Biochimica et Biophysica Acta 1788 (2009) 2481–2488

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Structural and functional analysis of extracellular loop 2 of the Na^+/H^+ exchanger

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ARTICLE INFO

Article history:
Received 9 April 2009
Received in revised form 30 September 2009
Accepted 6 October 2009
Available online 14 October 2009

Keywords:
Cation transport
Membrane
Na^+/H^+ exchanger
pH regulation

ABSTRACT

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is an integral membrane protein that regulates intracellular pH (pHi) by removing one intracellular H^+ in exchange for one extracellular Na^+. It has a large 500 amino acid N-terminal membrane domain that mediates transport and consists of 12 transmembrane segments and several membrane-associated segments. Extracellular regions of this domain are believed to contribute to cation coordination, transport and sensitivity to inhibitors. In this study we characterized the structure and function of extracellular loop 2. Mutation of residues Pro153, Pro154 and Phe155 demonstrated that these residues were critical for efficient NHE1 function. Mutations to Ala resulted in decreases in cation affinity and in decreases in activity of the protein, these were more marked in both Pro154 and Phe155. NMR spectroscopy was used to characterize the solution structure of a peptide NAc-Gly150-Phe155-NH_2. The peptide showed at least three different conformers in solution due to cis–trans isomerization of the Thr^{152}–Pro^{153} and Pro^{153}–Pro^{154} peptide bonds. The trans–trans conformation appeared to be in an extended conformation, whereas the cis–trans conformation showed a propensity to form a beta turn. Our results show that the EL2 region is critical to NHE1 function and that a peptide of the EL2 region can adopt different structures in solution potentially forming a beta turn that is important in function of the full protein Mutation of Pro^{154} could disrupt the beta turn, affecting helix packing and the protein structure and function.

1. Introduction

The mammalian Na^+/H^+ exchanger isoform 1 is a ubiquitous integral membrane protein that regulates pHi by mediating removal of one intracellular proton in exchange for one extracellular sodium ion [1]. NHE1 thereby functions to remove excess intracellular acid while facilitating Na^+ entry into the cytosol [1]. Ten known isoforms of the Na^+/H^+ exchanger are designated NHE1-NHE10, [2,3]. NHE2-10 have more restricted tissue distributions than NHE1 and some have predominantly intracellular localizations (reviewed in [1]). Mammalian NHE1 plays a key role in regulation of cell pH, cell volume, and cell proliferation; and in metastasis of some kinds of tumor cells [1,4]. It also promotes cell growth and differentiation [5], is critical to cell motility in some cell types [6], and regulates sodium fluxes and cell volume after challenge by osmotic shrinkage [7]. The Na^+/H^+ exchanger also plays a critical causal role in heart hypertrophy and in the damage that occurs during heart ischemia and reperfusion. Inhibition of the exchanger with Na^+/H^+ exchanger inhibitors protects the myocardium [8–10].

Although the exact mechanisms of transport and inhibitor binding by NHE1 are not known, specific residues within several regions of the membrane domain as well as several extracellular loops and membrane-associated segments of NHE1 have been implicated as being important for ion binding and transport [reviewed in [2]]. The membrane-associated segment (EL5) is involved in the drug sensitivity and cation binding and transport [2,11]. Loop regions between α-helices can influence their arrangements and packing [12] and extracellular loops of membrane proteins have been shown to modulate their function [13].

TM IV immediately follows EL2 (Fig. 1). Numerous residues of TM IV have been implicated in NHE1 function and the extramembrane loops at either end of TM IV contain residues that are important for NHE1 function. Early experiments showed that three residues of EL2 affect both the drug sensitivity and the activity of the exchanger [14]. IL2, at the C-terminal end of TM IV, contains residues that may line the ion-transport pore and when mutated to Cys Arg180 and Gln181 are accessible by externally applied MTSET [15]. Recently, we determined the structure of TM IV which was predominantly not alpha helical, though structured [16]. Phe161, in the extracellular side of the bilayer, was a pore lining residue and Pro residues in the middle of the bilayer were critical to function [16,17]. However, in those studies we did not examine the preceding EL2. In this study we characterize EL2 in detail. We examined individual amino acids of this extracellular loop and their contribution to differential drug cation sensitivity and cation coordination. In addition we present the first NMR solution structure of an extracellular loop of a mammalian Na^+/H^+ exchanger.

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doi:10.1016/j.bbamem.2009.10.004

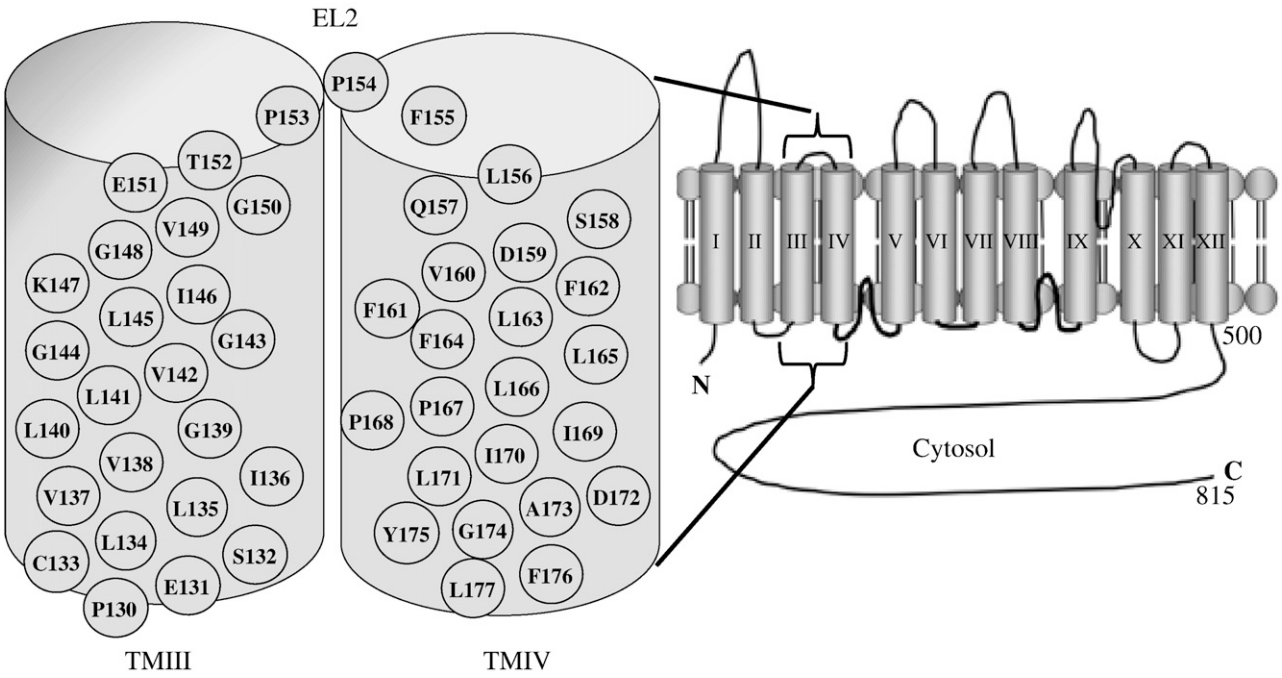

Fig. 1. Model of the NHE1 isoform of the Na^+/H^+ exchanger. The right panel indicates the topology of the Na^+/H^+ exchanger isoform 1 [15] while the left side is an enlargement of illustrating amino acids of TM III-EL2-TM IV.

