Interaction of N-terminal peptide analogues of the Na+,K+-ATPase with membranes


PII: S0005-2736(18)30080-4
Reference: BBAMEM 82727

To appear in:

Received date: 2 February 2018
Accepted date: 5 March 2018

Please cite this article as: Khoa Nguyen, Alvaro Garcia, Marc-Antoine Sani, Dil Diaz, Vikas Dubey, Daniel Clayton, Giovanni Dal Poggetto, Flemming Cornelius, Richard J. Payne, Frances Separovic, Himanshu Khandelia, Ronald J. Clarke, Interaction of N-terminal peptide analogues of the Na+,K+-ATPase with membranes. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Bbamem(2018), doi:10.1016/j.bbamem.2018.03.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Interaction of N-Terminal Peptide Analogues of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase with Membranes

Khoa Nguyen\textsuperscript{a}, Alvaro Garcia\textsuperscript{a,b}, Marc-Antoine Sani\textsuperscript{c}, Dil Diaz\textsuperscript{a}, Vikas Dubey\textsuperscript{d}, Daniel Clayton\textsuperscript{a}, Giovanni Dal Poggetto\textsuperscript{a}, Flemming Cornelius\textsuperscript{e}, Richard J. Payne\textsuperscript{a}, Frances Separovic\textsuperscript{c}, Himanshu Khandelia\textsuperscript{d}, and Ronald J. Clarke\textsuperscript{a,b,*}

\textsuperscript{a} School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia
\textsuperscript{b} The University of Sydney Nano Institute, Sydney, NSW 2006, Australia
\textsuperscript{c} School of Chemistry, Bio21Institute, University of Melbourne, VIC 3010, Australia
\textsuperscript{d} MEMPHYS, Center for Biomembrane Physics, University of Southern Denmark, DK-5230 Odense M, Denmark
\textsuperscript{e} Department of Biomedicine, University of Aarhus, DK-8000 Aarhus C, Denmark

Address correspondence to Assoc. Prof. Ronald J. Clarke, School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia. Tel.: 61-2-93514406; Fax: 61-2-93513329; E-mail: ronald.clarke@sydney.edu.au
Abstract
The Na\(^+\),K\(^+\)-ATPase, which is present in the plasma membrane of all animal cells, plays a crucial role in maintaining the Na\(^+\) and K\(^+\) electrochemical potential gradients across the membrane. Recent studies have suggested that the N-terminus of the protein’s catalytic \(\alpha\)-subunit is involved in an electrostatic interaction with the surrounding membrane, which controls the protein’s conformational equilibrium. However, because the N-terminus could not yet be resolved in any X-ray crystal structures, little information about this interaction is so far available. In measurements utilising poly-L-lysine as a model of the protein’s lysine-rich N-terminus and using lipid vesicles of defined composition, here we have identified the most likely origin of the interaction as one between positively charged lysine residues of the N-terminus and negatively charged headgroups of phospholipids (notably phosphatidylserine) in the surrounding membrane. Furthermore, to isolate which segments of the N-terminus could be involved in membrane binding, we chemically synthesized N-terminal fragments of various lengths. Based on a combination of results from RH421 UV/visible absorbance measurements and solid-state \(^{31}\text{P}\) and \(^{2}\text{H}\) NMR using these N-terminal fragments as well as MD simulations it appears that the membrane interaction arises from lysine residues prior to the conserved LKKE motif of the N-terminus. The MD simulations indicate that the strength of the interaction varies significantly between different enzyme conformations.

Keywords: poly-L-lysine; phospholipid membrane; lipid-protein interaction; phosphatidylserine; eosin; molecular dynamics simulations
1. Introduction

P-type ATPases are an enzyme family whose major role is transport of ions or phospholipids across biological membranes. A prominent member is the Na\(^+\),K\(^+\)-ATPase, which is expressed in all animal cells. It utilizes energy from ATP hydrolysis to transport 3Na\(^+\) ions out of and 2K\(^+\) ions into the cell per ATP molecule hydrolyzed. The electrochemical potential gradients of these ions, which the Na\(^+\),K\(^+\)-ATPase maintains across the cell membrane, are essential to fundamental cell functions such as solute transport and cell volume regulation \[1\]. Other prominent family members include the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, the gastric H\(^+\),K\(^+\)-ATPase, and the H\(^+\)-ATPases of plants and bacteria \[2\].

Because P-type ATPases are widely spread across different forms of cellular life and are crucial to fundamental cell functions, they are prime targets in disease treatment \[3\]. Indeed, a variety of pump inhibitors have been discovered or developed, and several have already been implemented in the treatment of pathological conditions, e.g. congestive heart failure, stomach ulcers, and malaria. However, the development of future drugs and therapies would be assisted by more detailed information on the structure and mechanism of all P-type ATPases.

Relatively recently it has become clear that many P-type ATPases possess regulatory R domains at their cytoplasmic N- or C-termini, which have an autoinhibitory effect on pump activity \[4-6\]. In addition to blocking ion translocation pathways, Yatime et al. \[3\] have suggested that peptide-based targeting of the R domains could represent a promising new strategy for therapeutic development, although their cytoplasmic location isn’t advantageous. Peptides mimicking the action of autoinhibitory domains have in fact been synthesized for the plasma membrane Ca\(^{2+}\)-ATPase \[7\] and the plant plasma membrane H\(^+\)-ATPase \[8\]. Here we report on peptides mimicking the N-terminus of the Na\(^+\),K\(^+\)-ATPase. In addition to any
possible effect of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase N-terminus on pump activity, evidence has also been presented that the first 40 amino acids of the N-terminus are involved in cell signaling, mediated by ouabain-induced calcium oscillations [9].

