation, the sample was divided into two. One sample was used to quantify the total NHE1 protein via western blotting. To the second sample IRDye800–maleimide (LI-COR) was added (final concentration 0.2 mM) which would react with any unblocked sulfhydryls of the introduced cysteine. Samples were separated by SDS-PAGE and we examined the protein reacting with the IRDye800–maleimide using the LI-COR system. MTSET accessibility = $100 - \frac{\text{Fluorescence in the presence of MTSET}}{\text{Fluorescence in absence of MTSET}}$. Readings were corrected for the amount of NHE1 immunoprecipitated by Western blotting for NHE1 with a monoclonal antibody against the HA tag.

2.6. Na^+/H^+ exchange activity

Na^+/H^+ exchange activity was measured as described earlier [25] using a spectrofluorometer and the pH sensitive dye 2',7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM (Molecular Probes Inc., Eugene, OR, USA). Ammonium chloride induced an acute acid load as described earlier [39]. The initial rate of Na^+ (135 mM)-induced recovery of cytosolic pH was measured as described earlier [25]. Buffering capacities of stable cell lines did not vary and the initial recovery rate measured was from equivalent initial pHs. Wherever indicated, NHE activity was corrected for the level of protein expression and for the targeting of the protein to the cell surface. To determine reactivity with externally reactive sulfhydryl compounds, cells were treated with MTSET or MTSES using a two-pulse acidification assay. Cells were treated with ammonium chloride two times and allowed to recover twice in NaCl containing medium [35]. The first pulse was in the absence of inhibitor and the second pulse was performed after the cells were treated with either 10 mM MTSET or MTSES for 10 min in NaCl containing buffer. The starting pH of recovery was equivalent in the two pulses. The rate of recovery in the absence of inhibitor was

compared to the rate in the presence of inhibitor. The calculation used for residual activity was

$$\% \text{ residual activity} = \frac{(\text{pH change after Reagent})}{(\text{pH change w/o Reagent})} \times 100\%.$$

Results are shown as mean \pm SE. Statistical significance was determined using a Mann–Whitney *U* test.

2.7. Peptide synthesis, purification and preparation

Linear and cyclic peptides representing EL4 (sequence acetyl-YHLFEFANYEHVGVGIVDI-amide, and disulfide-cyclized acetyl-CGYHLFEFANYEHVGVGIVDIFLGFLC-amide), were purchased from GL Biochem (Shanghai) Ltd. The peptide was purified by HPLC using in house services of the Institute for Biomedical Design. The identity of the peptide was confirmed by using matrix-assisted laser desorption ionization mass spectrometry and by sequential assignment of the NMR spectra.

Samples for NMR structure determination contained 1.0 mM of purified peptide dissolved in DMSO- d_6 . The linear and cyclic peptide samples were at temperatures of 30 °C and 35 °C, respectively. Spectra were referenced so that the residual DMSO (CH_3) $_2$ peak is at 2.50 ppm. 2D ^1H - ^1H DQF-COSY, TOCSY, and NOESY spectra were taken on either 500 MHz or 800 MHz NMR spectrometers. Spectra were processed with NMRPipe [40] and sequential assignment [41] was performed in NMRView [42].

3. Results

3.1. Characterization of EL4 mutants

Fig. 1 illustrates a model of EL4 and shows the amino acids present. EL4 consists of residues Y274–I291. It contains several acidic residues and a mixture of hydrophilic and hydrophobic amino acids. EL4 was

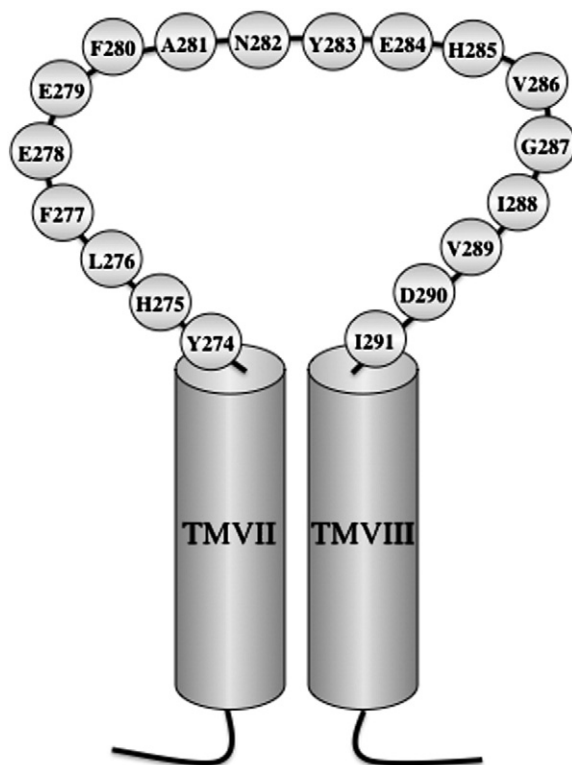


Fig. 1. Models of EL4 of the NHE1 isoform of the Na^+/H^+ exchanger. Topological model of the EL4 of the transmembrane domain of the NHE1 isoform of the Na^+/H^+ exchanger. TM segment numbering is based on cysteine accessibility studies [22].

initially studied by a combination of site specific mutagenesis and cysteine scanning mutagenesis. The acidic residues of EL4, E278, E279, E284 and D290, were changed to their corresponding non-charged amino acids, E to Q and D to N in individual mutant proteins. Also, all the amino acids of EL4 were changed to cysteine in the background of the cysteineless NHE1 (cNHE1) protein. We first determined the expression levels, targeting and activity of the mutant NHE1 proteins. Fig. 2A–C illustrates these results. Expression levels were determined by western blotting with antibody against the HA tag on the Na^+/H^+ exchanger protein. Fig. 2A demonstrates that all the mutant proteins were expressed. As shown earlier [35], NHE1 was expressed as a fully glycosylated and a partial or de-glycosylated protein and these two immunoreactive bands were present in all stable cell lines. Expression of several of the mutants was reduced. Most notably, the E279Q and Y283C protein levels were less than 10% of the level of cNHE1. The mutants Y274C, L276C, V286C and I291C also had reduced expression that was between 50 and 30% of cNHE1. Most other mutants had some slight decrease in expression compared to controls, though a few (D290N, H275C, F277C, E279C, F280C) were equivalent to cNHE1. For both the E279Q and the V286C mutants, aside from their lower levels of expression, it was notable that a larger percentage of the expressed protein was present as the unglycosylated form.

