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Poulsen, Hanne; Nissen, Poul; Mouritsen, Ole G.; Khandelia, Himanshu

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Protein Kinase A (PKA) Phosphorylation of the Na+/K+-ATPase Opens an Intracellular C-terminal Water Pathway Leading to the Third Na+−binding site in Molecular Dynamics Simulations

Hanne Poulsen1, Poul Nissen1, Ole G. Mouritsen2 and Himanshu Khandelia3*

From1 PUMPKIN – Centre for Membrane Pumps in Cells and Disease, Aarhus University, Aarhus C, Denmark

2MEMPHYS – Center for Biomembrane Physics, University of Southern Denmark, Odense, Denmark.

*Running title: Phosphorylation of the sodium pump opens C-terminal ion pathway

To whom correspondence should be addressed: Himanshu Khandelia, MEMPHYS: Center for Biomembrane Physics, University of Southern Denmark, Odense, Denmark. Phone: +4565503510 Fax: +4565504048, E-mail: hkhandel@memphys.sdu.dk

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Background: There is an ongoing debate on whether and how the ion pump NKA can be regulated by a PKA kinase.

Results: Phosphorylation of the PKA target S936 opens an intracellular ion pathway leading to the ion-binding sites

Conclusion: PKA phosphorylation has a drastic impact on NKA structure and dynamics

Significance: The molecular mechanism of PKA regulation of NKA has been described for the first time.

SUMMARY

Phosphorylation is one of the major mechanisms for posttranscriptional modification of proteins. The addition of a compact, negatively charged moiety to a protein can significantly change its function and localization by affecting its structure and interaction network. We have used all-atom Molecular Dynamics (MD) simulations to investigate the structural consequences of phosphorylating the Na+/K+- ATPase (NKA) residue S936, which is the best characterized phosphorylation site in NKA, targeted in vivo by Protein Kinase A (PKA) (1-3). The MD simulations suggest that S936 phosphorylation opens a C-terminal hydrated pathway leading to D926, a transmembrane residue proposed to form part of the third sodium ion-binding site (4). Simulations of a S936E mutant form, for which only subtle effects are observed when expressed in Xenopus oocytes and studied with electrophysiology, does not mimic the effects of S936 phosphorylation. The results establish a structural association of S936 with the C-terminus of NKA and indicate that phosphorylation of S936 can modulate pumping activity by changing the accessibility to the ion-binding site.

Ion gradients across cellular membranes sustain electrical excitability, secondary transport, energy storage and cell volume regulation. The plasma membrane contains numerous proteins, e.g. the ion channels that depend directly on the ion gradients created by ion pumps. Cation pumps such as the NKA use the energy of ATP hydrolysis to transport ions against their concentration gradients across the membrane. The NKA extrudes three Na+ ions in exchange of two K+ ions for each molecule of ATP hydrolyzed.

The NKA α-subunit has the conserved features of canonical P-type ion-ATPases, including cytoplasmic nucleotide, phosphorylation and acutator domains and intramembrane ion-binding sites. X-ray crystallography has revealed the structure of NKA in the potassium bound form (5,6). In the sodium-bound pump, the two potassium-binding sites reorganize to achieve high sodium affinity, but the binding site for the third sodium ion remains a matter of debate (4,5,7).

PKA phosphorylation is one of several mechanisms for regulating NKA activity (1-3). S936 lies near the lipid-water interface, and in vitro phosphorylation depends on detergent, but antibodies raised against a peptide with the phosphorylated S936 show that the site is phosphorylated in vivo in response to PKA activation (reviewed in (8)).

In various cell types, induction of PKA and phosphorylation of NKA have been found to
correlate inversely with NKA activity, while the amount of pumps at the plasma membrane remaining unaffected, suggesting that phosphorylation of S936 directly lowers pump activity (3,9-14). Pump trafficking may, however, also be affected by PKA phosphorylation in some cell types, apparently via a cAMP independent pathway responding to the intracellular sodium levels (15,16).

