

The π -Helix Formation between Asp³⁶⁹ and Thr³⁷⁵ as a Key Factor in E₁-E₂ Conformational Change of Na⁺/K⁺-ATPase

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Summary

Molecular modeling of the H₄-H₅-loop of the α_2 isoform of Na⁺/K⁺-ATPase in the E₁ and E₂ conformations revealed that twisting of the nucleotide (N) domain toward the phosphorylation (P) domain is connected with the formation of a short π -helix between Asp³⁶⁹ and Thr³⁷⁵. This conformational change close to the hinge region between the N-domain and the P-domain could be an important event leading to a bending of the N-domain by 64.7° and to a shortening of the distance between the ATP binding site and the phosphorylation site (Asp³⁶⁹) by 1.22 nm from 3.22 nm to 2.00 nm. It is hypothesized that this shortening mechanism is involved in the Na⁺-dependent formation of the Asp³⁶⁹ phospho-intermediate as part of the overall Na⁺/K⁺-ATPase activity.

Key words

Computer modeling • Molecular dynamics simulations • Molecular structure • Na⁺/K⁺-ATPase • E₁/E₂ conformation

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Introduction

Na⁺/K⁺-ATPase (EC 3.6.3.9) transports Na⁺ and K⁺ ions against an electrochemical gradient across the plasma membrane (Skou 1957). Na⁺/K⁺ transport by the sodium pump is intimately connected to conformational changes of the α -subunit (\approx 110 000 Da) of Na⁺/K⁺-ATPase and is energized by ATP. ATP hydrolysis proceeds *via* a Na⁺-dependent formation of an aspartyl³⁶⁹ phospho-intermediate and a K⁺-activated hydrolysis of it (Lutsenko and Kaplan 1995, Kaplan 2002). Analogous processes occur in other P-type ATPases, such as H⁺/K⁺-ATPase and Ca²⁺-ATPase of sarcoplasmic reticulum (Kaplan 2002, Toyoshima and Inesi 2004). The three-dimensional (3D) structure of the latter enzyme has been elucidated in several conformational states (Toyoshima and Inesi 2004). Structural 3D information of Na⁺/K⁺-ATPase, however, is relatively vague. Consistent with the long distance of about 3 nm between the ATP binding site at the N-domain and Asp³⁶⁹ in the E₁ conformation (Ettrich *et al.* 2001, Tejral *et al.* 2007), the various preparations of the isolated H₄-H₅-loop can bind ATP only but not hydrolyze it, or to phosphorylate the phosphorylation site (Gatto *et al.* 1998, Costa *et al.* 2003). An intrinsic *p*-nitrophenylphosphatase of these preparations (Tran and Farley 1999) is unrelated to the catalytic activities of the whole enzyme (Krumtschid *et al.* 2004).

An attractive option to understand the so-called

hinge movement, i.e. how the distance between N- and P- domains of the α -subunit is shortened, is computer modeling based on homology and similarity with proteins of known structures. Since the high resolution 3D structures of the Ca^{2+} -ATPase pump from sarcoplasmic reticulum in both E_1 and E_2 conformations are known (Toyoshima *et al.* 2000, Toyoshima and Nomura 2002), it is possible to deduce by restraint-based comparative modeling the analogous 3D structure of the H_4 - H_5 -loop of Na^+/K^+ -ATPase which has about 30 % identity and 65 % similarity with Ca^{2+} -ATPase.