2. Materials and methods

2.1. Materials

PWO DNA polymerase was from Roche Molecular Biochemicals, Mannheim, Germany. LIPOFECTAMINE™ 2000 Reagent was from Invitrogen Life Technologies, Carlsbad, CA, USA. Anti-HA-antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). BCECF-AM was from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON), Sigma or BDH (Toronto, ON). EMD87580 was a generous gift of Merck, Darmstadt, Germany.

2.2. Site-directed mutagenesis

Mutations were made to an expression plasmid containing a tagged human NHE1 isoform of the Na^+/H^+ exchanger. The plasmid pYN4+ contains the cDNA of the entire coding region of human NHE1. It has a C-terminal hemagglutinin (HA) tag that we have previously shown does not affect activity [16]. PWO DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) was used for amplification and site-directed mutagenesis was performed using the Stratagene (La Jolla, CA, USA) QuikChange™ site directed mutagenesis kit as recommended by the manufacturer. Mutations changed the indicated amino acids to alanine and were designed to create or remove a new restriction enzyme site for use in screening transformants (Table 1). The fidelity of DNA amplification was confirmed by DNA sequencing.

2.3. Cell culture and stable transfection

Stable cell lines of all mutants were made as described earlier [17] via transfection with LIPOFECTAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). AP-1 cells are a Chinese hamster ovary cell line that lacks an endogenous Na^+/H^+ exchanger. They were used to examine NHE1 activity and were routinely grown in a humidified atmosphere of 5% CO_2 and 95% air in α-MEM supplemented with 10% (v/v) bovine growth serum, 25 mM Hepes, penicillin (100 U/ml) and streptomycin (100 μg/ml), pH 7.4 at 37 °C. The transfection and selection of stable cell lines was carried out as described previously and transfection was done with LIPOFECTAMINE™ 2000 Reagent [16]. Briefly, 1.0×10^6 cells were seeded in 60 mm Petri dish, in 4 ml of growth media. Cells were grown until 90% confluent and transfected with 10 μg of wild type or mutant plasmids. Post-transfection cells were trypsinized, diluted 10 or 100 times with α-MEM medium and plated in 100 mm dishes in α-MEM media containing 800 μg/ml geneticin (G418) that was used to maintain selection pressure without acute acid load selection. After the initial selection, single clones of transfected cells were obtained and stable cell lines were maintained in 400 μg/ml G418 and were regularly re-established from frozen stocks at passage numbers between 5 and 15. Results shown are from at least two independently obtained clones for each mutant cell line.

2.4. SDS-PAGE and immunoblotting

Western blot analysis was used to confirm NHE1 expression [17]. For each Western blot, 100 μg of each total lysate was resolved on 10%

Table 1
Oligonucleotide primers for site-directed mutagenesis.

Mutation	Sequence	Site
Pro153Ala	5′GGTGTAGGCGAGAC**gg**C**g**CCCTTCCTGCAGTCCGAC3′	*Nar*I
Pro154Ala	5′GGTGTAGGCGAGACgCC**gg**C**C**TTCCTGCAGTCCGACGTCTTC3′	*Nae*I
Phe155Ala	5′GGTGTAGGCGAGACACCCCCC**gc**C**C**TaCAGTCCGACGTCTTCTTCCTC3′	−*Pst*I

Mutated nucleotides are in lower case letters. Mutated codons are indicated in bold. Restriction sites removed (−) or added are underlined. In each case the forward direction of the primer pair is illustrated.

SDS-PAGE gel and transferred onto a nitrocellulose membrane. Immunoblot analysis with anti-HA antibody tag confirmed NHE1 expression in samples of cell lysates of transfected AP-1 cells [17]. The Amersham enhanced chemiluminescence Western blotting and detection system was used to detect immunoreactive proteins. Densitometric analysis of X-ray films was carried out using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

2.5. Cell surface expression

Cell surface expression was measured as described earlier [16,17] to ensure that all mutant proteins were properly targeted to the cell surface. Cells were labeled with Sulpho-NHS-SS-Biotin (Pierce Chemical Company, Rockford, IL, USA) and immobilized streptavidin resin was used to remove surface labeled Protein Equal amounts of the total and unbound proteins were analyzed by 10% SDS-PAGE followed by Western blotting against the HA tag. Relative amounts of NHE1 on the cell surface were calculated by comparing both the 110-kDa and the 95-kDa forms of NHE1 in the total and unbound fractions. It was not possible to efficiently elute biotin labeled proteins bound to immobilized streptavidin resin for direct measurement of extracellular NHE1 protein.

2.6. Na^+/H^+ exchange activity

NHE1 activity was measured using a PTI Deltascan spectrofluorometer as described earlier [18]. Stably transfected cells were seeded on glass coverslips (2×10^5 cells per coverslip) and grown until they reached 80–90% confluency. The coverslip was then transferred to a cuvette holder with constant stirring at 37 °C. The cells were loaded with 0.15 μg/ml 2′,7-bis (2-carboxyethyl)-5 (6) carboxyfluorescein-AM (BCECF-AM) and incubated in "Normal buffer" containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4 at 37 °C. Normal buffer is nominally bicarbonate free. Intracellular acidosis was induced by NH_3/NH_4^+ prepulse/withdrawal (3 min in "Normal buffer" containing 50 mM NH_4Cl, pH 7.4), followed by withdrawal for 30 sec in "Na^+-free buffer": (135 mM *N*-methyl-D-glucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4). Intracellular pH (pH_i) recovery was in "Normal buffer" allowing the cells to recover for at least 3 min. There were no differences in buffering capacities of stable cell lines (not shown). Following pH_i recovery for every experiment a three-point pH calibration curve was made using the K^+/nigericin method with Na^+-free calibration buffers (5 mM *N*-methyl-D-glucamine, 135 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES, at pH 6, 7, and 8) and 10 μM nigericin [11]. Buffering capacity of cells was determined as described earlier [11,19] and proton flux via NHE1 protein was determined using the slope of the first 20 seconds of the recovery period. Other kinetic parameters of the Na^+/H^+ exchanger were determined essentially as described earlier [11,19]. Because we earlier found that growth conditions affect the absolute level of NHE1 activity, experiments comparing the activities of wild type and NHE1 mutants were done in pairs or groups, so that all comparisons of activity were done with cells were grown to the same degree of confluence and with the same media. For some experiments Na^+ and Li^+ concentrations were varied while maintaining osmolarity with *N*-methyl-D-glucamine. In other experiments we investigated the inhibitor sensitivity of some mutants. Cells were treated with the highly selective NHE1 inhibitor EMD87580 at varying concentrations [20]. EMD87580 was dissolved in water and the inhibitory effect of EMD87580 was measured using a dual-pulse acidification assay. In this assay, cells were treated with ammonium chloride two times as described above and allowed to recover in NaCl containing medium following each pulse. The first pulse and recovery is in the absence of EMD87580 while the second pulse recovery is in the presence of inhibitor. To calculate residual activity during recovery from acidosis the following formula was used

$$\%\ \text{residual activity} = \frac{\text{proton flux after(Reagent)}}{\text{proton flux w/o(Reagent)}} \times 100\%$$

