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The three-dimensional structure of notexin, a presynaptic neurotoxic phospholipase A_2 at 2.0 Å resolution

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The three-dimensional structure of notexin has been solved by molecular replacement methods. The structure has been refined at 2.0 Å resolution to a crystallographic R-value of 16.5% with good stereo-chemistry. The core of the protein is very similar to other phospholipase A_2s (PLA_2s) but several parts of the molecule are distinctly different. The most significant differences from PLA_2s from bovine pancreas and rattlesnake occur in the stretches 56–80 and 85–89. Residue 69, which has been shown to be important for phospholipase binding, has a different conformation and different interactions than in other known PLA_2s. The Cα positions for residues 86–88 differ by about 6 Å from both the bovine and the rattlesnake enzyme. The crystals contain no Ca^{2+} ions. Instead, a water molecule occupies the calcium site.

X-ray crystallography; Molecular replacement; Refinement; 3D structure; Phospholipase A_2; Snake venom

1. INTRODUCTION

Notexin is a presynaptically neurotoxic and myotoxic protein from the venom of the Australian tiger snake *Notechis scutatus scutatus* and was the first PLA_2 toxin to be characterized in terms of amino acid sequence [1]. The molecule is a single peptide chain of 119 residues cross-linked by 7 disulphide bridges. The toxin causes rapid death through asphyxiation by presynaptic blockade of transmission across the neuromuscular junctions of the breathing muscles. Other peripheral cholinergic junctions are affected as well. Beside this blocking action on nerve terminals notexin shows a strong direct myotoxic action upon intra-muscular injection. However, the presynaptic activity of notexin is so potent that intravenous injection of lethal doses into mice kill the animal before other symptoms appear.

The presynaptic effects of notexin are characterized by gradual reduction to a total cessation of acetylcholine release from the poisoned nerve terminals. Irregular spontaneous release of transmitter producing short bursts of miniature end plate potentials, sometimes of giant amplitude, are usually observed at some stage prior to the total shutdown. Ultrastructural examination of nerve terminals at an early stage of poisoning by notexin revealed a reduction in the content of synaptic vesic les and the presence of vesicles of abnormally large size [2]. At later stages of intoxication the nerve terminals were shrunken and devoid of vesicles and the axolemma showed numerous omega-shaped identations which were thought to represent arrested vesicle reformation. The blocking action of notexin was thus attributed to impaired recycling of vesicles rather than to direct interference with the release mechanism.

The functional differences between notexin and other PLA_2s are not obviously attributable to differences in amino acid sequences. Therefore we have initiated a site-directed mutagenesis investigation. To place these studies on a firm basis we have determined the three-dimensional structure of notexin.

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2. MATERIALS AND METHODS

Notexin purified as described [3] was crystallized using hanging drops. The best crystals were obtained when a protein solution with half of the precipitant concentration of the well was equilibrated against 1.4 M ammonium sulphate buffered with 50 mM CHES-buffer at pH 7.8 with 2% dioxane present.

The space group of the crystals was determined from diffraction data collected on a Xentronics multiwire area detector (Nicolet) mounted on a Rigaku rotating anode. The collected frames were evaluated using the Buddah program [4] and a trigonal cell was compatible with all measured reflections. The data-set from a 90° oscillation was further analysed on a Silicon graphics 4D workstation using the Precess program by W. Furey (Pittsburg). The space group was found to be $P3_121$ or the enathiomorph $P3_221$. The cell dimensions were a=b=74.6 Å, c=49.0 Å and β=120°. The crystals contain one monomeric notexin molecule per asymmetric unit, and are very similar to those described earlier by Kannan and co-workers [5], although the crystallization conditions are somewhat different.

