1. Summary

The problems studied in this work are related to the question how small molecules affect the conformation of proteins upon functional binding. The complexes of two proteins were examined by molecular dynamics simulation.

1. The conformational effects of binding allosteric effectors on human hemoglobin (HbA). Lacking crystallographic data, we examined whether oxy-HbA is also able to bind allosteric effectors. We successfully determined the structure of the complex, and unraveled significant tertiary changes due to effector binding in the case of three allosteric effectors. The stability of the structures shows that the models are reliable. The analysis of the binding site in oxy-HbA showed specific like in the deoxy (T) state, but different in structure from it. The analysis of the subunit interfaces showed that effector binding decreased the stability of the HbA tetramer in the deoxy(T) and increased in the oxy(R) state.

2. The effects of binding antagonists in complexes in two kinds of Ca-Calmodulin (CaM) complexes: CaM-2TFP and CaM-2DPD. The x-ray structures of the two complexes were similar in the literature, but the dissociation constants differed by two orders of magnitude showing DPD a better antagonist. This contradicted the x-ray results. Based on the analysis of the structures of 12ns molecular dynamics simulations we found out, that the residues interacting with antagonists differ from those in the x-ray structure: the structure of the CaM-2DPD complex became more compact during the dynamics. According to electrostatic Poisson-Boltzmann calculations the desolvation terms are more favourable in case of DPD. Analysing the interactions of the amino acids, we can tell that the reason of the TFP's less efficiency is that its positive charge cannot form charge-charge interaction with the surrounding polar amino acids because the charged group is on a too short carbon chain. Therefore TFP molecule blocks these polar parts to interact with the solvent.

2. Introduction

Computational methods are widely used for the interpretation of the physical and chemical properties of solid and fluid systems based on their atomic structures. The molecular dynamics simulation (MDS) methods are especially important for the atomic level examination of the structure and interactions of biomolecules, which is the object of the thesis. Based on the atomic structures obtained from x-ray crystallography or NMR spectroscopy the methods use the classical Newtonian equations of motion to yield the atomic fluctuations at physiological circumstances. One advantage of using this approach is that the special experimental circumstances of the experimental methods can be corrected using MDS. Therefore the determination of the structure representing the native condition is possible. However, this is only one result of the simulations. It is even more important that every bond's flutuation can be obtained. The fluctuations of the atoms in the structure -conformational dynamics- has special properties, which are characteristic for a molecule or for part of a molecule. The proper conformational dynamics of biological macromolecules is an important prerequisite of their biochemical activity. The team where I did my PhD work, examines the dynamics of proteins by experimental and computational methods. My PhD work focuses on how small molecules –antagonists, allosteric effectors- change the molecular structure and dynamics of proteins in relation to their biological function. One system what I studied was human hemoglobin (HbA), where the biological function is regulated oxygen binding. It is known that allosteric effectors binding in the central cavity of the tetramer modify the oxygen binding. I was supposed, -based on X-ray crystallographic data- that effectors bind to the oxygen free „T" state and decrease the oxygen affinity. Recent literature results show that allosteric effectors can also regulate the oxygen affinity in oxygen-binding „R"-state HbA. However, there is no x-ray crystallographic structure of the R-state effector-binding complex has been published. At the

beginning of my work, I joined the studies aimed at determining the effect of allosteric effector binding to both R and T state HbA on the molecular structures and conformational dynamics.

In the second part of my work, I studied the Ca-regulated signal transducer protein, calmodulin (CaM). This protein can activate a great number of enzymes and therefore takes part in many biochemical processes. The blocking of CaM-target binding is one important approach in pharmaceutical drug design. The focus of my work was the examination of optimal properties of CaM antagonists using MDS and Poisson – Boltzmann free energy calculations.

3. Specific aims

The main question of my doctoral work was, how functionally binding small molecules (antagonists, allosteric effectors) affect to conformational properties of proteins. This general problem was studied with relation to two proteins.

3.1. HbA-allosteric effector complexes

The goal of this work was to examine wether R –state HbA, similarly to the T-state HbA, can form stabil complex structure with allosteric effectors. Three important effectors were examined: DPG, IHP and RSR13 We started from docking these molecules, and then examined if the structure proves to be stable energetically and dynamically. In this way we obtained the structure of the R –state HbA – allosteric effector complex. After this, our goal was to find parameters, which are related to the functionality of the new structure, and allow to compare it with the T state HbA – effector complexes. In cooperative oxygen binding the interaction and coupling of the interfaces plays an important role. Therefore the H-bonds, hydrophobicity and the, way of binding water molecules in the interface were examined.