The rate of recovery from acid load is compared ± inhibitor. Results are shown as mean ± SE and statistical significance was determined using a Wilcoxon–Mann–Whitney Rank Sum test. IC50s were calculated as described earlier [21]. Differences between IC50s of mutants was determined by a Holm–Sidak *post hoc* test.

2.7. Peptide synthesis and purification

An EL2 peptide consisting of amino acids Gly^{150} to Phe^{155} of human NHE1 (sequence, GETPPF; acetyl-capped N terminus, amide-capped C terminus) was synthesized by the Alberta Peptide Institute at >95% purity. Purity was assessed by HPLC and identity confirmed using sequential assignment of NMR data and by matrix-assisted laser desorption ionization mass spectroscopy.

2.8. NMR spectroscopy and structure calculations

Samples for NMR spectroscopy contained 5 mM EL2 peptide, 0.1 M KCl, 10 mM imidazole, and 0.25 mM DSS-d_6 in 95% H_20/5% D_20. The pH was adjusted to 5 without consideration of deuterium effects on the pH electrode. 1D ^1H NMR spectra and 2D ^1H–^1H ROESY (250 ms mix) spectra were obtained on the NANUC Varian 800 MHz spectrometer. 2D TOCSY (60 ms mix) and natural abundance ^{13}C HSQC spectra were obtained on a Varian 500 MHz spectrometer. Spectra were processed using NMRPipe [22] and peak picking and assignment were done manually in NMRVIEW [23]. Distance restraints for structure calculation were obtained from the ROESY spectrum peaks. Peaks were calibrated based on peak intensity, and sorted into distance ranges of either strong (1.8–2.8 Å), medium (1.8–3.2 Å) or weak (1.8–5.0 Å). Structures were calculated in the python scripting interface of XPLOR-NIH 2.19 [24]. Several rounds of simulated annealing and refinement were performed. Initially, restraints with frequent violations greater than 0.5 Å were examined, and either lengthened or modified. In later rounds, violations greater than 0.1 Å were examined. 50 structures were calculated each round, and the structures with no NOE violations greater than 0.1 Å were kept.

3. Results

Fig. 1 illustrates a general model of the Na^+/H^+ exchanger [15] and an enlargement of TMIII-EL2-TM IV is also shown. Based on our and other reports of the importance of TM IV we conducted further investigation of this region. In particular, we characterized amino acids Pro^{153}, Pro^{154} and Phe^{155} of the extracellular loop 2. Prolines may be particularly important in membrane proteins, disrupting α-helices, and introducing flexibility [17,25]. Mutations were made to an expression plasmid containing a full length, tagged human NHE1 isoform of the Na^+/H^+ exchanger. Initially, experiments determined whether these mutant forms of the Na^+/H^+ exchanger expressed and targeted properly. Western blotting using anti tag (HA) antibodies was used to characterize the proteins expression (Fig. 2A). AP1 cells transfected with plasmid containing the HA-tagged wild type NHE1 resulted in the presence of an immunoreactive species of 110 kDa, plus a smaller band at 95 kDa that represents an immature form of the exchanger that is not fully glycosylated [16]. Untransfected cells (Lane 1, AP1) show no such immunoreactivity. The level of expression relative to the wild type Na^+/H^+ exchanger is shown below each mutant. The Pro153Ala mutant had significantly decreased expression ($P<0.05$), 78% of the wild type, while the protein level of Pro154 and Phe155Ala mutants was not significantly different than that of wild type.

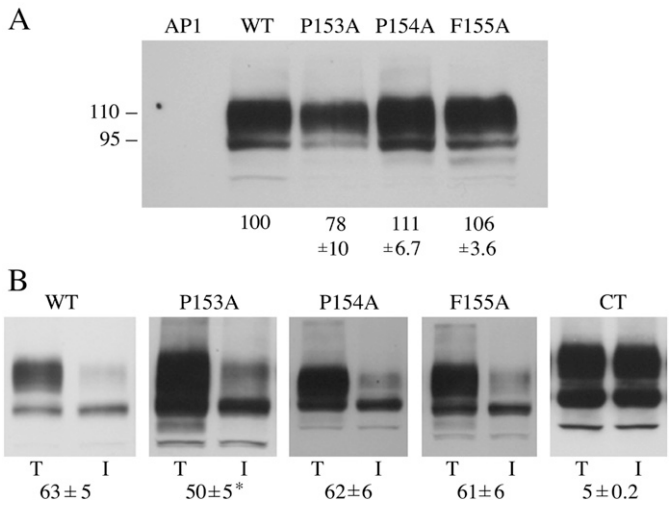

Fig. 2. Western blot analysis of cell extracts from stable cell lines expressing wild type and mutants of NHE1. (A) Western blot of cell extracts from AP-1 cells transfected with HA-tagged wild-type NHE1 (WT) or transfected with NHE1 mutants Pro153Ala (P153A), Pro154Ala (P154A) and Phe155Ala (F155A). Anti-HA antibody was used to detect tagged NHE1 protein and the amount of NHE1 protein was quantified. Numbers underneath the lanes indicate the mean value (±SD) of the sum of densitometric scans of both 110 kDa and 95 kDa bands relative to wild-type NHE1 for at least three experiments. (B) Plasma membrane targeting of the Na^+/H^+ exchanger in AP-1 cells transfected with wild-type NHE1 (WT) and with the NHE1 mutants Pro153Ala (P153A), Pro154Ala (P154A) and Phe155Ala (F155A). Sulfo-NHS-SS-biotin-treated cells were lysed and their proteins were solubilized and subsequently treated with streptavidin agarose to bind labeled protein as described in Materials and methods. Equal samples of total lysates (T) and unbound (representing intracellular) lysates (I) were run on SDS-PAGE and blotted with anti-HA antibody to identify NHE1 Protein Non-specific (CT) refers to an experiment in which non-specific binding to streptavidin-agarose beads was measured by following the standard procedure without labeling cells with biotin. The percent of the total NHE1 protein localized to the plasma membrane is indicated. The results are mean ± standard error for at least 4 determinations. * Indicates significantly reduced plasma membrane targeting in comparison to wild type NHE1 ($P<0.05$).

