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Comparative Modeling of the H_4-H_5-Loop of the α_2-Isoform of Na^+/K^+-ATPase α-Subunit in the E_1 Conformation

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Summary

Restraint-based comparative modeling was used for calculation and visualization of the H_4-H_5-loop of Na^+/K^+-ATPase from mouse brain (*Mus musculus*, adult male brain, α_2-isoform) between the amino acid residues Cys^{336} and Arg^{758} in the E_1 conformation 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 P-domain is composed of a typical Rossman fold. The ATP-binding site was found on the N-domain to be identical in both α_2- and α_1-isoforms. The phosphorylation Asp^{369} residue was found in the central part of the P-domain, located at the C-terminal end of the central β-sheet. The distance between the α-carbon of Phe^{475} at the ATP-binding site and the α-carbon of Asp^{369} at the phosphorylation site is 3.22 nm. A hydrogen bond between the oxygen atom of Asp^{369} and the nitrogen atom of Lys^{690} was clearly detected and assumed to play a key role in maintaining the proper structure of the physphorylaton site in E_1 conformation.

Key words

Computer modeling • Molecular dynamics simulations • Na^+/K^+-ATPase • E_1 conformation

Introduction

Na^+/K^+-ATPase (EC 3.6.1.37 in the enzyme classification system, TC 3.A.3 group in the transporter classification system) is an enzyme exporting sodium and importing potassium ions across the plasma membrane against a concentration gradient. The enzyme belongs to the P_2-subtype of the P-type ATPase superfamily and is formed by the assembly of three subunits, designated as thecatalytic α-subunit with 10 transmembrane segments (≈110 kDa), the heavily glycosylated β-subunit (≈55 kDa) and the γ-subunit (≈ 7 kDa) (Forbush *et al.* 1978, Collins and Leszyk 1987). All transport and catalytical enzyme functions are attributed to the α-subunit which is known to adopt two main conformations, so-called E_1 and E_2, the molecular structure of which was broadly studied e.g. (Abbott *et al.* 1991). However, the molecular mechanism of the enzyme function is not fully understood at present.

Ca^{2+}-ATPase is another member of the P_2-

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subtype of the ATPase family, transporting ions across the plasma membrane against a concentration gradient. The crystal structure of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SERCA) at 2.6 Å resolution with bond Ca^{2+} ions revealed that the cytoplasmic region consists of three well-separated regions – the N-domain (nucleotide-binding), the P-domain (phosphorylation) with the Asp^{351} phosphorylation residue and the A-domain (actuator or anchor) (Toyoshima *et al.* 2000).

Progress in computer modeling has recently offered an attractive option in protein structure analysis: computer modeling of the protein structure based on homology and similarity with proteins of already known structures. In addition, computer modeling is also a powerful tool for visualizing not only the structure of protein segments but also the segmental motion, which aids in our understanding of the molecular mechanism of the enzyme function. Knowledge of the protein structure from crystals is, thus, an advantage for the description of the protein structure and for modeling of molecular dynamics.

Na^+/K^+-ATPase has a high 30 % identity and 65 % similarity with SERCA. In our earlier paper (Ettrich *et al.* 2001), we calculated and visualized the three-dimensional model of the H_4-H_5-loop of the α_1-isoform of the α-subunit of Na^+/K^+-ATPase in E_1 conformation (between Leu^{354} and Leu^{773}) from pig kidney in E_1 conformation by restraint-based comparative modeling using the homology with the crystal structure of SERCA in E_1 conformation (Toyoshima *et al.* 2000). The developed model was verified using combination of advanced biophysical and molecular biology techniques (Kubala *et al.* 2002, Krumscheid *et al.* 2003, Kubala *et al.* 2003, Lánský *et al.* 2004). Based on the restraint-based comparative modeling, the ATP-binding site was localized in the N-domain (Arg^{378}–Arg^{589}). This site is clearly separated from the P-domain (Leu^{354}–Asn^{377} and Ala^{590}–Leu^{773}) where the phosphorylation site (Asp^{369}) is located (Ettrich *et al.* 2001). The complete structure of the ATP-binding site which contains eight amino acid residues (Glu^{446}, Phe^{475}, Lys^{480}, Gln^{482}, Lys^{501}, Gly^{502}, Phe^{548}, Cys^{549}) (Kubala *et al.* 2003) was determined as well as the important role of the hydrogen bond between Arg^{423} and Glu^{472} for stabilization of the ATP-binding pocket (Lánský *et al.* 2004).

