STRUCTURAL BIOLOGY ON THE SODIUM PUMP: A COMBINED APPROACH LEADING TO A FULL CHARACTERIZATION OF THE CATALYTIC DOMAIN

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Keywords:
Na^+/K^+-ATPase, ATP binding, molecular modeling, Raman spectroscopy, fluorescence spectroscopy

Abstract
In this paper we summarize our previous work on the catalytic part of Na^+/K^+-ATPase. The nucleotide-binding domain of the subunit of mouse brain of Na^+/K^+-ATPase was expressed and isolated from *Escherichia coli* cells. The secondary structure of the expressed domain was experimentally determined by UV circular dichroism and Raman spectroscopy. By computer modeling was generated a three-dimensional model with and without docked ATP and predicted amino acids involved in the ATP binding site. ATP binding of wild type was followed by Raman difference spectroscopy and point mutants were measured by fluorescence spectroscopy with TNP-ATP. The set of eight amino acids residues was identified to form the complete ATP recognition site.

Na^+/K^+-ATPase (EC 3.6.1.37) is an integral membrane protein. This enzyme consists of two subunits, the catalytic subunit and the associated glycoprotein subunit [1]. The subunit contains 10 transmembrane segments with a large cytoplasmic loop, which is located between helices H_4 and H_5, where the ATP binding site and the phosphorylation site are localized [2]. This H_4-H_5 loop was shown to preserve a rigid and self-supporting structure [3] and is able to bind nucleotide triphosphates [4]. On the basis of the recently solved tertiary structure of Ca^{2+}-ATPase, the complete 3D structure of the large cytoplasmic loop between the fourth and fifth transmembrane segment (H_4-H_5 loop) of the a subunit of Na^+/K^+-ATPase, beginning with Lys^{354} and ending with Lys^{773}, was modeled using the method of restraint-based comparative modeling [5]. Due to the relatively high degree of homology of more than 50 % with respect to the cytoplasmic loop of Ca^{2+}-ATPase and the sufficient amount of background information, contributed by different labelling techniques of certain residues and kinetic studies, it was possible to create a model in which all amino acids were modeled convincingly. We have shown that the ATP binding site and the phosphorylation site are located on two different, well-separated

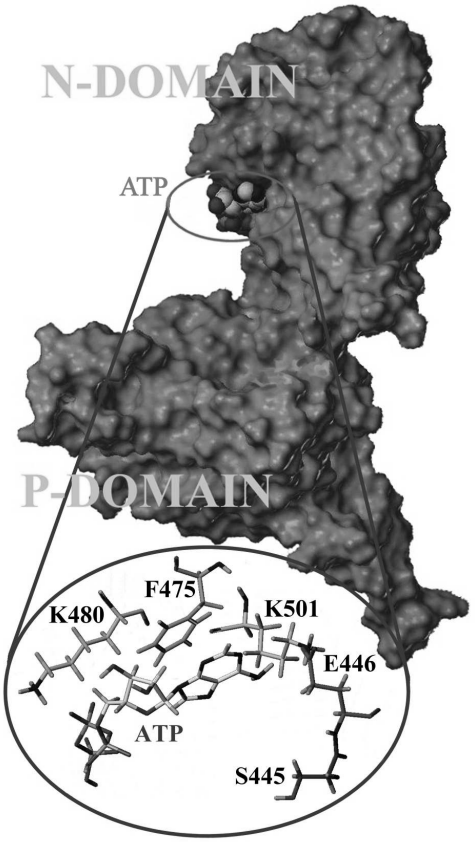

Fig. 1. N and P-domain of the H_4-H_5 loop of Na^+/K^+-ATPase [5]. In the inset are important amino acids for the ATP-binding.

domains, which together form the large cytoplasmic loop. For these domains we propose to use the following names: domain P comprises the N-terminal and C-terminal ending of the H_4-H_5 loop and contains Asp^{369}, the residue of phosphorylation surrounded by a highly negatively charged surface; and domain N, which binds nucleotides. We also show that there is evidence for only one ATP binding site on the N domain of the H_4-H_5 loop, and we were able to specify Cys^{549}, Phe^{548}, Glu^{505}, Lys^{501}, Gln^{482}, Lys^{480}, Ser^{477}, Phe^{475} and Glu^{446} as parts of the ATP binding site

with Lys^{501} located in the depth of the positively charged binding pocket (Fig. 1).