Mutation of membrane associated amino acids can affect surface targeting of the Na^+/H^+ exchanger [16]. Therefore, we examined intracellular targeting of the NHE1 expressing cell lines. After cells were treated with sulfo-NHS-SS-biotin, labeled proteins of lysates were bound to streptavidin-agarose beads. To identify NHE1 protein, we used Western blotting with anti-HA antibody and examined equal amounts of total cell lysates and unbound lysates. This revealed the relative amounts of tagged intracellular NHE1 protein. Fig. 2B illustrates examples of the results and a summary of at least 4 experiments. A majority of the wild-type NHE1 protein was present on the plasma membrane, Pro154Ala and Phe155Ala proteins had similar level of plasma membrane localization in comparison with the wild type protein. However, Pro153Ala did have slightly, but significantly reduced plasma membrane targeting compared to the wild-type NHE1 protein. We noted that non-specific binding of proteins to streptavidin agarose beads was approximately 5%, therefore, our values of NHE1 on the cell surface are an overestimate by this amount. We also analyzed the subcellular distribution of the fully glycosylated and partially or de- glycosylated forms of NHE1 separately (Supplementary Table 1). This analysis showed that the partially glycosylated form of NHE1 was mostly intracellular and the surface targeting of fully glycosylated NHE1 was therefore 5–10% higher than the values given in Fig. 2B. The P153A mutant, was still significantly decreased in its targeting relative to the other mutants.

Since we observed that these mutations did not greatly impair expression or surface targeting of the protein, we examined the activity of the mutant exchangers in comparison with the wild type protein. Fig. 3 shows both the uncorrected activity (grey bars) and the activity corrected for expression and surface processing (black bars) relative to wild-type NHE1 for the three mutants. All three mutants

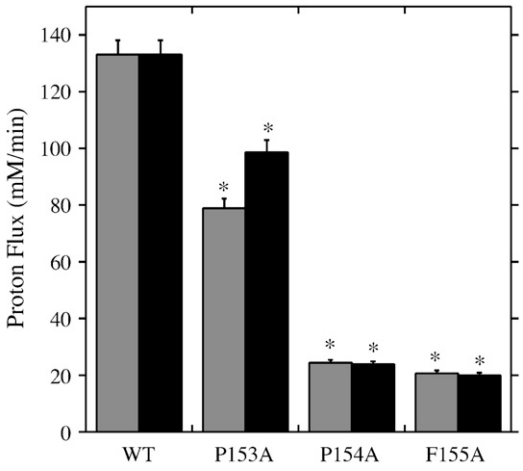

Fig. 3. Na^+/H^+ exchanger activity of AP1 cells stably transfected with control and NHE1 mutants. NHE activity was measured in 135 mM NaCl as described in Materials and methods in stable cell lines expressing wild type NHE1 (WT) and NHE1 mutant proteins Pro153Ala (P153A), Pro154Ala (P154A) and Phe155Ala (F155A). Initial pH's of the mutant cells after acidification were 6.04, 6.06, 6.07 and 6.07 for the mutant cell lines, respectively, and were not significantly different from one another. Grey bars indicated uncorrected values of NHE1 activity. Black bars indicate activity after correction for the level of expression and surface targeting relative to the WT. Results are relative to the value of the wild type NHE1 protein and are the mean ± the S.E. of 5–7 determinations from two independently made cell lines. * Indicates significantly different from the wild type at $P<0.01$.

had significantly decreased activity relative to wild-type NHE1. The Pro153Ala mutant was decreased the least of these mutants. When correcting for expression levels and targeting this decrease was reduced.

EMD87580 is a potent and selective NHE1 inhibitor [20]. Alterations in sensitivity to inhibition have been reported earlier upon mutation of amino acids in transmembrane segments of NHE1 and may indicate important alterations in cation binding or coordination by the protein [2,26]. To determine whether alterations in NHE1 inhibitor sensitivity occurred with the amino acids mutated in this study, we initially tested the effect of 0.7 μM EMD87580 on the mutants. We have earlier determined that this concentration of EMD87580 inhibits approximately 40–50% of the activity of the NHE1

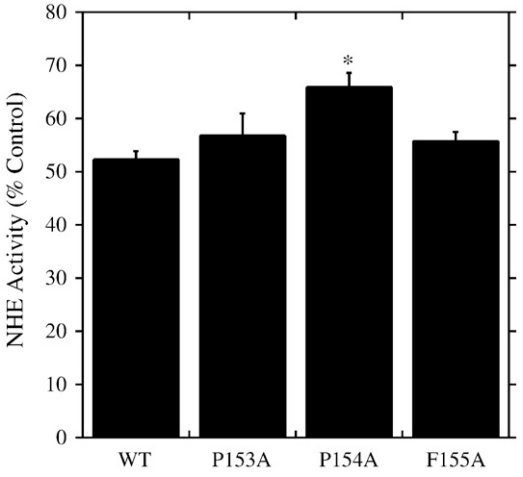

Fig. 4. Effect of EMD87580 on activity of wild type and NHE1 mutant proteins. NHE1 activity was determined in 135 mM NaCl. The activity was determined in a dual pulse assay in which cells were acidified twice and allowed to recover twice. The relative rates of proton flux were determined and the second pulse in the presence of 0.7 μM EMD87589, was compared to the first. NHE1 (WT); Pro153Ala (P153A); Pro154Ala (P154A); Phe155Ala (F155A). The results are the mean ± the S.E. of at least 12 determinations. * Indicates significantly different from the wild type at $P<0.05$.

protein [18,21]. The initial pH of recovery varied nominally by an average of less than 0.04 pH units between wild type and mutants. The difference between the starting pH of the first and second pulse varied nominally by an average of 0.009 pH units. Fig. 4 shows the results comparing the rate of proton flux of the second pulse in the presence of EMD87580 with that of the proton flux in the absence of EMD87580. The Pro154Ala mutant had decreased level of inhibition by EMD87580 in comparison to the wild type. To further investigate these results in more detail we characterized the effectiveness of a wide range of concentrations of EMD87580 on the mutant NHE1 proteins relative to the control (not shown). In these tests there was also no significant difference in the starting pHi of recovery or between the starting pHi in the first and second pulse of recovery. The value of wild type NHE1 IC50 was 0.64 μM. The IC50s of Pro153Ala and Pro155Ala were 0.84 and 0.83 μM respectively, which were not significantly different from the wild type. The resistance of the Pro154Ala mutant was increased more than that of the others and it had an IC50 of 1.27 μM, a two-fold increase over that of the wild type and was the only statistically significant change ($P<0.05$).