Recent data [10,11] suggest that the cytoplasmic N-terminus of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase may electrostatically interact with the surrounding membrane, stabilizing the enzyme in the E2 state relative to E1 and causing an autoinhibition. The recent findings provide strong support for earlier biochemical data which also implicated the N-terminus in controlling the enzyme’s conformational distribution [12-18]. It is known that in kidney the Na\textsuperscript{+},K\textsuperscript{+}-ATPase is surrounded by significant amounts of the anionic lipids phosphatidylserine and phosphatidylinositol, i.e., 13 and 6 mol% of total phospholipid, respectively [19]. The N-terminus of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, on the other hand, is particularly rich in the positively charged amino acid residue lysine [11]. A reasonable hypothesis is that the E2 state is stabilized by electrostatic interaction between positively charged lysine side chains of the protein’s N-terminus and negatively charged lipid headgroups surrounding the protein. Indeed, preliminary molecular dynamics (MD) simulations of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase with the non-crystallographically resolved N-terminus added [10] support to this suggestion. So too do UV/visible absorbance data using the voltage-sensitive probe RH421 to monitor membrane binding and poly-L-lysine (PLL) as a model of the N-terminus [11]. To further investigate the validity of this hypothesis and more precisely determine the possible location of N-terminus-membrane interaction, here we describe membrane binding studies using both poly-L-amino acids and synthesized fragments of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase N-terminus. Furthermore, we have investigated the dependence of interaction of the N-terminus with the membrane on the protein’s conformational state using MD simulations.
2. Materials and methods

2.1 Enzyme and reagents

Na\(^+\),K\(^+\)-ATPase-containing membrane fragments from the outer medulla of pig kidney were purified as described by Klodos et al. [20]. They were stored in pH 7.4 buffer containing 25 mM imidazole, 250 mM sucrose and 1 mM EDTA. The specific ATPase activity at 37°C and pH 7.4 was measured according to Ottolenghi [21]. The activity of the preparation used was 1086 μmol ATP hydrolysed h\(^{-1}\) (mg of protein\(^{-1}\)) at saturating substrate concentrations and the protein concentration was 6.2 mg mL\(^{-1}\), determined according to the Peterson modification [22] of the Lowry method [23] using bovine serum albumin (BSA) as a standard.

N-(4-Sulfobutyl)-4-(4-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and was used without further purification. RH421 was added to Na\(^+\),K\(^+\)-ATPase-containing membrane fragments from an ethanolic stock solution. The dye spontaneously partitions into the membrane.

Origins of the reagents used were as follows: NaCl (suprapure, Merck, Kilsyth, Australia), KCl (analytical grade, Merck), MgCl\(_2\)·6H\(_2\)O (analytical grade, Merck), poly-L-lysine (PLL) hydrochloride (MW 15,000 – 30,000 g mol\(^{-1}\), Sigma), poly-L-arginine hydrochloride (MW 15,000 – 70,000 g mol\(^{-1}\), Sigma), poly-L-glutamic acid sodium salt (MW 15,000 – 50,000 g mol\(^{-1}\), Sigma), EDTA (99%, Sigma), tris(hydroxymethyl)aminomethane (99%, Alfa Aesar, Heysham, UK), imidazole (≥99%, Sigma), eosin Y (C.I. 45380, BDH, Kilsyth, Australia), L-histidine (≥99.5%, Fluka, Castle Hill, Australia), NaOH (analytical grade, Merck), ethanol (analytical grade, Merck), chloroform (≥99.0%, Uvasol, Merck) and HCl (0.1 N Titrisol solution, Merck). 1-palmitoyl-d31-2-oleoyl-phosphatidylcholine (d31-POPC), 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylserine sodium salt (DOPS), dioleoylphosphatidylglycerol.
sodium salt (DOPG), dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidic acid sodium salt (DOPA) were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

2.2 Vesicle preparation

For the preparation of unilamellar lipid vesicles, lipids were first dissolved in chloroform at a concentration of 3 mM. All solutions were prepared by weight, using the chloroform density of 1.48 g mL$^{-1}$. Appropriate volumes (again measured by weight) of the chloroform solutions of each lipid were mixed to obtain the required mole %, with a final total volume 3 mL. Chloroform was then removed from each mixture by rotary evaporation at 40°C at 474 mbar and maximum rotation speed. After no visible traces of chloroform could be detected in the flask, the resulting lipid film was dried for a further 30 min at 10 mbar. The lipid film was then hydrated by the addition of buffer (30 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.2) to obtain a final lipid concentration of 3 mM and sonicated in a sonic bath for 60 s to ensure complete lipid resuspension. The suspension was finally extruded 11 times through a 0.1 μm Nucleopore polycarbonate membrane using an Avanti Mini-Extruder (Alabaster, AL, USA) at room temperature to break apart multilamellar vesicles and obtain a suspension of unilamellar vesicles.

2.3 Amino acid sequence analysis

Sequences of the main catalytic α1 subunit of the Na$^+$,K$^+$-ATPase were obtained from the protein database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/protein/). All available entire vertebrate sequences were aligned using the MUSCLE program [24] within the MEGA7 suite of evolutionary genetics programs [25].
2.4 Eosin fluorescence measurements

All fluorescence measurements were carried out using an RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with 1 cm pathlength quartz microcuvettes. 1000 μL of pH 7.4 buffer (2.2 mM Tris and 0.1 mM EDTA), 39 μL of Na⁺,K⁺-ATPase-containing membrane fragments (6.2 mg mL⁻¹ in 25 mM imidazole, 250 mM sucrose and 1 mM EDTA, pH 7.4) and 2.9 μL of eosin (11 μM in water) were consecutively added to the cuvette. Fluorescence intensity values for each λ<sub>ex</sub> of every buffer were averaged over 5 individual measurements. The temperature was maintained at 24°C via a circulating water bath. The value of λ<sub>em</sub> was 550 nm (bandwidth 5 nm) with an OG530 cut-off filter (Schott, Mainz, Germany) in front of the photomultiplier. At each λ<sub>ex</sub>, 490 nm and 535 nm, the apparent background fluorescence was subtracted prior to calculating the fluorescence ratio, \( R = \frac{F_{490}}{F_{535}} \). The background level was determined at λ<sub>ex</sub> = 400 nm, at which eosin doesn’t undergo excitation.

The final Na⁺,K⁺-ATPase concentration in the cuvette was 230 μg/ml. This concentration was chosen based on previous studies by Skou and Esmann [26, 27] in order to obtain an initial equilibrium between eosin molecules protein-bound in the neighboring aqueous solution, which is perturbed when the enzyme undergoes a conformational transition between E2 and E1 states. This allows the observed fluorescence shifts on PLL addition to be maximized. Measurements were performed in a buffer containing a low Tris concentration of 2.2 mM, which was chosen based on previous measurements [10] showing that this low concentration stabilizes the enzyme initially in the E2 state.

2.5 RH421 UV-visible absorbance measurements

UV-visible absorbance measurements were carried out with a UV-2450 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) using quartz semi-micro cuvettes. To reduce
scattering contributions to the measured absorbance spectra of membrane-bound RH421, Na\(^+\),K\(^+\)-ATPase-containing membrane fragments, PLL or peptide fragments were added to both the reference and sample cuvettes. To further reduce any effects of scattering on the measured spectra, an ISR-2200 double-beam integrating sphere attachment (Shimadzu, Kyoto, Japan) was used. This reflects scattered light back into the photomultiplier and prevents it from being erroneously recorded as an absorbance. The bandwidth was 5 nm.