In the meantime, the crystallographic structure of α_2 N-domain of porcine Na^+/K^+-ATPase at 2.6 Å resolution (Hakansson 2003) and high-resolution NMR solution structure of the N-domain of rat α_1 Na^+/K^+-ATPase (Hilge *et al.* 2003) were published and the original SERCA 1EUL structure at 2.6 Å resolution (Toyoshima *et al.* 2000) was in the year 2004 replaced by the 1SU4 structure at 2.4 Å resolution. Consequently, the new templates increased significantly the chance for the model refinement. In addition, our previously presented model of the α_1-isoform has to differ from the α_2-isoform due to the difference in the primary structure, but also did not include the initial helical part of the P-domain which seems to play an essential role in the enzyme structure-function relations. In this paper, thus, we present the three-dimensional model of the H_4-H_5-loop of the α_2-isoform of Na^+/K^+-ATPase α-subunit in the E_1 conformation starting already from Cys^{336}. The aim of this paper is not only to present the structure of the α_2-isoform but also to suggest the molecular mechanism of the enzyme phosphorylation and especially to describe in more detail the key enzyme binding sites in the E_1 conformation.

Methods

Rough secondary structure prediction, template choice and multialignment

The primary structure sequence was retrieved from the ExPASy server (UniProtKB/TrEMBL http://www.expasy.ch/). The Q3UHK5 target sequence of 1020 amino acids for the Na^+/K^+-ATPase α_2-isoform subunit precursor from mouse brain (*Mus musculus*, adult male brain) was chosen. Five amino acids at the beginning of this sequence coresponding to the translated RNA sequence, which do not occur in the native form (Ovchinnikov Yu *et al.* 1986), are not included in our further numbering. The difference in numbering of our new model behind the Ser^{494} residue as the consequence of one lacking amino acid in the α_2-isoform compared with the α_1-isoform was taken in account.

The PSIPRED program used for rough secondary structure prediction (Jones 1999, McGuffin *et al.* 2000, Bryson *et al.* 2005) represents a highly accurate secondary structure prediction method which performs the analysis on the output obtained from PSI-BLAST (Position Specific Iterated – BLAST). The transmembrane helices were identified in the MEMSAT program (Jones *et al.* 1994, Jones 1998) predicting the secondary structure and topology of all-helical integral membrane proteins based on the recognition of topological models. The H_4-H_5-loop target sequence

Cys^{336}–Arg^{758} was determined.

Appropriate templates were found using GenTHREADER (Jones 1999, McGuffin and Jones 2003), a fast and reliable protein-fold recognition method, which uses a traditional sequence alignment algorithm to generate alignments which are then evaluated by a method derived from threading techniques. For the E_1 conformation modeling, Protein Data Bank accession codes 1Q3I (Hakansson 2003), 1MO7 (Hilge *et al.* 2003), 1SU4 (Toyoshima *et al.* 2000) template structures were chosen. Creation potential of a unique optimal sequence-based alignment that coincides with a structure-based alignment is the principal presumption of homology modeling. In contrast, each alignment program tries to maximize the number of alignable residues, although these may not be spatially superposable. These intrinsic limitations and error sources were taken into account when estimating the degree of confidence of the particular model. Multialignment in ClustalX program (Thompson *et al.* 1997) was prepared. The pairwise identities and similarities of the target sequence and the particular template were determined using BLAST.

Protein modeling, model validation and visualization

Using target sequence, templates and restrictions described above, the H_4-H_5-loop model in the E_1 conformation was generated in MODELLER (Sali and Blundell 1993, Fiser *et al.* 2000). This computer program models the three-dimensional structures of proteins by satisfaction of spatial restraints and is most frequently used for homology or comparative protein structure modeling. Because of differences in structures of N-domain templates of sodium and calcium pumps and the long gap in alignment for the Lys^{653}–Leu^{675} region, additional spatial restraints were used for modeling of these regions. Individual secondary structures of the H_4-H_5-loop were localized using the DSSP program (Definition of secondary structure of proteins given a set of 3D coordinates), which defines secondary structure, geometrical features and solvent exposure of proteins, given atomic coordinates in Protein Data Bank format (Kabsch and Sander 1983).