S936 is only few angstroms from the C-terminus, and we hypothesized that the phosphorylation may affect the C-terminal structure. In C-terminal mutants expressed in *Xenopus* oocytes, inwardly rectifying leak currents were observed in the presence of extracellular sodium (4,17,18) and similar though much smaller leak currents were observed with the phosphorylation-mimicking mutation S936E (8). However, the S936E mutation might underestimate the electrostatic and steric effects of S936 phosphorylation.

Here, the structural effects of S936 phosphorylation are investigated with Molecular Dynamics (MD) simulations. S936 lies on the cytoplasmic loop connecting TM8 and TM9, which is close to the C-terminal interaction network that controls a pathway to the ion-binding sites. We examine the effects of S936 phosphorylation on the C-terminal network, on the ion-binding sites, and on transmembrane structure and dynamics. Upon phosphorylation, water molecules enter an intracellular pathway that leads to the ion binding sites. The pathway is similar to that described in C-terminal mutants (4). The finding is significant because it is the first example of a structural effect of phosphorylation on the NKA that may explain the marked functional effects of NKA phosphorylation by PKA.

**EXPERIMENTAL PROCEDURES**

**Molecular Dynamics Simulations**

Simulations were implemented with the pig renal αβ dimer (PDB accession 3B8E (5)) embedded in a fully hydrated 111 Å x 111 Å x 190 Å 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid bilayer. POPC is a lipid well-known model lipid used to model membranes both in simulations and in experiments in a large variety of systems, ranging from protein-membrane interactions such as ion-channel (19) and ion-pump simulations (4,20-22) to drug binding assays (23). A POPC membrane is in the fluid phase at room temperature. Important physical properties of the membrane, such as membrane thickness, area-per-lipid and fluidity, all of which may impact protein-lipid interactions are captured accurately by the POPC lipid model. Finally, lipids diffuse in membranes on time scales of 100s of nanoseconds. Modeling a mixture of lipids will therefore bias the simulation towards their initial placement of lipids in the membrane.

Simulations were performed using GROMACS version 4.5.3 (24-26) using the modified Berger force field for lipids (27) and the OPLS-AA all atom force field for proteins (28). The dihedral angles of the Berger force field were re-optimized in order to permit their combination with the OPLS-AA protein force field (29). The method reproduced accurate free energy of transfer of amino acids between aqueous and hydrophobic lipid phases, and also yielded reasonable protein-lipid interactions for a range of systems, including transmembrane peptides and proteins such as OmpF. Furthermore, the Berger force field retains some of the philosophy of the OPLS united-atom force field in its treatment of Lennard-Jones interactions (27). The two publicly available force fields to run protein-membrane simulations in the NPT ensemble are CHARMM and the OPLS-Berger combination. Simulations run more efficiently with the latter because it uses united atoms for lipids. Since the original implementation of the OPLS-Berger force field combination, several other important studies have been carried out using the same force field combination, see for example (30-32), and reviewed in (33). Therefore, we use the OPLS-AA protein force field in combination with the Berger for field for lipids. It should be noted that depending upon the system of investigation, different protein force fields may perform differently, for example, in terms of predicting the transition states and the folding rates of proteins (34).