We computed the 3D model of the H_4 - H_5 -loop of the α_1 isoform of Na^+/K^+ -ATPase α -subunit from pig kidney in E_1 conformation between Leu³⁵⁴ and Leu⁷⁷³ (Ettrich *et al.* 2001) and verified the developed model using a combination of advanced biophysical and molecular biology techniques (Kubala *et al.* 2002, Krumscheid *et al.* 2003, Kubala *et al.* 2003, Krumscheid *et al.* 2004, Lansky *et al.* 2004). We also demonstrated that eight amino acid residues (Glu⁴⁴⁶, Phe⁴⁷⁵, Lys⁴⁸⁰, Gln⁴⁸², Lys⁵⁰⁰, Gly⁵⁰¹, Phe⁵⁴⁷, Cys⁵⁴⁸) in the N-domain form the ATP-binding site and that the shape of the pocket is stabilized by a hydrogen bond between Arg⁴²³ and Glu⁴⁷² (Lansky *et al.* 2004). Recently, we also computed a 3D model of the H_4 - H_5 -loop of Na^+/K^+ -ATPase α_2 isoform from mouse brain between amino acid residues Cys³³⁶ and Arg⁷⁵⁸ in its E_1 conformation (Tejral *et al.* 2007).

However, the molecular mechanism of the enzyme phosphorylation and phosphate transfer from the ATP-binding site to the phosphorylation site remains almost unknown. To understand the overall catalytic process on a molecular level, detailed 3D structures of Na^+/K^+ -ATPase H_4 - H_5 -loop conformations must be determined. This paper is focused on the determination of the structure of the H_4 - H_5 -loop of the Na^+/K^+ -ATPase α_2 isoform in E_2 conformation and compares it with the structure in the E_1 conformation.

Methods

The model was created using free software and developed as described previously (Tejral *et al.* 2007). The Q3UHK5 sequence in the length of 1020 amino acids for the Na^+/K^+ -ATPase α_2 isoform subunit precursor from mouse brain (*Mus musculus*, adult male brain) was chosen.

After the transmembrane helix topology prediction in the MEMSAT program (Jones *et al.* 1994,

Jones 1998), a rough secondary structure based on protein sequence was predicted using the PSIPRED program (Jones 1999a, McGuffin *et al.* 2000, Bryson *et al.* 2005). The resulting H_4 - H_5 -loop target sequence Cys³³⁶-Arg⁷⁵⁸ was determined. Appropriate templates were found using GenTHREADER (Jones 1999b, McGuffin and Jones 2003). For modeling of the E_1 conformation of the H_4 - H_5 -loop (Tejral *et al.* 2007), the structures 1Q3I (Hakansson 2003), 1MO7 (Hilge *et al.* 2003), 1SU4 (Toyoshima *et al.* 2000) and of the E_2 conformation 1Q3I (Hakansson 2003), 1MO8 (Hilge *et al.* 2003), 1KJU (Xu *et al.* 2002) templates were used.

The multialignments of target sequence and templates chosen for particular conformation was prepared in the ClustalX program (Thompson *et al.* 1997) and is described in a previous paper (Tejral *et al.* 2007). The model in both E_1 and E_2 conformations was generated in MODELLER (Sali and Blundell 1993, Fiser *et al.* 2000). For modeling, additional spatial restraints were introduced; for details, see (Tejral *et al.* 2007). Individual secondary structures arising from generated 3D models of the H_4 - H_5 -loop were localized using the DSSP program (Kabsch and Sander 1983). The calculated model was verified using the PROCHECK (Morris *et al.* 1992, Laskowski *et al.* 1993) and Verifi3D programs (Bowie *et al.* 1991, Luthy *et al.* 1992). The main E_1 - E_2 conformational transition changes were determined with the Dyndom program (Hayward and Lee 2002). The native structure of the H_4 - H_5 -loop was created in GROMACS suit of programs (Lindahl *et al.* 2001, Van der Spoel *et al.* 2005). The water cubic box with periodic binding conditions and minimum distance of 1.5 nm between the solute and the box and with the SPC model for water molecules (Eisenberg and McLachlan 1986) utilizing force field G43a1 were used. The water-box energy was minimized by means of conjugate-gradient method with the tolerance of $100 \text{ kJ.mol}^{-1}.\text{nm}^{-1}$.

