COMPUTER MODELING

Protein structure and dynamics determined by protein modeling combined with spectroscopic techniques

Varga F, Kolacna L, Amler E

Department of Protein Structure, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague. **evzen.amler@lfmotol.cuni.cz**

Abstract

Beside of the protein crystals, another attractive option in protein structure analysis has recently appeared: computer modeling of the protein structure based on homology and similarity with proteins of already known structures. We used the combination of computer modeling with spectroscopic techniques, such as steady-state or time-resolved fluorescence spectroscopy or Raman spectroscopy, and with molecular biology techniques. This method could achieve reliable results comparable with resolution obtained from crystal structures.

Molecular modeling of the ATP site within the H_4-H_5-loop revealed eight amino acids residues, namely besides the previously reported amino acids Asp^{443}, Lys^{480}, Lys^{501}, Gly^{502} and Arg^{544}, also Glu^{446}, Phe^{475} and Gln^{482}, which form the complete ATP recognition site. Moreover, we proved that a hydrogen bond between Arg^{423} and Glu^{472} supported the connection of two opposite halves of the ATP-binding pocket. Similarly, the conserved residue Pro^{489} is important for the proper interaction of the third and fourth -strands, which both contain residues that take part in the ATP-binding *(Ref. 34)*.

Key words: computer modeling and molecular dynamics simulations, fluorescence spectroscopy and time-resolved fluorescence spectroscopy, Na^+/K^+-ATPase, point mutations.

Preparation of crystals and their analysis is the most straightforward way to resolve the structure of proteins. However, there are several obstacles and disadvantages which hinder a broader application of such a method. First, protein crystal preparation is usually tedious, very tricky and time-consuming problem which, moreover, remains frequently unresolved, namely in case of membrane integral proteins. Second, although the analysis of protein crystals is able to bring a valuable idea about the protein structure, the resolved structure is static and the information about the dynamics and, consequently, about the molecular mechanism of the protein function, is often difficult to receive. Third, the structure of some regions of protein molecule could remain unknown even after crystallization because it cannot be seen in any of the available X-ray structures, for example due to their high flexibility. Although application of NMR seems to be a plausible alternative to the analysis of protein crystals, also this method has several limits, namely the size of the analyzed protein.

Progress in computer modeling has recently offered another attractive option in protein structure analysis: computer modeling of the protein structure based on homology and similarity with proteins of already known structures. A serious objection, verification of the developed model, can be solved in combination with application of biophysical techniques and molecular biology. In addition, computer modeling is also a powerful tool to visualize not only a structure of protein segments but also a segmental motion which facilitates understanding of the molecu-

Department of Protein Structure, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Laboratory of Biophysics, Department of Experimental Physics, Faculty of Science, Palacky University in Olomouc, Department of Biophysics, 2nd Faculty of Medicine, Charles University in Prague and Department of Tissue Engineering, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Address for correspondence: E. Amler, RND, PhD, Charles University in Prague, 2nd Faculty of Medicine, Department of Biophysics, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic.
Phone: +420.257296350, Fax: +420.257296355

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lar mechanism of the enzyme function. Knowledge of the protein structure from crystals is, thus, an advantage for description of protein structure and for modeling of molecular dynamics. This paper is focused on Na+/K+-ATPase structure-function relationship using combination of computer modeling with advanced biophysical techniques and molecular biology. We used fluorescence spectroscopy techniques, protein X-ray crystallography, and molecular modeling (mainly molecular dynamics) as principal tools.

Na^+/K^+-ATPase is an enzyme exporting sodium and importing potassium ions across the plasma membrane against the concentration gradient. Such a transport requires energy, which is gained by the ATP hydrolysis. Now, it is generally accepted that this enzyme is formed by the assembly of two subunits, designated as alpha (~ 110 000 Da) and beta (~ 55 000 Da). If the enzyme is purified from kidney, they are accompanied by a small peptide (~ 7000 Da), often referred to as gamma-subunit, and these three are present in 1:1:1 ratio (1, 2).

