ACADEMIC PRESS

Biochemical and Biophysical Research Communications 297 (2002) 154–159

BBRC

www.academicpress.com

Phe[475] and Glu[446] but not Ser[445] participate in ATP-binding to the α-subunit of Na^+/K^+-ATPase

Martin Kubala,[a,b,1] Kateřina Hofbauerová,[a,c,d,1] Rüdiger Ettrich,[a,d,e] Vladimír Kopecký Jr.,[a,b,d] Rita Krumscheid,[f] Jaromír Plášek,[b] Jan Teisinger,[a] Wilhelm Schoner,[f] and Evžen Amler[a,*]

[a] *Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic*
[b] *Institute of Physics, Charles University, Ke Karlovu 5, CZ- 121 16 Prague 2, Czech Republic*
[c] *Department of Physical and Macromolecular Chemistry, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic*
[d] *Department of Biochemistry, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic*
[e] *Institute of Physical Biology, University of South Bohemia, Zámek 136, CZ-37333 Nové Hrady, Czech Republic*
[f] *Institut für Biochemie und Endokrinologie, Justus-Liebig-Universität, Frankfurter Strasse 100, D-35392 Giessen, Germany*

Received 12 August 2002

Abstract

The ATP-binding site of Na^+/K^+-ATPase is localized on the large cytoplasmic loop of the α-subunit between transmembrane helices H_4 and H_5. Site-directed mutagenesis was performed to identify residues involved in ATP binding. On the basis of our recently 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 fluorescent analog TNP-ATP, however, indicated that Ser[445] is close to the binding site, although it does not participate in binding.

Keywords: Na^+/K^+-ATPase; Fluorescence spectroscopy; ATP-binding site; TNP-ATP

Na^+/K^+-ATPase (EC 3.6.1.37) is an integral plasma membrane protein that transports sodium and potassium ions against an electrochemical gradient. This enzyme consists of two subunits. The catalytic α-subunit with a molecular mass of about 112,000 Da is responsible for all known transport and catalytic functions of associated glycoprotein. The β-subunit is an associated glycoprotein with a molecular mass of about 35,000 Da excluding oligosaccharides. The ATP-binding site has been localized on the large cytoplasmic loop of the α-subunit between transmembrane helices H_4 and H_5 (H_4–H_5-loop) [1].

This H_4–H_5-loop as well as the whole α-subunit of Na^+/K^+-ATPase shows a high sequence identity or homology with the Ca^{2+}-ATPase of the sarcoplasmic reticulum (32.8% identity and 53.3% similarity). The tertiary structure of the Ca^{2+}-ATPase has been recently solved at 2.6 Å resolution by X-ray crystallography [2]. Based on this finding and using restraint-based comparative modeling, we have recently calculated the three-dimensional structure of the large cytoplasmic loop of the Na^+/K^+-ATPase [3]. The model clearly shows that the H_4–H_5-loop contains only one ATP-binding site. This model was also confirmed by Raman and CD spectroscopy [4]. The ATP-binding site has been 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 [3].

Several amino acid residues, e.g., Lys[480] or Lys[501] [5,6] have previously been reported to be essential for the interaction with ATP and its binding. Using erythrosin 5-isothiocyanate, Cys[549] has also been shown to be part of the low affinity ATP-binding site [7]. Other amino acids, such as Arg[544], Asp[555], Glu[556], Asp[565], and Glu[567],

* Corresponding author. Fax: +420-2-4106-2249.
E-mail address: amler@biomed.cas.cz (E. Amler).
[1] Main authors contributed equally to this work.

0006-291X/02/$ - see front matter
PII: S0006-291X(02)02089-2

were discussed to have an influence on the activity of the whole enzyme [8]. In addition, the X-ray structure comparison of Ca^{2+}-ATPase with the sequence of the Na^+/K^+-ATPase has suggested that Ser^{445} and Glu^{446} are part of the ATP-binding site [8] as well as a key role of the Phe^{475} (Phe^{487} in Ca^{2+}-ATPase), highly conserved in P_2-type ATPases [2,9].

Our three-dimensional model [3] has also revealed a positively charged binding pocket interacting with ATP formed by Glu^{446}, Glu^{505}, Lys^{501}, Glu^{482}, Lys^{480}, Ser^{477}, and also by Phe^{475}. The latter amino acid may interact with the purine moiety while Glu^{446} forms a hydrogen bond to the NH_2 hydrogen donor of ATP. However, according to our model, Ser^{445} does not participate in ATP binding. To test this discrepancy as well as to verify the role of Glu^{446} and Phe^{475}, site-directed mutagenesis of all three residues has been performed.