Results

Three-dimensional model of the H_4 - H_5 -loop of Na^+/K^+ -ATPase in E_1 and E_2 conformations

Using restraint-based comparative modeling, structures of the H_4 - H_5 -loop of Na^+/K^+ -ATPase from mouse brain (*Mus musculus*, adult male brain, α_2 isoform) between Cys³³⁶ and Arg⁷⁵⁸ in both E_1 and E_2 conformations were calculated. Notably, the new 3D model was constructed based not only on similarity with Ca^{2+} -ATPase, but also on known structures of Na^+/K^+ -

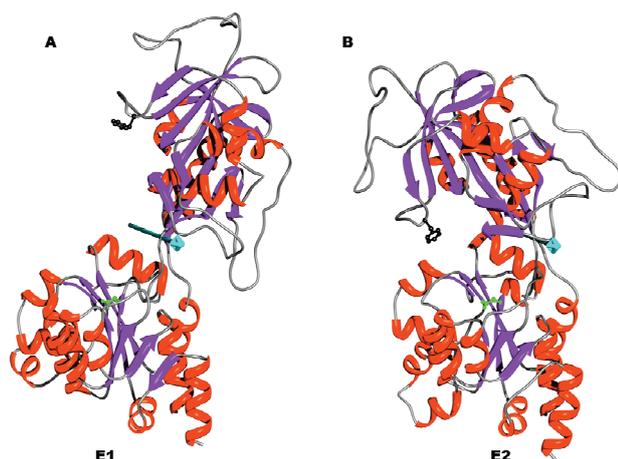


Fig. 1. The structure of Na⁺/K⁺-ATPase from mouse brain (α_2 isoform) in E₁ (A) and E₂ (B) conformations. The N-domain bent toward the P-domain by 64.8 °.

ATPase segments. The 1Q31 sequence (Hakansson 2003) is porcine (*Sus scrofa*) N-domain Na⁺/K⁺-ATPase α_2 isoform crystal structure. 1MO7 and 1MO8 structures (Hilge *et al.* 2003) represent the N-domain of Na⁺/K⁺-ATPase α_1 isoform from rat (*Rattus norvegicus*) determined by high resolution NMR – the 1MO7 in E₁ and 1MO8 E₂ conformation. The 1SU4 sequence represents the crystal structure at 2.4 Å resolution of the Ca²⁺-ATPase from sarcoplasmic reticulum (SERCA1a) of rabbit hind leg muscle (*Oryctolagus cuniculus*, skeletal muscle) in the E₁ conformation (Toyoshima *et al.* 2000); the 1KJU Ca²⁺-ATPase E₂ conformation structure (Xu *et al.* 2002) was determined by cryoelectron microscopy. Structures without any bound ions were used only.

The calculated structure (Fig. 1) clearly suggests no significant differences between the α_2 isoform of mouse brain (between Cys³³⁶ and Arg⁷⁵⁸) and the α_1 isoform of pig kidney (between Leu³⁵⁴ and Leu⁷⁷³) (Ettrich *et al.* 2001) of the H₄-H₅-loop of Na⁺/K⁺-ATPase in E₁ conformation (Fig. 1A). The structure consists of two well separated parts. The N-domain is formed by a seven-stranded antiparallel β -sheet with two additional β -strands and five α -helices sandwiching it. The ATP-binding site localized on the N-domain was found to be identical in both α_2 - and α_1 -isoforms. The P-domain comprises a typical Rossman fold. The P-domain is created by amino acid residues of the two sub-domains (Lys³⁴⁷-Asn³⁷⁷ and Ala⁵⁸⁹-Asn⁷⁴⁶) clearly separated from the N-domain (Arg³⁷⁸-Arg⁵⁸⁸). The phosphorylation site with the Asp³⁶⁹ phosphorylation residue is localized in the central part of the P-domain, located at the C-terminal end of the central β -sheet. The calculated E₁ structure is

