Chapter 5

Concluding remarks

In this thesis several Molecular Dynamics (MD) simulations have been applied to phospholipase A$_2$ (PLA$_2$). These simulations were used to investigate the behavior of PLA$_2$ in several environments. These are an aqueous environment (chapter 3), an aggregated substrate environment (chapter 3), and an aggregated environment where a substrate binding to the protein is simulated (chapter 4). The behavior of the protein in these different environments is described in the following sections followed by a discussion of the binding of phospholipid substrates to the protein. This chapter ends with an outlook and suggestions for further investigations.

5.1 Aqueous versus aggregated environment

The difference in behavior of PLA$_2$ between an aqueous and an aggregated environment was studied by performing two simulations. The simulation in an aqueous environment is that of PLA$_2$ free in solution (the pw simulation in chapter 3). The simulation in an aggregated environment is that of PLA$_2$ in the presence of a monolayer of 1,2-diacyl-sn-glycerol-3-phosphocholine (DDPC).

In order to generate a starting conformation for the simulation of the protein and the interface, the optimal starting orientation was calculated. The rotation of a protein does not take place within the time scale of molecular dynamics simulations. Therefore a simple algorithm, calculating the nonbonded interaction energy between a rigid protein and a rigid monolayer, was used. The result of these calculations is an energy map as a function of protein orientation. This map showed three low energy regions. One of these regions, the largest, was an orientation where the opening of
the hydrophobic cleft was oriented towards the monolayer surface. This orientation was then selected for the subsequent MD simulation of the protein and monolayer. This docking method seems to work quite well for this system. The main question is then if it will also work for other systems. It is likely that the validity of the method depends on the type of objects being docked onto each other. Two large surfaces involving many interactions may give reliable results, but surfaces with small contact areas will be more sensitive to the details of the interactions included in the docking method. Specifically, the incorrect treatment of water may cause artefacts in such cases.

The differences in conformation of PLA in aqueous environment and aggregated environment are clear but not large. The most important difference between structures in these two environments is the conformation of the N-terminal helix. Where the N-terminal helix shows some unfolding motions in aqueous environment, the helix is stable in the presence of the monolayer. In the aggregated environment, the N-terminal helix is rotating in a direction more perpendicular to the monolayer and more oriented towards the calcium binding loop. Through the connection of the N-terminal helix to the rest of PLA the monolayer side of the protein, where the surface loops are located, also shows a conformational change. The result of this change is a different conformation of the opening of the hydrophobic cleft of PLA. The size of the opening of the hydrophobic cleft is slightly smaller compared to that of PLA free in solution.

In the simulation of PLA and aggregated substrate a strong interaction existed between protein and monolayer. Both surface loops of the protein (residues 17 to 23 and residues 62 to 72) show an overall favorable interaction with the monolayer, and thus are supposed to be very important for the binding of PLA to the monolayer. While most residues show a favorable interaction with the monolayer, some residues, Lys62, Phe63 and Leu64, have a repulsive interaction with the monolayer. This can be an explanation for the fact that the enzymatic activity of PLA increases upon deletion of residues 62-66.9

When the protein approaches the monolayer, the latter shows a reorganisation. The phospholipids aggregate more strongly “underneath” the protein and their head groups orient preferentially with the phosphate directed towards the positively charged calcium ion and the N-terminal helix.

5.2 Substrate binding

After a stable conformation of PLA attached to a monolayer is formed (see chapter 3), the next step of the action of PLA is the binding of individual substrates to
the protein. Because the time scale of substrate binding (between a millisecond and seconds) is not within the nanosecond time scale of present MD simulations, a simulation was setup in such a way that a single phospholipid substrate molecule was forced into the active site. This method applies a constraint force between two selected groups of atoms, the substrate and the active site, and constrains the center of mass of these two groups with respect to each other. The constraint is only applied between the centers of mass of each group and thus gives the involved atoms maximum freedom. By slowly decreasing this constraint distance, the substrate is moved into the active site. The result of this ‘pulling’ simulation is thus a conformation of a substrate present in the active site. An unconstrained MD simulation was conducted in order to study the stability of the result of the ‘pulling’ simulation.

The result of the ‘pulling’ simulation is very satisfactory. The resulting conformation of the substrate in the active site corresponds to what is expected from X-ray conformations of inhibited PLA2. Nevertheless, the constraint forces needed to move the phospholipid into the active site are very high and far from equilibrium due to the fast pulling speed. The conformation of the head group shows perfect agreement with the experimentally available crystal structures. The two oxygen atoms of the phospholipid have replaced two water molecules and have become ligands of the calcium ion. Another fact that supports the correct binding mode of the phospholipid is the catalytic triad. The Asp-His-H2O triad is formed during the ‘pulling’ simulation and remains stable throughout the complete unconstrained simulation. The following step, the chemical catalysis reaction, is now possible.

