Crystallographic investigation of the dependence of calcium and phosphate ions for notexin

Bengt Westerlund[a,*], Markku Saarinen[1,b], Bertil Person[a], S. Ramaswamy[b], David Eaker[a], Hans Eklund[b]

[a] *Department of Biochemistry, Biomedical Centre, S-751 23 Uppsala, Sweden*
[b] *Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Centre, S-751 49 Uppsala, Sweden*

Received 16 December 1996

Abstract The crystal structure of the neurotoxic phospholipase A_2, notexin, revealed three binding sites for sulphate ions which were suggested to be phosphate binding sites of importance for the activity of the toxin. The present investigation shows that the sulphate ion bound to the major binding site alters the structure of residues 60–75. In the absence of sulphate and phosphate, the structure of this loop has a conformation which partly resembles the non-neurotoxic PLA_2s. The affinity of notexin for phosphate is 17 μM, as measured by the increase in fluorescence at 345 nm. Since the concentrations of phosphate and sulphate ions in blood plasma are 3 and 1 mM, respectively, the binding site must be occupied under physiological conditions. This major sulphate/phosphate binding site explains the specific affinity labelling by pyridoxal phosphate. Pyridoxal phosphate binds to this anion binding site which allows the reaction with Lys-88 or Lys-89. The structure of notexin in the presence and absence of Ca^{2+} shows only small local structural differences.

Key words: Phospholipase A_2; Notexin; Structure; Phosphate; Calcium; Pyridoxal phosphate; Toxin

1. Introduction

The venom of the Australian tiger snake *Notechis scutatus scutatus* contains a number of different proteins. Among the various proteins are the neurotoxic phospholipases A_2 (PLA_2) that are well characterized exemplified by notexin and notechis II-5 [1–3]. Notexin is a myotoxic (muscle degenerative), presynaptically active neurotoxic PLA_2. It is a basic, monomeric protein consisting of 119 amino acid residues cross-linked by seven disulphide bridges. Notexin impairs the release of acetylcholine from cholinergic synapses [4–6] and causes rapid death through asphyxiation. The toxicity of notexin is dependent upon its PLA_2 activity [7].

Although the sequences of more than 50 different phospholipases (PLA_2s) are known and the structures of approx. 15 PLA_2s have been solved it is difficult to deduce the basis for their special functions by merely comparing their sequences or structures. Notexin is the only neurotoxic PLA_2 for which the structure has been solved [8]. The crystal structure of notexin is similar to those of other PLA_2s and it does not seem to be significantly different from the non-neurotoxic ones apart from lacking the extra α-helix, amino acid residues (62–66) which is present mainly in pancreatic phospholipases, e.g. bovine pancreatic PLA_2 [9,10].

It is well known that all PLA_2s are dependent on Ca^{2+} for activity, but nothing has been published about a dependence on other ions. Therefore, we have as a first step toward elucidating the importance of this anion-binding site measured the affinity of notexin for phosphate ion and compared the structure of notexin in the presence and absence of phosphate and calcium ions, respectively. The main anion binding site explains the specific modification of Lys-88 and Lys-89[2] with pyridoxal phosphate.

2. Materials and methods

2.1. Binding assay

The affinity of notexin for phosphate was measured by the increase in fluorescence at 345 nm. Quantitative measurements of binding of phosphate were carried out with an Aminco spectrofluorometer equipped with a 500 W xenon lamp. The excitation wavelength was 280 nm and the emission was scanned between 250 and 450 nm. The bandwidth in both cases was 5 nm. The sample contained 1.4 μM toxin purified as described [1] and the phosphate concentration was varied from 1 to 1000 μM in a total volume of 1.5 ml 0.1 M NH_4Ac, pH 7.4. In this assay, tryptophan was used as the reporter group [12]. Treatment of the binding data was performed with a program using the algorithm of Marquart [13,14].

2.2. Modification with pyridoxal phosphate

15.5 mg (≈1 μmol) lyophilized notexin, dissolved in 0.75 ml degassed 50 mM *N*-ethylmorpholine-HAc pH 7.5, was mixed in a 3 ml glass vessel with stirring (reacti vial) with 500 μg (≈2 μmol) PLP dissolved in 100 μl of the same buffer. After 40 min in room temperature, 100 μl 0.2 mM (20 μmol) NaBH in 1 mM NaOH was added and the reaction proceeded in 10 min. The modified notexin was desalted on a PD-10 column equilibrated in 50 mM NH_4Ac pH 6.7 and lyophilized.