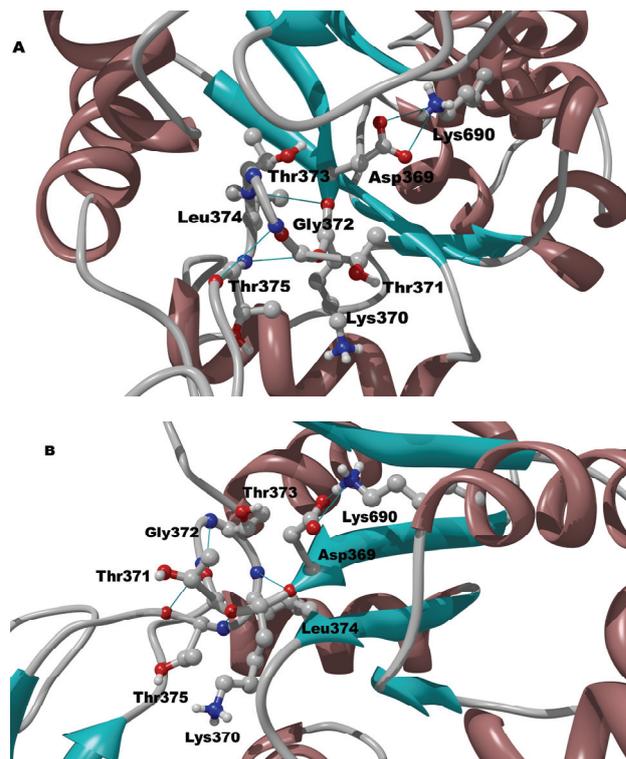


Fig. 2. The structure of Na⁺/K⁺-ATPase from mouse brain (α_2 isoform) phosphorylation site in the E₁ conformation. A. and B. represent different perspectives, the viewpoints in 2 and 3 are identical. A hydrogen bond between the O atom of Asp³⁶⁹ and the N atom of Lys⁶⁹⁰ detected in the E₁ conformation disappeared in the E₂ conformation (see Fig. 3A, B).

in good agreement with our previous model (Ettrich *et al.* 2001) as well as with published NMR and crystal structures of the Na⁺/K⁺-ATPase N-domain (Hakansson 2003, Hilge *et al.* 2003). Moreover, our transmembrane segment determination is in agreement with published data (Purhonen *et al.* 2006).

The main objective, however, was to analyze the enzyme E₁-E₂ conformational transition. The calculated structures of the H₄-H₅-loop of mouse brain enzyme H₄-H₅-loop between Cys³³⁶ and Arg⁷⁵⁸ in E₁ and E₂ conformations, presented in Fig. 1A, B, demonstrate clearly structural changes upon this conformational transition. Obviously, a significant part of the H₄-H₅-loop underwent an important structural change. While the N-domain remained virtually conserved during the conformational transition, the P-domain changed considerably. Notably, the most striking difference between the E₁ and E₂ conformations is visible in the hinge region between the N- and the P-domain. Structural changes are important for twisting of the N-domain toward the P-domain and for shortening of the distance between the α -carbons of Asp³⁶⁹ and Phe⁴⁷⁵ from 3.22 to 2.00 nm and also in bending of the N-domain by 64.7°.

