Scientific Report

concerning the implementation of project 8/02.05.2018 in the period May 2018 - December 2019

Electrophysiological analysis of *K. pneumoniae* NhaB mutants

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Introduction

All organisms require mechanisms for the regulation of the intracellular Na^+ concentration, as excessive amounts can lead to cell stress and death [1]. One system through which the intracellular Na^+ concentration is regulated is represented by membrane transporters called Na^+/H^+ exchangers or antiporters, which exchange n Na^+ ions with m H^+ ions [2]. If $n = m$, no net electrical charge is carried over the membrane and the exchanger is *electroneutral*. If $n < m$ (or, possibly, $m < n$), net charge is transported over the membrane and the exchanger is *electrogenic*.

In addition to Na^+ homeostasis, Na^+/H^+ exchangers also play a role in the regulation of intracellular pH and cell volume [3]. As the processes influenced by Na^+/H^+ exchangers are both interlinked and sensitive, a fine balance has to be achieved by the regulation of exchange activity. Excessive Na^+ export activity, though potentially beneficial through the reduction of the amount of intracellular Na^+, can lead to over-acidification of the cell [1, 4]. At the other extreme, excess H^+ export in order to compensate for an acidic cytoplasm can lead to undesired accumulation of high amounts of Na^+ inside the cell. As a first consequence, Na^+/H^+ exchange activity is highly regulated through what we have shown to be a simple mechanism [1, 4] that relies on the competition between Na^+ and H^+ for a single binding site. As a second consequence, many organisms employ several Na^+/H^+ exchange systems, some electrogenic, some electroneutral, each tuned for a particular optimum range of operation.

One such organism is the pathogenic bacterium *Klebsiella pneumoniae*, whose genome encodes for 4 Na^+/H^+ exchangers belonging to the NhaA family (KpNhaA1, KpNhaA2, both electrogenic), the NhaB family (KpNhaB, electrogenic) and the NhaP family (KpNhaP2, electroneutral). In a previous study, we have analysed the activity of *K. pneumoniae* Na^+/H^+ exchangers and, in particular KpNhaB [5]. We have shown that, despite belonging to a different transport superfamily compared to the better investigated NhaA exchangers, KpNhaB follows a similar competition-based mechanism. Furthermore, we were able to do a full electrophysiological characterization of KpNhaB and determine kinetic parameters for the wild type protein. Our study [5] represented the first detailed characterization of an NhaB-class Na^+/H^+ exchanger. However, the NhaB family still remains sadly under-investigated. In particular, only few studies have tried to identify essential amino acid residues in NhaB proteins [6, 7]. All of them have assessed function through an assay involving the dequenching of a fluorescent dye, acridine orange or 9-amino-6-chloro-2-methoxyacridine (ACMA), which has its limitations, in particular regarding the dynamic range and the quantitative information that can be obtained [4].

The aim of the present project is to investigate the effect of several point mutations on the function of KpNhaB. All mutations will be performed on Asp or Asn residues thought to be present in transmembrane

helices of KpNhaB, according to the alignment of the sequence of KpNhaB to the putative transmembrane domains of NhaB from *V. alginolyticus* [6] (Figure 1). Asp or Asn residues present in membrane helices were chosen as these residues are commonly found in the binding sites of Na^+/H^+ exchangers [1, 4]. Furthermore, mutation of Asp147 in the NhaB exchanger from *V. alginolyticus* (VaNhaB) was shown to impair transport function [8].