A data-set to a resolution of 2.0 Å was collected. A total of 32,518 reflections were collected and were merged to 10,387 unique reflections using the Rotavata/Agrovata procedures (CCP4, Daresbury,

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                1         10         20         30         40         50         60
                |<--- αA --->|   |<-αB>|                   |<-------αC--------->|--
B  QAGLNSR ALWQFNGMIK CKIPSSEPLL DFNNYGCYCG LGGSGTPVDD LDRCCQTHDN CYKQAKKLDS
N          NLVQFSYLIQ CANHGKRPTW HYMDYGCYCG AGGSGTPVDE LDRCCKIHDD CYDEAGKKG.
R          SLVQFETLIM KIAG.RSGLL WYSAYGCYCG WGGHGLPQDA TDRCCFVHDC CYGKAT..D.
C          -l-qf--mI- ---------- -y--YGCyCG -Gg-g-p-D- -DrCC--Hd- CY--a-----


            70         80         90         100        110        120
  -αD->|         |<-β->|<-β->|    |<---------αE------>|
B CKVLVDNPYT NNYSYSCSNN EITCSSENNA CEAFICNCDR NAAICFSKV. P.YNKEHKNL DK.KNC
N C.....FPKM SAYDYYCGEN GPYCRNIKKK CLRFVCDCDV EAAFCFAKA. P.YNNANWNI DTKKRCQ
R C.....NPKT VSYTYSEENG EIICGG.DDP CGTQICECDK AAAICFRDNI PSYDNKYWLF PP.KDCREEPEPC
C ---------- --Y------- ---C------ c----C-CD- -AaiCf---- --Yn------ -----C-------
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Fig. 1. The amino acid sequences of bovine PLA_2 (B), notexin (N) rattlesnake PLA_2 (R). The consensus sequence (C) is based on the PLA_2sequences present in the NBRF data bank, where capital letters indicate totally conserved residues and small letters indicate almost conserved residues. The numbering is in accordance with Renetseder et al. [12].

England). The R-factor for the merging was 6.3% and the final dataset contained 95% of all reflections to 2.0 Å resolution.

On the basis of sequence similarities (42% identities between bovine PLA_2 and notexin; Fig. 1), a model was originally built with Frodo [6] using the refined bovine PLA_2 [7] as a base (BP21 in the Brookhaven protein data bank). It was guided regarding gaps, etc., and also by the PLA_2 from the Western rattlesnake [8] (PP21 in the Brookhaven protein data bank). The model was adjusted using the O program [9] before using it in the molecular replacement investigations.

The rotation function search gave essentially only one significant peak both with the Polarrfn, Almn programs (CCP4, Daresbury, England) and Xplor [10]. The best solutions were further refined by Patterson correlation (PC) refinement of Xplor. A translation search was performed using the best solution from the PC refinement using both possible enathiomorph space groups. The space group $P3_121$ gave a peak about twice as high as the next highest peak and twice as high as the highest one from the $P3_221$ space group. This best translation solution gave good packing interactions except for a few side chains which were modelled before refinement using the O program [9] to avoid close contacts. The starting model gave an R-factor of 50% at 2.0 Å resolution, 40% for the 5–4 Å resolution range.

A simulated annealing refinement cycle of the model using the slow cool procedure of Xplor lowered the R-factor to 32% at 2.0 Å resolution. The calculated 2Fo-Fc map after this refinement revealed some errors made in the model which were adjusted. Then, cycles of positional refinement, temperature factor refinement and model-building were performed. The model contained errors in some of the loops which were corrected by model building. When most of the protein was positioned correctly the R-factor for all of the reflections in the 5.0–2.0 Å resolution range was at 27% and the map began to look very good with holes in the middle of the density for most of the aromatic rings. 50 well-defined water molecules were added and additional cycles of refinement and model building were performed until no significant peak was observed in the Fo-Fc maps. The final model contains 125 water molecules. The coordinates are deposited at the Brookhaven data bank.

3. RESULTS

The notexin structure has been refined to an R-factor of 16.5% for all reflections in the resolution range 5–2Å. The root mean square deviation from standard bond length is 0.013 Å and 2.7° for bond angles. The final electron density map is well defined (Fig. 2) with the exception of a few long side chains at the surface of the molecule – Glu-40, Met-70, Glu-79, Asn-80, Lys-123, Lys-124 and Gln-127 which have weak density.