To gain insights into the nature of the effects of the mutations, we examined their affinity for Na^+ or Li^+ relative to that of the wild type. Ammonium chloride was used to induce acidification and varying levels of NaCl or LiCl were used to allow recovery. Differences in activity between the groups were not due to differences in initial pH following acidification induced by ammonium chloride. Fig. 5A illustrates the effects of altering the Na^+ levels on the activity of the protein. All the mutants had less activity than wild type. The Pro153Ala had the most activity of the mutants while the Pro154Ala and Phe155Ala mutants were reduced more in activation in comparison with the wild type. The K_m for the wild type, Pro153Ala, Pro154Ala and Phe155Ala was 73.3, 74.2, 66.6, and 72.3 mM respectively, which indicated that the affinity for Na^+ was not greatly altered compared to the wild type. The V_{max} values of the wild type and Pro153Ala, Pro154Ala and Phe155Ala mutants were 196, 138, 111 and 56 mM/min, respectively, suggesting substantial apparent decreases for the mutants. It should be noted that saturating concentrations of NaCl were not necessarily reached due to osmotic considerations with the cells, so the V_{max} values should be noted as estimates only.

Similar experiments were done using LiCl instead of NaCl (Fig. 5B). In this case, the proton transport rates were much lower than that of Na^+. The V_{max} for wild type, Pro153Ala, Pro154Ala and Phe155Ala mutants was 66, 71, 51 and 19 mM/min, respectively, indicating a possible decrease in the V_{max} of transport for the Pro154Ala mutant and a reduction in activity in the Phe155Ala mutant. The K_m for these proteins was 22, 29, 30 and 23 mM, respectively, which again illustrated that the Li^+ transport affinity of the Pro154Ala mutant was not greatly altered. It was noted that for the wild type NHE1 protein and the three mutants, by the LiCl concentration of 70 mM activity was maximal and further increases in LiCl did not stimulate NHE1 activity further. It has been previously reported that lower concentrations of LiCl are required for activation of NHE1 in comparison to NaCl concentrations [21,27].

The peptide representing the predicted extracellular loop 2 of NHE1 was synthesized with an N-terminal acetate and C-terminal amide cap to remove charges at the termini that would not be present in the sequence when in the full protein. NMR spectra were acquired with a sample containing 0.1 M KCl; however, the extracellular loop would normally be exposed to a higher concentration of Na^+ compared to K^+ in its native environment. The 1D NMR spectra of a single sample in 0.1 M NaCl was the same as spectra in 0.1 M KCl (data not shown), so the spectra from multiple samples with KCl were used for analysis.

Trial 1D NMR spectra were acquired at 10, 20, and 30 °C. Spectra acquired at 10 °C allowed for better resolution of peaks near the water peak, and the 2D spectra for analysis were obtained at this temperature. It is also expected that lower temperatures would stabilize any nascent structure in the peptide. Due to the small molecular weight of the peptide (about 700 Da), NOESY spectra

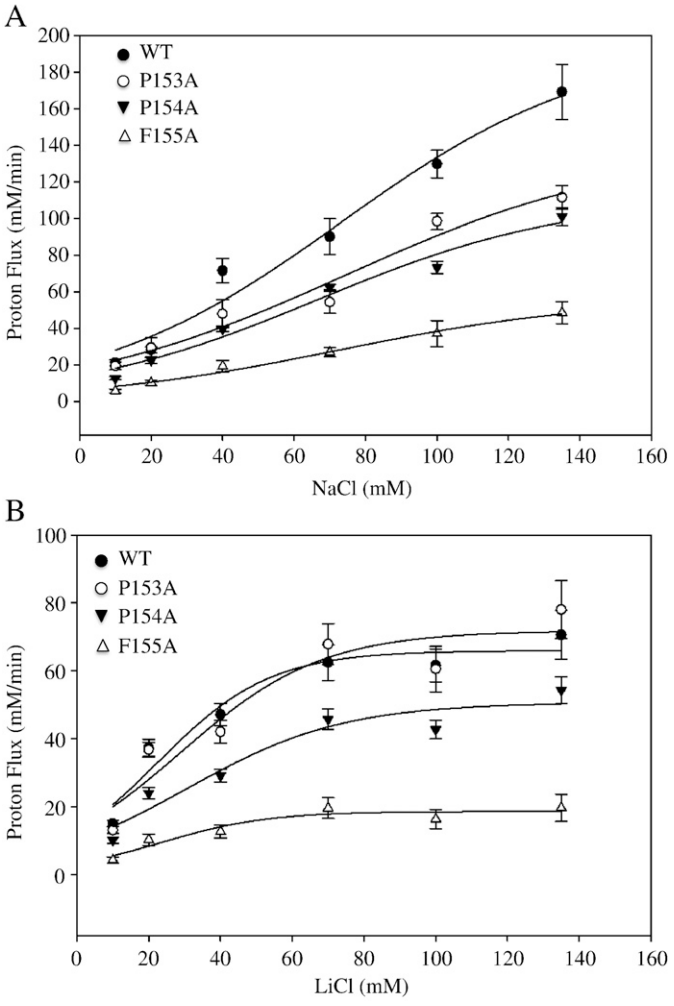

Fig. 5. Effect of different Na^+ and Li^+ concentration on activity of wild type and NHE1 mutant proteins. NHE1 activity was determined as described in the "Materials and methods" and is shown without correction for expression levels or targeting. (A) Effect of different Na^+ concentrations (10, 20, 40, 70, 100 and 135 mM) on NHE1 activity. (B) Effect of different Li^+ concentrations (10, 20, 40, 70, 100 and 135 mM) on NHE1 activity. All the results are the mean ± the S.E. of at least 8 determinations.

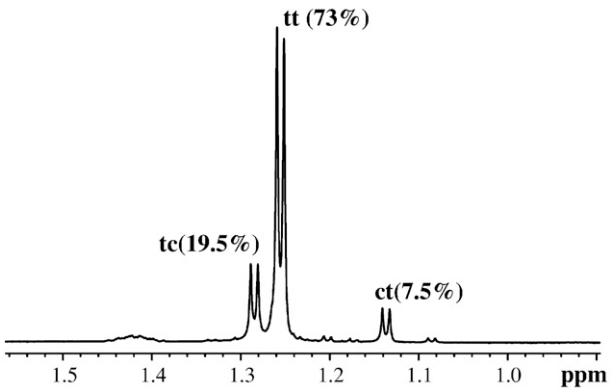

Fig. 6. Region of 1D NMR spectrum showing cis–trans isomerization in the EL2 peptide. The threonine methyl region from the 1D spectrum of the EL2 peptide is shown, with the methyl peaks labeled with the conformation of the peptide it belongs to: trans–trans (tt), trans–cis (tc), or cis–trans (ct). The relative percentages of each from integration of the peaks are shown in brackets.

crosspeaks would have intensities near zero, and so a ROESY spectrum was obtained instead.