The created model was evaluated in PROCHECK and Verifi3D. In PROCHECK, the percentage of amino-acid residues in disallowed regions of the Ramachandran plot as well as the *g* factor of individual residues was evaluated. The PROCHECK analyses (Morris *et al.* 1992, Laskowski *et al.* 1993) provide an idea of the stereochemical quality of all protein chains in a given PDB structure. They highlight protein regions with unusual geometry and provide an overall assessment of the structure as a whole. In Verifi3D, the 3D-1D score was monitored. Verifi3D provides a reliable method for protein three-dimensional structure verification by comparison of the model with its own amino-acid sequence using 3D profile (Bowie *et al.* 1991, Luthy *et al.* 1992). The native structure of the H_4-H_5-loop was created in GROMACS suite of programs (Lindahl *et al.* 2001, Van der Spoel *et al.* 2005). The water cubic box with periodic bonding conditions and the minimal distance between the solute and the water box of 1.5 nm and for water molecules the SPC (Simple Point Charge water) (Eisenberg and McLachlan 1986) model utilizing field of force G43a1 were used. The initial water-box energy was minimized by means of conjugate-gradient method with the tolerance of 100 kJ $mol^{-1}nm^{-1}$.

Results

Template choice and sequence similarity and alignment

For our modeling, the Q3UHK5 for the Na^+/K^+-ATPase α_2-isoform subunit precursor from mouse brain (*Mus musculus*, adult male brain) was chosen. The rough secondary structure was predicted using PSIPRED program after the transmembrane helices were identified in the MEMSAT program. The resulting target sequence Cys^{336}–Arg^{758} of the H_4-H_5-loop with short extracellular parts of transmembrane helices was determined. The characteristic phosphorylating sequence DKTGTLT with the Asp^{369} phosphorylation site was identified. Appropriate templates were found (see Methods) and three templates extracted from the Protein Data Bank were used. The 1Q3I structure (Hakansson 2003) represents the crystallographic structure of the N-domain of the porcine (*Sus scrofa*) Na^+/K^+-ATPase α_2- isoform in the E_1 conformation, determined at 2.6 Å resolution. The 1MO7 structure (Hilge *et al.* 2003) is the N-domain of the Na^+/K^+-ATPase α_1-isoform from rat (*Rattus norvegicus)* in the E_1 conformation, determined by high-resolution NMR. The 1SU4 sequence structure represents the Ca^{2+}-ATPase from sarcoplasmic reticulum (SERCA1a) of rabbit hind leg muscle (*Oryctolagus cuniculus*, skeletal muscle), replacing the original 1EUL sequence (Toyoshima *et al.* 2000).

The alignment, prepared in ClustalX program and used for further modeling is shown in Fig. 1. The slow-accurate mode with a gap opening penalty of 10 and a gap extension penalty of 0.2 for the global alignment