There are no major structural differences in the C-terminal and ion-binding region of the low-resolution pig renal structure (3B8E) and the higher resolution shark enzyme (6) (PDB accession code 2ZXE), other than the presence of two extra water molecules near the ion binding sites in the latter. The water molecules appeared spontaneously in the binding site during our simulations. All residue numbering follows the sequence of the pig renal protein. D926 and E954 were kept protonated, as argued earlier (4). All other aspartate and glutamate residues were deprotonated, and all arginine and lysine residues
carried a charge of +1. The simulation cell was kept electrostatically neutral by using Na\(^+\) ions. The simulations were implemented in the isobaric–isothermal ensemble. Temperature coupling was performed using the Berendsen thermostat (35) separately for the protein, lipids and the solvent with a reference temperature of 310 K, and a time constant of 0.1 ps for all subgroups. A semi-isotropic pressure coupling was applied using Berendsen barostat (35). A coupling constant of 1.0 ps and a reference pressure of 1.0 bar was used in all directions. Since the compressibility, \(\kappa\), for the systems was not known, the value for pure water, \(4.5 \times 10^{-5}\) bar\(^{-1}\) was used. For the production run, the leap-frog integrator (36) was used with a time step of 2 fs. All bonds containing hydrogen were constrained using the LINCS algorithm (37,38), and water molecules were constrained with SETTLE (39). Periodic boundary conditions were applied in all directions. A neighbor list with a 10 Å cut-off was used for non-bonded interactions and was updated every 20 fs. The van der Waals interactions were truncated with a cut-off of 10.0 Å and the electrostatic interactions were treated with the Particle Mesh Ewald (PME) (40) method using default parameters. The center of mass translation was removed at every step of the simulation. Trajectories were sampled every 10 ps. Simulations were run for at least 60 ns. For calculation of ensemble-averaged properties, the first 20 ns of the production run of each simulation were discarded. The analysis was carried out using GROMACS or custom-made programs. Visualization and snapshots were rendered using VMD (41).

The wild-type system was equilibrated using harmonic constraints on lipids, water and protein. The constraints were sequentially released, followed by constraint-free equilibration for 20 ns. The final conformation from this equilibrated system was used to start the production runs for the wild type (WT), the S936E mutant, and the protein with S936 phosphorylated (S936PHOS). Two copies of the S936E and S936PHOS simulations were implemented to collect better statistics. Most of the results from the two copies were identical. Simulation snapshots will be presented only for one copy for brevity. Although the simulation time scales are indeed short by current standards, they still successfully capture the phenomena of interest (opening of water pathway), which takes place on timescales of 10s of nanoseconds. For robust results, each system was simulated twice, and the results were reproducible. Furthermore, the simulations are in good agreement with electrophysiological data, which indicates that the risk of drawing invalid conclusions was minimized despite the short simulation timescales.

**Electrophysiology**

Pumps were expressed in oocytes from *Xenopus laevis* by co-injecting cRNAs encoding ouabain insensitive hsNKA\(\alpha\)2 and hsNKA\(\beta\)1 (4). After 1–3 days at 19 °C, oocytes were loaded with sodium in 95 mM Na, 90 mM sulfamic acid, 5 mM HEPES, 10 mM TEACl, 0.1 mM EGTA, pH 7.6. Two-electrode voltage-clamping was performed in 115 mM Na, 110 mM sulfamic acid, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 5 mM BaCl\(_2\), 10 mM HEPES, 1 \(\mu\)M ouabain, pH 7.4. To determine pre-steady-state currents, a series of 200 ms voltage steps was run with and without 10 mM ouabain, and single exponentials were fitted to the difference traces to determine the amount of charges moved (Q) and the inverse time constants (R).

**RESULTS**

In oocytes, the mutation of the PKA targeted serine (number 940 in the human a2 expressed) to glutamate led to increased leak currents in the presence of sodium at negative membrane potentials: an effect reminiscent of the effect of C-terminal mutations, but much milder (4,17). The C-terminal mutants studied so far furthermore exhibit severely reduced sodium affinity and altered kinetics of extracellular sodium binding and release (4,17,42). These effects are not observed for the S940E mutant, which has extracellular sodium affinity and relaxation rate constants similar to the wild-type values as determined from pre-steady-state currents after expression in *Xenopus* oocytes (Fig. 1). Alpha2 expressing oocytes were treated with forskolin and IBMX to activate PKA as previously described (43,44). Phosphorylation was not observed under these conditions.