The overall structure of notexin is, as expected, very similar to other homologous PLA_2s (Fig. 3). We have compared the structure of notexin to the bovine and rattlesnake venom PLA_2s [7,8] for which structures are available in the protein data bank (Fig. 3). The secondary structure of notexin is essentially the same as for other PLA_2s. The four main helices of PLA_2s (αA, αB, αC and αE as defined in [7]) are present also in notexin as well as a short carboxyl end helix. Furthermore, a number of conserved side chains in the core have practically identical positions. This concerns the active site His-48, Asp-49 and Asp-99 as well as residues 5, 9, 27, 28, 29, 35, 37, 42, 44, 45, 51, 52, 73, 84, 95, 96 and 98. Side chain interactions with main chain atoms are to some extent conserved in all the PLA_2s. Gln-4 forms hydrogen bonds to main chain atoms of the peptide bond between residue 72 and 73, probably to stabilize the position of Tyr-73, which is hydrogen bonded to Asp-99, which in turn is hydrogen bonded to the active site, His-48. This Asp is also hydrogen bonded to Tyr-52 in notexin and the other PLA_2s. Tyr-28 is hydrogen bonded to main chain carbonyl oxygens in the calcium loop. Asp-42 binds three main chain nitrogen atoms; the binding to N-38 is the only helix capping in the protein. Two other tyrosine–main chain interactions are also conserved involving Tyr-25 and Tyr-113. In notexin the side chains of Gln-10, Asn-13, Asp-74, Asn-86 and Arg-

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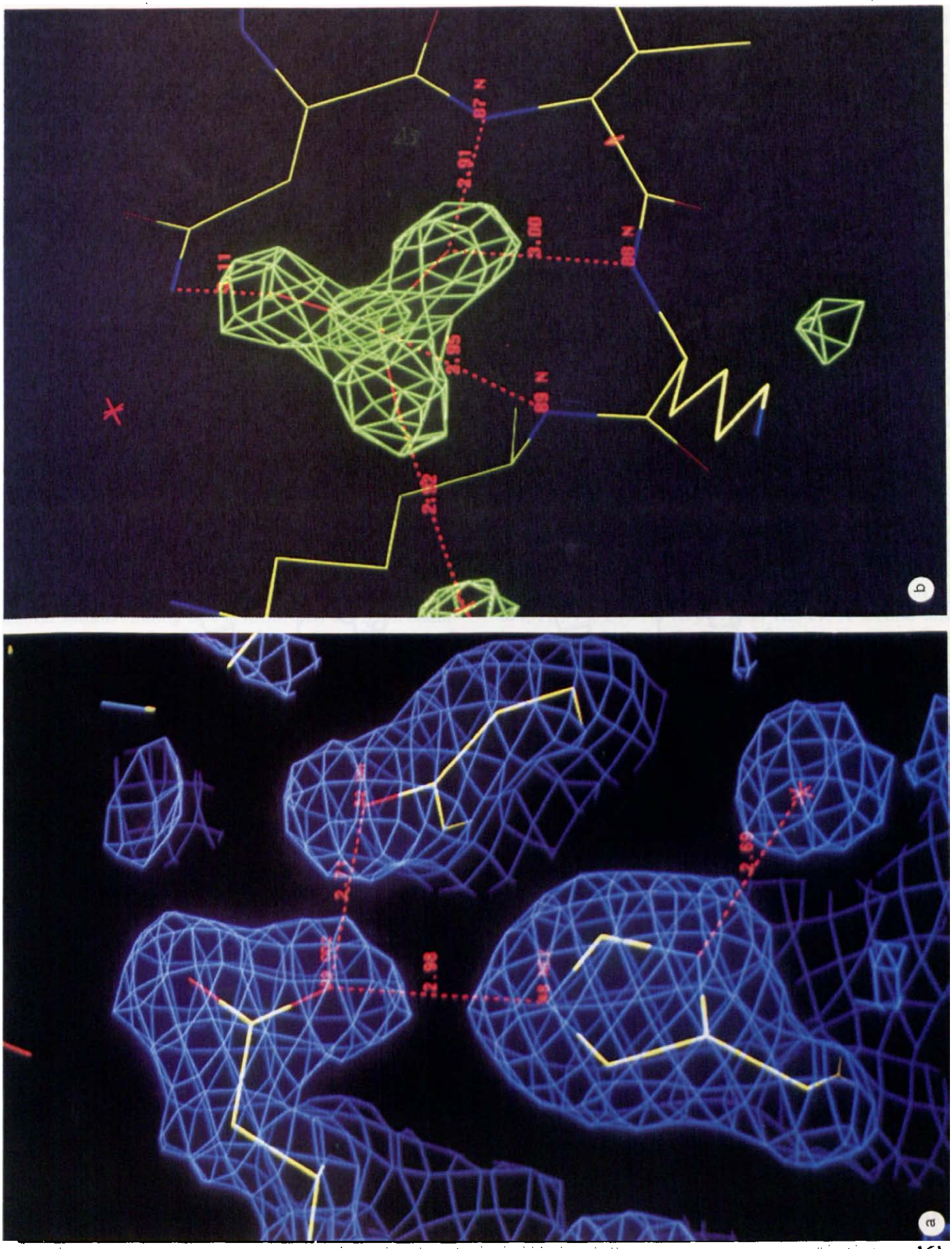
Fig. 2. (a) Final electron density (2Fo-Fc) map of active site residues His-48, Tyr-52, Asp-99 and surrounding residues in notexin. The nucleophilic water at the catalytic centre is at the lower right side. Hydrogen bonds are shown as dotted red lines. (b) Difference electron density (Fo-Fc) observed during refinement and interpreted as a sulfate ion.