In a next step the H^4-H^5 loop sequence (Leu^{354}-Ile^{778}) was prepared from the sequence of the a subunit of mouse brain Na^+/K^+-ATPase by polymerase chain reaction (PCR) [6]. The purified DNA of the a subunit was amplified by PCR with PCR primer pairs for the desired sequence and TFL polymerase. The product was purified on agarose gel. This vector was digested with *Bam*HI and *Eco*RI and subcloned into the linearized pGEX-2T at the site location downstream from the GST coding sequence. The ligated DNA was transformed into competent *Escherichia coli* DH5 cells. The stop codon at Lys^{605} was introduced using a Stratagene Quick-Change Kit for site-directed mutagenesis. The *E. coli* transformants were selected on Luria-Bertani agar containing ampicillin (50 mg/mL) and positive clones were finally sequenced. The protein was expressed as in a previous study [7]. In a next step a model of the N domain of mouse brain Na^+/K^+-ATPase from Leu^{354}-Ile^{604} was generated by analogy to the above mentioned model for pig kidney Na^+/K^+-ATPase [6]. After docking of ATP to domain N, changes in the secondary structure were affecting only residues close to the binding site; the overall changes were smaller than 2%. The Raman amide I band of the Raman spectra was analyzed by two different methods for estimation of the secondary structure: least square analysis (LSA) and four reference intensity profiles (4-RIP) with a spectrum of a solvent (Table I). Both methods gave the same results within the maximal calculated deviation of 5 and 3 %, respectively. Raman spectra can also provide additional information. Two bands at 826 and 848 cm^{-1} are assigned to the Tyr (Y1 + Y16a) Fermi dublet. The intensity ratio I^{848}/I^{826} is an indicator of the Tyr environment. We attained a value of 0.4. This indicated that the hydroxyl groups of all three Tyr residues were donors of strong hydrogen bonds (i.e., Tyr^{467}, Tyr^{481}, and Tyr^{535} are buried under the accessible surface), which completely agreed with our model. The Trp Fermi doublet intensity ratio I_{1360}/I_{1340} is a sensitive marker of the amphipathic environment of the aromatic ring. In our case the band at about 1360 cm^{-1} was overlapped by the strong band at 1342 cm^{-1}, which made the estimation of the ratio impossible. However, the absence of the strong band at 1360 cm^{-1} indicated a hydrophilic environment for the two Trp residues. This was in good agreement with the presence of the strong band at 755 cm^{-1}, whose intensity is sensitive to the amphipathic environment of the indole ring. Our spectra thus indicated no hydrophobic interaction of Trp with neighboring residues. The fact that our protein contains only Trp^{385} and Trp^{411} enabled us to fit the band corresponding to the W17 normal mode for two bands with positions at 874 and 880 cm^{-1}. The W17 mode is sensitive to NH-H bond donation. The band at 874 cm^{-1} showed hydrogen bonding and thus corresponds to Trp^{385} in our model. The Trp^{411}, represented by the band at 880 cm^{-1}, does not take part in hydrogen bonding. The shift of the Phe breathing vibration mode from 1003 to 1002 cm^{-1} in the difference spectrum, in addition to ATP, was also observable. We can interpret these spectral changes as local changes of the domain N conformation caused by the ATP binding. Changes of the Phe band were in good agreement with the presence of Phe^{475} and Phe^{548} in the binding site found in our model. Thus, we can say that our recombinant protein corresponds to domain N in mouse brain Na^+/K^+-ATPase and docking of ATP proposed changes in the range of 1-2 % of the overall secondary structure, which is in agreement with our experimental findings [6].

Table I. Secondary structure of domain N [6] obtained by modeling, circular dichroism (program Varselec and CDNN) and Raman spectroscopy (method LSA and 4-RIP).

Method	-helix	-sheet	-turn	other
Model	29.6	31.2	20.4	18.8
Varselec[a]	25.5	27.5	-	31.5
CDNN	26.2	21.5	20.7	31.2
LSA	31.6	34.8	19.1	14.5
4-RIP[a]	30.3	34.9	-	34.8

[a] -turns are included in other structure

Site-directed mutagenesis was performed to identify residues involved in ATP binding [8]. On the basis of our above developed model of this loop, Ser^{445}, Glu^{446}, and Phe^{475} were proposed to be close to the binding pocket. Replacement of Phe^{475} with Trp and Glu^{446} with Gln profoundly reduced the binding of ATP, whereas the substitution of Ser^{445} with Ala did not affect ATP binding. Fluorescence measurements of the fuorescent analog TNP-ATP, however, indicated that Ser^{445} is close to the binding site, although it does not participate in binding. All point mutants of our construct F475W, E446Q, and S445A were expressed as GST-fusion proteins in *E. coli* and purifed. The dissociation constant of ATP to the H_4-H_5-loop from Na^+/K^+-ATPase is about three orders of magnitude higher than of TNP-ATP. Competition for the binding sites between ATP and TNP-ATP was used to characterize the binding of ATP to the fusion proteins. The presence of ATP changed signifcantly the fuorescence intensity of TNP-ATP when titrated in the presence of all the mutants used in our study. The decrease in fuorescence intensity in the presence of ATP indicated that some binding sites were occupied by ATP and allowed us to calculate the dissociation constants for ATP. We observed an increase of the value of the dissociation constant from 6.2 ± 0.7 mM for WT to 19 ± 2 or 14 ± 3 mM for E446Q or F475W, respectively, suggesting an inhibition of ATP-binding. Contrary to TNP-ATP-binding, this effect was not observed for the mutation S445A.