The limited effect of the S940E mutation in the electrical recordings correlates poorly with the inhibition of NKA observed upon PKA phosphorylation (9,10). A likely reason for this discrepancy is that the introduction of glutamate does not fully replicate the larger steric and electrostatic effect of explicit phosphorylation, as previously reported for a cyclin dependent kinase
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(45). Treatment of oocytes with the adenylyl cyclase activator forskolin did not alter the pump characteristics (results not shown). It was previously reported that NKA phosphorylation in oocyte homogenates, where PKA was activated by cAMP, depended on detergent treatment (46), suggesting that forskolin may be insufficient to initiate phosphorylation of NKA in the oocytes.

To study the structural effects of explicit phosphorylation of the PKA site in the sodium pump, we therefore implemented MD simulations of the pig renal α1-β dimer in a fully hydrated POPC membrane, examining WT pump as well as pumps with the PKA target site S936 phosphorylated.

In the WT protein, the S936 side chain is hydrogen-bonded to the carboxylate of E996 (Fig. 4A), which is 20 residues upstream of the C-terminus on helix M10. Phosphorylation of S936 breaks the hydrogen bond and electrostatically repels E996. In the WT, E996 also interacts with R1003, but phosphorylation leads to an electrostatic salt bridge between S936PHOS and R1003. The resultant movement of the M8-M9 loop displaces M8 slightly away from the third sodium-binding pocket and rearranges the interaction network in the ion-binding pockets.

Two residues in the ion-binding region, Y771 and N923, are close together in all of the four available crystal structures of NKA (PDB IDs 3A3Y, 3KDB, 3N23 and 2ZXE). In the MD simulations of the wild type pump, the side chain of Y771 even hydrogen bonds with the backbone of N923 and vice versa (Fig. 2A). The movement caused by S936 phosphorylation in the MD simulations breaks the Y771-N923 interaction, and the Y771 side chain occasionally hydrogen bonds to the backbone of T807 that is part of the first Na⁺ binding site.

The reorientation of the transmembrane (TM) helices in S936PHOS is captured in the changes in the angle between helices M7 and M8 in Fig. 3. The radial and axial tilt angles between M7 and M8 increase, resulting in the opening up of helices M5, M7 and M8 to a cavity that becomes hydrated as far as residue D926 (Fig. 4 and Supplementary movies 1, 2, and 3, coloring scheme same for movies and Fig. 4). In the WT, no water enters during the simulations.

To examine if the limited functional effect of the S936E (S940E) mutation (Fig 1A) correlates with a limited structural effect of the mutation compared to the phosphorylation, we performed MD simulations of the S936E mutant. In S936E, the S936E-R1003 interaction and the S936E-E996 repulsion are much weaker compared to S936PHOS, owing to the lower size and charge (-1) of S936E compared to S936PHOS (-2). Therefore, the movement of the M8-M9 loop observed in S936PHOS is much smaller with S936E, and consequently, as observed with the WT, water does not enter between M5, M7 and M8.

In the WT and S936E simulations, the Y1016 side chain is bridged to the protonated D926 residue by a single water molecule not observed in the crystal structures (Fig. 4A). Phosphorylation increases the distance between Y1016 and D926 significantly (Fig. 5A), and the space between the two residues is filled by a large number of water molecules (Fig. 4A). The radial distribution functions of D926 and water (Fig. 5B) illustrate that the phosphorylated enzyme has a peak at 1.8 Å and maximal values at 3-5 Å from D926, indicating substantial hydration, while the WT and S936E simulations only show peaks at 1.8Å and 3Å as characteristic of a single water molecule (Fig. 5B)

It is interesting to note that the interactions of the C-terminal Y1016 residue with K766 and R933 seen in the crystal structure and in the WT and S936E simulation also remain largely unaffected by S936 phosphorylation.