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 [10,11]. We used the construct Leu^{354}–Ile^{604}, further denoted as wild type (WT).

Materials and methods

All chemicals were of the highest purity available. DNA sequencing was accomplished using an automated sequencer (ABI Prism).

Construction of the protein expression vector pGEX–H_4–H_5. The H_4–H_5-loop sequence was prepared by polymerase chain reaction from the sequence of the α-subunit from mouse brain Na^+/K^+-ATPase. The construction of the expression plasmid pGEX-2T containing the cDNA for H_4–H_5-loop (Leu^{354}–Ile^{604}) was described previously [4]. Site-directed mutagenesis was performed using polymerase chain reaction with the QuickChange Kit (Stratagene, USA) according to manufacturer's protocol. The sequence of the primer used for site-directed mutagenesis was S445A: 5′-CA GTA GCG GGA GAT GCT GCC GAG TCG GCG CTC TTA AAG TGC-3′; E446Q: 5′-GTA GCG GGA GAT GCT TCC CAG TCG GCG CTC TTA AAG TGC-5′; F475W: 5′-CCA AGA TAG TGG AGA TTC CTT GGA ACT CCA CCA ACA AGT ACC-3′. The altered nucleotides are underlined.

Expression and purification of the H_4–H_5-loop. An overnight culture (3 ml, *Escherichia coli* BL21 cells) was diluted into 1 L fresh LB medium containing ampicillin (50 μg/ml) and incubated to OD 0.8 at 600 nm (8 h) at 37 °C. After addition of IPTG (0.1 mM), the culture was incubated overnight at 30 °C. Cells were collected by centrifugation at 4 °C and resuspended in 50 ml TENG buffer (50 mM Tris–HCl, pH 7.5; 1 mM EDTA; 100 mM NaCl; 10% glycerol; 1% NP-40; and 1 mM DTT) with protein inhibitors (1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 20 μg/ml aprotinin). The cells in the suspension were disrupted using a French press. Cell debris were centrifuged at 4 °C and 15,000*g* for 25 min. The supernatant was loaded onto a 2 ml glutathione–Sepharose column (equilibrated with TENG buffer), incubated at 4 °C for 1 h, and rinsed with 180 ml buffer (20 mM Tris–HCl, pH 7.4; 140 mM NaCl). The fusion protein was eluted by addition of 2 ml of 50 mM Tris–HCl, pH 8, containing 10 mM glutathione [12,13]. Samples were dialyzed overnight at 4 °C against an excess of 50 mM Tris–HCl, pH 7.5. The protein purity was checked by 12 % (w/v) SDS–PAGE.

TNP-ATP binding to recombinant H_4–H_5-loop. The fluorescence probe TNP-ATP (2′,3′-*O*-(2,4,6- trinitrophenyl) adenosine 5′-triphosphate, disodium salt; Molecular Probes, USA) is sensitive to the polarity of its environment [14]. Its binding to the protein is detected as an increase of fluorescence intensity [15]. Aliquots of 100 μM TNP-ATP were subsequently added to 1 ml of 50 mM Tris–HCl, pH 7.5, containing either 1.6 μM glutathione *S*-transferase (GST)-fusion protein, GST alone or no protein, and then gently stirred for about 1 min after each addition. The fluorescence intensity was measured at 22 °C using the FluoroMax-2 (Jobin Yvon/Spex) fluorometer in 0.4 × 1 cm quartz cuvettes. Excitation and emission wavelengths were 462 and 527 nm, respectively, both the excitation and emission bandpass were 10 nm, and fluorescence intensity at each step was integrated for 5 s.