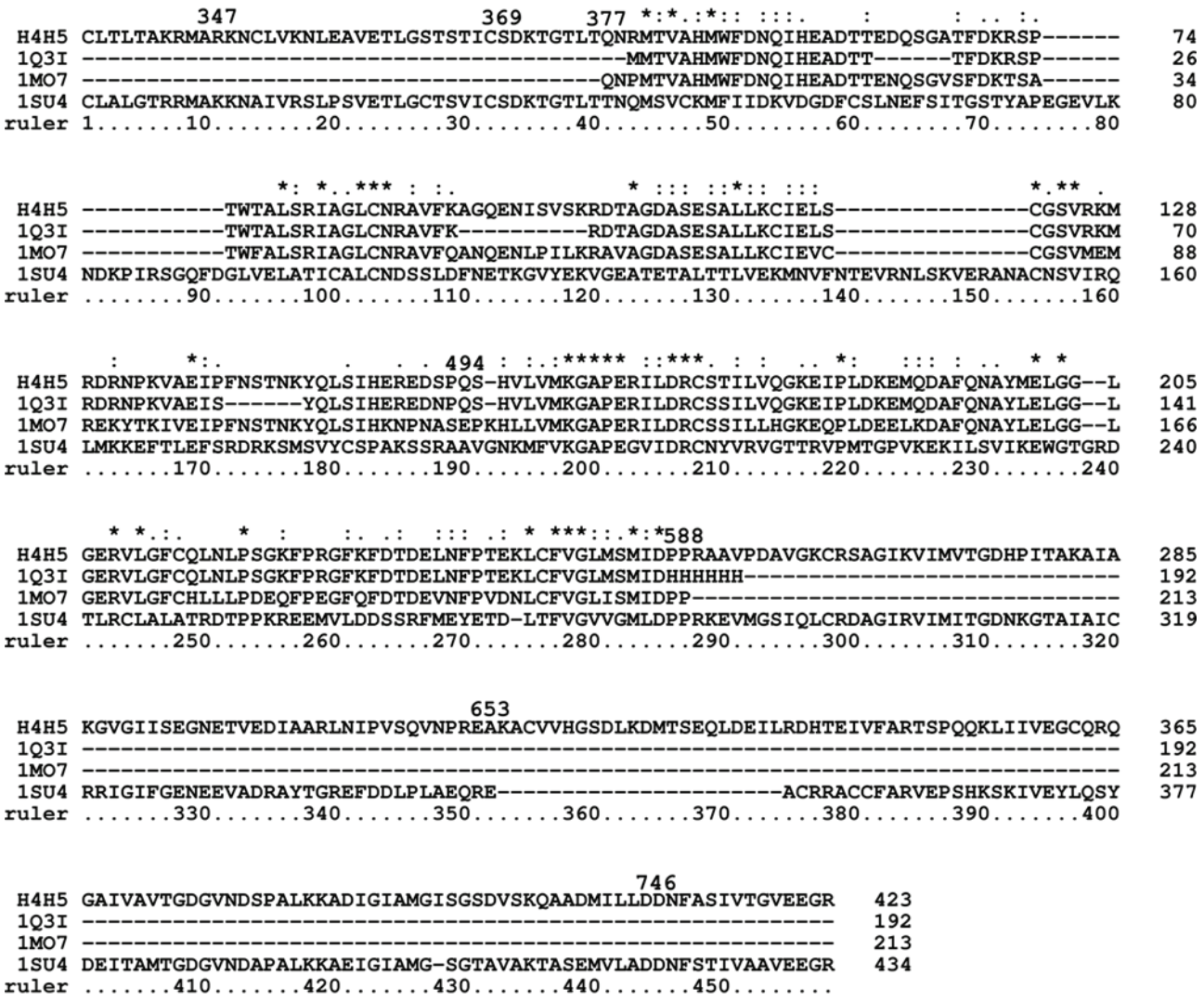

Fig. 1. The sequence alignment used for modeling of Na^+/K^+-ATPase from mouse brain (α_2- isoform) at E_1 conformation. The sequence alignment of the Na^+/K^+-ATPase α_2-isoform subunit precursor from mouse brain (*Mus musculus*, adult male brain) target sequence (Q3UHK5) and chosen template sequences: the crystallographic structure of α_2 N-domain of porcine Na^+/K^+-ATPase at 2.6 Å resolution (1Q3I), high-resolution NMR solution structures of the N-domain of rat α_1 Na^+/K^+-ATPase (1MO7) and SERCA 1SU4 crystal structure at 2.4 Å resolution.

was used, as well as the Gonnet series protein weight matrix and hydrophobic penalties for the amino acids GPSNDQEKR. The pairwise identities and similarities in BLAST of the target sequence and the particular template were: for 1Q3I sequence 89 % identity and 91 % homology; for 1MO7 sequence 73 % identity and 85 % homology; for 1SU4 sequence 34 % identity and 50 % homology.

Modeling and structure validation

Because of differences in the structure of N-domains and in the Lys^{653}–Leu^{675} region, additional spatial restraints were used for modeling of these regions. The N-domain Ca^{2+}-ATPase structure 1SU4 differed from those determined for Na^+/K^+-ATPase. Because of inexplicitness, the Ca^{2+} template was excluded and only the Na^+/K^+-ATPase N-domain templates were used for modeling of the region roughly corresponding to the N-domain – the crystal structure 1Q3I (Hakansson 2003) and the NMR structure 1MO7 (Hilge *et al.* 2003). In our alignment, there is a gap in the sequence between Lys^{653} and Leu^{675}. The lacking template structure was substituted with the PSIPRED secondary structure data and GenTHREADER template data. Using target sequence, templates and restrictions described above, the H_4-H_5-loop model in the E_1 conformation was generated in MODELLER. Individual secondary structures of the H_4-H_5-loop were localized using the DSSP program.

The created model was evaluated in PROCHECK and Verifi3D. The Ramachandran plot of