2.91
3.00
2.95
2.92
2.77
2.68
2.98

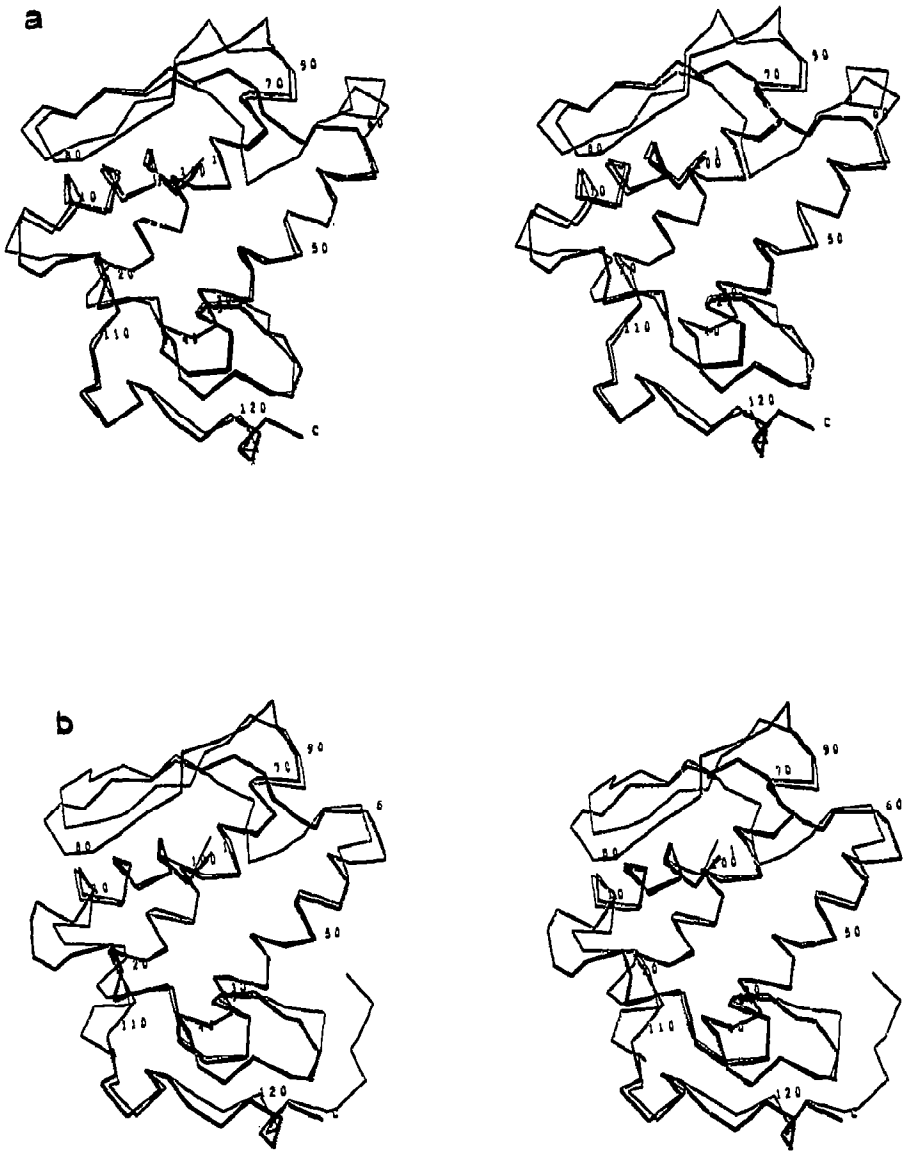


Fig. 3. The main chain conformation of notexin (thick lines) is practically the same as in the bovine (a) and rattlesnake (b) enzymes for the the four largest helices, particularly residues 1–12, 22–29, 37–54 and 90–108. These residues constitute most of the closely superimposable residues between notexin and the rattlesnake PLA_2. 57 Cα atoms can be superimposed with an rms difference between the two proteins of 0.59 Å. The main chain conformation of notexin is closer to the bovine PLA_2since the residues of the calcium binding loop are also very similar; these proteins also have the disulfide bridges in common. 76 Cα atoms in these two proteins can be superimposed with an rms difference of 0.53 Å. The pleated sheet hairpin is present in all PLA_2s but is positioned slightly differently with respect to the helical core.

93 also form hydrogen bonds to main chain atoms. These interactions are not present in the other PLA_2s.

The main chain of notexin deviates most from the other molecules in the region 59–89, where there are differences in chain length. Notexin is six residues shorter than the bovine PLA_2 and the rattlesnake enzyme is eight residues shorter than bovine PLA_2. Particularly, the stretch 86–89 deviates from both of the other structures by about 6 Å. This was not expected, since the number of residues here is the same as in bovine PLA_2 and is a single residue less in the rattlesnake PLA_2.

The stretch 59–71 is very different between bovine PLA_2 and notexin due to differences in chain length, but it also differs significantly from rattlesnake PLA_2 with the same number of residues for this part. The αD helix in bovine PLA_2 is not present in notexin due to the deletion of six residues in this region. Pro-68 is present in both notexin and the rattlesnake PLA_2, but the side chains are about 8 Å away from each other when the core is superimposed. Pro-68 was found to be in the *cis* conformation in notexin but not in the rattlesnake PLA_2.

Residue 69, which has been shown to be important for phospholipase binding [11], has a different confor-

mation in notexin than in the other PLA_2s and has different interactions than for other known PLA_2s. Its side chain interacts with Asn-1, Gln-4 and a sulfate ion.

The antiparallel β-structure has globally different positions in the different molecules and equivalent Cα atoms in notexin deviate by as much as 4 Å from the positions in the rattlesnake protein (residue 81). The turn after the β-sheet has the same number of residues in bovine PLA_2 and notexin, but is one residue shorter in the rattlesnake PLA_2. This turn in notexin differs significantly from those in the other two PLA_2s. Residues 15–20 in notexin also differ from the two other PLA_2s.

