X-ray crystallographic studies on structure and action of phospholipase A2.
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7. Summary and conclusions

7.1. The chapters of this thesis

In this thesis we attempted to shed some more light on structural aspects of the action of phospholipase A₂ (PLA₂). The enzymes studied in this thesis all belong to the class of extracellular PLA₂'s, but as mentioned in the introductory chapter, we have reasons to believe that results obtained with such enzymes, at least partially, also hold for the intracellular PLA₂'s, which turned out to play important roles in the regulation of various cellular responses.

In the first chapter we give a short overview over the most important functional and structural properties of pancreatic phospholipases A₂.

In chapter 2 we describe the re-determination of the structure of porcine pancreatic phospholipase A₂ at 2.64 Å resolution using molecular replacement methods. This is an extended version of the paper by Dijkstra et al. (1983a). In contrast to an earlier report (Drenth et al., 1976) and as expected from the high degree of homology in amino acid sequence, the new structure displayed great structural similarity with the bovine enzyme determined by Dijkstra et al. (1978, 1981a). During the refinement it became obvious, that the crystals contained the mature enzyme and not the pro-enzyme that had been set to crystallize. The geometry of the active site of the porcine enzyme is virtually identical to that of the bovine enzyme. The largest differences, including the disappearance of a short helix, are found in a region close to the N-terminus, comprising residues 58-70. This observation is very intriguing, because within this range of 13 residues, the only amino acid difference is the substitution of Val 63 in the bovine enzyme by a Phe in the porcine enzyme. It also illustrates the difficulties involved in the prediction of secondary structures from amino acid sequence information only. As pointed out by Dijkstra et al. (1983b), a paradox is created by the finding that proteins with as little as 20% identity in the amino acid sequence display a very similar folding (e.g. sperm whale myoglobin and the subunits of horse haemoglobin), whereas such a small local change in amino acid properties, as requested by the substitution of a Val by a Phe within a peptide of 13 residues, can lead to a completely different conformation of the surrounding region.
The comparison of the structures of bovine and porcine PLA2, with special emphasis on the interface recognition site (IRS) as proposed by Dijkstra et al. (1981b) did not clearly reveal other specific structural differences which might explain the observed differences in kinetic properties between the two enzymes. Therefore we ascribe these differences mainly to the more positively charged IRS of the porcine enzyme. This hypothesis is strongly supported by the observation that a semisynthetic bovine PLA2 with Arg instead of Asn at position 6 (Van Scharrenburg et al., 1981) shows kinetic properties very similar to those of the porcine enzyme.

In chapter 3 we describe the determination and refinement of the structure of bovine phospholipase A2 covalently modified with p-bromo-phenacyl-bromide. Volwerk et al. (1974) had shown that this reagent specifically modifies His 48 of the active site covalently. The modified enzyme becomes inactive due to the inability to bind monomeric substrates and Ca\(^{2+}\). However, its interaction with aggregated substrates is virtually unaffected. Crystals of this derivative had grown under the same conditions as for the native enzyme, and were found to be nearly isomorphous with those of the bovine pro-enzyme. Detailed knowledge of the interactions of the modifying group with the active site of phospholipase A2 may provide a good starting point for the design and development of phospholipase A2 inhibitory drugs. Such drugs are very much sought after because of their possible effects and other unwanted cellular responses to exogenous stimuli involving the liberation of arachidonic acid by intracellular PLA2's. Therefore, the structure of the modified enzyme was determined at 2.5 Å resolution and refined to a crystallographic R-factor of 0.25. Data were collected from one single crystal of 0.2 x 0.2 x 0.2 mm\(^3\) by the screenless oscillation film method. Comparison of the refined model with the structure of the native enzyme shows that no great conformational changes occur in the active site and the hydrophobic residues surrounding it. As expected the modifying group was found to make favourable interactions with the hydrophobic wall surrounding the active site, especially with the side chains of the absolutely conserved residues Phe 5 and Phe 106, and the disulfide bond between Cys 29 and Cys 45. Large conformational differences, however, were found for the residues 63-71, which are some 15 - 20 Å removed from the modified residue. How these long range effects are transmitted is not completely clarified yet, but it appears that small differences in hydrogen bonding systems play an important role. It is noteworthy that the differing region of the
structure coincides with that part which has also a different conformation in the porcine enzyme, and due to disorder is not visible in the structures of the bovine pro-enzyme (Dijkstra et al., 1982) and of the N-terminally transaminated bovine enzyme (Dijkstra et al., 1984). A possible function of the apparent conformational freedom of this part of the structure of pancreatic phospholipases with respect to their action will be given further below.

In chapter 4 we present the results obtained with crystals of bovine pancreatic phospholipase A2 grown under standard conditions in the presence of a non-hydrolysable glycerosulfate substrate-analogue. Also in this case the crystals turned out to be nearly isomorphous with those of the bovine pro-enzyme. Unfortunately, no substrate-analogue molecule could be detected and the change in crystal form most probably was not due to binding of the substrate-analogue in the active site. Additionally, we found that also in this case the region of residues 63-72 is so disordered or mobile that there is no interpretable electron density for this part of the structure. Taken together, these findings suggest that, under the crystallization conditions used, this substrate-analogue does not bind to the active site of bovine PLA2, but rather interacts with residues at the surface of the molecule, inducing a crystal form different from that of the native enzyme alone.