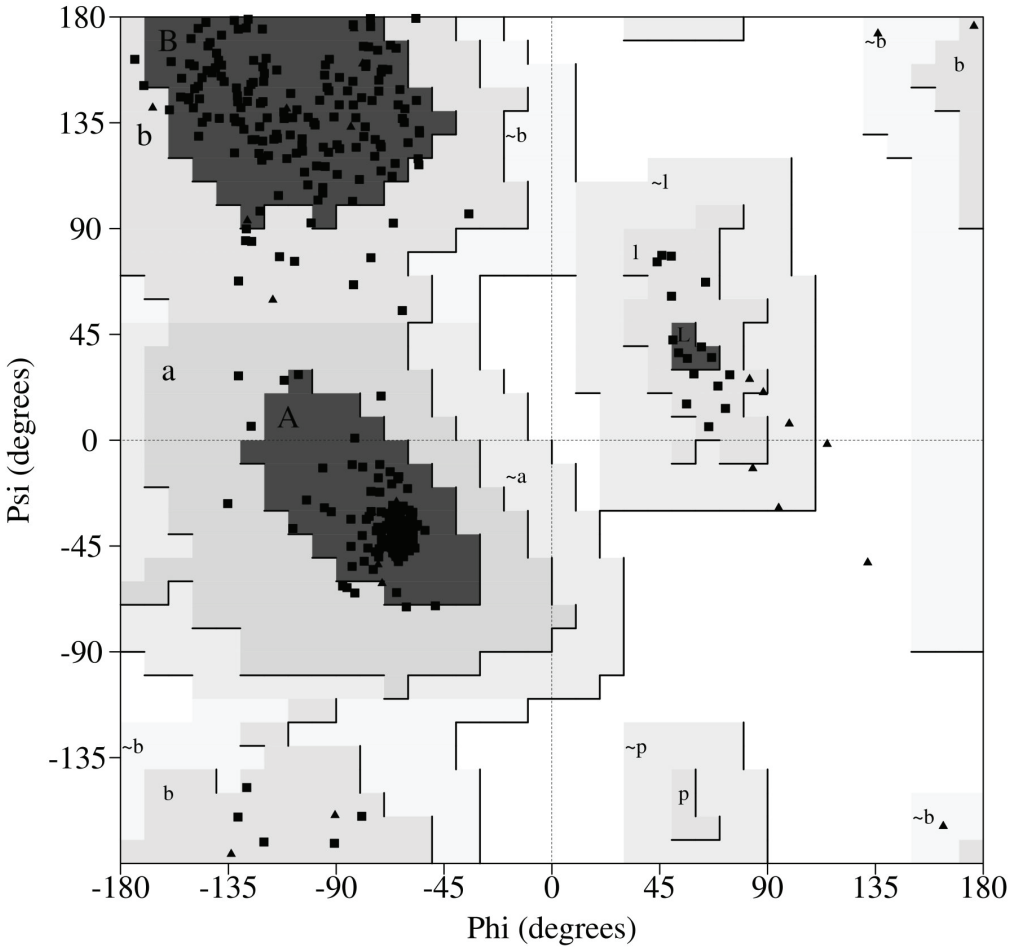

Fig. 2. The Ramachandran plot of predicted structure of Na^+/K^+-ATPase from mouse brain (α_2 isoform). The good quality of the model is shown by 89 % residues in most favored regions (A, B, L) and 11 % residues in additional allowed regions (a, b, l, p). Glycine residues are shown as triangles.

the predicted structure calculated with PROCHECK, shown in Fig. 2, revealed a very good quality of the model with 89 % of residues in the most favored regions and 11 % of residues in additionally allowed regions. No residues were in the disallowed regions. The overall *g* factor of the structures was –0.12. The calculated 3D profile score was 69.5. Consequently, the native structure of the H_4-H_5-loop was created in GROMACS suit of programs.

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

Using restraint-based comparative modeling, the H_4-H_5-loop of Na^+/K^+-ATPase of mouse brain (*Mus musculus*, adult male brain, α_2-isoform) between amino acid residues Cys^{336} and Arg^{758} in the E_1 conformation was calculated and visualized (Fig. 3). Despite the good general agreement with our previously reported three-dimensional structure of the H_4-H_5-loop of the Na^+/K^+-ATPase α-subunit from pig kidney (α_1-isoform) Leu^{354} and Leu^{773} (Ettrich *et al.* 2001), the newly computed model of the α_2-isoform differed from the model of the α_1-isoform for several reasons. Naturally, the newly employed templates improved the structure prediction. In addition, the primary sequence of the α_2-isoform slightly differed due to the missing Lys^{495} residue. This residue, in fact, is close to the ATP-binding site. Compared to our previous model of the α_1-isoform of Na^+/K^+-ATPase, we also included in our structure the initial and terminal extracellular parts of transmembrane helices (Cys^{336}–Arg^{346} and Phe^{747}–Arg^{758}).