Calculation of dissociation constant of binding of TNP-ATP to fusion proteins. The signal of buffer and protein (if present) was collected before the addition of TNP-ATP and this value was subtracted from all further raw data as a background. Corrections for volume dilution of both protein and probe were also done. Fluorescence intensity was normalized so that a fluorescence of 1 μM free TNP-ATP (i.e., in the absence of protein) was equal to unity. The dependence of fluorescence intensity on the concentration of TNP-ATP was fitted to the equation describing a model with one binding site per protein molecule

$$F = [P] + \frac{1}{2}(\gamma - 1)\left([P] + [E] + K_D - \sqrt{([P] + [E] + K_D)^2 - 4[P][E]}\right),$$

where F is the normalized fluorescence intensity, $[P]$ is the concentration of TNP-ATP, $[E]$ is the concentration of the enzyme, γ is the enhancement of fluorescence intensity of the bound probe relative to free probe and K_D is the dissociation constant. The enhancement factor γ was 7 ± 0.7. All parameters except K_D were kept constant during the fitting procedure. All values are presented as means ± SEM from at least three independent measurements.

Measurement of binding of ATP to fusion proteins. GST-fusion proteins were incubated with TNP-ATP in the presence of ATP (2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate, disodium salt; Sigma, USA) at pH 7.5. A competition between the fluorescence probe and ATP resulted in a lower rate of binding of TNP-ATP to the protein (i.e., lower fluorescence intensity) compared to experiments where no ATP was present. The experimental procedure was the same as that for measuring TNP-ATP-binding to proteins, except that the measurements were performed in 50 mM Tris–HCl, 20 mM ATP, pH 7.5 (buffer pH was adjusted to 7.5 after addition of ATP). The ATP was diluted upon addition of protein so that the initial concentration of ATP was 19.4, 19.6, 19.7, and 19.1 mM for WT, S445A, E446Q, and F475W, respectively.

Calculation of the dissociation constant of ATP binding to fusion proteins. The signal of buffer, ATP, and protein was collected before addition of TNP-ATP and this value was subtracted from all further raw data as a background. Corrections for volume dilution of ATP, protein, and probe concentrations were done. The fluorescence intensity was normalized as described above. The dependence of fluorescence intensity on the concentration of TNP-ATP was fitted to the equation, in good approximation describing a model where the fluorescence probe and ATP compete for the same binding site

$$F = [P] + \frac{1}{2}(\gamma - 1)\left([P] + [E] + K_P + [A]\frac{K_P}{K_A} - \sqrt{\left([P] + [E] + K_P + [A]\frac{K_P}{K_A}\right)^2 - 4[P][E]}\right)$$

where $[A]$ represents the concentration of ATP, K_P the dissociation constant of TNP-ATP, and K_A the dissociation constant of ATP. All parameters except K_A were kept constant during the fitting procedure. The dissociation constant of TNP-ATP was estimated as described above. All values are presented as means ± SEM from at least three independent measurements.

Molecular modeling. Computational site-directed mutagenesis was performed on our recently published model structure [3] using the program TRITON [16].

Results and discussion

Based on molecular modeling, we have recently proposed a three-dimensional structure of the H_4–H_5-loop of Na^+/K^+-ATPase [3], with the residues Phe^{475} and Glu^{446} being an integral part of the ATP-binding site (Fig. 1). First, we tested whether F475W, E446Q, and S445A substitutions of these residues may affect the three-dimensional structure of the H_4–H_5-loop and its binding affinity. Computational site-directed mutagenesis did not show any significant effect on the structure of the ATP-binding site (Fig. 2). However, there is no computational method available to date that would predict the effect of the mutation on the protein structure with absolute certainty. Therefore, the secondary structure was also checked by vibrational spectroscopy, which did not reveal any structural change nor aggregation of the protein (data not shown).