To quantify more precisely shifts in the absorbance spectrum of membrane-bound RH421 induced by interaction with PLL or peptide fragments, similar to the procedure outlined above for eosin, we utilised a ratiometric approach. In this case the absorbance ratio was determined by dividing the absorbance at 440 nm by the absorbance measured at 540 nm, i.e., 
\[
R = \frac{A_{440}}{A_{540}}
\]
This method eliminates any variation due to small differences in RH421 concentration.

2.6 Static light scattering experiments

Measurements of the static light scattering of Na\(^+\),K\(^+\)-ATPase-containing membrane fragments were recorded using an RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with 1 cm pathlength quartz microcuvettes. The \(\lambda_{\text{ex}}\) and \(\lambda_{\text{em}}\) values were both set to either 826.4 nm (for Na\(^+\),K\(^+\)-ATPase membrane fragments) or 824.2 nm (for vesicles), which corresponds to a high intensity line in the spectrum of the instrument’s Xe arc lamp. Light scattering was detected at 90\(^\circ\) to the incident light beam. The bandwidths of the incident and scattered light were set at 5 nm using the instrument’s monochromators.

2.7 Experimental data analysis

Nonlinear regression fitting of experimental data was carried out via the commercially available program Prism 7.02 (GraphPad Software Inc., La Jolla, CA). Plots of the
experimental data and fitted curves were prepared using Origin 7 (OriginLab, Northampton, MA).

2.8 Peptide synthesis, HPLC purification and ESI-MS analysis

Peptides (1-20: GRDKYEPAAVSEHGDKKAK, 21-40: KERDMDELKKEVSMDHKLS, 41-60: LDELHRKYGTDLSRLTPAR and 13-40: HGDKKKAKKERDMDELKKEVSMDHKLS) from the N-terminus of the α-subunit of pig kidney Na⁺,K⁺-ATPase were prepared at 100 μmole scale using Fmoc solid-phase peptide synthesis (SPPS) on Wang resin to yield the C-terminal acid. After the first residues were loaded using HBTU/DMAP chemistry the peptides were elongated on a CEM Liberty Blue automated microwave peptide synthesiser (USA, NC) [4 min coupling cycle; 2 min coupling (90°C); 1 min deprotection (90°C); 1 min associated washes and liquid handling] using a 5-fold excess of Fmoc amino acid, Oxyma and DIC. 25 μmoles of each peptide was cleaved from the resin and deprotected with a trifluoroacetic acid (TFA)-based cleavage solution (TFA/TIPS/H₂O, 95:2.5:2.5) for 2 h at room temperature, then worked up with diethyl ether, and lyophilised to yield the crude peptide. All target Na⁺,K⁺-ATPase peptides were purified using acetonitrile and H₂O buffer systems (0.1% TFA) using a Waters 2535 Quaternary gradient Module system and a 2489 UV/Vis detector (230/280 nm) with a Waters Sunfire, C18 OBD, 5 μm, 19 x 150 mm preparative column (20 mL min⁻¹); Na⁺,K⁺-ATPase 1-20 (0-30% B over 30 min, other Na⁺,K⁺-ATPase peptides (5-35% B over 30 min). The identity of the peptides was confirmed on a Shimadzu UPLC-MS 2020 ESI instrument (LC-M20A pumps/SPD-M30A diode array detector) in positive mode (0.1% formic acid). The purity of the peptides was assessed on a Waters Acquity UPLC system (Acquity C18 BEH 1.7 μm 2.1 x 50 mm column) at 0-50% B, 5 min at 0.6 mL min⁻¹. Refer to Supplementary Information for detail on peptide masses, ESI-MS identification, purity and yields.
2.9 Peptide dialysis, salt transfer concentration determination

Prior to addition of synthetic N-terminal peptide fragments 1-20, 21-40 or 41-60 to Na\(^+\),K\(^+\)-ATPase membrane preparations, the membrane-binding TFA counterions of the basic amino acid residues (lysine, arginine and histidine) were removed by dialysis in the buffer used for subsequent spectroscopic measurements. For the separation 500 – 1,000 g mol\(^{-1}\) cut-off Spectra/Por cellulose ester dialysis tubing was used (John Morris Scientific, Chatswood, Australia). After dialysis the peptide concentration was checked using the Peterson modification [22] of the Lowry method [23] with BSA as a standard utilising the Total Protein Kit, micro Lowry, Peterson’s modification (Sigma).

For NMR experiments on N-terminal peptide fragment 13-40 (5.3 mg peptide) the TFA counterion was transferred to the chloride ion by three cycles of lyophilisation from 1.5 ml of 5 mM HCl [28] with three further lyophilisation cycles performed from 50% acetonitrile/water to yield a fluffy white peptide. UPLC-MS analysis was performed on an aliquot of the final sample to confirm no acid-induced modification had occurred during the salt transfer procedure.

2.10 MD simulations

All simulations were carried out using GROMACS (v. 5.1.4) [29-33]. A modified CHARMM36 force field with recently developed virtual sites parameters for lipids was used to model all system components [34-37]. The virtual sites parameters enabled the use of a 5 fs time step. We constructed an asymmetric lipid bilayer with ~600 lipids using CHARMM-GUI [38-42] with POPC, POPS and cholesterol in the ratio 48:12:40%. POPS is present only in the cytoplasmic leaflet. The system was explicitly hydrated using the 3-site TIP3P water model with ~61000 water molecules. Periodic boundary conditions were enabled. For non-
bonded interactions, neighbor searching was performed within the cutoff of 12 Å and updated after 20 steps. Using a force-switch function, van der Waals interactions were smoothly turned off between 10 Å and 12 Å. Electrostatic interactions were calculated using the particle-mesh Ewald (PME) method [43, 44]. After energy minimization, the systems were equilibrated for 25 ns, and finally a production run of 200 ns was performed. For each conformation state, 3 copies of the system with different distributions of the POPS lipids were simulated. A temperature of 310 K was maintained using Nosé-Hoover thermostat [45, 46] during the production runs. The Parrinello-Rahman barostat [47] was used for semi-isotropic pressure coupling. The Linear Constraint Solver algorithm (LINCS) was used to constrain all covalent bonds [48].

The first 30 residues of the N-terminus of the E₁:P·ADP·3Na⁺ state (PDB ID: 3WGU) [49] and the E₂·P·2K⁺ state (PDB ID: 3KDP) [50] were modeled using QUARK [51, 52], which is an ab-initio computation based structure prediction server. The crystal structures used for the current simulations belong to the same organism Sus scrofa (pig). Residue D369 was manually phosphorylated using previously developed parameters [53]. The modeled N-termini were carefully aligned in the input structures before starting the simulations. The depth of the pump in the membrane was estimated using the Orientations of Proteins in Membranes (OPM) server [54]. An ion concentration of 150 mM was used to ionize the system. Simulation snapshots were rendered using VMD [55].