Sequential assignment was performed using the TOCSY and ROESY spectra. Spectra showed triplicate peaks from all of the residues except the N-terminal glycine, resulting from the slow cis–trans isomerization of the peptide bonds preceding the two prolines. Fig. 6 shows a portion of the 1D spectrum containing the Thr methyl peaks from the three conformations. Integration of the threonine methyl peaks in the 1D spectrum gives percentages of about 73, 19.5, 7.5% for the three conformations. X-Pro peptide bond conformations were determined from the X-Hα to Pro Hα or Hδ connectivities. The major conformation was identified as trans–trans, and the second as trans–cis. The third conformation (cis–trans) was not investigated further due to spectral overlap and the low intensity of the peaks. A cis–cis conformation could not be seen.

Contacts from the ROESY spectra between Pro153 and Phe155 suggest a beta turn in the trans–cis conformation. A strong Pro Hα-Phe HN contact can be seen, which would suggest a beta-turn at residues $T^{152}PPF^{155}$. Other contacts, between the Pro and Phe rings, suggest there could be an interaction between them, possibly a CH-π interaction, which could help stabilize the cis bond and promote the turn formation [28]. There are also weak contacts between the Phe and Glu residues, which could result from the turn formation. The interaction of the Phe and Pro rings is supported by the splitting of the Phe Hβ proton peak, from which 2 different values of the $^3J_{\alpha\beta}$ coupling constants can be measured (~5 and 12 Hz), suggesting restricted movement of the side chain. In contrast, the trans–trans conformation has only a single peak with a $^3J_{\alpha\beta}$ of ~7.6 Hz. There do not appear to be any contacts in the trans–trans conformation that support formation of any secondary structure.

Structures were calculated separately for the trans–trans and trans–cis conformations. Structures for the third conformation were not calculated, due to the lack of interresidue restraints. Secondary structure assignment using the PROMOTIF v2.0 [29] suggests that beta-turns occur in some of the structures calculated in both the trans–trans and trans–cis peptides. 35 of 46 trans–cis peptide structures contained beta-turns at $T^{152}PPF^{155}$, with some structures showing close contacts between the Phe and Pro rings. The flexible nature of a small peptide would allow it to sample a large number of conformations, with the result that the spectra would show an average of these conformations. The restraints obtained from the ROESY spectrum are thus also an average of the conformations the peptide is experiencing. The variation in the structures could be reflective of this flexibility, or of the low number of intermolecular restraints available for structure calculation, but the presence of crosspeaks in the ROSEY still suggest that a turn is being formed. 24 of the 44 structures in the trans–trans conformer contained beta-turns at $G^{150}ETP^{153}$, however, due to a lack of appropriate interresidue contacts, it is unlikely that a stable turn is being formed. Fig. 7 shows a calculated structure for each conformation.

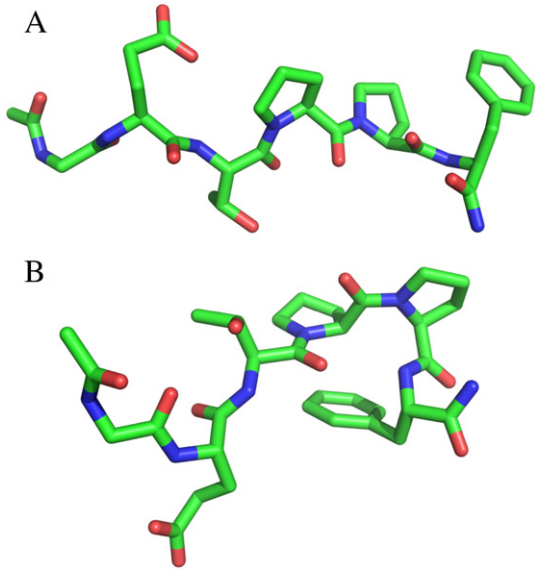

Fig. 7. Structures of EL2 peptide calculated from ROESY distance restraints. Single structures from the ensembles of structures calculated for the (a) trans–trans and (b) trans–cis conformation of the peptide.

4. Discussion

Studies on the membrane domain of the NHE1 isoform of the Na^+/H^+ exchanger have suggested that specific amino acids of the TM segments IV, VII, IX and XI are critical in the function of the protein [2,18,30,31]. Specific mutations of TM IV in particular can alter the Na^+ affinity and sensitivity to inhibition [2,32]. It is known that the loop regions at either end of transmembrane segments also contain residues that are important for NHE1 function [2,14,33]. In potassium channels and in the *E. coli* Na^+/H^+ antiporter NhaA, residues extending past the plane of the lipid bilayer have also been suggested to be important in attraction of cations, facilitating their transport [34,35]. Studies have suggested TM IV and associated amino acids are important in cation coordination and inhibitor efficacy [14,16,27,32,36]. In the present study we therefore further characterized three residues of the extracellular loop 2 involved in the NHE1 structure, function and drug recognition.

Of the amino acids 153–155 it was clear that both Pro154 and Phe155 are critical to NHE1 function. Though mutation of either residue did not cause a great aberration in either targeting or expression of the protein, mutation of either causes nominally larger decreases in activity than mutation of Pro153. The only significant effects on resistance to inhibition by EMD87580 were via mutation of Pro154, further suggesting it had a critical role in NHE1 function. Fig. 8 shows a comparison of the amino acid sequences of EL2 and nearby sequences of TM III and IV. Pro154 was completely conserved in all vertebrate species examined while Phe155 was more variable and Pro153 was usually, but not always, conserved. Phe155 was not well conserved, though a bulky hydrophobic residue was always present in this position. Neither EL2 nor this general region were conserved at all in invertebrate species such as Drosophila. It is generally agreed that conservation of a particular region or of an amino acid is suggestive of a more critical function, which is in agreement with our results. Of the other amino acids studied, the Pro153Ala mutation decreased both the protein expression level and the surface targeting comparing with the wild type. This result indicated that although Pro153 is not as critical to NHE1 activity as Pro154 and Phe155, its mutation affects NHE1 expression levels and targeting.

In the case of Phe155Ala mutation, there were no significant changes among the protein expression level and surface targeting however, activity was reduced and EMD87580 sensitivity was slightly decreased. Thus the Phe155 residue was not essential for NHE1 structure and function but mutation to Ala resulted in a decrease in activity of NHE1. It may be that a bulky hydrophobic residue is required at this position for full function (Fig. 8).