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

Restraint-based comparative modeling

When the three-dimensional structure (0.26 nm resolution) of the Ca^{2+}-ATPase pump of sarcoplasmic reticulum (SERCA) became known (3, 4), the possibility arose to deduce by restraint-based comparative modeling the analogous three-dimensional structure of the H_4–H_5 loop of Na^+/K^+–ATPase. SERCA is a close relative of Na^+/K^+–ATPase in the P-type ATPases superfamily; their sequences show 30 % identity and 65 % similarity. In analogy to Ca^{2+}-ATPase, cryo-electron microscopy on crystals of Na^+/K^+-ATPase with 9 Å resolution (5) showed three large domains exposed to the cytoplasm. Sweadner and Donnet (6) analyzed the structure of SERCA in E_1 conformation in terms of corresponding residues in Na^+/K^+-ATPase. They estimated that all residues known as cleavage sites for trypsin, chymotrypsin or pronase were located on the surface of the enzyme, except for those on the H_9-H_{10}-loop. Fragments obtained by Fe^{2+}-oxidative cleavage of Na^+/K^+-ATPase were also consistent with the structure found for SERCA. Furthermore, they confirmed that the position of all cysteine residues, which were successfully labeled in experiments with Na^+/K^+-ATPase, corresponded to the residues on the surface of the enzyme. These findings support the idea that the topologies of SERCA and Na^+/K^+-ATPase are very similar.

We calculated and visualized the H_4-H_5 loop of Na^+/K^+-ATPase (7). The ATP-binding site was localized on the so-called N-domain (Arg^{378}-Arg^{589}), which is clearly separated from the P-domain where the phosphorylation site (Asp^{369}) resides. The docking of ATP as a substrate into the active site was explored with the AUTODOCK program. It revealed existence of single ATP-binding site only and suggested amino acid residues that could be involved in the interaction with the substrate. It appeared to be in quite good agreement with recently published NMR and crystal structures of the Na^+/K^+-ATPase N-domain (8, 9), which allowed further rectification of the model (10).

Model verification

A soluble part of the Na^+/K^+-ATPase containing exclusively the H_4–H_5 loop with the ATP-binding site has been expressed in several laboratories (11, 12). The H_4–H_5 loop sequence was prepared by polymerase chain reaction from the sequence of the alpha-subunit from mouse brain Na^+/K^+-ATPase and inserted into pGEX-2T plasmid, which contains the GST-tag on the N-terminus. The expressed H_4-H_5 loop-GST fusion protein is lacking the interactions with other domains of the sodium pump and is not affected in its structure by Na^+– or K^+-dependent conformational changes of the transmembrane part. Moreover, it is well known that this loop has a self-supporting structure and retains the ability to bind ATP (11, 13) and to hydrolyze very slowly p-nitrophenylphosphate in Mg^{2+}-dependent way (12). Higher expression level in *E. coli* was observed when the STOP-codon was inserted on the position 605. Thus, we used the construct Leu^{354}-Ile^{604} (roughly the N-domain).

Using site-directed mutagenesis, we performed mutations of selected residues on this construct and evaluated the effect of these mutations on the nucleotide binding. Thus, we tested whether the predicted amino acid residues represent the active binding site. Effect of performed mutations was evaluated by the binding of fluorescent analog of ATP, 2'(or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (further referred to as TNP-ATP), to the isolated N-domain containing the point mutation. Previously, we estimated that some mutations could affect only TNP-ATP binding but not ATP binding. Therefore competitive displacement of TNP-ATP by ATP was used to test the influence of the point mutation on the binding of pure ATP.

ATP-binding detection using the fluorescence analog of the nucleotide

Fluorescence analysis represents a powerful tool for investigating the interaction of biopolymers with various ligands. Unfortunately, the ligands of interest usually do not emit strong fluorescence, and/or the biopolymers themselves exhibit fluorescence that may exceed considerably the weak ligand emission (e.g. when proteins containing multiple aromatic acids are studied). To overcome this problem, fluorescent analogs of many important ligands have been synthesized that are capable of emitting bright fluorescence in a spectral range that is far from that of the biopolymer autofluorescence. Nearly three decades ago, Hiratsuka and coworkers demonstrated that TNP-ATP is sensitive to the environment polarity, which can be used for various assays of ATP binding to (14–18). The assay on ATP binding to Na^+/K^+-ATPase was confirmed by Moczydlowski and Fortes (19) who found that upon binding of TNP-ATP to the enzyme the probe fluorescence has increased and the fluorescence-enhanced data have been a reliable measure of TNP-ATP binding to Na^+/K^+-ATPase. We derived an equation that makes it possible to treat the fluorescence of TNP-ATP-stained protein as the explicit function of total probe concentration in the examined sample (20). Thus it has been possible to obtain the value of the dissociation constant from the titration experiment without using the Scatchard plot that has been suboptimal from the point of view of mathematical statistics.