The simulations were carried out with an N-terminus modeled based on a secondary structure prediction server, because the disordered N-terminus did not generate an assignable density in the crystal structures. Further changes in the secondary structure of the N-terminus peptide may occur on longer timescales. However, the interaction of the N-terminus with the membrane is dominated by long-lived electrostatic interactions that anchor the lysine residues to the membrane surface, and thus limit the conformational flexibility of the peptide. The
simulations were set up so that the initial distance between the N-terminus and the membrane surface was the same in the E₂P₁2K⁺ and the E₁P·ADP·3Na⁺ simulations, so we are confident that the results are independent of the initial configurations of the simulations.

2.11 Solid-state NMR spectroscopy

NMR sample preparation: d31-POPC and POPS were co-solubilized at a 7:3 molar ratio in chloroform/methanol (3:1 v/v) prior to solvent rotary evaporation and further drying under high vacuum overnight. The lipid film was then resuspended with Milli-Q water and aliquoted in two Eppendorf tubes, one of which contained the dry ATPase N-terminal 13-40 peptide fragment to give a lipid to peptide molar ratio of 15:1. The solutions were freeze-dried overnight and resuspended in buffer (20 mM MOPS pH 7.4, 0.5 mM EDTA) to reach 60% w/w hydration level, followed by five freeze-thaw cycles and the multilamellar vesicles (MLVs) were transferred into a 4 mm zirconia NMR rotor with vespel spinning cap.

Solid-state NMR experiments: The ³¹P and ²H solid-state NMR experiments were conducted at 25°C on a Bruker 400 MHz NMR equipped with HXY 4 mm MAS probe. ³¹P static and magic angle spinning (MAS) experiments were performed at 161 MHz, using a ca. 46 kHz single pulse excitation with ca. 50 kHz SPINAL64 ¹H decoupling, a 3 s recycle delay, a spectral width of 125 kHz, 8k complex points acquisition zero-filled to 16k points and line broadening ranging from 25 Hz to 100 Hz. Spectra were externally referenced to 0 ppm using H₃PO₄ prior to each MAS experiment. MAS experiments were acquired with proton decoupling using a single-pulse experiment and static spectra were acquired using a Hahn echo pulse sequence [56] with Exorcycle phase cycling on the π pulse. T₁ and T₂ relaxation experiments [56] were performed at 6 kHz spinning speed using an inversion-recovery pulse sequence and a Hahn echo pulse sequence with Exorcycle phase cycling on the π pulse, respectively. T₁ and T₂ values were obtained from single exponential fitting of
the $^{31}$P single resonance intensity using Gnuplot 4.6 (http://www.gnuplot.info) in-built least-squares fitting functionality.

Static $^2$H spectra utilized a solid echo sequence [57] at 61.5 MHz with 38 kHz excitation, 8192 complex points, 250 kHz spectral width, 0.5 second recycle delay and a total echo delay of 40 μs. Pake-pattern powder spectra were dePaked to resolve quadrupolar splitting at 0° orientation using weighted Fourier transformation [58] added as part of the NMRPipe 8.1 distribution. Reported lipid acyl chain order parameters ($S_{CD}$) were calculated from $^2$H (or D) quadrupolar splitting ($\Delta \nu_Q$) by $S_{CD} = (2/3 A_Q) \Delta \nu_Q$, where $A_Q$ is the C-D quadrupolar coupling constant of 168 kHz.

3. Results

3.1 Amino acid sequence analysis

To first determine which amino acid residues in the N-terminus of the Na$^+$,K$^+$-ATPase $\alpha_1$ subunit are potentially physiologically relevant we carried out a sequence alignment of all available vertebrate sequences. Alignments of the N-termini up to the start of the first transmembrane helix are shown in Figure 1. The propeptide sequence of the first 5 amino acid residues, which is cleaved prior to activation of the protein, has been omitted from the figure. The numbering of the residues is based on the human sequence and starts from the first amino acid residue after the propeptide. All conserved amino acid residues are highlighted in yellow. One region of note is the conserved sequence $^{30}$L$^{31}$K$^{32}$K$^{33}$E. The linkage between $^{32}$K (lysine) and $^{33}$E (glutamic acid) is the so-called T$_2$ site of rapid cleavage by trypsin when the enzyme is in the E1 state [12-15]. In the E2 state this site is initially protected from trypsin attack and is only cleaved subsequent to cleavage at an arginine at position 438 (the T$_1$ site). This indicates that the LKKE region of the N-terminus must be
involved in some reorganization associated with the E1-E2 conformational change. A further lysine residue, $^{49}\text{K}$, is conserved across all the vertebrate sequences presented, which include both bony and cartilaginous fish, amphibians, birds and mammals. Scanzano et al. [18] showed from mutagenesis studies that both of these sites modulate the E1-E2 conformational transition.

Another obvious point from the alignment (see Fig. 1) is that, although not in the same positions for all species, within the peptide sequence 13-25, lysine (K) is by far the most dominant amino acid residue. An analysis of the frequency of lysines in the N-terminus up to the conserved LKKE motif yielded values ranging between 22.9% ($\text{Oncorhynchus mykiss}$, rainbow trout, $\alpha_1$-subunit) and 36.4% ($\text{Rhabdosargus sarba}$, goldlined seabream, $\alpha_1$ subunit). This range can be compared to the frequency of lysines in the sequences of 1,021 unrelated proteins of 5.7% [59]. The lysine frequency in the N-terminus of the Na$^+$,K$^+$-ATPase $\alpha_1$ subunit is at least 4 times higher than this value. This appears to indicate a significant evolutionary pressure to increase the lysine composition of this part of the protein for a particular functional purpose. The high lysine content also provides a justification for using poly-L-lysine as an experimental analogue of the protein’s N-terminus in some of the following experimental studies.
Figure 1: Sequence alignment of the N-terminus of the α₁ isoform of the catalytic α-subunit of the Na⁺,K⁺-ATPase from vertebrates. Conserved residues are highlighted in yellow. The numbering of the residues is based on the *Homo sapiens* sequence.