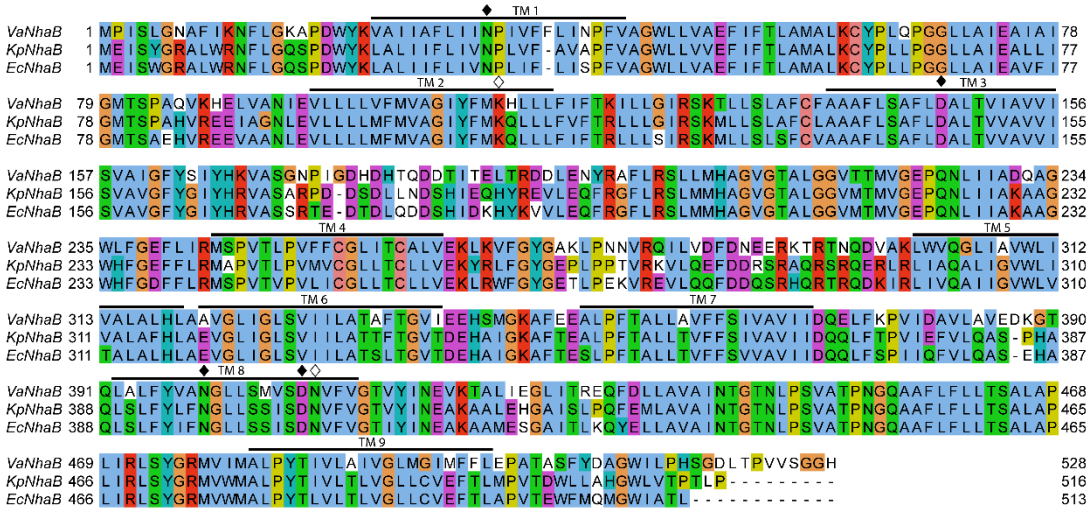

Figure 1. Residues that will be mutated in KpNhaB are shown by black diamonds. TM denotes putative transmembrane helices. White diamonds denote residues that will be mutated in case there is no significant effect of performing any of the initial set of mutations. VaNhaB = NhaB exchanger from *V. alginolyticus*. EcNhaB = NhaB exchanger from *E. coli*.

The mutants that will be produced in this project are: N32A, D146A, D146E, D404A, D404E, N396A, for a total of four mutations of two Asp residues (to either the similarly charged Glu residue or to a non-polar Ala) and two mutations of two Asn residues to a non-polar alanine. Following production of these mutants, they will be reconstituted into proteoliposomes and characterized by solid supported membrane (SSM)-based electrophysiology, an investigative technique highly suitable for analysis of prokaryotic transporter function [9] that we also employed for characterization of the KpNhaB WT [5].

As SSM-based electrophysiology is the main investigation technique used in this project, it is worthwhile to provide an overview of the information that can be obtained following its use. The technique [9] involves adsorbing proteoliposomes containing the transporter of interest on a hybrid phospholipid-octadecanethiol bilayer formed on a gold surface (electrode). The established system has a high mechanical stability and can be subjected to rapid exchanges of solution without loss of the absorbed proteoliposomes. Furthermore,

using the SURFE2R N1 instrument (see Methods section), the deposited proteoliposomes are stable over several days, and electrodes can be stored at 4 ^{o}C in between measurements.

Following rapid substrate concentration jumps, transport activity can be activated and transient currents recorded. The information derived from these currents regards the following [10]:

1. The polarity of the currents indicates the direction of charge transfer. Thus, a negative current shows transfer of negative electrical charge towards the interior of the proteoliposomes (or net positive charge towards the outside). Positive currents represent transfer of positive charge towards the interior of the proteoliposomes (or negative charge to the outside);
2. The peak current, or maximum amplitude of the recorded currents is an indicator of transport activity [11, 12];
3. The time course of the transient currents allows their classification into two categories [13]. We call "pre-steady-state" the currents with a fast decay time that is usually independent of substrate concentration. Those currents that decay slower and have decreasing time constants with increasing substrate concentration will be referred to as "steady-state". Note that all recorded currents are transient currents, due to the capacitively coupled membrane of the SSM [9, 12].

The present report details the first and second phases of the project. The aim of the first phase was the production of the 6 KpNhaB mutants. As shown below, **all objectives of Phase 1 were successfully completed and functional proteoliposomes of all mutants were obtained.** The aim of the second phase was the electrophysiological investigation of all mutants to establish eventual differences compared to the WT protein. **All objectives of Phase 2 were also successfully achieved.** The project can proceed to Phase 3 where additional analysis and kinetic modelling will be performed.