In chapter 5 we compare the crystal structure of bovine pancreatic phospholipase A2, refined at 1.7 Å, with that of the dimeric enzyme from the venom of a rattle snake (Crotalus atrox), refined at 2.5 Å resolution. The latter has been reported to be active in a dimeric form only. The global folding patterns of the two enzymes are very similar, except for regions around insertions and deletions. The three major helices and much of the polypeptide chain without secondary structure form a "homologous core", which is nearly identical in both cases. Also the two-stranded beta-structures are strikingly similar in both enzymes, although their orientation with respect to the remainder of the molecule differs due to the absence of Cys 11 and Cys 77 in the snake venom enzyme. In the pancreatic enzymes these residues form a disulfide bridge. The atoms of the absolutely conserved residues His 48, Tyr 52, Tyr 73 and Asp 99, which form an also conserved internal hydrogen-bonded network, and the hydrophobic wall surrounding it, are virtually superimposa-
ble in the two structures. The highly conserved Ca\(^{2+}\)-binding loop has different conformations due to the absence of Ca\(^{2+}\) and the formation of dimer stabilizing interactions in the venom enzyme crystals.

An interesting feature is the deletion of 8 amino acids in the region corresponding to residues 56-65 of the pancreatic enzyme. The resulting shortening of the chain allows for the dimer formation of the venom PLA2. A possible functional significance of this difference will be given further below. A somewhat puzzling observation from a functional point of view is the fact that the access of substrates to the active site is substantially shielded by inter-subunit contacts in the dimer of the snake venom PLA2. Together with the absence of a bound Ca\(^{2+}\)-ion, this finding suggests that the observed conformation is not necessarily that of a catalytically active dimer.

In chapter 6 we present the first results obtained with data from a \textit{FAST Area Scanning TV-detector} (FAST) diffractometer. With this device diffraction pattern intensities can be collected at much higher rates and with higher efficiency than with ordinary diffractometers and without the need for time consuming digitization and evaluation of large numbers of oscillation films. Crystals of bovine pancreatic PLA2 grown under normal conditions (50\% v/v MPD) and subsequently soaked in a 30\% v/v methanol solution were chosen as a test case. Lipid binding to crystals of bovine phospholipase A\(_2\) under standard crystallization conditions is found to be extremely poor and might be improved by a more polar environment. Therefore we transferred crystals grown under standard conditions to a solution containing methanol instead of the MPD in the same buffer solution. The crystals remained intact under these conditions. Intensity differences were observed on precession photographs, while the cell dimensions were not noticeably affected. To examine the structural differences leading to these intensity differences, a three-dimensional data set to high resolution (1.75 Å) was collected with the FAST-diffractometer. The data obtained from this instrument were judged to be of good quality based on R\(_{\text{sym}}\)-values (on F) in the range of 0.033 - 0.068 for the various partial data sets and an overall R\(_{\text{merge}}\)-value of 0.034 (on F) after scaling. The R-factor between the data from normal crystals collected on a CAD4-diffractometer and the new data was 0.146 on some 7400 structure factors. A similar comparison of CAD4- and FAST-data
for crystals from MPD-solutions only gave an R-factor of 0.064.

The refinement of the bovine PLA2 model against the new data brought the R-factor from an initial value of 0.276 down to 0.183. No major conformational changes with respect to the starting model were found, but rather small changes spread over the whole molecule, amounting to an r.m.s. difference in atomic positions of 0.66 Å for the 958 protein non-hydrogen atoms, including the Ca$^{2+}$-ion. The largest fraction of this difference comes from a reorientation of a few side-chains at the surface of the molecule. Thus, significant differences in diffraction pattern intensities may be due to only minor structural changes.

7.2. General conclusions

The three-dimensional structure of secretory phospholipases A$_2$ from three different species are now known: The bovine pancreatic enzyme and its pro-enzyme, the porcine pancreatic enzyme and the enzyme from the venom of the rattle snake *Crotalus atrox*. These three enzymes show high degrees of homology in their amino acid sequences, which are even surpassed by their structural similarities. Significant structural differences were mainly due to insertions or deletions.

In the remainder of this chapter we want to address two important questions:
1) How is the interaction of the enzyme with monomeric substrates?
2) What can we say about the interaction of the enzymes with aggregated substrates?

Concerning the first question, we have shown that the active sites of all PLA2's with known three-dimensional structure at this time are virtually identical. This means that most probably the interactions of these enzymes with monomeric substrates are identical. Unfortunately, from our studies with the glycerosulfate analogue we could not draw a firm conclusion on how an inhibitor very similar to an actual substrate molecule binds to the active site of phospholipase A$_2$. The only result pertaining to this issue comes from our studies with the p-bromo-phenacyl modified bovine enzyme. There we have shown that Phe 5, Phe 106 and the disulfide bond between Cys 29 and Cys 45 very probably play an important role in the hydrophobic interaction between enzyme and monomeric substrate molecules. Especially the involvement of the absolutely conserved disulfide bond is very interesting.