Although the analysis here is restricted to vertebrates, this does not mean that the LKKE motif or the high lysine content of the N-terminus are exclusively vertebrate phenomena. In fact many invertebrates also exhibit similar characteristics in their Na⁺,K⁺-ATPase N-termini. To mention just a few specific examples, the echinoderm *Acanthaster planci* (the crown-of-thorns starfish) and the cnidarian *Hydra vulgaris* (the fresh-water polyp) also possess the LKKE motif. The molluscs, *Doryteuthis opalescens* (the opalescent inshore squid) and *Crassostrea virginica* (the eastern oyster), as well as the crustacean *Halocaridina rubra* (the Hawaiian red shrimp) also possess Na⁺,K⁺-ATPase N-termini which are very rich in lysines, often together with glycines. *Doryteuthis opalescens* even has six consecutive lysine residues. The LKKE motif and the high lysine content of the N-terminus are also not isoform specific. Each of the α₁-α₄ isoforms of the *Homo sapiens* Na⁺,K⁺-ATPase catalytic subunit, which show tissue-specific expression levels, possess the LKKE motif. Based on this wide distribution of the LKKE motif and the lysine-rich N-terminus across different animal species and tissues, one must conclude that they don’t have a tissue- or organ-specific function. Whatever the role of the lysine-rich N-terminus is, it must be associated with a fundamental process at the individual cell level or cellular communication level.

3.2 Effect of poly-L-lysine on the E1-E2 distribution of the Na⁺,K⁺-ATPase

From previous experiments [10, 26, 27, 60] it is known that a high fluorescence excitation ratio, $R = F_{490}/F_{535}$, of the probe eosin is characteristic of Na⁺,K⁺-ATPase in the E2 state, whereas a low value is characteristic for the E1 state. Titration of the enzyme in the
presence of eosin was found to cause a significant drop in $R$ (see Fig. 2), from an initial value of $\sim0.6$ to a saturating value at high PLL concentrations of $\sim0.25$. This indicates that PLL causes a shift in the enzyme’s conformation away from E2 and towards E1. The effect appears to have saturated at a PLL concentration of $\sim50$ µg/ml, which corresponds to a concentration of lysine residues of 0.3 mM.

In a previous study [10], we showed that increasing the ionic strength, $I$, of the buffer solution also causes a similar significant shift from the E2 to the E1 state. However, the ionic-strength-induced shift saturates at an $I$ of $\sim20–30$ mM, i.e., at a concentration range 2 orders of magnitude greater than the effect seen here for PLL. Therefore, the PLL effect cannot simply be interpreted as an ionic strength effect. The high local positive charge density of PLL with its sequence of closely spaced positively charged lysine residues must be allowing a relatively strong interaction with either the Na⁺,K⁺-ATPase itself or its surrounding membrane.

![Figure 2: Effect of poly-L-lysine (PLL) concentration on the fluorescence ratio, $R$, of eosin noncovalently bound to pig kidney Na⁺,K⁺-ATPase. $R$ is defined as the fluorescence intensity ratio using excitation wavelengths of 490 nm and 535 nm, i.e., $R = F_{490}/F_{535}$, at an emission](image)

Figure 2: Effect of poly-L-lysine (PLL) concentration on the fluorescence ratio, $R$, of eosin noncovalently bound to pig kidney Na⁺,K⁺-ATPase. $R$ is defined as the fluorescence intensity ratio using excitation wavelengths of 490 nm and 535 nm, i.e., $R = F_{490}/F_{535}$, at an emission
wavelength of 550 nm. A decrease in $R$ corresponds to a decrease in the proportion of the enzyme in the E2 state and hence an increase in the proportion in the E1 state [9]. The solid line represents a non-linear least squares fit to the experimental data using a hyperbolic saturation curve. The $K_{0.5}$ value determined was 13 (± 2) µg/ml. The limiting values of $R$ at zero and saturating concentrations of PLL were determined to be 0.592 (± 0.009) and 0.18 (± 0.01), respectively. The Na$^+$.K$^+$-ATPase and eosin concentrations were 230 µg/ml and 29 nM, respectively. The buffer composition was 2.2 mM Tris, 0.1 mM EDTA, pH 7.4, 24ºC. The error bars represent the standard deviations of five individual measurements.

In another recent publication [11] it was shown using the voltage-sensitive dye RH421 that PLL is able to bind to the surface of Na$^+$.K$^+$-ATPase-containing membrane fragments. To test the lipid specificity of this interaction we have, therefore, utilized the same procedure and investigated the interaction of PLL with pure lipid vesicles of varying lipid composition. Furthermore, rather than just use PLL as an analogue of the Na$^+$.K$^+$-ATPase’s N-terminus we have chemically synthesized different fragments of the N-terminus to identify the amino acid residue origin of any interaction with the membrane (see Section 3.5).

3.3 Interaction of polyamino acids with Na$^+$.K$^+$-ATPase-containing membranes

To test the interaction of PLL with Na$^+$.K$^+$-ATPase-containing membrane fragments we have applied a static light scattering-based assay. Addition of PLL to a suspension of Na$^+$.K$^+$-ATPase-containing membrane fragments causes a significant increase in light scattering (see Fig. 3). The increase in light scattering can be explained by the binding of the positively-charged PLL polymer to the membrane surface, thus neutralizing the negative charges of phospholipid headgroups (e.g. phosphatidylserine), decreasing electrostatic repulsion between the membrane fragments and allowing them to aggregate via van der
Waals forces. The results shown in Figure 3 indicate a critical coagulation concentration of approximately 18 μM of lysine residues, which corresponds to a mass concentration of PLL of 3 μg/ml.

A very similar increase in light scattering is observed if poly-L-arginine (PLA) is added to Na⁺,K⁺-ATPase-containing membrane fragments (see Fig. 3). However, the addition of negatively charged poly-L-glutamic acid (PLG) causes no observable increase in light scattering when added at the same concentration level. These results support the hypothesis, described in the previous paragraph, that positive charges on the polymer are necessary for interaction with the membrane fragments. The results shown in Figure 3 indicate that PLA is slightly more effective than PLL at inducing aggregation of the membranes. For PLA the critical coagulation concentration is ~ 13 μM of arginine residues, which corresponds to a mass concentration of PLA of 2.5 μg/ml. The lower concentration of PLA necessary to induce membrane aggregation compared to PLL could perhaps be explained by the fact that the positive charge of arginine is delocalized between two amino groups, whereas the positive charge of lysine is localized on a single amino group. The greater charge delocalization of arginine would be expected to reduce the Born energy barrier for insertion into a nonpolar lipid membrane, thus allowing it to penetrate more deeply into the membrane, as MD simulations predict [61]. A deeper penetration of arginine than lysine into the membrane could produce a slightly stronger membrane interaction of PLA than PLL so that a lower concentration of PLA is required to induce membrane aggregation.
Figure 3: Effect of polyamino acids (red squares ■ = poly-L-arginine (PLA), black circles ● = poly-L-lysine (PLL), blue triangles ▲ = polyglutamic acid (PLG)) on the light scattering of a suspension of Na⁺,K⁺-ATPase-containing membrane fragments. The observed absolute change in light scattering, ΔI, is expressed relative to the initial level of light scattering, I₀, prior to polyamino acid addition. The concentration of each polyamino acid is expressed as a micromolar concentration of monomer units, i.e., amino acid residues, [AA], using a molecular weight in the middle of the range given by the supplier. The Na⁺,K⁺-ATPase concentrations were 40 µg/ml. The buffer composition was 30 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.2, 24°C. Measurements were performed at a wavelength of 826 nm and a bandwidth of 5 nm.