Materials and Methods

Materials

Genetic constructs

The KpNhaB WT gene (Strain 342, Uniprot accession number: B5XQ77) codon-optimized for expression in *E. coli* and inserted in the pET-21d(+) expression vector was used as a template for site-directed mutagenesis.

Bacterial strains

All cloning work was performed in the *E. coli* XL-1 Blue strain (Agilent Technologies, Santa Clara, CA, USA). Expression of proteins for production purposes was performed in the *E. coli* BL21(DE3) strain.

Chemicals

Unless otherwise stated, chemicals were produced by Carl Roth (Karlsruhe, Germany). 1,2-diphytanoyl-sn-glycero-3-phosphocholine and *E. coli* polar lipids were produced by Avanti Polar Lipids (Alabaster, AL, USA). Ultrapure water was produced using a Millipore MiliQ water purification system (Merck Millipore, Darmstadt, Germany). Bradford reagent was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Methods

Site-directed mutagenesis

Mutagenesis was performed using the Quikchange kit from Stratagene (Agilent Technologies, Santa Clara, CA, USA). In brief, the technique uses a supercoiled double stranded DNA plasmid as template. Two oligonucleotide primers were designed for each mutant (Table 1) containing the desired mutated bases in the middle part of the primer. The primers were extended by temperature cycling using PfuUltra DNA polymerase (Agilent Technologies, Santa Clara, CA, USA), resulting in a product containing staggered nicks. Following temperature cycling (Table 2), the product was treated with the enzyme *DpnI* (New England Biolabs, Ipswich, MA, USA) in order to digest the methylated DNA template. The resulting DNA was then used to transform XL1-Blue *E. coli* competent cells. These were grown overnight on ampicillin-agar plates, after which clones were selected in order to check for success of the mutation. DNA Mini preps were performed using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany).

Sequences of mutated constructs were verified by sequencing performed by Eurofins Genomics (Ebersberg, Germany). Primers used for sequencing were standard Eurofins primers T7: TAA TAC GAC TCA CTA TAG GG and T7term: CTA GTT ATT GCT CAG CGG T.

Table 1. Mutagenic primers used for obtaining KpNhaB mutants. Capital letters denote bases unchanged from the template sequence.

Mutant	Mutation	Primer sequence
N32A	AAC-gcC	GATTATTTTTCTGATTGTGgcCCCGCTGGTGTTCGCGGTTGC
		GCAACCGCGAACACCAGCGGGgcCACAATCAGAAAAATAATC
D146A	GAC-GcC	CCTGAGCGCGTTTCTGGcCGCGCTGACCGTGGTTGC
		GCAACCACGGTCAGCGCGgCCAGAAACGCGCTCAGG
D146E	GAC-GAa	CCTGAGCGCGTTTCTGGAaGCGCTGACCGTGGTTGC
		GCAACCACGGTCAGCGCtTCCAGAAACGCGCTCAGG
N396A	AAC-gcC	GCCTGTTCTACCTGTTTgcCGGTCTGCTGAGCAGCATCAGC
		GCTGATGCTGCTCAGCAGACCGgcAAACAGGTAGAACAGGC
D404A	GAT-GcT	GGTCTGCTGAGCAGCATCAGCGcTAACGTGTTCGTTGGCACCG
		CGGTGCCAACGAACACGTTAgCGCTGATGCTGCTCAGCAGACC
D404E	GAT-GAg	GGTCTGCTGAGCAGCATCAGCGAgAACGTGTTCGTTGGCACCG
		CGGTGCCAACGAACACGTTcTCGCTGATGCTGCTCAGCAGACC

Table 2. Temperature cycling program for KpNhaB mutations.

Step no.	Temperature (°C)	Time (s)	No. of cycles
1.	95	30	-
2.	95	30	30
3.	70	30	
4.	72	480	
5.	72	600	-

Protein overexpression and purification

Transformation of chemically competent cells was performed via heat shock (42 °C), using 50 ng of DNA.