An earlier report demonstrated that in rat NHE1, the dual mutation Pro157Ser/Pro158Phe (corresponding to human Pro153/Pro154) markedly reduced the sensitivity to another NHE1 inhibitor EIPA, 7 fold [14]. We observed that mutation of either residue had a smaller affect on inhibitor sensitivity than that study. This could be due to either the different inhibitor used, EMD87580 vs. EIPA, or because we mutated individual amino acids and not both prolines simultaneously. The dual Pro157Ser/Pro158Phe reported earlier [14] did not affect Na^+ affinity but did affect maximal activity of the protein. Our study found that mutation of Pro154 had a marked inhibitory effect on cation affinity in addition to transport rate, while

148	GVGET	PPF	LQSDV	160	Human [44]
148	GVGEK	PPF	LQSEV	160	Rabbit [45]
148	AVGET	PPF	LQSEV	160	Porcine [46]
148	GVGET	PPI	LQSEV	160	Bovine (#Q28036)
156	GVGET	PPV	LHSDT	168	Amphiuma [47]
139	AIGEK	APV	LHYEL	151	Flounder [48]
221	GVGES	PPL	LQSDI	233	Ornithorhynchus (XM_001518234)
129	AVGET	PPV	LNSDV	141	Xenopus [49]
115	VIGEE	PPV	LDSQL	128	Oncorhynchus [50]
140	AVGEK	PPI	LKSDI	152	Coturnix (ABJ88911)
154	GSWKR	EEV	FSPMG	166	Drosophila (NM 134647)

Fig. 8. Alignment of amino acid sequences of EL2 and surrounding region of various NHE1 proteins. The alignment was made either manually or using the program "DNA Strider." The EL2 region is shaded where identical to that of human NHE1. References [44–50] are indicated and where they were not available the accession number of the protein sequence was given.

mutation of Pro153 did not. Our results therefore suggest the second Pro of the pair in EL2 is more important than the first in determining vertebrate NHE1 function.

Because Pro residues are known to significantly influence the secondary conformations of proteins, they may contribute to the differential drug sensitivity of the NHE isoforms. Prolines tend to be helix breakers [25,37] within TM segments. In addition it has been suggested that membrane proteins accumulate Pro residues at the end of their TM segments in order to expose select amino acids at the interface of molecular recognition events, while allowing stable association and native folding [38]. It is also interesting to note that TM IV itself has a pair of Pro residues, Pro167 and Pro168 that are critical to function of the protein [17]. However in contrast to these prolines, the requirement for a Pro at position 153 and 154 is not absolute. Substitutions could be made that retained NHE1 activity.

Proline residues have a high preference of forming the cis isomer of the peptide bond preceding it due to the cyclic nature of proline, relative to other amino acids, which are mainly in the trans form [39]. The structure of the peptide exhibited at least three conformations observed in solution due to the cis–trans isomerization of the peptide bonds preceding the proline residues, with a possible fourth conformation not observed, due to a low population and/or peak overlap. When in the trans–cis conformation, the peptide is able to form a beta turn, and this suggests that the sequence in the full protein could also adopt a turn. Also, the sequence involved appears to be favorable in forming a turn with a cis–peptide bond [40]. The formation of a turn would support its predicted location in the topology model of NHE1, as a short loop between two transmembrane helices. The beta-turn structure allows for a 180-degree reversal of peptide backbone facilitating re-entry into the membrane bilayer. A short peptide would be quite flexible, with a large population of the peptide in a trans–trans and extended conformation, so the actual conformation in the protein would likely also depend on the distances between the ends of the two connecting helices as well as its interactions with residues nearby in the protein.

The ability of the peptide sequence to adopt a turn conformation might explain the results in the mutagenesis experiments. If the sequence forms a turn in the full protein, then the mutation of the critical Pro154 to Ala could make the formation of the turn less favorable, due to the decreased preference for the cis bond previous to the mutated residue. Conformational changes in the TM helix packing have been implicated in the bacterial Na^+/H^+ exchanger activation [41,42] and dynamics simulations suggest that the sequences of interhelical loops may be involved in stabilizing helix interactions [43]. The change from a beta-turn to an extended conformation in the extracellular loop could affect the packing of the transmembrane helices, which could result in a less active protein. The observed decrease in inhibition by EMD87580 could be due to a decrease in binding of the inhibitor, due to a change in structure of its binding pocket on mutation of the loop region. The drug could be binding in a pocket that includes the loop, so that a mutation would change the structure of the binding pocket, or the drug could bind in a pocket which is indirectly affected by mutations in the loop which could affect the helix packing.

In summary our results show that EL2 is important in NHE1 function, likely playing a role in maintaining appropriate conformation of the protein, possibly by influencing helix packing. Pro154 and Phe155 are both critical residues of this extracellular loop though significant function of the protein still remained after their mutation. Future studies will characterize the role of other extracellular loops of the protein.

Acknowledgements

This work was supported by funding from the Canadian Institutes of Health Research (CIHR) to LF and BDS. BL is supported by a CIHR Strategic Training Grant in Membrane Proteins and Cardiovascular Disease. LF is supported by an AHFMR Scientist Award. BDS receives support as a Canada Research Chair in Structural Biology. We would like to thank the Canadian National High Field NMR Centre (NANUC) for their assistance and use of the 800 MHz spectrometer. Operation of NANUC is funded by the Canadian Institutes of Health Research, the Natural Science and Engineering Research Council of Canada, and the University of Alberta. We are grateful to Kathy Trieber (U of AB, Dept. of Biochemistry) for her assistance in statistical analysis of samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2009.10.004.

References

[1] L. Fliegel, Molecular biology of the myocardial Na^+/H^+ exchanger, J. Mol. Cell Cardiol. 44 (2008) 228–237.
[2] E.R. Slepkov, J.K. Rainey, B.D. Sykes, L. Fliegel, Structural and functional analysis of the Na(+)/H(+) exchanger, Biochem. J. 401 (2007) 623–633.
[3] S.H. Lee, T. Kim, E.S. Park, S. Yang, D. Jeong, Y. Choi, J. Rho, NHE10, an osteoclast-specific member of the Na^+/H^+ exchanger family, regulates osteoclast differentiation and survival [corrected], Biochem. Biophys. Res. Commun. 369 (2008) 320–326.
[4] R.A. Cardone, V. Casavola, S.J. Reshkin, The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis, Nat. Rev. Cancer. 5 (2005) 786–795.
[5] 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.
[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] L. Shrode, A. Cabado, G. Goss, S. Grinstein, Role of the Na^+/H^+ antiporter isoforms in cell volume regulation. in: L. Fliegel (Ed.), The Na^+/H^+ Exchanger, R.G. Landes Company, 1996, pp. 101–122.
[8] M.E. Malo, L. Fliegel, Physiological role and regulation of the Na^+/H^+ exchanger, Can. J. Physiol. Pharmacol. 84 (2006) 1081–1095.
[9] 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.