3.4 Interaction of poly-L-lysine with lipid vesicles

In order to test the lipid specificity of the interaction of lysine residues with the membrane we utilized phospholipid vesicles to allow complete control over the lipid composition. This was performed using the same light scattering assay as in the previous
section. Vesicles were prepared containing both pure DOPC and mixtures of 85 mol% DOPC and 15 mol% of either DOPS, DOPG, DOPA or DOPE.

The observed results clearly show that the presence of either phosphatidylserine (PS), phosphatidylglycerol (PG) or phosphatidic acid (PA) in the membrane significantly increases the observed changes in light scattering, indicating a significant enhancement of the interaction of PLL with the membrane due to the presence of the negatively charged PS, PG or PA headgroups (see Fig. 4). In contrast, vesicles composed of pure phosphatidylcholine (PC) or of 85% PC and 15% phosphatidylethanolamine (PE), which both have a net neutral charge at pH 7.2, showed no increase in light scattering on the addition of PLL. It appears, therefore, that the presence of lipids with a negatively charged headgroup are essential for the binding of lysine residues to the membrane surface, and that a composition of 15 mol% anionic phospholipid is sufficient to observe such an interaction.

At low concentrations of PLL, i.e., [Lys] < 5 µM (or 0.8 µg/ml PLL), the time course of the light scattering change observed using DOPC/DOPS, DOPC/DOPG or DOPC/DOPA vesicles occurs as a monotonic increase. However, at [Lys] > 5 µM a slower subsequent smaller drop in light scattering occurs which proceeded over a time scale of up to an hour. Mixing the sample after one hour didn’t produce any increase in scattering, indicating that the slow drop is not due to sedimentation. Measurements on anionic lipid vesicles loaded with a fluorescent dye have shown that at elevated concentrations of PLL dye leakage occurs [62]. Reuter et al. [62] interpreted this result as being due to penetration of PLL through the membrane, leading to membrane defects and vesicle disruption. Thus, it seems likely that the drop in light scattering which we observe at high PLL concentrations is due to vesicle lysis. It appears that a polymer concentration equivalent to 5 µM of lysine is a critical concentration necessary to allow bilayer disruption in the membrane. In contrast, in the measurements described in the previous section, where PLL was added to Na⁺,K⁺-ATPase membrane
fragments, no slow drop in light scattering was observed after the initial increase. The Na\(^+\),K\(^+\)-ATPase membrane fragments, thus, appear to be more resistant to disruption than pure lipid vesicles, possibly due to their high protein content and the presence of cholesterol.

Figure 4: Effect of poly-L-lysine on the light scattering of suspensions of lipid vesicles (0.15 mM total lipid) composed of 100% DOPC (blue triangles ▲), 85% DOPC and 15% DOPE (orange inverted triangles ▼), 85% DOPC and 15% DOPS (black circles ●), 85% DOPC and 15% DOPG (red squares ■), and 85% DOPC and 15% DOPA (green diamonds ♦). The observed absolute change in light scattering, ΔI, is expressed relative to the initial level of light scattering, I\(_0\), prior to PLL addition. The concentration of PLL is expressed as a micromolar concentration of monomer units. The buffer composition was 30 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.2, 24°C. Measurements were performed at a wavelength of 824 nm and a bandwidth of 5 nm.

3.5 Interaction of peptide fragments with Na\(^+\),K\(^+\)-ATPase-containing membranes

Tests of the effect of chemically synthesized N-terminal fragments of the Na\(^+\),K\(^+\)-ATPase on the absorbance spectrum of RH421 noncovalently bound to Na\(^+\),K\(^+\)-ATPase-
containing membrane fragments showed that fragments 1-20 and 21-40 caused a red shift (to longer wavelengths) of the RH421 absorbance spectrum. As described under Materials and Methods, the shift was quantified by the absorbance ratio $R = A_{440}/A_{540}$ (see Fig. 5), i.e., a decrease in $R$ signifying a red shift. In contrast, fragment 41-60 caused no significant change in the value of $R$ over the concentration range studied. The decrease in the value of $R$ caused by fragments 1-20 and 21-40 is consistent with the interaction of positive charge with the surface of the membrane, as evidenced by analogous shifts of the RH421 fluorescence excitation spectrum of membrane bound dye caused by the membrane binding of inorganic cations such as Ca$^{2+}$, Mg$^{2+}$, Ba$^{2+}$, Sr$^{2+}$ and La$^{3+}$ [63]. Fragments 1-20 and 21-40 both contain 4 positively charged lysine residues, whereas fragment 41-60 only contains 1 lysine residue. Therefore, the observed absorbance shifts strongly suggest that fragments 1-20 and 21-40 interact with the membrane surface via their lysine residues.

The drop in $R$ observed on addition of fragment 21-40 occurred over roughly a tenfold smaller concentration range than that observed on addition of fragment 1-20. This suggests that fragment 21-40 interacts more strongly with the Na$^+$.K$^+$-ATPase membrane fragments than fragment 1-20. It is worth noting that fragment 21-40 contains the conserved sequence LKKE, suggesting that this sequence could be a contributor to interaction with the membrane. To test this hypothesis further we have carried out molecular dynamics simulations of membrane-embedded Na$^+$.K$^+$-ATPase and more detailed spectroscopic investigations via solid-state NMR.
Figure 5: Effect of N-terminal peptide fragments on the absorbance ratio, $R$, of RH421 noncovalently bound to pig kidney Na$^+$,K$^+$-ATPase. $R$ is the absorbance ratio at wavelengths of 440 nm and 540 nm, i.e., $R = A_{440}/A_{540}$. The plotted values, $R_N$, represent the normalized $R$ value based on $R = 1.215 (\pm 0.008)$ in the absence of peptide fragments. The solid lines represent non-linear least squares fits to the experimental data using a hyperbolic curve, except when there was no significant trend. In this case the solid line represents the average $R_N$ value. The $K_{0.5}$ values for fragments 1-20 and 21-40 were determined to be 0.03 (± 0.01) $\mu$g/ml and 0.0016 (± 0.0006) $\mu$g/ml. The Na$^+$,K$^+$-ATPase and RH421 concentrations were 40 $\mu$g/ml and 384 nM. The measurements were performed in a solution containing 30 mM Tris, 150 mM NaCl, 1 mM EDTA and 5 mM MgCl$_2$, pH 7.2. The error bars represent the standard deviations of seven or more individual measurements.