We next examined the targeting of the mutant proteins to the cell surface. Cell surface biotinylation experiments were done as described earlier [25]. The results are shown in Fig. 2B. The mutant proteins targeted well to the cell surface. Though minor variations occurred in the efficiency of targeting, these were not nearly as large as we have observed earlier with mutations of transmembrane regions of the NHE1 protein [34,35].

Determination of the activity of the EL4 mutants showed that most of the mutants had reduced activity relative to either the wild type or cysteineless NHE1 protein. With the exception of the G287C and I288C mutants, all of the activity was less than half of that of the control (Fig. 2C). The uncorrected activity of the E279Q, Y274C, L276C, Y283C and V286C mutants was less than 10% of the control level and these mutants were considered inactive or nearly so, and were not used for further study. For the E279Q and the Y283C much of the decrease in measurable activity was due to a decrease in the expression level of the protein, though the residual activity was too low for further characterization.

We determined the effect of MTSET and MTSES on the activity of the remaining active cysteine mutants. The results are shown in Fig. 3. Mutant F277C was exquisitely sensitive to inhibition by positively charged MTSET, but not to inhibition by negatively charged MTSES. Mutants F280C, N282C and E284C were all also partially sensitive to inhibition by MTSET while only mutant F280C was partially sensitive to inhibition by MTSES.

It is possible that some Cys residues were reactive with MTSES or MTSET but that this was not inhibitory to NHE1 activity. We therefore measured the accessibility of the active cysteine mutants. The results are shown in Fig. 4. Residues that were inhibited by MTSET when changed to Cys (F277, F280, N282, E284) were more accessible to MTSET and incubation with MTSET blocked reactivity with IRDye800-maleimide. The F280C mutant protein was less accessible than the F277C, N282C and E284C containing proteins, but was more accessible than other residues.

3.2. Characterization of EL4 protein

We purchased two chemically synthesized peptides containing the EL4 sequence for high-resolution NMR spectroscopy. The first peptide was a linear peptide containing Y274–I291 of EL4, with N-terminal acetyl and C-terminal amine caps to remove the charges at the peptide termini. A second peptide of the sequence acetyl-CG-Y274-L296-C-amine was also synthesized. Additional residues on the C-terminal end of this second peptide corresponded to the slight difference in prediction of the locations of the transmembrane helices between the topology models proposed by [22] and [23]. The peptide also contained disulfide-linked cysteines at the termini to produce a cyclic peptide, which may restrict