[10] 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.
[11] 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.
[12] 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.
[13] 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.
[14] A. Khadilkar, P. Iannuzzi, J. Orlowski, 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.
[15] 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.
[16] E.R. Slepkov, 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.
[17] E.R. Slepkov, 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.
[18] 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.
[19] N.L. Silva, H. Wang, C.V. Harris, D. Singh, L. Fliegel, Characterization of the Na^+/H^+ exchanger in human choriocarcinoma (BeWo) cells, Pflugers Archiv. Eur. J. Physiol. 433 (1997) 792–802.
[20] L. Chen, C.X. Chen, X.T. Gan, N. Beier, W. Scholz, M. Karmazyn, Inhibition and reversal of myocardial infarction-induced hypertrophy and heart failure by NHE-1 inhibition, Am. J. Physiol. Heart Circ. Physiol. 286 (2004) H381–387.
[21] E. Slepkov, J. Ding, J. Han, L. Fliegel, Mutational analysis of potential pore-lining amino acids in TM IV of the Na(+)/H(+) exchanger, Biochim. Biophys. Acta 1768 (2007) 2882–2889.
[22] 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.
[23] B.A. Johnson, R.A. Blevins, NMRView: a computer program for the visualization and analysis of NMR data. J. Biomol. NMR 4 (1994) 603–614.
[24] C.D. Schwieters, J.J. Kuszewski, N. Tjandra, G.M. Clore, The Xplor-NIH NMR molecular structure determination package, J. Magn. Reson. 160 (2003) 65–73.
[25] M.S. Sansom, Proline residues in transmembrane helices of channel and transport proteins: a molecular modelling study, Protein Eng. 5 (1992) 53–60.
[26] 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.
[27] J. Orlowski, Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger, J. Biol. Chem. 268 (1993) 16369–16377.
[28] B. Dasgupta, P. Chakrabarti, G. Basu, Enhanced stability of cis Pro–Pro peptide bond in Pro–Pro–Phe sequence motif, FEBS Lett. 581 (2007) 4529–4532.
[29] E.G. Hutchinson, J.M. Thornton, PROMOTIF—a program to identify and analyze structural motifs in proteins, Protein Sci. 5 (1996) 212–220.
[30] T. Reddy, J. Ding, X. Li, B.D. Sykes, J.K. Rainey, L. Fliegel, Structural and functional characterization of transmembrane segment IX of the NHE1 isoform of the Na^+/H^+ exchanger, J. Biol. Chem. 283 (2008) 22018–22030.
[31] B.L. Lee, X. Li, Y. Liu, B.D. Sykes, L. Fliegel, Structural and functional analysis of TM XI of the NHE1 isoform of the Na^+/H^+ exchanger, J. Biol. Chem. 284 (2009) 11546–11556.
[32] L. Counillon, J. Noel, R.A.F. Reithmeier, J. Pouyssegur, Random mutagenesis reveals a novel site involved in inhibitor interaction within the fourth transmembrane segment of the Na^+/H^+ exchanger-1, Biochemistry 36 (1997) 2951–2959.
[33] S. Wakabayashi, T. Hisamitsu, T. Pang, M. Shigekawa, Mutations of Arg440 and Gly455/Gly456 oppositely change pH sensing of Na^+/H^+ exchanger 1, J. Biol. Chem. 278 (2003) 11828–11835.
[34] C. Hunte, E. Screpanti, M. Venturi, A. Rimon, 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.
[35] D.A. Doyle, J. Morais Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, R. MacKinnon, The structure of the potassium channel: molecular basis of K+ conduction and selectivity [see comments], Science 280 (1998) 69–77.
[36] S.F. Pedersen, S.A. King, E.B. Nygaard, R.R. Rigor, P.M. Cala, NHE1 inhibition by amiloride- and benzoylguanidine-type compounds. Inhibitor binding loci deduced from chimeras of NHE1 homologues with endogenous differences in inhibitor sensitivity, J. Biol. Chem. 282 (2007) 19716–19727.
[37] D.J. Barlow, J.M. Thornton, Helix geometry in proteins, J. Mol. Biol. 201 (1988) 601–619.
[38] M. Orzaez, J. Salgado, A. Gimenez-Giner, E. Perez-Paya, I. Mingarro, Influence of proline residues in transmembrane helix packing, J. Mol. Biol. 335 (2004) 631–640.
[39] M.W. MacArthur, J.M. Thornton, Influence of proline residues on protein conformation, J. Mol. Biol. 218 (1991) 397–412.
[40] D. Pal, P. Chakrabarti, Cis peptide bonds in proteins: residues involved, their conformations, interactions and locations, J. Mol. Biol. 294 (1999) 271–288.
[41] E. Olkhova, E. Padan, H. Michel, The influence of protonation states on the dynamics of the NhaA antiporter from Escherichia coli, Biophys. J. 92 (2007) 3784–3791.
[42] L. Kozachkov, K. Herz, E. Padan, Functional and structural interactions of the transmembrane domain X of NhaA, Na^+/H^+ antiporter of Escherichia coli, at physiological pH, Biochemistry 46 (2007) 2419–2430.
[43] M.B. Ulmschneider, M.S. Sansom, A. Di Nola, Properties of integral membrane protein structures: derivation of an implicit membrane potential, Proteins 59 (2005) 252–265.
[44] C. Sardet, A. Franchi, J. Pouysségur, Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter, Cell 56 (1989) 271–280.
[45] C.-M. Tse, S.R. Brant, M.S. Walker, J. Pouyssegur, M. Donowitz, Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na^+/H^+ exchanger isoform (NHE-3), J. Biol. Chem. 267 (1992) 9340–9346.
[46] R.F. Reilly, F. Hildebrandt, D. Biemesderfer, C. Sardet, J. Pouyssegur, P.S. Aronson, C.W. Slayman, P. Igarashi, cDNA cloning and immunolocalization of a Na^+/H^+ exchanger in LLC-PK1 renal epithelial cells, Am. J. Physiol. 261 (1991) F1088–F1094.
[47] L.A. McLean, S. Zia, F.A. Gorin, P.M. Cala, Cloning and expression of the Na^+/H^+ exchanger from Amphiuma RBCs: resemblance to mammalian NHE1, Am. J. Physiol. 276 (1999) C1025–1037.
[48] S.F. Pedersen, S.A. King, R.R. Rigor, Z. Zhuang, J.M. Warren, P.M. Cala, Molecular cloning of NHE1 from winter flounder RBCs: activation by osmotic shrinkage, cAMP, and calyculin A, Am. J. Physiol. Cell Physiol. 284 (2003) C1561–1576.
[49] S. Busch, Cloning and sequencing of the cDNA encoding for a Na^+/H^+ exchanger from *Xenopus laevis* oocytes (X1-NHE), Biochim. Biophys. Acta 1325 (1997) 13–16.
[50] F. Borgese, C. Sardet, M. Cappadoro, J. Pouyssegur, R. Motais, Cloning and expression of a cAMP-activated Na^+/H^+ exchanger: evidence that the cytoplasmic domain mediates hormonal regulation, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 6765–6769.