3.2 CaM-antagonista komplexek

During my work I examined the structure of two CaM-antagonists complexes: CaM complexed with TFP, which is known possible as a pharmacon in neurology, and CaM complexed with DPD, which was designed for cardiac beat regulation. The starting point of this work was that the published x-ray structures of the two CaM-antagonist complex (in 1:2 stochiometry) are very similar, but their dissociation constants are significantly different. The dissociation constant in CaM-2TFP is 1-8μM, and in CaM-2DPD it is 18nM. According to the structures of the antagonists, the DPD molecules can only form van der Waals and hydrophobic interactions with CaM, but TFP has one positive charge, therefore its binding should be favorably supported by charge-charge interactions. However the dissociation constant shows that DPD binds to CaM stronger than TFP. The x-ray

crystallographic results can not explain this significant difference. The first question was, wether the two structures are really similar at 300K in water and in the presence of counterions. Secondly, we would have liked to examine in these conditions, why TFP binds weaker, though it has a positively charged group and DPD has only hydrophobic and polar groups? We hoped, that we can draw general conclusions, concerning the kind of optimal structural properties needed to block the activity of CaM.

4. Methods

4.1 Molecular mechanics and molecular dynamics approach

In this approach the energy is defined as the sum of the interaction energy between atom-pairs, therefore the starting point of this algorithm is the molecular structure obtained from x-ray or NMR spectroscopy. In this approach the interactions and movements of electrons are neglected (Born-Oppenheimer approach). One considers three types of interactions: covalent, van der Waals, and electrostatic. These parameters define the forcefield. The most commonly used forcefields are: GROMOS, AMBER, CVFF, ESFF, CHARMM. In our calculations we used CHARMM (Chemistry at HARvard of Molecular Mechanics) forcefield, which defines the potential energy (ϕ) the following way:

$$\phi = E_b + E_\theta + E_\varphi + E_\varpi + E_{vdw} + E_{el} \quad (1)$$

where the first four members are the kovalent binding energy terms (b: bond length, θ: angle, φ: dihedral angle, ϖ: out of plane angle), the last two members are the van der Waals and the electrostatic energy terms. In molecular dynamics the classical equation of motions are solved for atoms and molecules at every timestep, in order to get the time evolution of the system. The thermodynamical properties (temperature, pressure) are considered in Maxwell-Boltzmann approximation. The equation of motion from the Newton's second law is the following:

$$-\nabla_{r_j}\phi(r) = m_i \frac{d^2 r}{dt^2} \quad 1 \le i \le N \qquad (2)$$

Where $\phi(r)$ is the potential energy function, $\nabla_{r_j}\phi$ is the gradient of the potential energy function, m_i is the mass of the atom i. Equation (2) shows the link between the potential energy and Newton's second law.

4.2 Analysis of the trajectory

The primary result of the MDS is the fluctuation of the atomic coordinates during the simulation time. This series of data is the trajectory and characterized with different parameters. The first important parameter is the “root mean square deviation” ” (RMSD). This shows the average motion of the atoms during the trajectory related to a reference structure. In equilibrium, the RMSD fluctuates around a constant value. The radius of gyration shows the average distance between the atoms and the centre of mass. This parameter also related to the volume of the molecule in a globular approximation. The fluctuation of the specific parts in the molecule is described by the RMSF (root mean square fluctuation). This parameter is comparable with the B-factors obtained from x-ray crystallography, therefore it allows to compare the results of MDS with the experimental results. Another important parameter, the solvent accessible surface area (SASA) was calculated the following method. A sphere with 1.4 Å (representing the water molecule) probe radius is poured on the surface of the molecule and the areas touched by the probe are summarized. The free energy of the binding can be calculated with the subtraction of the free energy of the solvated antagonist and the solvated protein from the solvated complex. This way the hydrophobicity of atomic regions can also be characterized. These calculations would need the knowledge of the absolute free energy functions. Instead of those functions we described the free energy change using the method described by Karplus. The electrostatic potential functions were described using Poisson-Boltzmann equation. The calculations were performed for several dielectric constant values. During the analysis, we examined, how the binding free energies of the two antagonists differ from each other in the structure obtained from x-ray crystallography and MDS.