The calculated structures of the mouse brain enzyme H_4-H_5-loop between Cys^{336} and Arg^{758} in the E_1 conformation are shown from different perspectives in Figs. 3A and 3B. The structure consists of two well-separated parts, corresponding to the previously described N and P domains. In the ribbon presentation (Fig. 3C), the detailed structure of the H_4-H_5-loop is clearly visible;

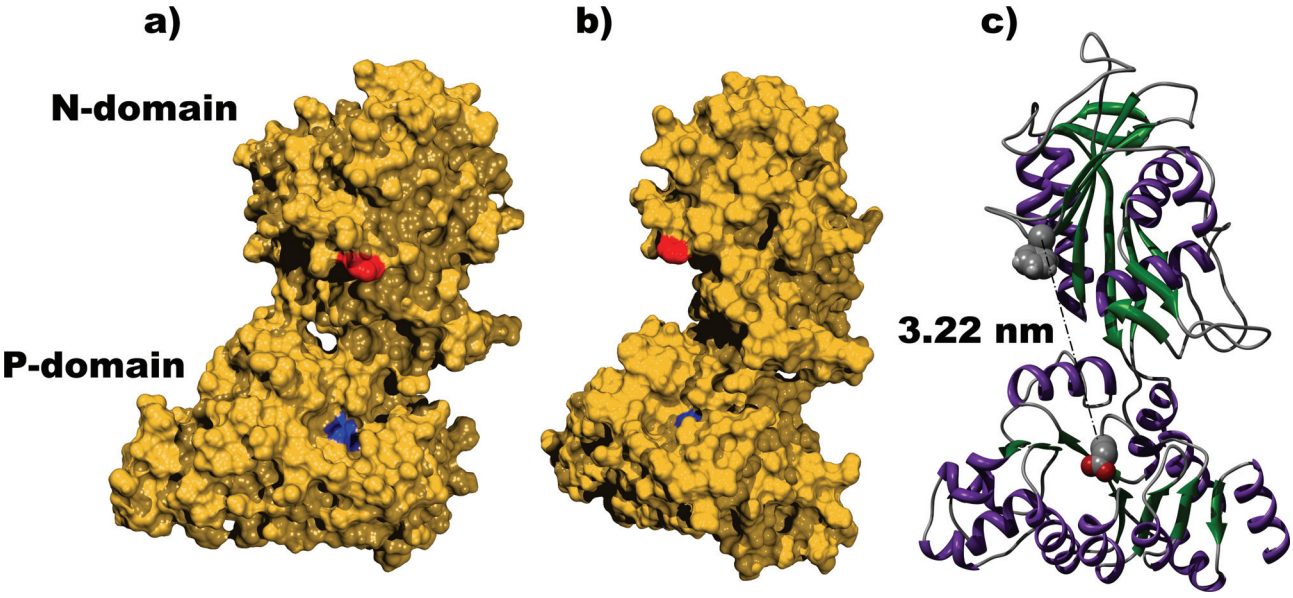

Fig. 3. The structure of Na^+/K^+-ATPase from mouse brain (α_2-isoform) at E_1 conformation (**a, b** – different perspectives in surface presentation; **c** – detailed structure in ribbon presentation; α-helices are shown in violet, β-sheets in green). The structure of the large cytoplasmic loop was calculated between Cys^{336} and Arg^{758}. The N-domain (Arg^{378}–Arg^{588}) where the ATP-binding site (Asp^{369}) is localized is clearly separated from the P-domain (Lys^{347}–Asn^{377} and Ala^{589}–Asn^{746}) where the phosphorylation site (Asp^{369}) is localized.