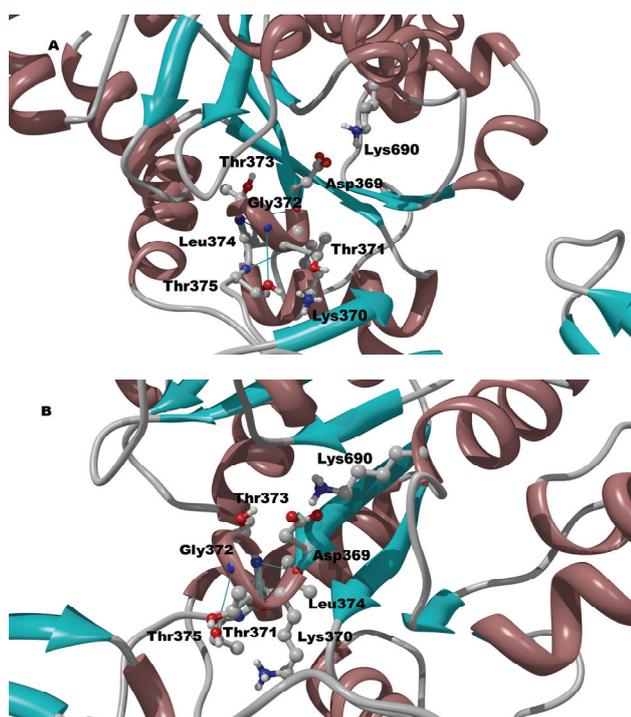


Fig. 3. The structure of Na⁺/K⁺-ATPase from mouse brain (α_2 isoform) phosphorylation site in the E₂ conformation. A. and B. represent different perspectives, the viewpoints in 2 and 3 are identical. A hydrogen bond between the O atom of Asp³⁶⁹ and the N atom of Lys⁶⁹⁰ detected in the E₁ conformation (see Fig. 2A, B) disappeared in the E₂ conformation accompanied by appearance of the short n-helix. Repositioning of Thr³⁷⁵ and the rearrangement of the hydrogen bond between the O atom of Thr³⁷⁵ and the N atom of Gly³⁷² from the O atom of Thr³⁷⁵ in the protein backbone to the O atom of Thr³⁷⁵ in the protein sidebone resulting in a change of the Thr³⁷⁵ sidebone position and in the formation of a helical structure at the phosphorylation site in E₂ conformation are closely connected with bending of the N-domain.

Structure of the phosphorylation site of Na⁺/K⁺-ATPase in E₁ and E₂ conformations

Additional structural differences are also detectable in the P-domain, namely at the phosphorylation site. Details of the 3D structure of the phosphorylation site in both main conformations (Figs 2A, B and 3A, B) show the P-domain to be composed of seven-stranded parallel β -sheets with eight short α -helices forming a typical Rossman fold flanked by one antiparallel β -strand. A hydrogen bond between the O atom of Asp³⁶⁹ and the N atom of Lys⁶⁹⁰ in the E₁ conformation (Fig. 2) disappears in the E₂ conformation (Fig. 3). This hydrogen bond breakage is due to the formation of so-called π -helical structure (Pauling *et al.* 1951) between the amino acid residues Asp³⁶⁹ and Thr³⁷⁵ in the E₂ conformation. Clearly, Thr³⁷⁵ and six amino acid residues between Asp³⁶⁹ and Thr³⁷⁵ play a key role in the E₁-E₂ conformational transition and formation of the

phosphorylation site structure in the E₂ conformation. This π -helix formation is strengthened by repositioning of the hydrogen bond between the O atom of Thr³⁷⁵ and the N atom of Gly³⁷² as well as the hydrogen bond formation between the N atom of Leu³⁷⁴ and the O atom of Lys³⁷⁰, or the N atom of Leu³⁷⁴ and the O atom of Asp³⁶⁹. Thr³⁷⁵ is localized just on the interface between the N- and P-domains.

Discussion

The 3D model of the H₄-H₅-loop of Na⁺/K⁺-ATPase from mouse brain (*Mus musculus*, adult male brain, α_2 isoform) between Cys³³⁶ and Arg⁷⁵⁸ in both E₁ and E₂ conformations was calculated. Modeling approach and comparison with the previous E₁ model (Etrich *et al.* 2001) have been discussed in detail elsewhere (Tejral *et al.* 2007). The new model clearly revealed that neither the effect of the amino acid residues localized between Cys³³⁶ and Leu³⁵⁴, nor the different subtype influence the 3D structure of the H₄-H₅-loop of Na⁺/K⁺-ATPase.

The modeling approach, utilizing template structures without any bound ions and other substances, resulted in the model enabling further investigation in the sense of docking particular substrates and cofactors and applying molecular dynamics approach to hydrated structures. The docking procedure can amount to undisputable advantages of the comparative modeling approach, allowing investigation of structures not obtainable in laboratory conditions (our previous unpublished data) and comparing the results with available crystallographic data. According to the best of our knowledge, this is the first description of the 3D E₂ conformation of the H₄-H₅-loop of Na⁺/K⁺-ATPase in such detail.