5. Results

5.1 Results on HbA

The first step of the work on HbA was examining the binding of DPG, IHP and RSR13 to the R state structure. When I joined the team, the three docked structures had already been determined. I started with performing 500ps and 2ns MDS on these structures. The simulation resulted in structural rearrangements as could be seen from the RMSD related to x-ray crystallography data. The high RMSD values show that all of the allosteric effector changed their own conformation and the structure of the protein. DPG and IHP binds to the same site in the central cavity of the R-state HbA. The structure of the complex is stabilized by hydrogen bonds formed with α2-arg141, α1-arg141, α2-lys99, α1-lys99 and with the bridging water molecules in the central cavity. The binding site is analoguos with that in T-state HbA. The binding site of RSR13 is different from the two natural allosteric effector's binding sites. Only the methyl-propionic acid forms a few H-bond with the α2-arg141, α1-lys99 and the water molecules in the central cavity. Its aromatic ring is connected to the β2-tyr35's aromatic ring. Furthermore it forms hydrophobic interactions with α2-leu29, α2-leu34, and β2-val34. The binding sites on the two α and one β subunit is analogous with the RSR13 binding site in T-state HbA. During the 500ps MDS none of the effectors leave their binding sites, which shows the stability of the docked structure. The RMSD time averages of the residues show, that apart from the terminal residues, the $\alpha_1$36-$\alpha_1$51, $\beta_1$41-$\beta_1$53, $\beta_1$81-$\beta_1$87, $\alpha_2$37-$\alpha_2$75, és a $\beta_2$47-$\beta_2$92 ranges in the DPG complex, $\beta_1$39-$\beta_1$49, $\beta_1$79-$\beta_1$84, és $\alpha_2$50-$\alpha_2$64 ranges in the IHP complex are also significantly mobile. This also shows that as a result of the effector binding the tertiary structure changes in the protein. In case of RSR13 the fluctuations have maximum in $\beta_1$39-$\beta_1$87 and $\beta_2$50-$\beta_2$80 regions. Comparing with the fluctuation of effector free R-state HbA, it appears that the fluctuations without effectors are less. According to these results, the binding of effectors causes changes in the tertiary structure and in the

dynamics. We think this plays an important role in the regulation of oxygen affinity.[1]

In the next step of the work 2ns molecular dynamics simulation was performed on the R and T state HbA in the presence and in the absence of chloride ions and on the R and T state HbA complexed with DPG. The most important result of the simulation was that DPG-binding changes the structure of the interface and also the interactions between the amino acids and the water molecules in the interface. After the simulation, the R state HbA in chloride free circumstance has an interdimeric interface of large volume, containing a large number of water molecules and few H-bonds. In the presence of chloride ions, the volume of the interdimeric interfaces decreases and the number of H-bond increases. The effect of DPG binding the number of H-bonds even more increases, but the number of the bridging water molecules decreases. The gap volume and the SASA does not change significantly. The effector binding causes less perturbation in the structure of R-HbA. The trend in the T state HbA is the opposite of the R state trend. The effector binding causes an increase on the gap volume and the water molecules within the interface and this decreases the stability of the tetrameric structure. These simulation results agree well with the group's experimental K_d values.[2]

5.2 The results on CaM

The structure of CaM after 12ns of simulation was examined by analyzing the RMSD, the radius of gyration and the atomic fluctuations. According to the RMSD of the two CaM antagonist complexes, the backbone of the protein became stable after ~5ns. The central helix of both complexes are stable during the 12ns MDS. Large rearrangements can be observed, however on the two Ca-binding domains, especially in case of the TFP complex. The structure of the antagonists also change significantly. In the case of the DPD complex, the change is more significant than in the case of the TFP complex. We also examined the the RMSD time averages per residue during the last nanosecond. The dynamic properties were more

significantly different from each other; the two kinds of antagonist influence the dynamics of the protein at different sites.

According to the atomic fluctuations (RMSF) and the B-factors obtained from the x-ray structure the structure including dynamics showed more mobility of TFP. Radius of gyration were also examined in case of both complexes. These show significant effect of MDS in the case of the CaM-2DPD complex. In the case of TFP, the change is not significant. Because TFP has a positive charge the CHARMM interaction energies were higher. The highest term was the electrostatic interaction. This term is low in the case of the neutral DPD. However, the comparison of van der Waals energy terms shows an opposite trend. We examined the H-bonds and the hydrophobic interactions. These show that the binding site changes in both cases during the dynamics. The change is more significant in the case of the TFP complex: TFP1 leaves the C-domain and will only be connected to the N-domain and the central helix, while TFP2 interacts only with the C-domain. In the N domain Glu11 and Glu14 has an important role.