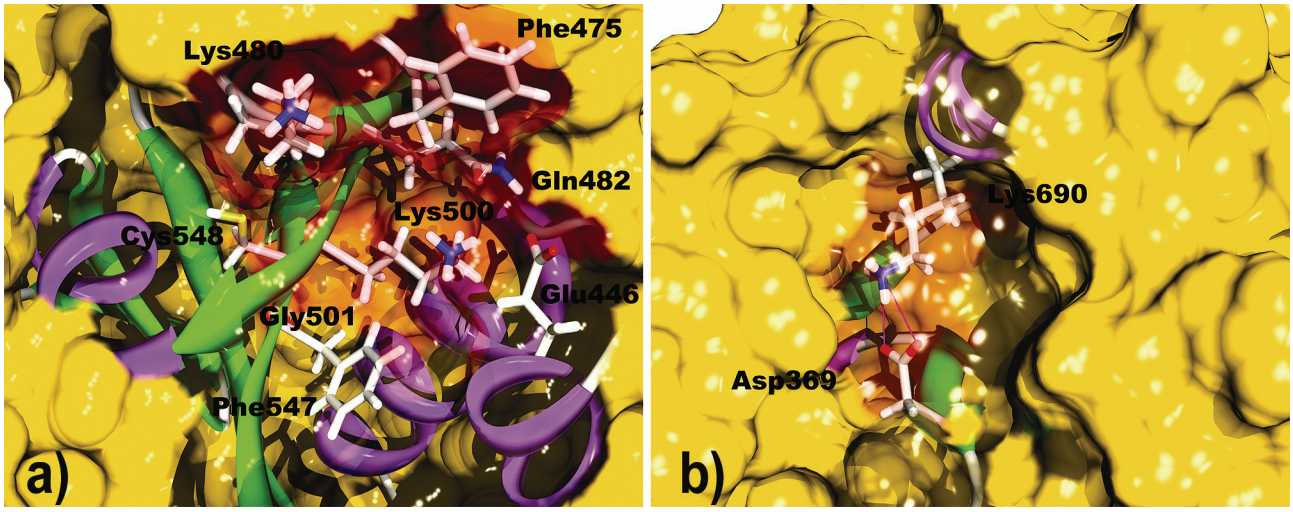

Fig. 4. Detailed structure of the ATP-binding site and phosphorylation site of Na^+/K^+-ATPase from mouse brain (α_2-isoform) at E_1 conformation in ribbon presentation. α-Helices are shown in violet, β-sheets in green. **a)** The ATP-binding site structure. The eight amino acids residues forming the ATP recognition site are shown. **b)** The phosphorylation site structure. The distance between Phe^{475} at the ATP-binding site and Asp^{369} at the phosphorylation site is 3.22 nm.

the distance between the α-carbon of Phe^{475} at the ATP-binding site and the α-carbon of Asp^{369} at the phosphorylation site is 3.22 nm. This is in good general agreement with our previously reported data. Nevertheless, the presented model suggests that either the effect of the amino acid residues localized between Cys^{336} and Leu^{354} or the different subtype or both seem to influence the three-dimensional structure of the H_4-H_5-loop.

Structure of the N-domain with ATP-binding site and the P-domain with phosphorylation site

The clearly separated larger the N-domain with the ATP-binding site was localized in the middle part of the sequence between amino acids Arg^{378} andArg^{588}. Its structure is formed by a seven-stranded antiparallel β-sheet with two additional β-strands and five α-helices sandwiching it. On the N-domain (Fig. 4A) the eight amino acid residues (Glu^{446}, Phe^{475}, Lys^{480}, Gln^{482}, Lys^{500}, Gly^{501}, Phe^{547}, Cys^{548}) forming the ATP-binding site

(Kubala *et al.* 2003) are marked. The P-domain, where the phosphorylation site (Asp^{369}) is localized, consists of two sub-domains – the C-terminal one connected to the fourth transmembrane helix (Lys^{347}–Asn^{377}) and the N-terminal one connected with the fifth transmembrane segment (Ala^{589}–Asn^{746}). The P-domain structure is composed of a seven-stranded parallel β-sheet with eight short α-helices made up to form a typical Rossman fold flanked with one antiparallel β-strand. The phosphorylation Asp^{369} residue was found in the central part of the P-domain, located at the C-terminal end of the central β-sheet. A clear advantage of our model seems to be the visualization of details of the phosphorylation site structure in the E_1 conformation. Among other hydrogen bonds, the crucial hydrogen bond between the oxygen atom of Asp^{369} and the nitrogen atom of Lys^{690} was clearly detected (Fig. 4B). This bond seems to play a key role in maintaining the proper structure of the physphorylaton site in E_1 conformation.

Discussion

The three-dimensional structure of the N-domain of the of the Na^+/K^+-ATPase α-subunit both from pig kidney and from mouse brain has already been broadly discussed in our previous papers (Ettrich *et al.* 2001, Kubala *et al.* 2002, Krumscheid *et al.* 2003, Kubala *et al.* 2003, Lánský *et al.* 2004). In this paper, however, we present the three-dimensional reconditioned model of the H_4-H_5-loop of the α_2-isoform of Na^+/K^+-ATPase α-subunit in E_1 conformation extended by 18 amino acid residues at the C-terminus and compare it with the previous one.