3.6 MD simulations of the Na$^+$,K$^+$-ATPase

Modeled starting structures of the E$_1$P-ADP·3Na$^+$ and E$_2$P$_i$2K$^+$ states based on published X-ray crystal structures and theoretically predicted N-terminus secondary
structures are shown in Figure S1. These structures were taken as initial configurations for the MD simulations. At the conclusion of the simulations it was found that the $E_1P$-ADP-3$\text{Na}^+$ and $E_2P_2$-2$\text{K}^+$ states differed significantly with respect to the interactions between the N-terminus and the cytoplasmic membrane leaflet. The N-terminus interacts more strongly in the $E_1P$-ADP-3$\text{Na}^+$ state compared to the $E_2P_2$-2$\text{K}^+$ state. Almost all lysine residues present on the N-terminus 1-30 reside in close proximity to the membrane in the $E_1P$-ADP-3$\text{Na}^+$ state whereas very few lysine residues associate with the membrane in the $E_2P_2$-2$\text{K}^+$ state (see Fig. 6). Radial distribution functions and the electrostatic interaction energy of individual amino acids with the lipid bilayer (data not shown) suggest that the 5 lysine residues (K4, K16, K17, K20 and K21) bind strongly to the bilayer in the $E_1P$-ADP-3$\text{Na}^+$ state whereas K4 and K16 binds the membrane strongly in the $E_2P_2$-2$\text{K}^+$ state. It is worth noting that all of these membrane-interacting lysines occur before the conserved LKKE motif, which, in the pig sequence, occurs at positions 28-31. The electrostatic interaction energies of the $E_1P$-ADP-3$\text{Na}^+$ state with the membrane were determined to be $-686 \pm 79$ kJ mol$^{-1}$ for residues 1-30 and $-691 \pm 79$ kJ mol$^{-1}$ for residues 1-80 of the N-terminus. For the $E_2P_2$-2$\text{K}^+$ state the corresponding values were $-405 \pm 60$ kJ mol$^{-1}$ for residues 1-30 and $-411 \pm 60$ kJ mol$^{-1}$ for residues 1-80. This indicates that the contribution of residues 31 to 80 to the total electrostatic interaction energy with the membrane is small compared to that of only residues 1-30, in good agreement with the RH421 absorbance assay which shows negligible impact of the N-terminus fragment 41-60 on the absorbance ratio (see Fig. 5).

It should be pointed out that here we have used the calculated electrostatic energies purely in a comparative fashion as a means to determine which segments of the N-terminus are responsible for the interaction. The absolute magnitudes of the energies determined from the MD simulations are most likely not entirely reliable because of the difficulty in
incorporating screening from mobile counterions in the calculations. Based on previous experimental results [10], calculations based on the Guoy-Chapman theory (see Eq. 6 in [10]) indicate that the interaction energies are likely to be not more than \(\sim 10 \text{ kJ/mol}\).

Figure 6: Interaction between the N-terminus and lipid headgroups. Snapshots rendered from the last frame of three different copies of the simulations starting from different random distributions of PS lipids in the bilayer. PS lipids are enriched near the Lys residues. (A), (B) and (C): N-terminus of the sodium-bound \(E_1\) state. (D), (E) and (F): N-terminus of potassium-bound state \(E_2\). The pink and tan beads represent phosphorous atoms of POPS and POPC headgroups, respectively. The backbone of the lysine residues on the N-terminus are shown in red.

Apart from the stronger interaction with the membrane in the \(E_1\) state, we found an \(E_1\) exclusive salt-bridge between residues Lys38 and Glu231 (see Fig. S2). The importance of this glutamate residue (Glu233 in the rat sequence) was reported in an investigation of the interaction of the N-terminus with the rest of the \(\alpha\)-subunit in the rat enzyme [64, 65].
3.6 Solid-state NMR spectroscopy

Based on the UV/visible absorbance results, indicating membrane interaction of the 1-20 and 21-40 peptide fragments, and the MD simulations, suggesting the importance of membrane interaction with residues K16, K17, K20 and K21, we decided to synthesize a longer fragment, 13-40, which includes all of these residues plus the conserved LKKE motif and investigate its interaction with vesicles composed of both POPC and POPS via NMR spectroscopy.

The $^{31}$P isotropic chemical shift of d31-POPS (-0.12 ppm) was significantly shifted by -0.30 ppm in the presence of the ATPase N-terminal fragment while the POPC isotropic chemical shift (-0.58 ppm) changed by only 0.11 ppm (Fig. 7A), and both lipid signals were broadened by the presence of the peptide. These results indicate a preferential interaction between the cationic peptide and the anionic POPS lipids, in a similar fashion to behaviour observed for the cationic peptide cupiennin 1a, which has been found to preferentially interact with the anionic lipid headgroup phosphatidylglycerol [66]. The static $^{31}$P NMR experiments showed a reduction in the width of the powder pattern, with the chemical shift anisotropy (CSA) decreased from 27.8 ppm to 26.4 ppm and the formation of a significant isotropic peak (at ~ 0 ppm) in the presence of the peptide (Fig. 7B). The CSA reduction is usually due to an increase in the headgroup re-orientation and the isotropic phase indicates a lipid population re-orientating at faster rate than the CSA (μs – ms).

$T_1$ and $T_2$ $^{31}$P relaxation measurements also confirmed preferential interactions between the peptide and POPS (Fig. S3). Addition of peptide reduced $T_2$ to a greater extent for POPS ~66% (9.6 ms to 3.3 ms) than for POPC ~50% (11.4 ms to 5.8 ms). A decrease in $T_2$ is due to an increase in low frequency motions, such as lipid diffusion, whereas $T_1$ reports on higher frequency dynamics, mainly long axis lipid rotation. $T_1$ decreased ~30% for POPS
(0.82 s to 0.59 s) and ~20% for POPC (0.87 s to 0.69 s) with the peptide. The $T_1/T_2$ ratio was ~ 80 for both lipids but increased to ~180 for POPS and ~120 for POPC with the peptide, which indicates an increase in low frequency motions [57, 67]. The substantially greater increase for POPS indicates a stronger interaction of the N-terminal peptide fragment with the anionic phospholipid.