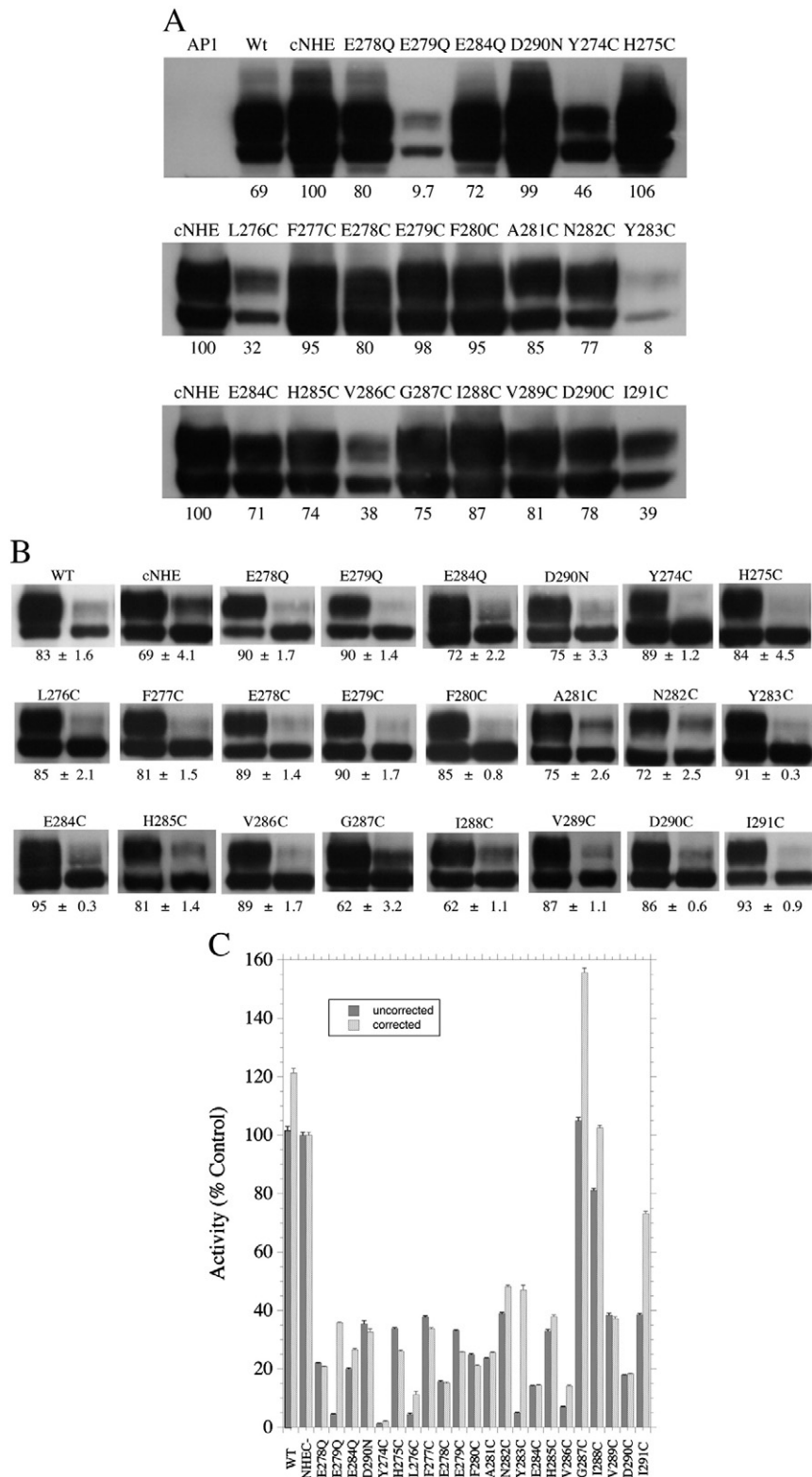


Fig. 2. Analysis of wild type NHE1 and mutant EL4 proteins. **A**, Western blot of whole cell lysates of stable cell lines expressing Na^+/H^+ exchanger EL4 mutants or control protein. Mutations are as indicated. 100 μg of total protein was loaded in each lane. Numbers below the lanes indicate the amount of NHE1 protein relative to wild type NHE. Mean values ($n = 3-4$) were obtained from densitometric scans of both the 110 and 95-kDa bands. AP-1 refers to AP-1 cells mock transfected. Wt, and cNHE1 refer to cells stably expressing wild type Na^+/H^+ exchanger protein and the cysteineless NHE1, respectively. **B**, Surface localization of NHE1 in AP-1 cells expressing control and EL4 mutants. Equal amounts of total cell lysate (left lane) and unbound intracellular lysate (right lane) were examined by Western blotting with anti-HA antibody to identify NHE1 protein. WT and cNHE1 are cell lines stably expressing wild type NHE1 and cysteineless NHE1, respectively. The percent of the total NHE1 protein found on the plasma membrane is indicated for each mutant. For Ct this indicates the amount of non-specific binding to streptavidin-agarose beads. Results are the mean \pm the S.E. $n =$ at least 4 determinations. Autoradiography exposure times were increased for mutants expressing lower levels of protein. **C**, Summary of the rate of recovery after an acute acid load of AP-1 cells transfected with wild type NHE1, cNHE, and EL4 Na^+/H^+ exchanger mutants. The mean activity of cNHE1 stably transfected with NHE1 was 1.7 Δ pH/min, and this value was set to 100% and other activities are a percent of those of cNHE. Values are the mean \pm SE of 6–10 determinations. Results are shown for mean activity of both uncorrected (black) and normalized for surface processing and expression levels (gray).

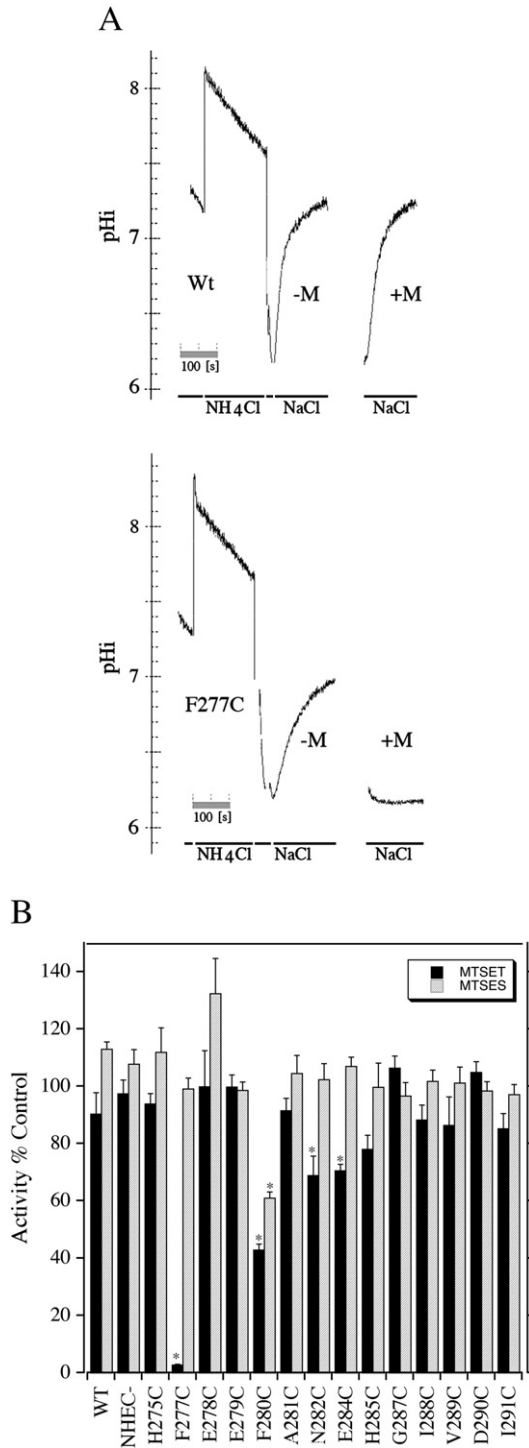


Fig. 3. Effect of sulphhydryl reactive compounds, MTSET and MTSES, on activity of cNHE1 and single cysteine NHE1 mutant containing cell lines. A, Example of results of effect of MTSET on activity of cNHE1 and F277C NHE1 mutant. NHE1 protein activity was assayed in stably transfected AP-1 cells as described in the “Materials and methods”. Activity was measured after two acid pulses. The first pulse illustrated was in the absence of MTSET. For ease of viewing, only the recovery from acidosis is shown for the second pulse, in which cells were treated with MTSET. NH_4Cl , treatment with ammonium chloride. After NH_4Cl treatment, there was a brief Na Free treatment to induce acidosis. NaCl, time when recovery from acidosis was in NaCl containing buffer, for the second pulse this contained MTSET as indicated and cells were pretreated with MTSET for 10 min prior to NH_4Cl induced acid load. B, Summary of effects of MTSET and MTSES on NHE activity of EL4 mutants. Activity was measured after dual ammonium chloride pulses as described in the “Materials and methods”. The second acidification was after cells were treated with 10 mM MTSET or MTSES. Results are presented as the % of activity of the second acid load, relative to the first. * indicates that the second recovery from acid load was significantly lower than the first at $^*P < 0.05$. Solid filled bars represent MTSET treatments and hatched bars are MTSES treatments.

the conformations adopted by the extracellular loop, and a glycine spacer at the N-terminal end to increase the distance between the ends of EL4. These were designed to link the N and C-terminals of the peptide, mimicking a putative association between transmembrane segments VII and VIII.