Modeling and E_1 conformation of the Na^+/K^+-ATPase

We calculated and visualized a new model of Na^+/K^+-ATPase α_2-isoform in E_1 conformation between amino acids Lys^{336} and Arg^{758}. The model of the α_2-isoform differed in some details from the previously reported three-dimensional structure of the H_4-H_5-loop of the Na^+/K^+-ATPase α_1-isoform from pig kidney, constructed for amino acid residues between Leu^{354} and Leu^{773} (Ettrich *et al.* 2001). Model verifications revealed a very good quality of our new model, as indicated by the Ramachandran plot. Notably, we found no residues in the disallowed regions and the overall *g* factor of the structures was –0.12. The model shows an even lower profile score than the template structure of Ca^{2+}-ATPase (69.5 for Na^+/K^+-ATPase compared 75.0 for the template). This suggests a very good quality of our fit and a high reliability of the model.

Compared with our previous model (Ettrich *et al.* 2001), the α_2-isoform was employed. In addition, the extended templates, but also the unlike alignment with SERCA and diverse modeling approach based on different software utilization were chosen. Additional spatial restraints based on the Na^+/K^+-ATPase N-domain structure and lacking template substitution for the gap in the Lys^{653}–Leu^{675} region, differently located comparing to our previous approach (Arg^{640}–His^{659}), were used. However, the secondary structure of both models differed only slightly, mainly due to differences in the numbering of our new model, starting after the Ser^{494} residue. This is due to the lacking amino acid residue Lys^{495} in the α_2-isoform compared with the α_1-isoform (Hakansson 2003). The tertiary structures of both isoforms show a high similarity including the distance between the phosphorylation and the ATP-binding sites.Ettrich *et al.* (2001) determined the distance of 2.5 nm between Asp^{369} and the γ-phosphate of docked ATP. The distance of the α_2-isoform between the α-carbon of Phe^{475} at the ATP-binding site and the α-carbon of Asp^{369} at the phosphorylation site was 3.22 nm. Because the size of phosphate molecules exactly matches the difference between the two measurements, our data are in good agreement with previous findings. Small divergences can be caused by modeling the E_1 conformation first without ATP. ATP-docking and E_2 conformation modeling will be the next step in our research. We assume a distance reduction in the E_2 conformation as the consequence the so-called hinge movement and bending of the N-domain toward the P-domain during the main E_1-E_2 conformational transition.

Structure of the ATP-binding site and the phosphorylation site of Na^+/K^+-ATPase

The N-domain (Arg^{378}–Arg^{588}) is the larger one and binds nucleotides. Its secondary structure showed a seven-stranded antiparallel β-sheet with two additional β-strands and five α-helices sandwiching it similarly as described previously (Ettrich *et al.* 2001). In addition, the model appeared to be in good agreement with published crystal structures of the Na^+/K^+-ATPase N-domain (Hakansson 2003). Previously described eight amino acid residues (Glu^{446}, Phe^{475}, Lys^{480}, Gln^{482}, Lys^{500}, Gly^{501}, Phe^{547}, Cys^{548}) (Kubala *et al.* 2003) engaged in ATP-binding form the positively charged binding pocket. The ATP-binding site was found to be identical in the α_2-

isoform as in the α_1-isoform previously described (Kubala *et al.* 2003). Because of being well characterized in our previous papers (Ettrich *et al.* 2001, Kubala *et al.* 2002, Krumscheid *et al.* 2003, Kubala *et al.* 2003, Lánský *et al.* 2004), we do not describe in detail the N-domain characterization and only can conclude it to be in good agreement with our previous results as well as with the NMR and crystallographic data (Hakansson 2003, Hilge *et al.* 2003).

Also, in agreement with previous results, the P-domain structure (Lys^{347}–Asn^{377} and Ala^{589}–Asn^{746}) is composed of a typical, previously described, Rossman fold (Aravind *et al.* 1998, Ettrich *et al.* 2001). The phosphorylation site (Asp^{369}) is situated in the C-terminal end of the central β-strand. The phosphorylation motif has a typical DKT motif initiating a short single-turn α-helical structure. This is a typical position for nucleotide-binding proteins containing a Rossman fold, previously described for the Ca^{2+}-ATPase (Toyoshima *et al.* 2000), conserved in the alignment. Around the prosphorylation residue, a highly negatively charged region accessible to the solvent is located. This motif and the helical structure is very important for coordination of the phosphate group and well adapted to bind both the magnesium cations as well as the negatively charged phosphate ion. The postulated hydrogen bond (Patchornik *et al.* 2000, Jorgensen *et al.* 2003) between Asp^{369} and Lys^{690} is clearly visible in the E_1 conformation. This hydrogen bond seems to play a key role in the enzyme structure in the E_1 conformation.

Last but not least, the model was developed using free software.

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