Physiol. Res. 53 (*Suppl. 1*): S187-S197, 2004

Protein Modeling Combined with Spectroscopic Techniques: an Attractive Quick Alternative to Obtain Structural Information

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Received February 10, 2004
Accepted March 5, 2004

Summary

Beside of the protein crystallography or NMR, 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 have used the combination of computer modeling with spectroscopic techniques, such as steady-state or time-resolved fluorescence spectroscopy, and with molecular biology techniques. This method could provide useful structural information in the cases where crystal or NMR structure is not available. 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 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 β-strands, which both contain residues that take part in the ATP-binding. Alternatively, molecular dynamics simulation combined with dynamic fluorescence spectroscopy revealed that 14-3-3 zeta C-terminal stretch is directly involved in the interaction of 14-3-3 protein with the ligand. Phosphorylation at Thr^{232} induces a conformational change of the C-terminus, which is presumably responsible for observed inhibition of binding abilities. Phosphorylation at Thr^{232} induces more extended conformation of 14-3-3zeta C-terminal stretch and changes its interaction with the rest of the 14-3-3 molecule. This could explain negative regulatory effect of phosphorylation at Thr^{232} on 14-3-3 binding properties.

Key words

Computer modeling • Molecular dynamics simulations • Fluorescence spectroscopy • Time-resolved fluorescence spectroscopy • Na^+/K^+-ATPase • 14-3-3 proteins • Point mutations

Introduction

Protein X-ray crystallography and nuclear magnetic resonance (NMR) are standard techniques commonly used to solve the structure of proteins. However, there are several obstacles and disadvantages, which in some cases hinder the application of such

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methods. First, protein crystallization 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 rather 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 protein crystallography, 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: comparative modeling of the protein structure based on homology and similarity with proteins of already known structures. A serious objection, the 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 molecular mechanism of the enzyme function. This paper is focused on two selected proteins the structure-function relationships of which has been successfully addressed in our laboratory using combination of computer modeling with advanced biophysical techniques and molecular biology, namely Na^+/K^+-ATPase and 14-3-3 proteins. We used fluorescence spectroscopy techniques and molecular modeling (comparative modeling and molecular dynamics simulations) 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 α (~ 110 000 Da) and β (~ 55 000 Da). If the enzyme is purified from kidney, they are accompanied by a small peptide (~ 7000 Da), often referred to as γ-subunit, and these three are present in 1:1:1 ratio (Forbush *et al.* 1978, Collins and Leszyk 1987).

The 14-3-3 proteins are a family of conserved regulatory molecules, which specifically bind to the phosphorylated proteins and peptides (for review see Fu *et al.* 2000, van Hemert *et al.* 2001). Through these binding interactions, the 14-3-3 proteins play key regulatory roles in signal transduction, cell cycle control, metabolism control and apoptosis. Many of 14-3-3 binding partners contain one of two consensus motifs, Arg-Ser-X-pSer-X-Pro, and Arg-X-Tyr/Phe-X-pSer-X-Pro, where pSer denotes phosphoserine. Many organisms express multiple isoforms; for example, in mammals seven isoforms have been identified. All 14-3-3 isoforms can form stable homo- and heterodimers (Liu *et al.* 1995).

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 became known (Toyoshima *et al.* 2000, Toyoshima and Nomura 2002), 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 (Hebert *et al*. 2001) showed three large domains exposed to the cytoplasm. Sweadner and Donnet (2001) 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 are 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, correspond 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 have calculated and visualized the H_4-H_5 loop of Na^+/K^+-ATPase (Ettrich *et al.* 2001). 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. 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 (Hilge *et al.* 2003, Hakanson 2003), which allowed further rectification of the model (Lánský *et al.* 2004).

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 (Gatto *et al*. 1998, Tran and Farley 1999). 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 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 (Kubala *et al.* 2002, Gatto *et al.* 1998) and to hydrolyze very slowly p-nitro-phenylphosphate in Mg^{2+}-dependent way (Tran and Farley 1999). 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), further denoted as wild type (WT).

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, the 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 fluorescent analog of ATP, 2' (or 3')-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (further referred to as TNP-ATP), is sensitive to the environment polarity, which can be used for various assays of ATP binding to macromolecules (Hiratsuka 1975, 1976, 1982, Hiratsuka and Uchida 1973, Hiratsuka *et al.* 1973). The assay on ATP binding to Na^+/K^+-ATPase was confirmed by Moczydlowski and Fortes (1981) 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 (Kubala *et al.* 2003a). 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 H_4-H_5 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. Again, we derived an explicit formula for the probe fluorescence intensity, which is suitable for nonlinear least-squares analysis (Kubala *et al.* 2004).