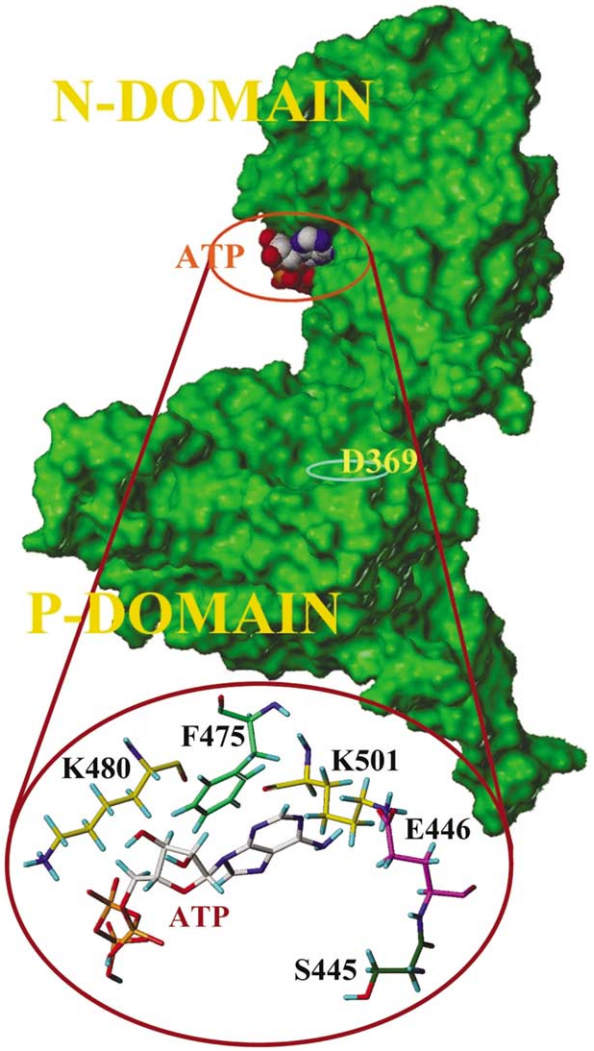

Fig. 1. Computer model of the H_4–H_5-loop of Na^+/K^+-ATPase [3] shows two well-separated domains (N- and P-domains). The ATP-binding site is localized on the N-domain, while the phosphorylation site (Asp^{369}) is on the P-domain. Some amino acids confirmed already previously to play a role in ATP-binding are shown in the inset. Our experiments showed that besides Lys^{480} and Lys^{501} [5,6], Phe^{475} and Glu^{446} but not Ser^{445} are relevant for ATP-binding. Solvent access is represented by a Conolly-type surface. Docking was explored with AutoDock [19].

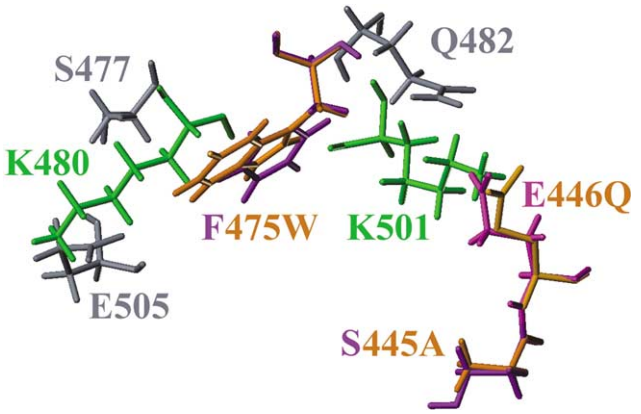

Fig. 2. Computational site-directed mutagenesis of Phe^{475}, Glu^{446}, and Ser^{445} predicts only minor changes in the structure of the ATP-binding site (in the absence of ATP as a ligand) and has no influence on the secondary and/or tertiary structure of the protein. Amino acids already confirmed experimentally are shown in green, while other amino acids proposed to play a role in ATP-binding according to a computer model are shown in gray.

Binding of TNP-ATP to the fusion protein

All the mutants F475W, E446Q, and S445A were expressed as GST-fusion proteins in *E. coli* and purified. To verify that the GST protein itself does not bind TNP-

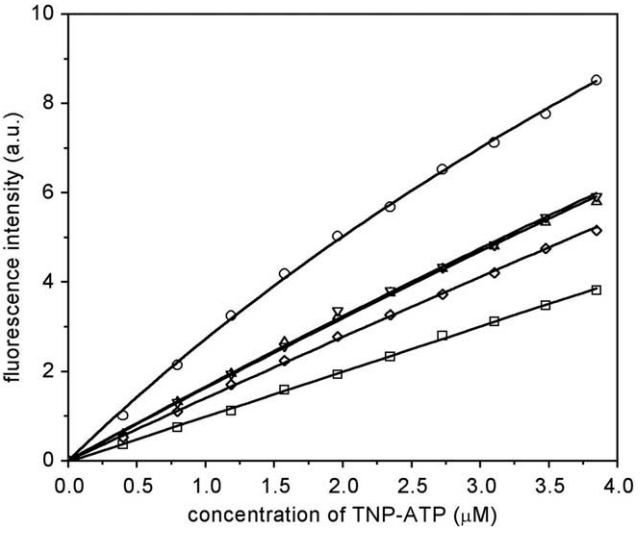

Fig. 3. Fluorescence intensity of TNP-ATP in 50 mM Tris–HCl, pH 7.5, in the absence (□), or in the presence of 1.6 μM WT (○), S445A (Δ), E446Q (▽), and F475W (◇) proteins. Binding of TNP-ATP to protein resulted in an increase of fluorescence intensity. Titration of TNP-ATP in the presence of GST resulted in an increase of fluorescence intensity that was indistinguishable from a control without GST.