1D ^1H NMR spectra were used to judge the quality of the samples of solubilized EL4 for structure determination. Spectra of the linear peptide in aqueous buffer (50 mM imidazole, 100 mM KCl, 10% D_2O , 0.25 mM DSS-d_6) suggested that the peptide was unstructured in aqueous solution. HN and H α peaks in the 1D spectra were distributed over a very narrow range, 8.0–8.4 ppm and 4.1–4.3 ppm, respectively. Addition of up to 20% TFE-d_3 did not affect the dispersion of the HN and H α peaks and again suggested that the peptide was unstructured. The peptide sample was lyophilized and reconstituted in DMSO-d_6 , where there appeared to be an improvement in the dispersion of the peaks, suggesting it may be more structured in DMSO. 2D NMR spectra were then acquired for assignment and structure determination. The cyclic peptide was found to be insoluble in H_2O and $\text{TFE/H}_2\text{O}$, but was soluble in DMSO. NMR spectra were also acquired for this peptide in DMSO.

Differences in the quality of the spectra could be observed between the linear and cyclic peptides. Spectra of the linear peptide contained relatively narrow peaks, while the spectra of the cyclic peptide contained broad peaks as shown by the amide regions of the 1D NMR spectra (Fig. 5). Both peptides could be fully assigned. The cyclic peptide also contains additional unassigned peaks that may have been either impurities or minor conformations of the peptide.

The NMR spectra suggest that despite the increased dispersion of the peaks in DMSO compared to aqueous solution, the peptide is still mostly unstructured in DMSO. There is poor chemical shift dispersion in the H α dimension, as shown by the 2D DQFCOSY spectra (Fig. 5) with the non-glycine H α peaks limited to 4.1–4.6 ppm in both the linear and cyclic peptides. The NOESY spectra of both peptides contained weak intraresidue HN–H α peaks and strong sequential H α –HN connectivities, and generally lack medium and long range connectivities, also indicative of poorly structured peptides. A comparison of H α chemical shift values with published random coil chemical shift values measured in H_2O [43] and DMSO [44] also suggest that the peptides are relatively unstructured in solution (Fig. 6). Continuous regions of large negative chemical shift differences suggest alpha-helical content, while positive values would suggest beta-sheet content. Neither of the peptides shows any contiguous segments with either strong negative or positive values, again suggesting that the peptides are relatively unstructured. The similarities in the chemical shifts and chemical shift differences between the linear and cyclic peptides suggest that they may also be adopting similar, if relatively unstructured, conformations, despite the restraint provided by the disulfide bond in the cyclic peptide.

4. Discussion

4.1. Critical residues of EL4

In this study we examined EL4 of the NHE1 isoform of the Na^+/H^+ exchanger, examining the residues critical in function, their accessibility and the structure of EL4. There has been some previous related work [22] that examined the topology of NHE1 and the accessibility of a few residues of EL4. Mutation of amino acids E278 to Cys resulted in a protein in which all these mutants were all shown to be accessible extracellularly. In addition that study examined the effect of external MTSET on NHE1 activity. They found some similar but some different results from our study. Similar to their study, we found slight inhibitory effects of MTSET on the N282C mutant protein. However, we found many other differences. In contrast to their results, we found no significant inhibitory effect of MTSET on the A281C mutant. It should be noted that the effect of MTSET that was found earlier was very small. Another difference between their study and ours is that we found significant effects of MTSET on the E284C protein and of both MTSET and MTSES on the

F280C protein which was not shown earlier. Further, we found that the F277C mutant protein (which was not examined in that study) was inhibited most strongly by MTSET. Why they did not detect effects of MTSET on the F280C protein is uncertain at this time. However we found a significant, reproducible inhibitory effect of MTSET and in addition, this residue was accessible to MTSET. We noted that accessibility of the F280C mutant protein was temperature sensitive, and was greatly reduced at 30 °C (not shown). It should be noted that the earlier study examined NHE1 activity using a radioisotope assay examining Na⁺ uptake. The present assay, examining rapid kinetics of NHE1 in a dual pulse system, would likely be more sensitive and detect changes in activity that might not be detectable using a radioisotope assay. Of note, we found that the F277C mutant protein was the most reactive protein with MTSET, in our accessibility assay (Fig. 4), further confirming the results of the activity measurements.

We also found that with the exception of mutant F280C, positively charged MTSET was inhibitory while negatively charged MTSES was without effect. If EL4 is an external region of the NHE1 protein involved in cation coordination, it would appear reasonable that addition of a positively charged compound would be more inhibitory, possibly repelling a cation at the mouth of the protein. In the case of the F280C mutant protein, while MTSET was greatly inhibitory, negatively charged MTSES was also strongly inhibitory, though not as much as MTSET. In this case, effects on ion passage by MTSES are likely due to a steric hindrance caused by the addition of the bulky compound and are not charge related. Another mutant protein, the F277C mutant, was the most strongly inhibited by MTSET. We suggest that this side chain of this amino acid was more directly pointing toward the mouth of the extracellular cation funnel of the NHE1 protein. Therefore, its linkage to the positively charged MTSET more effectively caused inhibition of cation coordination in the mouth of the NHE1 protein. Overall, our study suggests that the residues F277, F280, N282 and E284 form an important part of the extracellular pore of NHE1. Mutation of the adjacent residues L276 and Y283 to Cys caused an inactive protein, suggesting they may also play an important part of the extracellular pore of NHE1. While further study of these residues is called for, clearly the side chain of these amino acids must be within certain constraints that were not met by substitution to Cys.

We also made mutations to eliminate the negative charge on amino acids E278, E279, E284 and D290. In all cases activity was reduced but not eliminated. For the mutant protein with the E279Q mutation, the decrease in activity was largely due to a reduction in the level of protein expression. We had hypothesized that having negatively charged side chains, these amino acids might be critical in cation attraction. However for these amino acids this does not appear to be the case. Nevertheless, it is apparent that only amino acid E279 is important of these, and the effects were mainly on protein expression. An effect on protein conformation or processing may occur.