Molecular modeling of the E₁ and E₂ conformational states of the H₄-H₅-loop of the α_2 subunits of Na⁺/K⁺-ATPase from mouse brain and their comparison revealed two important structural aspects of this essential step for the Na⁺/K⁺-activated ATP hydrolysis: 1) the 3D structure of the N-domain remains conserved and hence rather rigid, and 2) there are significant conformational changes between the N-terminal and C-terminal subdomains of the P-domain taking place when the enzyme converts from the E₁ to the E₂ conformational state.

The main and the most striking change between the E₁ and E₂ conformations is the bending of the N-domain toward the P-domain by 64.7 ° (Figs 1A, B).

This rather huge movement is very probably the first inevitable step in the molecular mechanism of the enzyme phosphorylation, a process seen also in Ca²⁺-ATPase (Toyoshima and Mizutani 2004). Notably, such a bending brings the ATP-binding and phosphorylation sites closely together. The distance between the α -carbon of Phe⁴⁷⁵ at the ATP-binding site and the α -carbon of Asp³⁶⁹ at the phosphorylation site decreases by 1.22 nm after E₁-E₂ conformational transition: from 3.22 nm in the E₁ conformation (Fig. 1A) to 2.00 nm in the E₂ conformation (Fig. 1B). With regard to the distance of 2.00 nm in the E₂ conformation, ATP length of 1.00-1.50 nm depending on the temperature motion, and Asp³⁶⁹ and Phe⁴⁷⁵ sidebones total length of about 0.5 nm, mutual interaction of the ATP γ -phosphate with the phosphorylation site is possible. The aromatic ring of Phe⁴⁷⁵ and the adenine ring of ATP are probably parallel and a stacking interaction between their π -electron systems is important for the stabilization of ATP within the binding pocket (Etrich *et al.* 2001, Kubala *et al.* 2003).

The second key point in the phosphorylation process seems to be the conformational change of the P-domain. Interestingly, the basic structure of the P-domain remains conserved during the E₁-E₂ conformational transition. A rather significant difference, however, occurs in the structure of the phosphorylation site. The phosphorylation site (Asp³⁶⁹) has already been described as ³⁶⁹DKTGS/T³⁷³ motif (Lutsenko and Kaplan 1995, Kaplan 2002, Scheiner-Bobis 2002, Horisberger 2004). This motif is very important for the phosphate-group coordination. It is well suited to bind both Mg²⁺ as well as the negatively charged phosphate ion. The short helical structure and the hydrogen bond between Asp³⁶⁹ and Lys⁶⁹⁰ seem to play a key role in the conformational change (Figs 2 and 3). The postulated hydrogen bond (Patchornik *et al.* 2000, Jorgensen *et al.* 2003) between

the O atom of Asp³⁶⁹ and the N atom of Lys⁶⁹⁰ is clearly visible in the E₁ conformation but disappears in the E₂ conformation. This break of a hydrogen bond is apparently followed by the formation of the short π -helix between Asp³⁶⁹ and Thr³⁷⁵ in the E₂ conformation which is surprisingly missing in the E₁ conformation. This π -helix formation is strengthened by the hydrogen bonds between the N atom of Leu³⁷⁴ and the O atom of Lys³⁷⁰ or the N atom of Leu³⁷⁴ and the O atom of Asp³⁶⁹. Notably, Thr³⁷⁵ is localized just on the interface between the N- and P-domains. Consequently, repositioning of Thr³⁷⁵ and the rearrangement of the hydrogen bond between the O atom of Thr³⁷⁵ and the N atom of Gly³⁷² from the O atom of Thr³⁷⁵ in the protein backbone to the O atom of Thr³⁷⁵ in the protein sidebone resulting in a change of the Thr³⁷⁵ sidebone and the formation of a helical structure at the phosphorylation site in the E₂ conformation are closely connected with the bending of the N-domain. The N-domain bending toward the P-domain resulting finally in the formation of a closed E₂ conformation is also accompanied by N-domain twisting. The angle between the two domains decreases by about 20°. Such a structure is, thus, suitable for phosphate coordination and leads to enzyme phosphorylation.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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