DISCUSSION

MD simulations have been used to investigate the molecular effects of phosphorylation of S936 in NKA, a well-documented PKA substrate. The simulations show that in the E2P state, phosphorylation resulted in movement of the M8-M9 loop away from the functionally important C-terminus, causing an altered interaction network of loops close to the C-terminus that allowed for water to gain access to a cavity between helices M5, M7 and M8. Additionally, the simulations show that the phosphorylation caused rearrangements of residues suggested to be involved in Na⁺ binding, which may reflect a weakening of ion binding, so even though S936 is distant from the ion binding sites, ion-binding is likely to be affected by PKA phosphorylation.

The opening of the C-terminal pathway in the simulations upon S936 phosphorylation is similar to the opening seen with C-terminal mutations. Disruption of the C-terminal structure was previously shown to strongly affect both pre-
steady state and steady-state currents as measured with electrophysiology (4,17,18,47). Based on the leak currents in C-terminal mutants and MD simulations that showed water entering a C-terminal pathway, an alternative mechanism of pumping was earlier proposed which involved proton entry through the C-terminal pathway. The same pathway opens in the current set of S936PHOS simulations, suggesting that leak currents, similar to those in C-terminal mutants should be observed in the S936E mutant. The electrophysiological data presented here suggests that the S936E mutation has little or no effect on the pump properties; the mutant has affinity for extracellular sodium similar to the wild type and only leaks a little more in the presence of extracellular sodium, which is in contrast to the significant leak measured with C-terminal mutants under the same conditions. However, the electrophysiology data correlate well with the simulations of the same mutant showing that the C-terminal pathway does not open in S936E, unlike what was observed with the phosphorylated pump. Thus, the S → E mutation is unable to mimic the effects of a phosphorylation in the current case. The possible limitations of a glutamate mutation in mimicking phosphorylation have been documented earlier, e.g. that glutamate, unlike phosphothreonine in the activation loop of the protein cyclin dependent kinase 2 (45).

Of the proposed sodium pump phosphorylation sites, the PKA site is uniquely conserved – the consensus PKA recognition sequence, Arg-Arg-X-Ser/Thr-Φ (where Φ is hydrophobic), is Arg-Arg-Asn-Ser-Val/Leu/Ile in all pump isoforms from Caenorhabditis elegans to man. From different experimental setups, phosphorylation of the PKA site has been found in either NKAα1 (48), NKAα2 (49) or NKAα3 (13). PKA phosphorylation even appears to be conserved in another ATPase, namely the closely related colonic H,K-ATPase (50). All three isoforms are phosphorylated by PKA (51). So while different isoforms are certainly regulated differently by PKA depending e.g. on their cellular localization and interaction partners, our studies address the molecular mechanistic consequences on ATPase function of the PKA phosphorylation, and they will not be subject to variation between the isoforms or even between the different ATPases. We argue that our finding of PKA phosphorylation affecting the C-terminal configuration will be valid for all X,K-ATPases since they share both the PKA site and the characteristic C-terminus.

In the WT, the hydrophilic side chains of N923 and Y771 are stabilized by hydrogen bonding to the backbone atoms of each other. Although N923 and Y771 are proximal in structures, the internal hydrogen bonding arrangement is not apparent. Such a scheme of internal mutual hydrogen bonding could be generally applicable for stabilizing ion-binding hydrophilic residues in hydrophobic environments in absence of the bound ion.

The mutation Y1016F did not alter the sodium binding affinity of the protein in membrane extracts (42), while Y1016A reduced the apparent affinity 5-fold (42), suggesting that the hydroxyl group is not a primary determinant of sodium affinity, while the aromatic ring is required. It has been suggested that R933 and Y1016 interact via a cation-π bond and not via the Y1016 hydroxyl group (42,52). However, no configurations from the MD simulations support a direct cation-π interaction between R933 and Y1016 or Y1015, either stacked or “T” interaction, although the residues are proximal enough in the crystal structure to facilitate such interactions. Cation-π interactions in the E1 form or other intermediate states are also possible explanations to the importance of an aromatic side chain on the C-terminal residue.