Table 1
Dissociation constants for TNP-ATP- and ATP-binding to the GST–[Leu^{354}–Ile^{604}] fusion proteins are summarized

Protein	n	K_D (TNP-ATP) (μM)	$\Delta\Delta G$ (TNP-ATP) (kJ · mol^{-1})	n	K_D (ATP) (mM)	$\Delta\Delta G$ (ATP) (kJ · mol^{-1})
WT	3	3.1 ± 0.2	0	3	6.2 ± 0.7	0
S445A	4	12.4 ± 0.9	3.4	4	5 ± 1	−0.5
E446Q	5	12 ± 2	3.3	9	19 ± 2	2.7
F475W	3	22 ± 4	4.8	3	14 ± 3	2.0

The presented values are average ± SEM from n independent measurements. The difference in binding energies compared to WT ($\Delta\Delta G$) was calculated as described in [18].

ATP, the GST protein was incubated with TNP-ATP and the fluorescence was recorded. The fluorescence intensity of TNP-ATP in buffer was not different in the presence or absence of GST protein. Thus, there was no significant binding of TNP-ATP to GST observed over the whole range of TNP-ATP concentrations used in our study (Fig. 3). Accordingly, the GST–H_4–H_5-loop fusion protein was considered to be a suitable tool to study the binding of TNP-ATP to the ATP-binding site.

In contrast, incubation of all three mutants with TNP-ATP resulted in a clear and significant increase of the fluorescence intensity compared to GST in buffer or