The crystals contain no Ca^{2+} ions. Instead, a water molecule occupies the calcium site and forms hydrogen bonds to the ligand atoms. The conformation of the calcium loop is very similar to that of bovine PLA_2, which contains calcium, but differs slightly from that of rattlesnake PLA_2, which does not contain calcium.

Three clearly tetrahedral ions per molecule are present in the notexin structure and have been interpreted as sulfate ions, since the crystals were grown from 1.4 M ammonium sulfate. In one ion binding site the oxygen atoms are bound to Asn-86 and main chain nitrogen atoms of residues 87, 88 and 89 and to Asn-119 from another molecule. One water molecule completes the coordination (Fig. 2b). This sulfate ion has as low temperature factors as the best ordered protein parts. The second sulfate binding sit e is formed by the main chain nitrogen atoms of residues 1–4 and the side chain of Lys-69. A third sulfate ion is bound between two molecules in the crystals; to N-113 and Lys-16 in one molecule and to Asn-114 and Arg-17 in the other. The second and third sulfate ions have higher temperature factors (about 40 $Å^2$ compared to about 10 $Å^2$ for the first sulfate ion).

4. DISCUSSION

The structure of the core of the notexin molecule is very similar to that of other phospholipases, as expected. As regards the active site, model building studies of proteins as similar as the PLA_2s may be sufficient to discuss enzymatic properties. However, the PLA_2s have a much higher activity toward micelles than toward monomeric phospholipids and their activities differ considerably. Furthermore, many of the PLA_2s are highly toxic, whereas others are not, and the types of toxic effects also differ greatly. Therefore, it is necessary to study the surfaces of the PLA_2s by detailed crystallographic investigation. It also turns out that our model based on the homologous PLA_2s contained several large errors at the surface. Some of the differences were quite unexpected.

In the crystals there are two types of crystallographic symmetry, two-fold axes relating the molecules into dimers and three-fold screw axes relating these dimers. There is no biochemical evidence that notexin exists as a dimer in solution. On the contrary, all investigations clearly suggest that notexin works as a monomer. In the crystals the dimers do not have strong interactions and only a few of the hydrogen bonds formed between different molecules in the crystals are at the dimer interface. In the dimers the active site sides of the molecules are juxtaposed.

Several of the residues suggested by White et al. [11] to form the hydrophobic channel are the same in notexin. Leu-2, Phe-5 and Ile-9 are conserved, whereas Trp-19 is a Thr, Met-23 forms part of the cleft in notexin and Tyr-69 is a Lys in notexin. Tyr(Lys)-69 was suggested to form a part of a hydrophobic wall of the substrate channel and bind the *sn*-3 phosphate. There is a Lys in this position in both notexin and the rattlesnake PLA_2, but the positions of the NZ atoms differ by 12 Å. It is far from the substrate cleft in notexin where it instead interacts with Gln-4. The suggested interfacial binding surface [11] is very different in notexin and the cobra venom phospholipase. Residues 3, 6, and 31 are Val, Ser and Ala in notexin, as compared to Tyr, Lys and Arg in the cobra venom.

Positively charged residues that could be of importance for phospholipid interactions are distributed over the whole molecule. There are particularly three patches of positive residues, one in each corner of the triangular molecule. The first one is around the carboxyl end of αA, His-14, Lys-16, Arg-17 and His-21. A second one is dominated by the three consecutive lysines, residues 87, 88 and 89 and also contains Arg-85 and Arg-93. The third one is close to the carboxyl end with Lys-123, Lys-124 and Arg-125.

All three binding sites for sulfate ions in the notexin molecule are at open surface regions and are potential binding sites for negatively charged head groups of phospholipids. However, the strong binding site at the 87–90 loop points away from the proposed interfacial region, whereas the the second binding site is much more suitably positioned. It binds to residue 1 and 69, both of which have been shown to be involved in phospholipase activity. This site may bind the phosphate headgroup in order to or ient the phospholipids in a proper position for a subsequent transfer to the catalytic site.

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