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

The single ATP-binding site was identified on the N-domain (Fig. 1). It is constituted by the central part of the major cytoplasmic loop connecting transmembrane

helices 4 and 5 (roughly Arg^{378}-Arg^{589}) (Ettrich *et al.* 2001). Using the protein-reactive ATP analogs 2-azido-ATP and 8-azido-ATP (Tran *et al.* 1994a,b) 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) (Farley *et al.* 1984) and that fluorescence of FITC attached to this residue cannot be quenched by antifluorescein antibody (Linnertz *et al.* 1999) 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} (Scheiner-Bobis and Schreiber 1999) and Lys^{501} (Teramachi *et al.* 2002).

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 (Krumscheid *et al.* 2003). 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, has been used to evaluate changes of ATP and TNP-ATP dissociation constants caused by point mutations (Fig. 2). The estimated values of the dissociation constants for wild type protein (K_D = 3.1 ± 0.2 μM for TNP-ATP, and K_D = 6.2 ± 0.7 mM for ATP binding) (Kubala *et al.* 2002) were in good agreement with the results from other laboratories for similar proteins (Gatto *et al.* 1998, Tran and Farley 1999, Capieaux *et al.* 1993).

We have shown 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 (Kubala *et al.* 2002). 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 (Teramachi *et al.* 2002). 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.

Fig. 1. Computer model of the H_4-H_5-loop of Na^+/K^+-ATPase. The ATP-binding site is localized on the N-domain (Arg^{378}-Arg^{589}), which is clearly separated from the P-domain where the phosphorylation site (Asp^{369}) resides.

Another residue involved in the ATP binding pocket could be Gln^{482} as suggested by our computer model (Ettrich *et al.* 2001). 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 has resulted in a strong inhibition of both TNP-ATP and ATP binding (Kubala *et al.* 2003b). Molecular modeling proposed that the closest distance between this residue and ATP is 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 (Hilge *et al.* 2003). Residues Glu^{446}, Phe^{475} and Gln^{482} seem to be the most important ones for the interaction with ATP.

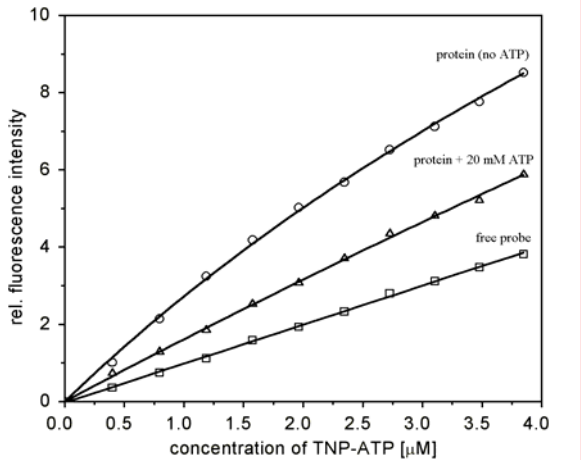

Fig. 2. Dependence of relative fluorescence intensity on TNP-ATP concentration. H_4-H_5 loop-GST-fusion protein (1.6 μM) was incubated with increasing concentration of TNP-ATP in 50 mM Tris-HCl, pH 7.5 (circles), or in 50 mM Tris-HCl + 20 mM ATP, pH 7.5 (triangles). For comparison, titration in 50 mM Tris-HCl in the absence of any protein is shown (squares) (Kubala *et al.* 2004). Binding of TNP-ATP to the protein results in an increase of fluorescence intensity. The presence of ATP in the solution inhibited this binding.

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 (Kubala *et al.* 2003b). Abu-Abed and coworkers (Abu-Abed *et al.* 2002) 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 (Fig. 3). This corresponds with the finding that mutations of Glu^{472} lead to a strong inhibition of Na^+/K^+-ATPase (Scheiner-Bobis and Schreiber 1999). 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} (Lánský *et al.* 2004). 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 (Lánský *et al.* 2004). Indeed, Pro^{489} is located in the loop connecting the third and fourth β-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 β-sheet. The third β-strand contains residues Lys^{480} and Gln^{482}, while the fourth β-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} (Patchornik *et al.* 2002) Lys^{480}, Lys^{501}, Gly^{502} and Arg^{544}, also Glu^{446}, Phe^{475} and Gln^{482} residues form 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 β-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} (Jacobsen *et al.* 2002), Asp^{710} or Asp^{714} (Ovchinnikov *et al.* 1987), 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.