The dissociation constant of ATP binding to the H4-H5 loop of Na^+/K^+-ATPase is about three orders of magnitude higher than that of TNP-ATP. This suggests a certain stabilizing role of the trinitrophenyl moiety of the fluorescence probe in the complex with the protein. Hence, one cannot exclude the possibility that the interaction of the trinitrophenyl moiety itself with the ATP-binding site is affected in our mutants. Therefore, we determined the dissociation constant of ATP-peptide complexes for all constructs as well.

Competition for the binding sites between ATP and TNP-ATP was used to characterize the binding of ATP to the fusion proteins. We derived an explicit formula for the probe fluorescence intensity, which is suitable for nonlinear least-squares analysis (21).

ATP-binding site of Na^+/K^+-ATPase

The single ATP-binding site was identified on the N-domain. It is constituted by the central part of the major cytoplasmic loop connecting transmembrane helices 4 and 5 (roughly Arg^{378}-Arg^{589} (7). Using the protein-reactive ATP analogs 2-azido-ATP and 8-azido-ATP (22, 23) it was possible to label and identify Gly^{502} and Lys^{480}, respectively, as possible recognition sites for ATP. The facts that ATP prevents modification of Lys^{501} by fluorescein 5'-isothiocyanate (FITC) (24) and that fluorescence of FITC attached to this residue cannot be quenched by antifluorescein antibody (25) led to the conclusion that Lys^{501} is localized in the depth of the ATP-binding pocket. Development of molecular biology brought a new efficient way for examining the role of individual amino acids in the interaction of the ATP with the enzyme. Mutagenesis studies confirmed the important roles of Lys^{480} (26) and Lys^{501} (27).

Our work provided a closer look at the ATP site and reveals the amino acids of the active site in the N-domain with ATP. The H_4-H_5-loop contains an N-terminal and a C-terminal subdomains of the P-domain and a bulky N-domain. C-terminal shortening of the H_4-H_5-loop-GST fusion proteins down to Leu^{576} removes the C-terminal subdomain of the P-domain and part of the N-domain has been reported to be without any effect on the affinity and the binding properties of the TNP-ATP (28). In other words, removal of two hundred amino acid residues between Leu^{576} and Leu^{777} did not significantly change the properties of the ATP binding site.

The fluorescent analog of ATP, the TNP-ATP, was used to evaluate changes of ATP and TNP-ATP dissociation constants caused by point mutations. The estimated values of the dissociation constants for wild type protein (KD = 3.1±0.2 μM for TNP-ATP, and KD = 6.2±0.7 mM for ATP binding) (13) were in good agreement with the results from other laboratories for similar proteins (11, 12, 29).

We showed that mutations of Phe^{475} and Glu^{446} resulted in substantial inhibition of TNP-ATP as well as ATP binding to the H_4-H_5-loop of Na^+/K^+-ATPase (13). In fact, our model reveals that the aromatic ring of Phe^{475} and the adenine ring of ATP are parallel at the distance of 0.3 nm. A stacking interaction between their π-electron systems is important for the stabilization of ATP within the binding pocket. The key role of this residue is also reflected by the substantial inhibition of the activity of the enzyme when it is mutated (27). The negatively charged Glu^{446} forms a hydrogen bond over a distance of 0.2 nm to the NH_2 hydrogen donor of the adenosine moiety. Because a hydrogen bond can be formed only over a very short distance, the exact position of this residue is crucial for the interaction with ATP.