4.2. Analysis of EL4 structure and function

High-resolution NMR analysis of the linear and cyclic EL4 peptides suggests that both peptides adopt mostly random-coil conformations in DMSO. DMSO has an intermediate dielectric constant (~46) [45] similar to the membrane interface [46], making it a reasonable mimetic of the environment experienced by the loop. A study by Katragadda et al. [47] showed that the NMR structures of isolated loops from bacteriorhodopsin in DMSO adopt structures equivalent to the known crystal structure of the protein. Other studies [48–50] including our own on EL2 of NHE1 [20], have also found structures in interhelical loops in aqueous solution or DMSO. However, this was not the case for EL4 of NHE1. It is unlikely that the length of the peptides we studied is the reason for the lack of structure, as the other studies on isolated loops have used peptides of similar lengths. The lack of structure could suggest that the loop is flexible, which could be important in the function of NHE1. Alternatively, the loop may only be structured in the full protein, where interactions with

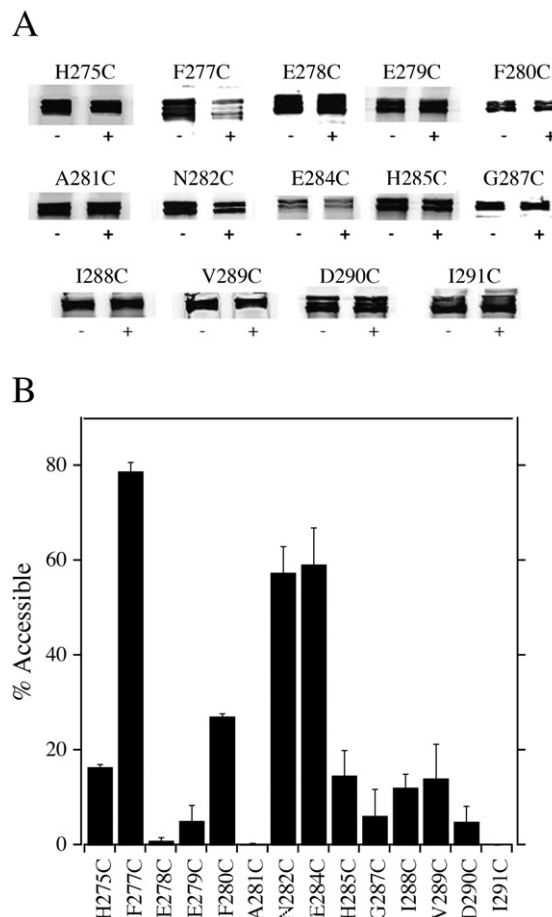


Fig. 4. Accessibility of residues of EL4 to reactivity with MTSES. The accessibility of the residues of EL2 to reactivity with MTSET was measured as described in the "Materials and methods". A, Examples of results illustrated MTSET blocking of reactivity with IRDye800-maleimide. Prior reaction with MTSET blocks the reactivity with IRDye800-maleimide. + indicates reacted with MTSET prior to reaction with IRDye800-maleimide. B, Summary of the results illustrating calculated accessibility of residues of EL4. Results are the mean ± SE of at least 3 experiments.

other extracellular regions of the protein and with the membrane bilayer could restrict the conformation of the loop. However to address this possibility we examine the EL4 peptide with its ends tethered by a disulfide bond. Even in this case we did not find a structured peptide. In the three-dimensional model of NHE1 proposed by Landau et al. [23] EL4 connects the transmembrane helices 5 and 6 of this model (corresponding to TM VII and TM VIII of the other model). The Landau et al. model does not suggest any interactions with each other. The lack of structure in the loop could be to allow the helices to be some distance apart from each other.

Our data are somewhat contradictory to a recent 3D model which did not place VII and VIII surrounding the catalytic core of the protein [51]. However, that model did not directly provide evidence for the location of these transmembrane segments and was largely based on homology modeling. We have earlier demonstrated that TM VII possesses several amino acids that when changed to Cys, are sensitive to derivatization with MTSET and affect protein function [24,25]. In addition, mutation of residues of TM VII altered sensitivity to NHE1 specific inhibitors [25]. Additionally, E262 of TM VII is exquisitely sensitive to mutation and changing that residue to an Asp, alters the affinity of the NHE1 protein to Li⁺ but not for Na⁺, suggesting that this amino acid is involved in cation coordination [19]. TM VII also has a flexible middle region from amino acids G261–S263 which may allow conformation changes associated with movement during transport [25,52]. These data all support a role for this transmembrane segment in NHE1 function which is consistent with a functional role for EL4 in the pore of the NHE1 protein.