Protein expression and purification were performed as previously described for KpNhaB WT [5]. In brief, bacteria were grown at 37 °C in LB medium, containing (per liter) 10 g tryptone, 5 g yeast extract and 10 g NaCl. The medium also contained 100 μg/mL carbenicillin. Cells were grown until they reached an optical density at 600 nm (OD_{600}) of 0.6 – 0.8 and then expression was induced by addition of IPTG (0.75 mM). Cells were collected after 2.5 hours via centrifugation.

Everted membrane vesicles were prepared via a French Press pressure cell [14] and resuspended in buffer containing Tris 10 mM (titrated to pH 7.5 with HCl), 250 mM sucrose and 140 mM choline chloride. Total protein concentration was determined according to the Bradford method [15].

Purification was performed by using Immobilized Metal Affinity Chromatography (IMAC) employing Ni-NTA Agarose (Qiagen, Hilden, Germany). Protein concentration was determined spectrophotometrically at 280 nm.

Western blot

SDS-PAGE and Western blot were performed essentially as described [16]. The marker used was PageRuler Protein Ladder 10 to 180 kDa (Thermo Fisher Scientific, Bonn, Germany). Antibodies used were PentaHis (Qiagen, Hilden, Germany) and polyclonal goat anti-mouse immunoglobulin (P0447 Dako, Agilent Technologies, Santa Clara, CA, USA). Chemiluminescence was used for detection employing SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Bonn, Germany).

Functional reconstitution into proteoliposomes

Reconstitution of proteins into proteoliposomes was performed as previously described for KpNhaB WT [5] at a lipid-to-protein ratio (LPR) of 10 (w:w).

Differential scanning fluorimetry

Differential scanning fluorimetry was employed to assess the thermal stability of investigated KpNhaB variants. In brief, glass capillaries were loaded with the detergent-solubilized proteins at a concentration of 0.5 mg/mL in buffer containing 100 mM KCl, 5 mM $MgCl_2$, 25 mM KCH_3COO (pH 4) and 0.03% n-dodecyl β-D-maltoside (DDM). The capillaries were placed in the thermal plate of a Prometheus NT.48 instrument (NanoTemper Technologies, Munich, Germany). A temperature ramp between 20 and 95 °C at a change rate of 1 °C/min was applied to the protein samples and the emission fluorescence was continuously followed at 350 and 330 nm upon excitation at 280 nm with a power setting of 10%.

A melting curve was recorded over the investigated temperature range. Thermal unfolding events could be detected and the melting temperature (T_m) was determined from the inflection point of every melting curve using first derivative analysis.

Fluorescence measurements of transporter activity using acridine orange

Fluorescence measurements were performed using a Hitachi F4500 Fluorimeter (Hitachi High-Technologies Corporation, Tokyo, Japan) at λ_{exc} = 495 nm and λ_{em} = 530 nm. The measurement buffer contained 10 mM MES (titrated to the indicated pH using Tris), 140 mM choline chloride, 5 mM $MgCl_2$, 2

μM acridine orange. Vesicles containing 100 μg total protein were added and their acidification (and thus fluorescence quenching) was induced by addition of 5 mM Tris-D-lactate. After reaching steady-state fluorescence, dequenching was induced by addition of 50 mM Na^+. Finally, the pH gradient was dissipated by addition of 20 mM NH_4Cl.

Fluorescence measurements of membrane potential using oxonol VI

In order to establish whether some of the prepared mutants were electrogenic (and, thus, generated a transmembrane potential), the potential sensitive dye oxonol VI was employed [17]. Measurements were performed using a Hitachi F4500 Fluorimeter at the wavelengths λ_{ex} = 614 nm and λ_{em} = 646 nm. Detailed experimental procedures are described in our recently published paper [10]. In brief, the dye responds to positive-inside membrane potentials by an increase of dye fluorescence. In our experiments, this was observed as a rapid increase towards a maximum value (F_{max}), followed by a slow decrease caused by what we attribute to be an inherent leakiness in the proteoliposomes. The membrane potential was dissipated by addition of uncoupler (monensin 1 μM or SF6847 1 μM), dropping fluorescence to a baseline (F_0). The value $(F_{max} - F_0)/F_0$ was plotted and used to assess stoichiometry.