Molecular mechanism of the regulation of 14-3-3 binding properties

The crystal structures of human 14-3-3 zeta and tau isoforms illustrated the conserved fold of the 14-3-3 proteins, where each monomer is composed of nine antiparallel helices, and two monomers form cup-shaped dimers with a large deep channel in the center running the length of the dimer. The walls of the channel contain amphipathic grooves that are ~30 Å long, and residues lining the grooves are mostly conserved among the different isoforms (Liu *et al.* 1995). Several modes of regulation through 14-3-3 binding have been proposed. They range from the control of the cellular localization of the target protein as has been observed for protein phosphatase Cdc25C, Cdc2/Cyclin B1 complex, or the insulin-regulated Forkhead transcription factors FKHRL1, AFX, to altering the intrinsic catalytic activity of the target protein as has been demonstrated for serotonin N-acetyltransferase (Obšil *et al.* 2001).

The main goal of our research was to understand the molecular mechanism of the regulation of 14-3-3 binding properties. Recently, we have studied the role of the C-terminal stretch in the regulation of binding properties of 14-3-3zeta. 14-3-3 C-terminal stretch is a flexible region with relatively high sequence variability among 14-3-3 isoforms. The structure of this region of 14-3-3 molecule is unknown because it cannot be seen in any of the available 14-3-3 X-ray structures, presumably due to its flexibility (Liu *et al.* 1995, Obšil *et al.* 2001). It has been speculated that 14-3-3 C-terminal stretch is involved in the regulation of ligand binding. X-ray structure of 14-3-3zeta suggested that its C-terminal stretch might interact with the cluster of basic residues at the top of the ligand binding groove (Liu *et al.* 1995).

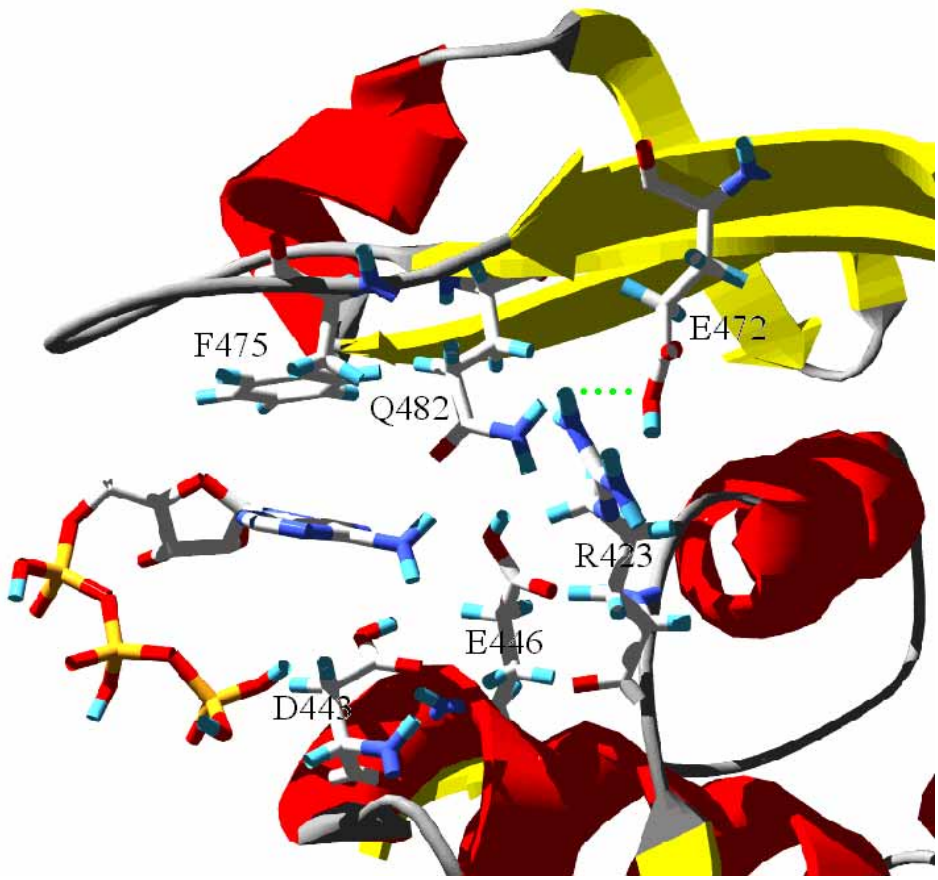

Fig. 3. Hydrogen bond between Arg^{423} and Glu^{472} supports the shape of the ATP binding pocket. Hydrogen bond between Arg^{423} and Glu^{472} over a distance of 0.17 nm brings the stretch of amino acids containing residues Phe^{475}, Lys^{480} and possibly also Glu^{482} (not shown) close to other residues involved in ATP binding, such as Glu^{446} and Lys^{501} (not shown).