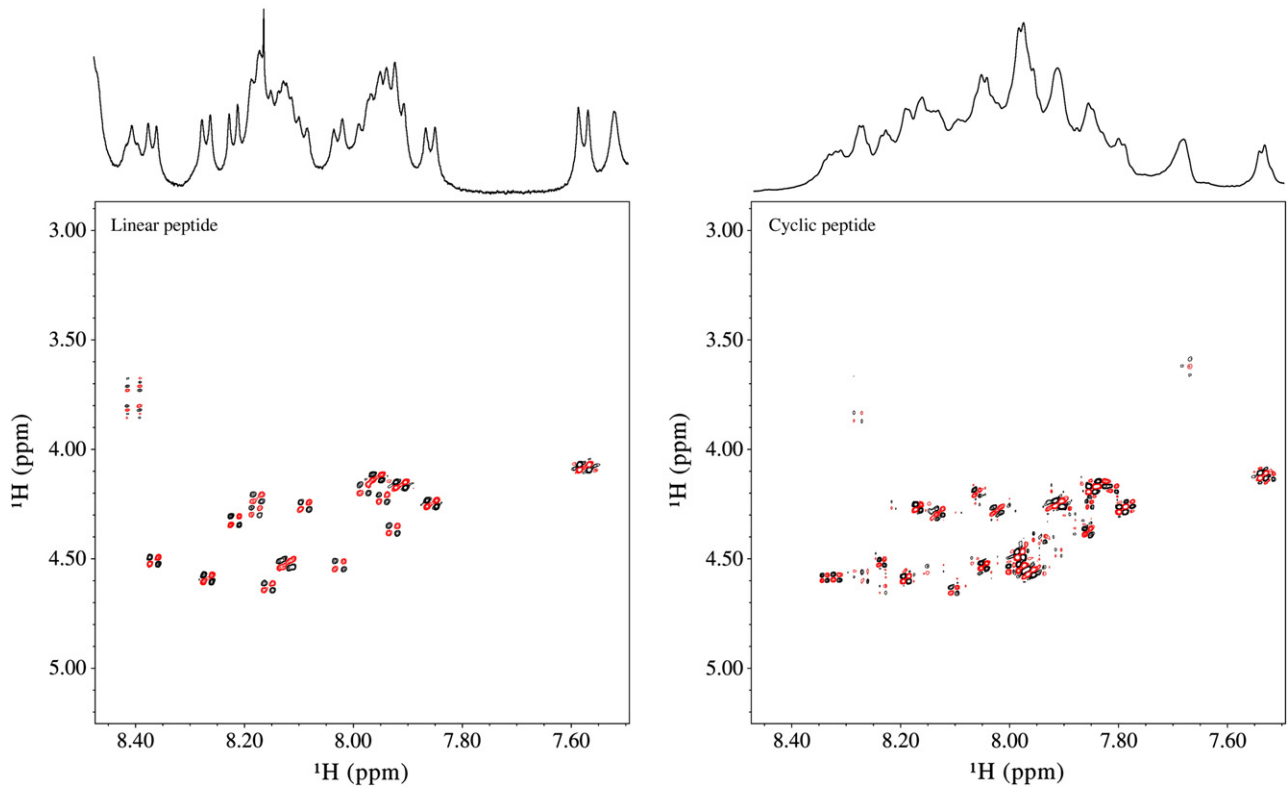


Fig. 5. 1D ^1H and 2D ^1H – ^1H DQF-COSY spectra of the linear and cyclic EL4 peptides in DMSO. The HN–H α “fingerprint” region of the 2D DQF-COSY spectra of the linear and cyclic peptides is shown. Above are the corresponding HN regions of the 1D NMR spectra of the peptides. Spectra for the linear peptide were acquired at 500 MHz and 30 °C, and 800 MHz and 35 °C for the cyclic peptide, and referenced to the DMSO peak at 2.50 ppm.

4.3. Conclusion

Overall, our results support a model in which TM VII is important in the catalytic core of the protein and amino acids 274–284 of EL4 make

an extension upward from TM VII to make up part of the mouth of the protein. A flexible connection from TM VII to TM VIII may allow TM VII to make conformational changes associated with its postulated role in transport [52]. F277, F280, N282, E284 are in particular shown

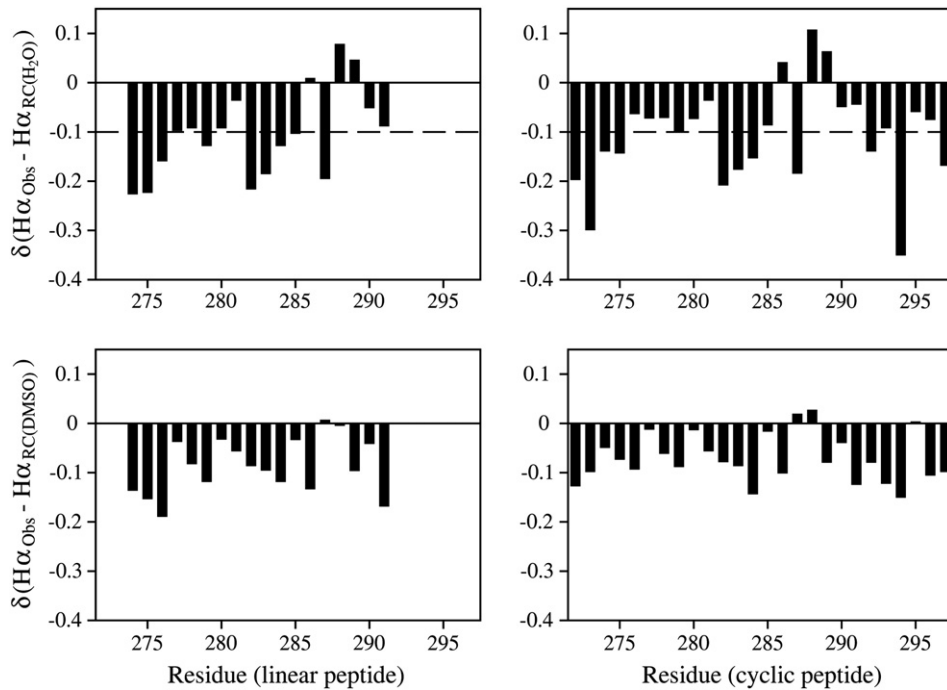


Fig. 6. Chemical shift index values for the linear and cyclic EL4 peptides in DMSO. Differences in H α chemical shift between observed H α values for the peptides and published random coil H α chemical shifts in water [53] and DMSO [44] are shown per residue. Contiguous regions of large negative secondary shifts suggest helical content while large positive secondary shifts suggest beta sheet content. Regions exceeding the cutoff value of -0.1 ppm (dotted line) when using random coil values determined in water suggest helical content [54].

to be critical as demonstrated by the inhibitory effect shown when mutated to Cys and reacted with positively charged MTSET.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamem.2012.06.021>.