Formation of a membrane potential via the activity of the transporter was performed by using proteoliposomes loaded with different concentrations of Na^+ (as Na_2SO_4) which were then diluted into solutions containing variable amounts of Na^+. To eliminate the ΔpH formed by transporter activity, nigericin was added to the fluorescence cuvette at 500 pM concentration.

The stoichiometry of KpNhaB WT was determined essentially as previously described [18]. Briefly, for a strictly coupled antiporter that exchanges Na^+ and H^+, $\Delta\tilde{\mu}_{Na+} = \Delta\tilde{\mu}_{H+}$, or, using electrical units, $\Delta pNa + \Delta\Psi = n(\Delta pH + \Delta\Psi)$. When $\Delta pH = 0$, $\Delta pNa = (n - 1)\Delta\Psi$.

SSM-based electrophysiology

SSM-based electrophysiological experiments were performed using the SURFE^{2}R N1 instrument (Nanion Technologies, Munich, Germany). A brief description of the technique and the information provided by it is presented in the introduction of this document.

SSM experiments were performed as previously described [5]. In brief, solution exchange was performed between a solution not containing the Na^+ substrate (non-activating solution, NA) and a solution containing Na^+ (activating solution, A), using an NA-A-NA exchange protocol, with each solution flowing for 1 s over the sensor. All solutions contained 25 mM MES, 25 mM HEPES, 25 mM Tris and 5 mM $MgCl_2$ titrated to the desired pH with HCl or KOH. In addition, NA solutions contained 300 mM KCl, whereas A solutions contained instead *x* mM NaCl and (300-*x*) mM KCl.

Unless otherwise stated, most measurements were performed under symmetrical pH conditions over the proteoliposomes membrane ($pH_i = pH_o$). In some cases, measurements were performed under asymmetrical pH ($pH_i \neq pH_o$), by initially incubating the SSM in a resting solution (R) for 20-30 minutes prior to the NA-A-NA solution exchange. With the exception of pH, the R solution was identical to the NA solution, so that the Na^+ concentration jump was achieved under a pH gradient.

In every case, the section of the recorded current traces corresponding to the NA-A solution exchange (ON transient) was taken for analysis. The peak current amplitude of the ON transient was taken as representative of Na^+/H^+ exchange activity.

Results and discussion

The following section lists the objectives of the project in the order in which they are present in the execution plan of the project and describes the steps undertaken for the achievement of each objective. Objectives 1.1 – 1.4 correspond to the first phase of the project, while Objectives 2.1 – 2.8 correspond to the second phase of the project. Also, besides the initially stated objectives of Phase 2, further experiments were performed, as described in the "Additional experiments – Phase 2" section.

Objective 1.1. Project management

The project management objective involved ensuring that all materials and required equipment were available for the well function of the project.

Thus, the oligonucleotide primers required for the site-directed mutagenesis reactions were designed. These were then ordered for synthesis with the help of Prof. Klaus Fendler from the Max Planck Institute of Biophysics in Frankfurt. For long term storage of biological sample a freezer capable of achieving temperatures in the range as low as -45ºC was ordered. Additionally, experimental protocols that will be used for the protein expression were reviewed in order to ensure availability of all necessary chemicals.

Objective 1.2. Obtaining plasmids containing mutants of KpNhaB in the expression vector pET21d

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 1.3. Expression of KpNhaB N32A, D146A, D146E, N396A, D404A and D404E in E. coli

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 1.4. Result dissemination

1.4.1. Creating and updating the project's website

The project's website was established at the address: http://octaviancalinescu.ro/pn-iii-p1-1-1-pd2016-0802/ . It contains the identification data for the project, the team's composition and the summary of the proposed research activity. In addition, the website lists the dissemination activities.