Recently it has also been reported that C-terminally truncated 14-3-3zeta exhibits increased binding to several tested ligands (Truong *et al.* 2002). Based on these observations it has been proposed that the C-terminus can function as a suppressor of unspecific interactions between 14-3-3 and inappropriate ligands. Moreover, C-terminal stretch of vertebrate 14-3-3zeta and tau isoforms contains a phosphorylation site at position 232, which is known to be phosphorylated both in vitro and in vivo by casein kinase I (Dubois *et al.* 1997). Phosphorylation has been suggested to be an important factor affecting properties of individual 14-3-3 isoforms (Fu *et al.* 2000).

In order to understand the role of C-terminal stretch in the regulation of 14-3-3 function, we have investigated the effect of phosphopeptide binding and phosphorylation at Thr^{232} on 14-3-3zeta C-terminal stretch conformation using time-resolved fluorescence spectroscopy and molecular dynamics simulations (Obšilová *et al.* 2004). Single Trp residue (Trp^{242}) have been placed at the end of the C-terminal stretch and exploited as an intrinsic fluorescence probe of the C-terminal stretch dynamics. Other tryptophan residues were mutated to phenylalanine. Time-resolved fluorescence of Trp^{242} showed that the C-terminal stretch (residues 229-245) changes conformation and increases its mobility upon phosphopeptide pRaf-259 binding. Addition of this ligand to 14-3-3 results in a broadening of the lifetime distribution of Trp^{242} suggesting that in the presence of the phosphopeptide the Trp^{242} undergoes different set of interactions compared to unliganded 14-3-3. Moreover, measurements of the emission anisotropies revealed different hydrodynamic properties of the Trp^{242} in the presence and absence of the phosphopeptide pRaf-259. The phosphopeptide binding induces conformational change resulting in higher flexibility of the C-terminal stretch where the Trp^{242} is located. This observation is consistent with the appearance of a new peak in Trp^{242} excited-state lifetime distribution, which might indicate that more flexible C-terminus "scans" larger environment in the presence of the phosphopeptide.

In addition, we have studied the conformational changes induced by phosphorylation at Thr^{232} located within the C-terminal stretch of 14-3-3zeta. We have shown that phosphorylation of 14-3-3zeta at Thr^{232} by casein kinase I significantly inhibits its ability to bind phosphopeptide pRaf-259. These observations are consistent with previously described negative effect of 14-3-3zeta phosphorylation at Thr^{232} on Raf-1 binding (Dubois *et al.* 1997). Phosphorylation at Thr^{232} causes appearance of a new peak in the lifetime distribution located between 200 ps and 300 ps. This effect is independent of the presence or absence of the ligand. The pattern of the rest of the lifetime distribution is almost unchanged by the phosphorylation. The increased heterogeneity of the phosphorylated 14-3-3w242 fluorescence decay suggests that the excited W242 is subjected to a new quenching interaction as a consequence of the conformational change induced by phosphorylation at Thr^{232}.

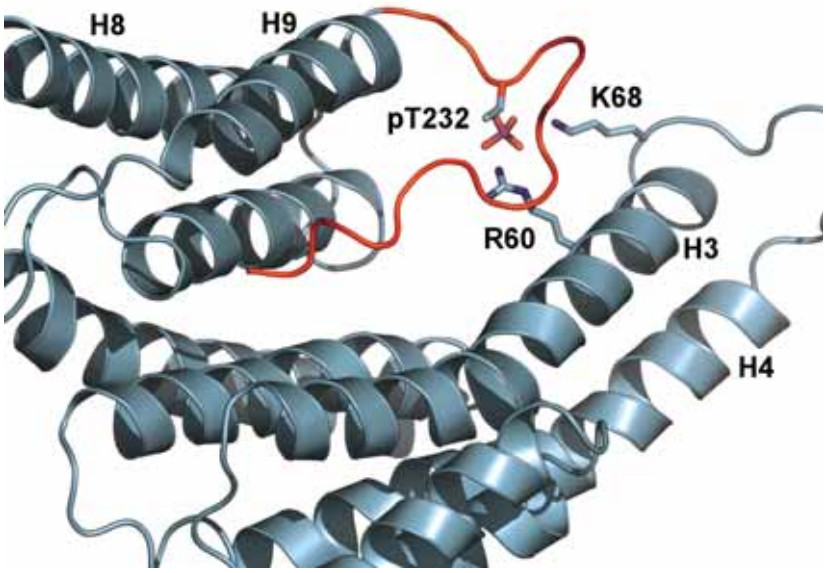

Fig. 4. Detailed view of the interactions between $pThr^{232}$ and positively charged residues located within the helix H3 of 14-3-3 molecule. The conformational behavior of 14-3-3zeta and Thr^{232} phosphorylated p14-3-3zeta was explored by analyzing the simulated trajectories (15 ns long MD simulations). The model with the end of the C-terminal stretch located within the ligand binding groove was used as a starting conformation. The representative conformations of 14-3-3zeta were obtained using cluster analysis.