In conclusion, we show that WT protein (Leu^{354}-Ile^{604}) binds ATP as well as its fluorescent analog TNP-ATP. The amino acids Phe^{475} and Glu^{446} play an important role in this interaction. Substitution of the phenylalanine residue 475 by tryptophan and glutamic acid 446 by glutamine affected severely the interaction with both ATP and TNP-ATP, as indicated by a positive change in the binding energy compared to WT. This is in good agreement, not only with our prediction from computer modeling but also with the sequence comparison of Ca^{2+}-ATPase with Na^+/K^+-ATPase. Both methods have suggested Phe^{475} and Glu^{446} to be part

of the ATP-binding site. We can observe stacking of the aromatic ring of phenylalanine that is parallel to the purine ring of ATP at a distance of 3 Å, in our predicted model structure. Substitution of Ser^{445} by alanine did not significantly affect ATP-binding. Nevertheless, the change in binding of the more bulky TNP-ATP molecule indicated the residue to be in close proximity to the ATP-binding site. In our predicted model structure, the carboxyl group of Glu^{446} forms a hydrogen bond over a distance of 2.0 Å to the NH_2 hydrogen donor of the adenosine moiety. The hydroxyl group of Ser^{445}, however, is about 7.5 Å away from the ATP molecule at the closest distance. Thus, a direct interaction seems unlikely and our model proves to be correct in this respect. As a result we have found beside the previously reported amino acid residues Lys^{480}, Lys^{501}, Gly^{502} and Cys^{549} another four amino acid residues, namely Glu^{446}, Phe^{475}, Gln^{482} and Phe^{548}, completing the ATP binding pocket of Na^+/K^+-ATPase. Moreover, mutation of Arg^{423} has also resulted in a large decrease of the ATP binding constant. This residue, localized outside of the binding pocket, seems to play a key role in supporting the proper structure and shape of the binding site, probably due to formation of the hydrogen bond with Glu^{472} over a distance of 1.7 Å. Breaking this hydrogen bond causes probably an instability in the stretch of amino acids containing the residues Phe^{475}, Lys^{480} or Gln^{482} within the binding pocket which are in proximity to the other residues involved in ATP binding, like Lys^{501} or Glu^{446} [9]. 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.

The support by the Grants No. 204/01/0254, 204/01/100 and 309/02/1479 of the Grant Agency of the Czech Republic and the Ministry of Education of the Czech Republic (No. MSM113100001, No. MSM123100001) is acknowledged.

References

1. P. L. Jorgensen, *Kidney Int.*, **29** (1986) 10-20.
2. M. Esmann, S. J. Karlish, L. Sottrup-Jensen and D. Marsh, *Biophys. J.*, **70** (1994) 182-193.
3. H. Linnertz, I. Mikšík, P. Kvasnička, E. Bertoli, L. Mazzanti, W. Schoner and E. Amler, *Eur. J. Biochem*., **251** (1998) 522-527.
4 T. Obšil, F. Mérola, A. Lewit-Bentley and E. Amler, *FEBS Lett.*, **426** (1998), 297-300.
5. R. Ettrich, M. Melicherčik, J. Teisinger, O. Ettrichová, R. Krumscheid, K. Hofbauerová., P. Kvasnička, W. Schoner and E. Amler, *J. Mol. Model.*, **7** (2001) 184-192.
6. K. Hofbauerová, V. Kopecký Jr., R. Ettrich, O. Ettrichová and E. Amler, *Biopolymers*, **67** (2002) 242-246.
7. T. Obšil, K. Hofbauerová, E. Amler, J. Teisinger, *FEBS Lett.*, **457** (1999) 311-315.
8. M. Kubala, K. Hofbauerová, R. Ettrich, V. Kopecký Jr., R. Krumscheid, J. Plášek, J. Teisinger, W. Schoner and E. Amler, *Biochem. Biophys. Res. Commun.*, **297** (2002) 154-159.
9. M. Kubala, J. Teisinger, R. Ettrich, K. Hofbauerová, V. Kopecký Jr., V. Baumruk, R. Krumscheid, J. Plášek, W. Schoner and E. Amler, *Biochemistry*, (2003) submitted.