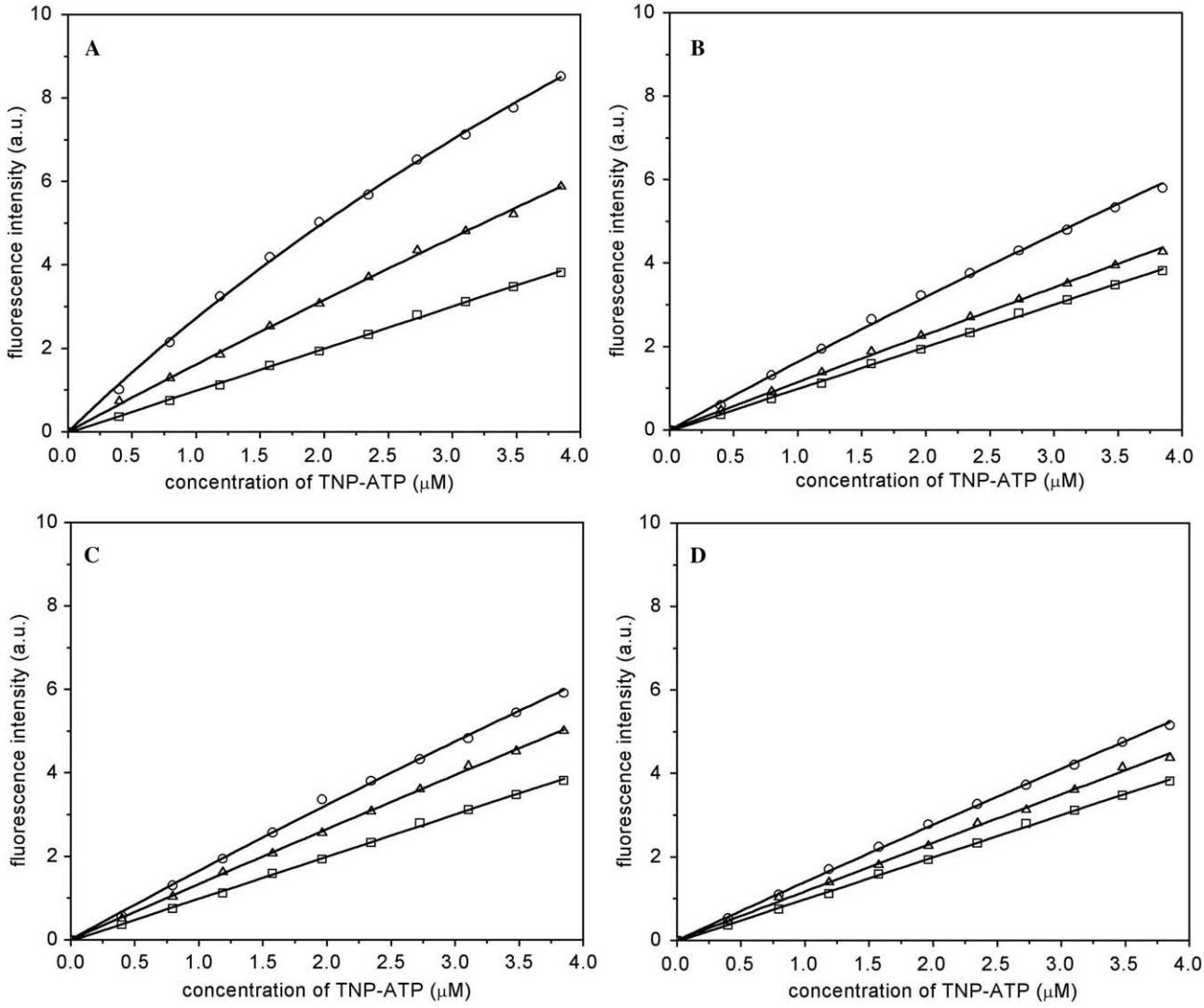

Fig. 4. Aliquots of TNP-ATP were added to 1.6 μM solution of (A) WT protein, (B) S445A, (C) E446Q or (D) F475W protein (bottom) in 50 mM Tris–HCl, pH 7.5 (○) or in 50 mM Tris–HCl + 20 mM ATP, pH 7.5 (Δ). For comparison, data of titration in absence of any protein are shown (□). Binding of TNP-ATP to the protein resulted in an increase of fluorescence intensity, while ATP competitively inhibited this binding.

buffer alone (see Fig. 3). The dissociation constants were calculated from at least three independent experiments. These values varied from $3.1 \pm 0.2\,\mu M$ for WT to 12.4 ± 0.9, 12 ± 2, and $22 \pm 4\,\mu M$ for S445A, E446Q, and F475W, respectively, suggesting that TNP-ATP binding for all mutants was significantly inhibited (Table 1).

Binding of ATP to fusion proteins

The dissociation constant of ATP to the H_4–H_5-loop from Na^+/K^+-ATPase is about three orders of magnitude higher than that of TNP-ATP (see Table 1). This suggests a certain stabilizing role of the trinitrophenyl moiety of the fluorescence probe in the complex with the protein. Hence, one may not exclude the possibility that the interaction of 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 (Fig. 4).

Competition for the binding sites between ATP and TNP-ATP was used to characterize the binding of ATP to the fusion proteins. Notably, the presence of ATP in buffer (without any protein) did not influence the fluorescence intensity of TNP-ATP. The presence of ATP, however, changed significantly the fluorescence intensity of TNP-ATP when titrated in the presence of all the mutants used in our study. The decrease in fluorescence 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 $6.2 \pm 0.7\,mM$ for WT to 19 ± 2 or $14 \pm 3\,mM$ 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 (Table 1). The dissociation constants for ATP ($6.2 \pm 0.7\,mM$) and TNP-ATP ($3.1 \pm 0.2\,\mu M$) obtained for the WT protein were in good agreement with recently published data for similar constructs [10,11,17].

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 (Table 1). 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.

In contrast with the prediction drawn from the X-ray structure of Ca^{2+}-ATPase, 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.

Acknowledgments

The work was supported by Grant Nos. 204/01/0254, 204/01/1001, and 309/02/1479 of the Grant Agency of Czech Republic, by the project WTZ CZE 00/033 of the Federal Republic of Germany, by the research plan of the Faculty of Science (MSM 113100001), and by the Volkswagen Foundation (I/74 679).

References

[1] J.B. Lingrel, T. Kuntzweiler, $Na^+,K^{(+)}$-ATPase, J. Biol. Chem. 269 (1994) 19659–19662.

[2] C. Toyoshima, M. Nakasako, H. Nomura, H. Ogawa, Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution, Nature 405 (2000) 647–655.

[3] R. Ettrich, M. Melicherčík, J. Teisinger, O. Ettrichová, R. Krumscheid, K. Hofbauerová, P. Kvasnička, W. Schoner, E. Amler, Three- dimensional structure of the large cytoplasmic H4–H5-loop of Na^+/K^+-ATPase deduced by restraint-based comparative modeling shows only one ATP-binding site, J. Mol. Model. 7 (2001) 184–192.