In order to model the possible conformation of the 14-3-3 C-terminal stretch we have used molecular dynamics simulations to study the conformational behavior of 14-3-3zeta and 14-3-3zeta phosphorylated at Thr^{232} (Fig. 4). Crystal structure of the 14-3-3zeta suggested that the end of the C-terminal stretch might be located within the ligand binding groove interacting with the cluster of basic residues at the top of the groove (Liu *et al.* 1995). We have used this model as a starting conformation for our molecular dynamics simulations. The analysis of the simulated trajectories showed that the end of both non-phosphorylated and Thr^{232} phosphorylated C-terminal stretch remained bound within the ligand binding groove during the whole up to 15 ns long simulations. This is consistent with our observation that phosphorylation had little effect on flexibility of the C-terminal stretch. The most significant differences between non-phosphorylated and Thr^{232}-phosphorylated 14-3-3 involve the conformation of the C-terminal stretch and flexible loops between helices. The rest of 14-3-3zeta molecule remains relatively unchanged during the simulations and close to the positions it has in the starting structure. Phosphorylation at Thr^{232} induces more open conformation of the C-terminal stretch, which in comparison with non-phosphorylated stretch interacts with the residues within the C-terminal part of helix H3. In both cases the end of the C-terminal stretch is located in the vicinity of the cluster of basic residues in the middle of the groove, close to the positions it has in the starting structure. The hypothesis that phosphorylation at Thr^{232} induces more extended conformation of the 14-3-3 C-terminus was also tested by performing 20 ns long molecular dynamics simulations of both phosphorylated and non-phosphorylated peptide corresponding to 14-3-3zeta C-terminal stretch (from Thr^{229} to Asn^{245}). These simulations revealed that phosphorylated peptide adopts more extended conformation. Time evolution of the radius of gyration, R_g, defined as the mass-weighted root mean square deviation of a collection of atoms from their common center of mass, revealed that in the case of phosphorylated peptide more extended conformations are sampled during the whole simulation. These results support the hypothesis that the C-terminal stretch is located within the ligand binding groove. Such position would mean that the C-terminal stretch has to be pushed away from the ligand binding groove by the ligand molecule and thus can become more flexible in agreement with our fluorescence data. Since we have shown that the C-terminal stretch changes its conformation and mobility upon phosphopeptide binding, it is reasonable to assume that phosphorylation-induced conformational changes within this region could be responsible for the negative regulation of 14-3-3 binding properties.

Our results show that 14-3-3zeta C-terminal stretch is directly involved in the interaction of 14-3-3 with the ligand. Phosphorylation at Thr^{232} induces a conformational change of the C-terminus which is presumably responsible for observed inhibition of binding abilities. In addition, molecular dynamics simulations suggest that phosphorylation at Thr^{232} induces more extended conformation of 14-3-3zeta C-terminal stretch and changes its interaction with the rest of the 14-3-3 molecule. This could explain negative regulatory effect of phosphorylation at Thr^{232} on 14-3-3 binding properties. These results indicate that the conformation of the C-terminal stretch and its phosphorylation by casein kinase I play an important role in the regulation of 14-3-3 binding properties.

In conclusion, computer modeling with molecular dynamics simulation is a powerful method for visualization of protein structures. Our results clearly show that computer modeling in combination with spectroscopic techniques, such as steady-state or time-resolved fluorescence spectroscopy, and with molecular biology techniques could provide useful structural information in the cases where crystal or NMR structures are not available. The indisputable advantage of computer modeling is significantly less time and cost requirements. In addition, beside of the structure visualization, molecular dynamics simulation enables understanding of the segmental motion and, thus could help to elucidate the protein function on the molecular level.

Acknowledgements

This work was supported by Grants 204/03/0714, 309/02/1479, and 204/01/1001 of the Grant Agency of the Czech Republic, by Grant B5011308 of the Grant Agency of the Czech Academy of Sciences, by Grant 245/2003/B-CH/PrF of the Grant Agency of the Charles University, by Research Projects 1K03020, MSM 1131 00001 and 1132 00001 of the Ministry of Education, Youth and Sports of the Czech Republic, and by Research Project AVOZ 5011922.

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