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References

- [1] M. Karmazyn, M. Sawyer, L. Fliegel, The Na(+)/H(+) exchanger: a target for cardiac therapeutic intervention, *Curr. Drug Targets Cardiovasc. Haematol. Disord.* 5 (2005) 323–335.
- [2] L. Fliegel, Regulation of the Na⁺/H⁺ exchanger in the healthy and diseased myocardium, *Expert Opin. Ther. Targets* 13 (2009) 55–68.
- [3] C. Harris, L. Fliegel, Amiloride and the Na⁺/H⁺ exchanger protein. Mechanism and significance of inhibition of the Na⁺/H⁺ exchanger, *Int. J. Mol. Med.* 3 (1999) 315–321.
- [4] S. Grinstein, D. Rotin, M.J. Mason, Na⁺/H⁺ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation, *Biochim. Biophys. Acta* 988 (1989) 73–97.
- [5] L. Shrode, A. Cardano, G. Goss, S. Grinstein, Role of the Na⁺/H⁺ antiporter isoforms in cell volume regulation, In: in: L. Fliegel (Ed.), *The Na⁺/H⁺ Exchanger*, R.G. Landes Company, 1996, pp. 101–122.
- [6] S.P. Denker, D.L. Barber, Cell migration requires both ion translocation and cytoskeletal anchoring by the Na–H exchanger NHE1, *J. Cell Biol.* 159 (2002) 1087–1096.
- [7] G. Busco, R.A. Cardone, M.R. Greco, A. Bellizzi, M. Colella, E. Antelmi, M.T. Mancini, M.E. Dell'Aquila, V. Casavola, A. Paradiso, S.J. Reshkin, NHE1 promotes invadopodial ECM proteolysis through acidification of the peri-invadopodial space, *FASEB J.* 24 (2010) 3903–3915.
- [8] C. Stock, R.A. Cardone, G. Busco, H. Krahling, A. Schwab, S.J. Reshkin, Protons extruded by NHE1: digestive or glue? *Eur. J. Cell Biol.* 87 (2008) 591–599.
- [9] S. Harguindey, G. Orive, J. Luis Pedraz, A. Paradiso, S.J. Reshkin, The role of pH dynamics and the Na⁺/H⁺ antiporter in the etiopathogenesis and treatment of cancer. Two faces of the same coin – one single nature, *Biochim. Biophys. Acta* 1756 (2005) 1–24.
- [10] A. Paradiso, R.A. Cardone, A. Bellizzi, A. Bagorda, L. Guerra, M. Tommasino, V. Casavola, S.J. Reshkin, The Na⁺–H⁺ exchanger-1 induces cytoskeletal changes involving reciprocal RhoA and Rac1 signaling, resulting in motility and invasion in MDA-MB-435 cells, *Breast Cancer Res.* 6 (2004) R616–R628.
- [11] M. Karmazyn, Q. Liu, X.T. Gan, B.J. Brix, L. Fliegel, Aldosterone increases NHE-1 expression and induces NHE-1-dependent hypertrophy in neonatal rat ventricular myocytes, *Hypertension* 42 (2003) 1171–1176.
- [12] L. Fliegel, Regulation of myocardial Na⁺/H⁺ exchanger activity, *Basic Res. Cardiol.* 96 (2001) 301–305.
- [13] R.M. Mentzer Jr., R.D. Lasley, A. Jessel, M. Karmazyn, Intracellular sodium hydrogen exchange inhibition and clinical myocardial protection, *Ann. Thorac. Surg.* 75 (2003) S700–S708.
- [14] H.J. Lang, Chemistry of NHE inhibitors, In: in: M. Karmazyn, M. Avkiran, L. Fliegel (Eds.), *The Na⁺/H⁺ Exchanger, From Molecular to Its Role in Disease*, Kluwer Academic Publishers, Boston/Dordrecht/London, 2003, pp. 239–253, 318 pp.
- [15] E. Murphy, D.G. Allen, Why did the NHE inhibitor clinical trials fail? *J. Mol. Cell. Cardiol.* 46 (2009) 137–141.
- [16] V. Munoz, F.J. Blanco, L. Serrano, The hydrophobic-staple motif and a role for loop-residues in alpha-helix stability and protein folding, *Nat. Struct. Biol.* 2 (1995) 380–385.
- [17] M.M. Zhao, R.J. Gaivin, D.M. Perez, The third extracellular loop of the beta2-adrenergic receptor can modulate receptor/G protein affinity, *Mol. Pharmacol.* 53 (1998) 524–529.
- [18] E.R. Slepko, J.K. Rainey, B.D. Sykes, L. Fliegel, Structural and functional analysis of the Na(+)/H(+) exchanger, *Biochem. J.* 401 (2007) 623–633.
- [19] R. Murtazina, B.J. Booth, B.L. Bullis, D.N. Singh, L. Fliegel, Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na⁺/H⁺ exchanger, *Eur. J. Biochem.* 268 (2001) 4674–4685.
- [20] B.L. Lee, X. Li, Y. Liu, B.D. Sykes, L. Fliegel, Structural and functional analysis of extracellular loop 2 of the Na(+)/H(+) exchanger, *Biochim. Biophys. Acta* 1788 (2009) 2481–2488.
- [21] A. Khadiilkar, P. Iannuzzi, J. Orłowski, Identification of sites in the second exomembrane loop and ninth transmembrane helix of the mammalian Na⁺/H⁺ exchanger important for drug recognition and cation translocation, *J. Biol. Chem.* 276 (2001) 43792–43800.
- [22] S. Wakabayashi, T. Pang, X. Su, M. Shigekawa, A novel topology model of the human Na⁺/H⁺ exchanger isoform 1, *J. Biol. Chem.* 275 (2000) 7942–7949.
- [23] M. Landau, K. Herz, E. Padan, N. Ben-Tal, Model structure of the Na⁺/H⁺ exchanger 1 (NHE1): functional and clinical implications, *J. Biol. Chem.* 282 (2007) 37854–37863.
- [24] J. Ding, R.W.P. Ng, L. Fliegel, Functional characterization of the transmembrane segment VII of the NHE1 isoform of the Na⁺/H⁺ exchanger, *Can. J. Physiol. Pharmacol.* 85 (2007) 319–325.
- [25] J. Ding, J.K. Rainey, C. Xu, B.D. Sykes, L. Fliegel, Structural and functional characterization of transmembrane segment VII of the Na⁺/H⁺ exchanger isoform 1, *J. Biol. Chem.* 281 (2006) 29817–29829.
- [26] S.M. Mitchell, E. Lee, M.L. Garcia, M.M. Stephan, Structure and function of extracellular loop 4 of the serotonin transporter as revealed by cysteine-scanning mutagenesis, *J. Biol. Chem.* 279 (2004) 24089–24099.