[4] K. Hofbauerová, V. Kopecký Jr., R. Ettrich, O. Ettrichová, E. Amler, Secondary and tertiary structure of nucleotide-binding domain of α-subunit of Na^+/K^+-ATPase, Biopolymers 67 (2002) 242–246.

[5] C.M. Tran, G. Scheiner-Bobis, W. Schoner, R.A. Farley, Identification of an amino acid in the ATP binding site of $Na^+/K^{(+)}$-ATPase after photochemical labeling with 8-azido-ATP, Biochemistry 33 (1994) 4140–4147.

[6] G. Scheiner-Bobis, S. Schreiber, Glutamic acid 472 and lysine 480 of the sodium pump α1 subunit are essential for activity. Their conservation in pyrophosphatases suggests their involvement in recognition of ATP phosphates, Biochemistry 38 (1999) 9198–9208.

[7] H. Linnertz, H. Kost, T. Obšil, A. Kotyk, E. Amler, W. Schoner, Erythrosin 5′-isothiocyanate labels Cys549 as part of the low-affinity ATP binding site of Na^+/K^+-ATPase, FEBS Lett. 441 (1998) 103–105.

[8] M.D. Jacobsen, P.A. Pedersen, P.L. Jorgensen, Importance of NaK-ATPase residue α1-Arg544 in the segment Arg544–Asp567 for high-affinity binding of ATP, ADP, or MgATP, Biochemistry 41 (2002) 1451–1456.

[9] P. Morsomme, C.W. Slayman, A. Goffeau, Mutagenic study of the structure, function and biogenesis of the yeast plasma membrane $H^{(+)}$-ATPase, Biochim. Biophys. Acta 1469 (2000) 133–157.

[10] C. Gatto, A.X. Wang, J.H. Kaplan, The M4M5 cytoplasmic loop of the Na,K-ATPase, overexpressed in *Escherichia coli*, binds

nucleoside triphosphates with the same selectivity as the intact native protein, J. Biol. Chem. 273 (1998) 10578–10585.
[11] C.M. Tran, R.A. Farley, Catalytic activity of an isolated domain of Na,K-ATPase expressed in *Escherichia coli*, Biophys. J. 77 (1999) 258–266.
[12] D.B. Smith, K.S. Johnson, Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase, Gene 67 (1988) 31–37.
[13] O. Ennis, R. Maytum, T. Mantle, Cloning and overexpression of rat kidney biliverdin IX α reductase as a fusion protein with glutathione *S*-transferase: stereochemistry of NADH oxidation and evidence that the presence of the glutathione *S*-transferase domain does not effect BVR-A activity, Biochem. J. 328 (1997) 33–36.
[14] T. Hiratsuka, Fluorescence properties of 2′ (or 3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate and its use in the study of binding to heavy meromyosin ATPase, Biochim. Biophys. Acta 453 (1976) 293–297.
[15] E.G. Moczydlowski, P.A.G. Fortes, Characterization of 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidine) adenosine 5′-triphosphate as a fluorescent probe of the ATP site of sodium and potassium transport adenosine triphosphatase. Determination of nucleotide binding stoichiometry and ion-induced changes in affinity for ATP, J. Biol. Chem. 256 (1981) 2346–2356.
[16] M. Prokop, J. Damborsky, J. Koca, TRITON: in silico construction of protein mutants and prediction of their activities, Bioinformatics 16 (2000) 845–846.
[17] E. Capieaux, C. Rapin, D. Thinès, Y. Dupont, A. Goffeau, Overexpression in *Escherichia coli* and purification of an ATP-binding peptide from the yeast plasma membrane $H^{(+)}$-ATPase, J. Biol. Chem. 268 (1993) 21895–21900.
[18] P.A. Pedersen, J.R. Jorgensen, P.L. Jorgensen, Importance of conserved α-subunit segment 709GDGVND for Mg^{2+} binding phosphorylation and energy transduction in Na,K-ATPase, J. Biol. Chem. 275 (2000) 37588–37595.
[19] G.M. Morris, D. Goodsell, R. Huey, A.J.J. Olson, Distributed automated docking of flexible ligands to proteins: parallel applications of AutoDock 2.4., Comput. Aided Mol. Des. 10 (1996) 293–304.