1.4.2. Conference attendance

The project's initial results and outlook were presented in this phase in the form of a poster at the 15th National Conference of Biophysics that took place in Bucharest between the 7th and the 10th of September 2018:

1. Patino-Ruiz, M., Ganea, C., Fendler, K., Călinescu, O., "The Na^+/H^+ Exchanger NhaB from Klebsiella pneumoniae – A Model System for Electrophysiological Studies.", 15th National Conference of Biophysics, 07-10 September 2018, Bucharest, Romania, ISSN 2248-0749.

The abstract of the presentation was published in the Book of Abstracts, ISSN 2248-0749. Additionally, the poster presentation received a "Best Poster" award from the organizers of the conference.

1.4.3. Articles published

The project's director work on mutations relevant to the Na^+/H^+ exchanger transport mechanism has led to the publication of an ISI article in The Journal of Biological Chemistry (2017 Impact Factor 4.25):

1. Patino-Ruiz, M., Dwivedi, M., Călinescu, O., Karabel, M., Padan, E., Fendler, K. 2018. "Replacement of Lys-300 with a glutamine in the NhaA Na^+/H^+ antiporter of Escherichia coli yields a functional electrogenic transporter", J. Biol. Chem. doi: 10.1074/jbc.RA118.004903.

Objective 2.1. Project management

The project management objective involved ensuring that all materials and required equipment were available for project. All necessary materials were acquired, including an extruder for the preparation of

liposomes of uniformous size. The budget was redistributed to insure adequate coverage of all expenses, including travel costs for work visits and the conference attendance.

Objective 2.2. Investigation of KpNhaB N32A

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 2.3. Investigation of KpNhaB D146A

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 2.4. Investigation of KpNhaB D146E

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 2.5. Investigation of KpNhaB N396A

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 2.6. Investigation of KpNhaB D404A

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 2.7. Investigation of KpNhaB D404E

The present objective was achieved. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Additional experiments – Phase 2

Besides the experiments described above, further additional experiments were performed in order to better elucidate the effect of mutations on KpNhaB. A detailed description has been reported to the contracting authority. For inquests please contact the project director directly at octavian.calinescu@umfcd.ro .

Objective 2.8. Result dissemination

2.8.1. Updating the project's website

The project's website at http://octaviancalinescu.ro/pn-iii-p1-1-1-pd2016-0802/ was updated with the current results of both Phase 1 and Phase 2.

2.8.2. Conference attendance

The project's results were presented in this phase in the form of a poster at the FEBS Advanced Lecture Course Biochemistry of Membrane Proteins – Structure, Trafficking, Regulation that took place in Bucharest between the 25th and the 30th of August 2019:

1. Patino-Ruiz, M., Ganea, C., Fendler, K., Călinescu, O., "Mutational study of the NhaB Na+/H+ exchanger from Klebsiella pneumoniae", FEBS Advanced Lecture Course Biochemistry of Membrane Proteins – Structure, Trafficking, Regulation, 25-30 August 2019, Budapest, Hungary.

2.8.3. Articles published

Our work on KpNhaB mutants has led to the publication of an ISI article in Scientific Reports (2018 Impact Factor 4.011):

1. Patino-Ruiz, M., Fendler, K., Călinescu, O. 2019. Mutation of two key aspartate residues alters stoichiometry of the NhaB Na+/H+ exchanger from Klebsiella pneumoniae, Sci. Rep. 9, 15390.

Conclusions

The first phase of the project was focussed on preparing all necessary materials for the activities of phase 2. In particular, all mutants of KpNhaB were obtained and overexpressed in *E. coli*. Everted membrane vesicles were prepared to test functionality of the mutants. These were then subjected to purification and the purified transporters were reconstituted into proteoliposomes. **All objectives of Phase 1 were successfully achieved.**

The second phase of the project aimed at characterizing all prepared KpNhaB mutants using SSM-based electrophysiology. **All objectives of Phase 2 were successfully achieved.** Additionally, another mutant, KpNhaB D146A/D404A was prepared after the results on the D146A and D404A mutant were analyzed and its activity was also measured.

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18.11.2019 Project director,

Conf. Dr. Octavian Călinescu