The ‘pulling’ simulation displays the importance of Tyr69. This residue binds to the phospholipid substrate at a very early stage of the simulation and forms a hydrogen bond which remains stable throughout the unconstrained simulation. The conformational changes of the side chain of Tyr69 suggest that this residue guides the substrate to the active site and ensures, through the early binding, a proper orientation of the phospholipid. Furthermore the conformational changes of Trp3 and Tyr69 during the ‘pulling’ simulation suggest that these residues function as a small flap covering the hydrophobic cleft of PLA2.

The conformation of the tail atoms of the phospholipid is not the same as that seen in X-ray structures of inhibitor-bound PLA2. There may be several reasons for this:
1. The location of the opening of the hydrophobic cleft of PLA2 is slightly changed (see simulation of PLA2 in an environment of aggregated substrate) resulting in a different conformation of the backbone.
2. The atomic structure of the inhibitors is not the same as the substrate in our simulations; this makes a good comparison difficult.
3. Inhibitors have an other function than substrates. As a result of that, the binding mode of an inhibitor and a substrate molecule to PLA2 do not have to be the same.
5.2.1 Rate of product accumulation

An expression was derived which relates the rate of product accumulation to the potential of mean force and the local diffusion constant of the phospholipid. Both these quantities can be calculated from constraint force simulations. The constraint force is equal to the derivative of the potential of mean force, and the local diffusion constant is related to the autocorrelation function of the constraint force. The data needed for these calculations is sampled from more than 17 ns MD simulation with a fixed constraint for several distances. The calculated maximum rate of product accumulation is \( 1.1 \cdot 10^7 \) phospholipid molecules s\(^{-1}\) protein molecule\(^{-1}\) is comparable to the experimentally determined value of \( 1.40 \) phospholipid molecules s\(^{-1}\) protein molecule\(^{-1}\). It should be noted that the error in the rate is expected to be of the same order as the rate itself. This is deduced from the errors measured from the constraint force experiments which are quite large. The errors originate from the limited sampling time of the constraint force simulations, and can thus be reduced by elongating these simulations.

5.3 Future experiments

As a result of the investigations presented in the previous chapters of this thesis, several ideas for future experiments will be suggested and discussed in this section.

We calculated the rate of product accumulation for phospholipase A\(_2\). However since the error of these calculations is quite large, extended simulations could be performed in order to increase the accuracy of the result. Instead of reducing the error, the influence of the substrate or protein on the catalytic rate can be investigated by using a different substrate or a mutated PLA\(_2\). Other forms of PLA\(_2\) can also be analyzed for their interaction with aggregated substrate.

The constraint force method that pulled the phospholipid into the active site resulted in a good substrate binding conformation to PLA\(_2\). Since such ligand binding is very interesting from a medicinal chemistry point of view, further investigation of this method is needed. An advantage here is that no external local bias is introduced into the system, which makes an automatic docking procedure feasible.

As the investigation of phospholipase A\(_2\) as described in the previous chapters ends with the substrate binding process, the most obvious next step is the analysis of the chemical reaction, i.e. the breakage of the sn-2 ester bond of the phospholipid substrate located in the active site of the protein. This process can be studied by using methods which combine quantum mechanics in MD simulations\(^{122,123}\) and should in this case involve a quantum-dynamical treatment of the proton transfer step from the catalytic water to histidine.
Besides these suggestions for other computer simulations, there are also some suggestions for real experiments. The most interesting is perhaps the validation of the movement of the N-terminal helix during monolayer binding. By mutation, it should be possible to create a disulfide bridge in such a way that the N-terminal helix is not allowed to change its orientation. By analyzing the substrate binding capability of such a mutated PLA$_2$ the validity of the suggested reorientation of the N-terminal helix can be tested. As it is expected from the simulations reported in this thesis, the binding of PLA$_2$ to aggregated substrates results in a reorientation of the N-terminal helix. When this reorientation is blocked for instance by introducing disulfide bridges, the binding mode of the protein towards aggregated surfaces might be reduced.

Another interesting experiment might be a mutation of Tyr$_{69}$ into another residue that can have the same function, i.e. guiding the phospholipid to the active site. If for example Tyr$_{69}$ is replaced by a lysine or the even smaller serine group, the specificity of the protein might be retained. As is expected from our simulations, the surface loop (residue 62 to 72) is flexible enough to compensate for the difference in position of the essential hydroxyl group. Another advantage may be that the phenyl group of tyrosine 69 that causes steric hindrance is removed, which results in an increased catalytic power of the protein.