- [27] M. Grunewald, D. Menaker, B.I. Kanner, Cysteine-scanning mutagenesis reveals a conformationally sensitive reentrant pore-loop in the glutamate transporter GLT-1, *J. Biol. Chem.* 277 (2002) 26074–26080.
- [28] B. Lopez-Corcuera, E. Nunez, R. Martinez-Maza, A. Geerlings, C. Aragon, Substrate-induced conformational changes of extracellular loop 1 in the glycine transporter GLYT2, *J. Biol. Chem.* 276 (2001) 43463–43470.
- [29] O. Capendeguy, P. Chodanowski, O. Michielin, J.D. Horisberger, Access of extracellular cations to their binding sites in Na, K-ATPase: role of the second extracellular loop of the alpha subunit, *J. Gen. Physiol.* 127 (2006) 341–352.
- [30] O. Capendeguy, J.D. Horisberger, The role of the third extracellular loop of the Na⁺, K⁺-ATPase alpha subunit in a luminal gating mechanism, *J. Physiol.* 565 (2005) 207–218.
- [31] N. Unwin, Acetylcholine receptor channel imaged in the open state, *Nature* 373 (1995) 37–43.
- [32] A. Miyazawa, Y. Fujiyoshi, N. Unwin, Structure and gating mechanism of the acetylcholine receptor pore, *Nature* 423 (2003) 949–955.
- [33] C. Hunte, E. Screpanti, M. Venturi, A. Rimón, E. Padan, H. Michel, Structure of a Na⁺/H⁺ antiporter and insights into mechanism of action and regulation by pH, *Nature* 435 (2005) 1197–1202.
- [34] E.R. Slepko, S. Chow, M.J. Lemieux, L. Fliegel, Proline residues in transmembrane segment IV are critical for activity, expression and targeting of the Na⁺/H⁺ exchanger isoform 1, *Biochem. J.* 379 (2004) 31–38.
- [35] E.R. Slepko, J.K. Rainey, X. Li, Y. Liu, F.J. Cheng, D.A. Lindhout, B.D. Sykes, L. Fliegel, Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na⁺/H⁺ exchanger, *J. Biol. Chem.* 280 (2005) 17863–17872.
- [36] A. Moor, X.T. Gan, M. Karmazyn, L. Fliegel, Activation of Na⁺/H⁺ exchanger-directed protein kinases in the ischemic and ischemic-reperfused rat myocardium, *J. Biol. Chem.* 27 (2001) 16113–16122.
- [37] K. Herz, A. Rimón, E. Olkhova, L. Kozachkov, E. Padan, Transmembrane segment II of NhaA Na⁺/H⁺ antiporter lines the cation passage, and Asp65 is critical for pH activation of the antiporter, *J. Biol. Chem.* 285 (2010) 2211–2220.
- [38] M. Michalak, L. Fliegel, K. Wlasichuk, Isolation and characterization of calcium binding glycoproteins of cardiac sarcolemmal vesicles, *J. Biol. Chem.* 265 (1990) 5869–5874.
- [39] N.L. Silva, H. Wang, C.V. Harris, D. Singh, L. Fliegel, Characterization of the Na⁺/H⁺ exchanger in human choriocarcinoma (BeWo) cells, *Pflugers Arch. Eur. J. Physiol.* 433 (1997) 792–802.
- [40] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, NMRPipe: a multidimensional spectral processing system based on UNIX pipes, *J. Biomol. NMR* 6 (1995) 277–293.
- [41] K. Wuthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986.
- [42] B.A. Johnson, Using NMRView to visualize and analyze the NMR spectra of macromolecules, *Methods Mol. Biol.* 278 (2004) 313–352.
- [43] D.S. Wishart, C.G. Bigam, J. Yao, F. Abildgaard, H.J. Dyson, E. Oldfield, J.L. Markley, B.D. Sykes, ¹H, ¹³C and ¹⁵N chemical shift referencing in biomolecular NMR, *J. Biomol. NMR* 6 (1995) 135–140.
- [44] A. Bundi, C. Grathwohl, J. Hockmann, R.M. Keller, G. Wagner, K. Wuthrich, Proton NMR of protected tetrapeptides TFA-Gly-Gly-L-X-L-Ala-OCH₃, where X stands for one of the 20 common amino acids, *J. Magn. Reson.* 18 (1975) 191–198.
- [45] W.M. Haynes, In: in: W.M. Haynes (Ed.), *CRC Handbook of Chemistry and Physics*, 92nd ed., CRC Press, Boca Raton, FL, 2011.
- [46] N. Bordag, S. Keller, Alpha-helical transmembrane peptides: a “divide and conquer” approach to membrane proteins, *Chem. Phys. Lipids* 163 (2010) 1–26.
- [47] M. Katragadda, J.L. Alderfer, P.L. Yeagle, Solution structure of the loops of bacteriorhodopsin closely resembles the crystal structure, *Biochim. Biophys. Acta* 1466 (2000) 1–6.
- [48] M. Bennett, J.A. Yeagle, M. Maciejewski, J. Ocampo, P.L. Yeagle, Stability of loops in the structure of lactose permease, *Biochemistry* 43 (2004) 12829–12837.
- [49] J. Wu, M. Feng, K.H. Ruan, Assembling NMR structures for the intracellular loops of the human thromboxane A2 receptor: implication of the G protein-coupling pocket, *Arch. Biochem. Biophys.* 470 (2008) 73–82.
- [50] K.H. Ruan, J. Wu, S.P. So, L.A. Jenkins, C.H. Ruan, NMR structure of the thromboxane A2 receptor ligand recognition pocket, *Eur. J. Biochem/FEBS* 271 (2004) 3006–3016.
- [51] E.B. Nygaard, J.O. Lagerstedt, G. Bjerre, B. Shi, M. Budamagunta, K.A. Poulsen, S. Meinild, R.R. Rigor, J.C. Voss, P.M. Cala, S.F. Pedersen, Structural modeling and electron paramagnetic resonance spectroscopy of the human Na⁺/H⁺ exchanger isoform 1, NHE1, *J. Biol. Chem.* 286 (2010) 634–648.
- [52] T. Reddy, X. Li, L. Fliegel, B.D. Sykes, J.K. Rainey, Correlating structure, dynamics, and function in transmembrane segment VII of the Na(+)/H(+) exchanger isoform 1, *Biochim. Biophys. Acta* 1798 (2010) 94–104.
- [53] D.S. Wishart, C.G. Bigam, A. Holm, R.S. Hodges, B.D. Sykes, ¹H, ¹³C and ¹⁵N random coil NMR chemical shifts of the common amino acids. I. Investigations of nearest-neighbor effects, *J. Biomol. NMR* 5 (1995) 67–81.
- [54] D.S. Wishart, B.D. Sykes, F.M. Richards, The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy, *Biochemistry* 31 (1992) 1647–1651.