Another residue involved in the ATP binding pocket could be Gln^{482}, as suggested by our computer model (7). This residue escaped the attention so far, perhaps because it is not conserved in the sequence of Ca^{2+}-ATPase. Interestingly, its replacement by leucine resulted in a strong inhibition of both TNP-ATP and ATP binding (30). Molecular modeling proposed that the closest distance between this residue and ATP was only 0.18 nm. The recently published structure of the N-domain estimated by NMR detected a hydrogen bond between Gln^{482} and adenosine moiety of ATP (9). Residues Glu^{446}, Phe^{475} and Gln^{482} seem to be the most important ones for the interaction with ATP.

Surprisingly, the strongest inhibition of ATP binding was observed when the guanidyl residue was missing in the R423L mutant, but the mutation of Asn^{422} and Ile^{417} had only a marginal effect (30). Abu-Abed and coworkers (31) predicted only a minor role of the corresponding stretch of residues in the Ca^{2+}-ATPase in the recognition of ATP. This is in accordance with the predictions from our model where these residues, including Arg^{423}, lie outside the ATP-binding pocket. However, Arg^{423} can form a hydrogen bond with Glu^{472} over a distance of 0.17 nm. This corresponds with the finding that mutations of Glu^{472} lead to a strong inhibition of Na^+/K^+-ATPase (26). To verify this hypothesis we mutated also Glu^{472} to see if this mutation would have a similar effect as the mutation of Arg^{423}. Indeed, estimated values for both TNP-ATP and ATP binding revealed strong inhibition in the binding of both ligands and the values matched within the range of error with values estimated for Arg^{423} (10). This finding strongly supports the suggestion that the hydrogen bond between Arg^{423} and Glu^{472} does exist. Breaking this hydrogen bond probably causes instability in the stretch of amino acids containing the residues Phe^{475}, Lys^{480} or Gln^{482} within the binding pocket, which are in the proximity of the other residues involved in ATP binding, such as Lys^{501} or Glu^{446}.

Even more dramatic changes were observed after mutation of the conserved residue Pro^{489}. We were not able to detect any TNP-ATP binding suggesting that this mutation substantially influenced the structure of the nucleotide-binding site (10). Indeed, Pro^{489} is located in the loop connecting the third and fourth beta-strands of the N-domain. Proline is the only residue that forces the peptidic backbone to adopt the cis-conformation. Therefore, its replacement by any other amino acid probably results in the change of the mutual position of the third and fourth beta-sheet. The third beta-strand contains residues Lys^{480} and Gln^{482}, while the fourth beta-strand contains the segment Lys^{501}-Ala^{503}. An appropriate mutual position of these residues is required for effective ATP recognition. Thus, the effect of the Pro^{489} mutation is rather indirect, similarly as discussed for Arg^{423} and Glu^{472} in the previous paragraph.

We also found that mutations S445A and E505Q had no significant effect on ATP binding. This indicates their minor role in ATP recognition. The fact that these mutations had a modest influence on binding of more bulky TNP-ATP indicates their close proximity to the binding site.

In conclusion, we showed that besides the previously reported amino acids Asp^{443} (32), Lys^{480}, Lys^{501}, Gly^{502} and Arg^{544}, also Glu^{446}, Phe^{475} and Gln^{482} residues formed the ATP recognizing pocket of Na^+/K^+-ATPase. The shape of this pocket is probably stabilized by a hydrogen bond between Arg^{423} and Glu^{472}. Mutations of Ile^{417}, Gln^{422}, Ser^{445}, Met^{500} and Glu^{505} did not affect the ATP binding. Molecular modeling of the ATP site within the H_4-H_5-loop reveals that the set of these eight amino acids residues forming the ATP recognition site is complete. Moreover, we have proved that a hydrogen bond between Arg^{423} and Glu^{472} supports the connection of two opposite halves of the ATP-binding pocket. Similarly, the conserved residue Pro^{489} is important for the proper interaction of the third and fourth beta-strands, which both contain residues that take part in the ATP-binding. However, we cannot exclude the possibility that certain amino acid interactions in the structural backbone contribute to the stabilization of the shape of this binding pocket as suggested for Arg^{423}, Glu^{472} and Pro^{489}. Several other amino acids such as Asp^{555}, Glu^{556}, Asp^{565}, Glu^{567} (33), Asp^{710} or Asp^{714} (34) were shown to be important for the activity of the whole enzyme. This effect may be explained rather by the influence on phosphorylation than on the binding of ATP itself.

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