According to Poisson-Boltzmann free energy calculations, in the case of each dielectric constant, after dynamics the binding free energy change was more favourable in CaM-2TFP. The direct charge-charge interaction was higher for TFP than for DPD in the case of the structure obtained both from x-ray crystallography and from MDS. However, the high desolvation term almost compensates the favourable direct interaction. These energy values did not show that the DPD binding would be more favourable. Therefore we examined in more details, how the amino acids surrounding the binding site contribute the ΔG. It seems that Glu11 and Glu14 approaches the piridyne N of the TFP1 and TFP2. A polarized methyl group also connects to the same N and this decreases the direct charge-charge interaction. This is also shown by the high desolvation term, which suggests that the interaction with water of glutamic acids would be more favourable. The carboxil oxygen of Glu10 is attracted by the TFP2, buti n this way this group gets nearer to the Met124 sidechain, which is an unfavourable effect. That shows why

the direct charge-charge interaction is not effective, it is accompanied an unfavourable desolvation term. Therefore the fitting of the two TFP molecule is problematic. The fitting of DPD becomes more favourable during the dynamics. Although the direct charge-charge interactions are much less favourable than in case of TFP complex, the favourable desolvation term compensates it. Interestingly, the desolvation term was more favourable in case of polar amino acids, where the appropriate contacts with the solvent could be formed. In order to determine the hydrophobic interactions, we examined the solvent accessible surface area in the structures in the presence and in the absence of antagonists we analysed the difference of the two SASA values especially for the hydrophobic amino acids. The high ΔSASA values show significant hydrophobic interactions. Our results show that DPD forms more hydrophobic contacts than TFP. Furthermore, these contacts become stronger in the case of CaM-2DPD and weaker in the case of CaM-2TFP.[3]

6. Conclusions (only the most important conclusions of the thesis is listed bellow)

6.1 HbA

In this work we examined if R-state HbA can form stabil complex with allosteric effectors. We also examined the binding site in the R state. After that we also examined, how the effector binding changes the interdimeric interfaces.

- R state HbA complexes with allosteric effectors are stable, and –similary to the T state- they also form specific effector binding sites in the central cavity. However, these binding sites are not the same in the R-state and in the T-state.
- The effector binding changed the stability of the tetramer differently in R and T state HbA. It decreased the stability in T state and increased in R state. In R state, the dimers bind stronger to each other due to the effector binding, while in T state it becomes weaker. This conclusion is supported by experimental K_D values of dissociation at the dimmer interface.
- There are large rearrangements in the interdimeric interfaces.

6.2 CaM

The goal of this work was to examine the efficiency of the CaM blocking in two CaM-antagonist (CaM-2TFP and CaM-2DPD) complexes with MDS and Poisson-Boltzmann free energy calculations. According to the literature, the two 1:2 CaM: antagonist complexes have very similar x-ray structure, but different dissociation constants. We examined the reason of this difference. We also would have liked to draw general conclusions, what properties are needed for blocking CaM efficiently.

- The results of the simulation (RMSD and the two structures) show that the two structures differ significantly after the dynamics. DPD complex became more compact. DPD fits more into the binding site due to its favourable hydrophobic interactions.

- The desolvation energy of binding site amino acids also show that DPD binds stronger to CaM. [3] The structure of TFP and the binding site does not allow the closer charge-charge interaction with the binding site of CaM. According to the structure and the free energy the molecule, which has a positive charge on a long chain can inhibit CaM most effectively.

7. Articles

7.1 My articles used for the basis of my thesis

1. M. Laberge, **I. Kövesi**, T. Yonetani, J. Fidy. R-state hemoglobin bound to heterotropic effectors: models of the DPG, IHP and RSR13 binding sites *FEBS Letters* 579, 627-632 2005

2. **I. Kövesi**, G. Schay, M. Laberge, T. Yonetani, J. Fidy. High pressure reveals that the stability of interdimeric contacts in the R- and T-state of HbA is influenced by allosteric effectors: Insights from computational simulations *Biochimica et Biophysica Acta-Proteins and Proteomics* 1764 (3): 516-521 2006

3. **I. Kövesi**, D. K. Menyhard, M. Laberge, J. Fidy. Interaction of antagonists with calmodulin: insights from molecular dynamics simulations. *J. Med.Chem* 51(11):3081-93 2008

7.2 Other article

Laberge M, **Kovesi I**, Yonetani T, Fidy J.
Normal mode analysis of the horseradish peroxidase collective motions: correlation with spectroscopically observed heme distortions. *Biopolymers.* 82(4):425-9 2006