![Figure 7](image)

**Figure 7:** Solid-state NMR data for d31-POPC/POPS (7:3) MLV (black solid line) and in the presence of the ATPase N-terminal fragment 13-40 (red dashed line) at a lipid to peptide molar ratio of 15:1. (A) $^{31}$P MAS spectra, (B) $^{31}$P static spectra, and (C) $^2$H order parameter profile at 25°C.

The peptide induced a slight ordering of the d31-POPC lipid acyl chain through the terminal methyl group as seen in the order profiles extracted from the $^2$H NMR experiments (Fig. 7C). The peptide may segregate the lipids, inducing more dynamic disordered domains enriched in POPS leading to the zwitterionic d31-POPC enriched domains with an increase in
acyl chain order. Similar behaviour has also been observed for the interaction of antimicrobial peptides with mixed zwitterionic/anionic lipid membranes [68].

4. Discussion

Previous studies [10, 11] have implicated an electrostatic interaction between the N-terminus of the α-subunit of the Na⁺,K⁺-ATPase and its surrounding membrane as an important contributor in determining the relative stabilities of different conformational states of the protein and possibly in its regulation. The present study was carried out in order to further define the specificity of the interaction and localise its site of origin.

Based on sequence analysis, light scattering assays using PLL as a model of the N-terminus and both UV/visible and NMR spectroscopic measurements using chemically synthesised fragments of the N-terminus, it appears highly likely that the interaction is due to an electrostatic attraction between positively charged lysine residues on the N-terminus and negatively charged phospholipid headgroups (notably phosphatidylinerine) in the surrounding membrane.

RH421 absorbance measurements using chemically synthesised peptide fragments of the N-terminus indicate that amino acid residues beyond 40 do not play any significant role in interaction with the membrane. Calculations of the electrostatic energy of interaction from MD simulations suggest that it is the first 30 amino acids which are predominantly responsible for membrane interaction. Thus, it appears that lysine residues located prior to the conserved LKKE motif are most likely the cause of membrane interaction. Based on this result, we investigated the interaction of a longer N-terminal peptide incorporating the LKKE motif and five lysine residues earlier in the sequence. An N-terminal fragment (residues 13-40) showed stronger interaction with POPS headgroups based on 31P solid-state NMR and
formation of an isotropic peak, which indicated severe disordering of the bilayer. However, 
$^2$H solid-state NMR showed that the peptide ordered the deuterated d31-POPC acyl chains, which indicates that POPS is segregated from d31-POPC by the lysine-rich peptide.

The positions of the lysine residues prior to the LKKE motif are not conserved across all vertebrate species (see Fig. 1). However, lysine is by far the most dominant amino acid in this region of the protein, and, if the purpose of the residues is interaction with a negatively charged membrane surface rather than with another part of the protein, there is no need for the positions of the lysines residues to be conserved. The contribution of the lysines to the positive charge density of the N-terminus is sufficient to produce an interaction, regardless of the precise position along the polypeptide chain.

Eosin fluorescence measurements using PLL as a model of the N-terminus indicate that interaction of lysine-rich polypeptides can shift the Na$^+$,K$^+$-ATPase conformational state. This is in agreement with mutational studies [64, 65] on the N-terminus as well as N-terminal truncation [69], which were also found to produce conformational shifts. Via Raman spectroscopy it was furthermore found that N-terminal truncation appeared to change the coupling between the protein and the membrane [69]. In the study of Daly et al. [70], however, it was found that deletion of the N-terminus up to and including residue $^{23}$K of the rat $^{\alpha_1}$ enzyme ($^{21}$K in pig) had no effect on the observed kinetic behaviour of the enzyme. This clearly indicates that this segment of the protein is not essential for activity. Nevertheless, it is important to point out that the mutational studies [18, 64, 65, 70] have all been performed with enzyme expressed in HeLa cells, not in the native lipid membrane environment which the enzyme would encounter, e.g., in the kidney. Plasma membrane composition and its physical properties are known to vary greatly between different cell lines [71]. If the lysine rich N-terminus of the Na$^+$,K$^+$-ATPase $\alpha$-subunit interacts with the membrane, as the results here suggest, the lack of an effect of deletion on activity in one
membrane environment does not preclude the possibility of a modulatory effect on the enzyme’s kinetics in a different membrane environment, particularly one such as the kidney with a high level of cytoplasmic anionic lipids. Lipid analysis of Na⁺,K⁺-ATPase-containing membrane fragments prepared from mammalian kidney in a similar fashion to those used in the present study have shown [72, 73] that on a molar basis they contain 51% phospholipids, 36% cholesterol, 5% acylglycerols and 9% free fatty acids. Of the phospholipids present 36% were determined to be PC, 28% PE, 18% sphingomyelin, 13% PS and 6% phosphatidylinositol. However, PS is known to be enriched in the inner cytoplasmic leaflet of the plasma membrane [74], so that the actual PS proportion of total phospholipid is likely to be close to 30% at the cytoplasmic face of the Na⁺,K⁺-ATPase in the preparation which we have used. Based on the results presented here, further mutational studies focusing on lysine residues prior to the LKKE motif but in other cell lines could provide further valuable information.

An important new finding presented here is that MD simulations support the hypothesis that interaction of the N-terminus with the membrane is dependent on protein conformation. Because no crystal structures of the Na⁺,K⁺-ATPase in the Na⁺-free unphosphorylated E1 state or the K⁺-free E2 unphosphorylated state are currently available and hence no MD simulations of these states are possible, this precludes a direct comparison with experimental results of Jiang et al. [10]. Nevertheless, based on the information currently available from experiment and MD simulation, it appears that the strength of interaction of the N-terminus with the membrane is greater in E₁P·ADP·3Na⁺ than E₂P·2K⁺, but greater in E₂ than E₁. The MD simulations also support previous findings [64, 65] that a salt bridge between Glu231 and Lys38 of the N-terminus (pig sequence numbering) is conformation dependent and could play a significant role in the kinetics of the protein’s conformational transitions.
Acknowledgement

R. J. C. and F.S. acknowledge with gratitude financial support from the Australian Research Council (Discovery Grants No. DP121003548, DP1501011112 and DP170101732 to R. J. C. and DP140102127 to F.S). The simulations were carried out on the Danish e-Infrastructure Cooperation (DeiC) National HPC Center with ABACUS 2.0, and on the Swiss PizDaint Supercomputer under the PRACE 14th Call, number 2016153468. H. K. and V. D. acknowledge financial support from the Lundbeckfonden.
References