Based on the evidence from MD simulations presented here, we suggest that PKA phosphorylation of S936 in NKA affects the structure of the C-terminus and promotes hydration of a cavity in the transmembrane region that connects the cytoplasm and the ion binding sites. The cavity has been previously identified to be similarly opened by mutations of residues in the C-terminal network that severely lower the pump’s sodium affinity. Our results can thus explain why PKA activation was reported to inhibit the NKA pumping activity (3,9-14), and we are currently pursuing a further structural and functional understanding of the role of NKA phosphorylation by PKA.
REFERENCES

This page contains references to various scientific articles and studies. The references are cited in the text and include details such as the authors, publication year, and journal names. Here are some examples of the references cited in the text:


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FIGURE LEGENDS

Figure 1: The voltage dependence of transient currents of S943E mutated Na/K-ATPase. (A) The charge translocation (Q/Qmax) of mutated and wild type pumps determined from the off pulses of a series of 20 mV voltage steps and fitted with Boltzmann curves. (B) Relaxation rate constants of mutated and wild type pumps.

Figure 2: Simulation snapshots showing hydrogen bonding between N923 on M8 and Y771 on M5 in (A) WT and (B) PHOS simulation at t=55 ns. The side chain of each residue is hydrogen bonded to the backbone of the other in the WT. Hydrogen bonds are shown as cyan springs. The hydrogen bond is broken as a result of M8 movement upon phosphorylation.
**Figure 3**: The relative axial and radial tilt angles between helices M7 and M8. The dashed lines correspond to the values in the starting pdb structure. The axial tilt simply represents the angle between the axes of the two helices. The radial tilt is the angle between the axis of M7 with the projection of the M7 axis on the plane through the M8 vector and the midpoint of the M7 axis. The two vectors describe the relative orientation of helices in the helix bundle. The magnitudes of both the radial and axial tilt angles increase in the PHOS simulations.

**Figure 4**: Simulations snapshots showing the entry of water between helices M5, M7 and M8 for the (A) WT and (B) PHOS simulation at 55 ns. Water molecules within 8 Å of D926 and within the M5-M7-M8 cavity are shown as purple spheres. K⁺ ions are shown as small red spheres. Y and D refer to residues Y1016 and D926. Helices M5, M7, M8 and M10 are shown in transparent green, black, black and blue respectively. The loop connecting M8 and M9, and the last 13 C-terminal residues are shown as orange ribbons. A larger number of water molecules populate the cavity between M5, M7 and M8 in the PHOS simulation. Please refer to Supplementary movies S1, S2 and S3 for dynamics of entry of water molecules.

**Figure 5**: Radial distribution functions of (A) Y1016 and (B) water around D926. The function calculates the local density of one group around the other in concentric spheres around the first group. It is thus a histogram made over the distance data between two groups over all trajectory frames. A 5-point running average is shown. The D-Y distance increases significantly in the PHOS mutations, and the resulting “gap” is filled with water molecules, which are manifest in the higher secondary peaks in (B), showing greater hydration of D926.
FIGURES

Figure 1

A

\[ \frac{Q}{Q_{\text{max}}} \]

\[ \text{Membrane Potential (mV)} \]

- S940E
- WT

B

\[ R (\text{s}^{-1}) \]

\[ \text{Membrane Potential (mV)} \]

- S940E
- WT
Figure 2

Phosphorylation of the sodium pump opens C-terminal ion pathway.
Phosphorylation of the sodium pump opens C-terminal ion pathway

Figure 3
Phosphorylation of the sodium pump opens C-terminal ion pathway.

Figure 4

A

K766 R933 S936 E996

M5 M7 M8 M10

B

K766 R933 SP936 E996

M5 M7 M8 M10
Phosphorylation of the sodium pump opens C-terminal ion pathway

Figure 5

A

B