2.3. Analysis of modified notexin

For purification, the modified notexin was dissolved in 2 ml 50 mM NH_4Ac, pH 5.0 and applied on a SP-Sephadex-25 cation exchanger equilibrated in 50 mM NH_4Ac, pH 5.0. Elution was performed with a linear gradient 0.5 l buffer A vs 0.5 l buffer B. Buffer A: 50 mM NH_4Ac, pH 5.0, and buffer B: 1 M NH_4Ac, pH 5.0. From the spectra of the observed fractions and the molar extinction coefficient of pyridoxal lysine at 325 nm ($\varepsilon_{325} = 10\,000$ [15]) it was possible to distinguish between mono- and di-derivatives. The fraction of interest was lyophilized and reduced and carboxymethylated with iodoacetic acid according to a standard procedure.

4 mg of the desalted and lyophilized RCM-notexin was dissolved in 1 ml 0.1 M NH_4HCO_3, pH 8.7, and mixed with 40 μl (1 mg/ml in water) *Staphylococcus* V-8 protease. After 3 h at 37°C under continuous stirring, an aliquot (75 μl) was removed and acidified with 40 μl

*Corresponding author.

[1] *In fond memory.*

Abbreviations: PLA_2, phospholipase A_2; PLP, pyridoxal phosphate.

[2] The numbering is in accordance with Renetseder et al. [11].

PII S0014-5793(96)01530-X

50% HAc. The reaction was complete according to HPLC analysis and the sample was lyophilized. The peptides were separated on a Vydac 4.8×250 mm RP-HPLC column and detected at 280 and 325 nm. The peptide containing the pyridoxal group was sequenced on an automated sequencer model ABI 476A according to the manufacturer's instructions.

2.4. Modelling of pyridoxal phosphate binding

Modelling of pyridoxal phosphate at the major sulphate/phosphate binding site was performed on a Silicon Graphics workstation using the O program [16]. The co-ordinates for other phospholipases than notexin were obtained from the Brookhaven protein data bank. The alignment of the different structures was achieved by superposing the α-helices C and E [9].

2.5. Structure determination of notexin in the presence of calcium ion

Notexin purified as described [1] was crystallized using hanging drops according to a slightly modified protocol described elsewhere [8]. In addition to this protocol, $CaSO_4$ was added to a final concentration of 5 mM. The crystals were trigonal of space group $P3_121$, and were isomorphous with Ca-free crystals of notexin with the cell dimensions $a=b=74.7$ Å and $c=48.9$ Å. A 91% complete data set to 2.1 Å resolution was collected. The structure was refined using the Ca-free structure as starting model using X-plor [17]. The final R factor for the reflections to 2.1 Å resolution was 16.9% and the R_{free} 22.9%.

2.6. Structure determination of notexin in the absence of sulphate/phosphate

Notexin purified as described [1], with an additional ion-exchange step on an LKB GlasPack SP-5PW 8×75 mm TSK column, was crystallized using hanging drops. Crystals grew when a protein solution with half of the precipitant concentration of the well was equilibrated against a 12% PEG 6000 solution in 0.4 M NH_4Cl containing 2% dioxane and buffered with 50 mM CHES at pH 9.0.

The diffraction data were collected on an R-axis imaging plate system mounted on a Rigaku rotating anode. 55 frames with 2° oscillation were collected and the data were processed using Denzo [18] and scaled using the program ScalePack [18]. The overall completeness of the data was 92.5% to 2.3 Å and between 2.38 and 2.30 Å it was more than 90% complete for reflections over 1σ. The R_{sym} for the data is 5.4% with more than 70% of the reflections observed more than once. The data were truncated using the CCP4 [19] suite of programs.

The crystals belong to the monoclinic space group $P2_1$ with the cell dimensions $a=39.5$ Å, $b=36.6$ Å, $c=42.6$ Å and $\beta=117.0°$, unlike notexin crystals grown in ammonium sulphate, which belong to the trigonal space group $P3_121$. The present structure has one molecule in the asymmetric unit.

The structure was determined by molecular replacement using the program Amore [20] using the refined notexin structure as the search model. The complete protein model was used, but without hetero

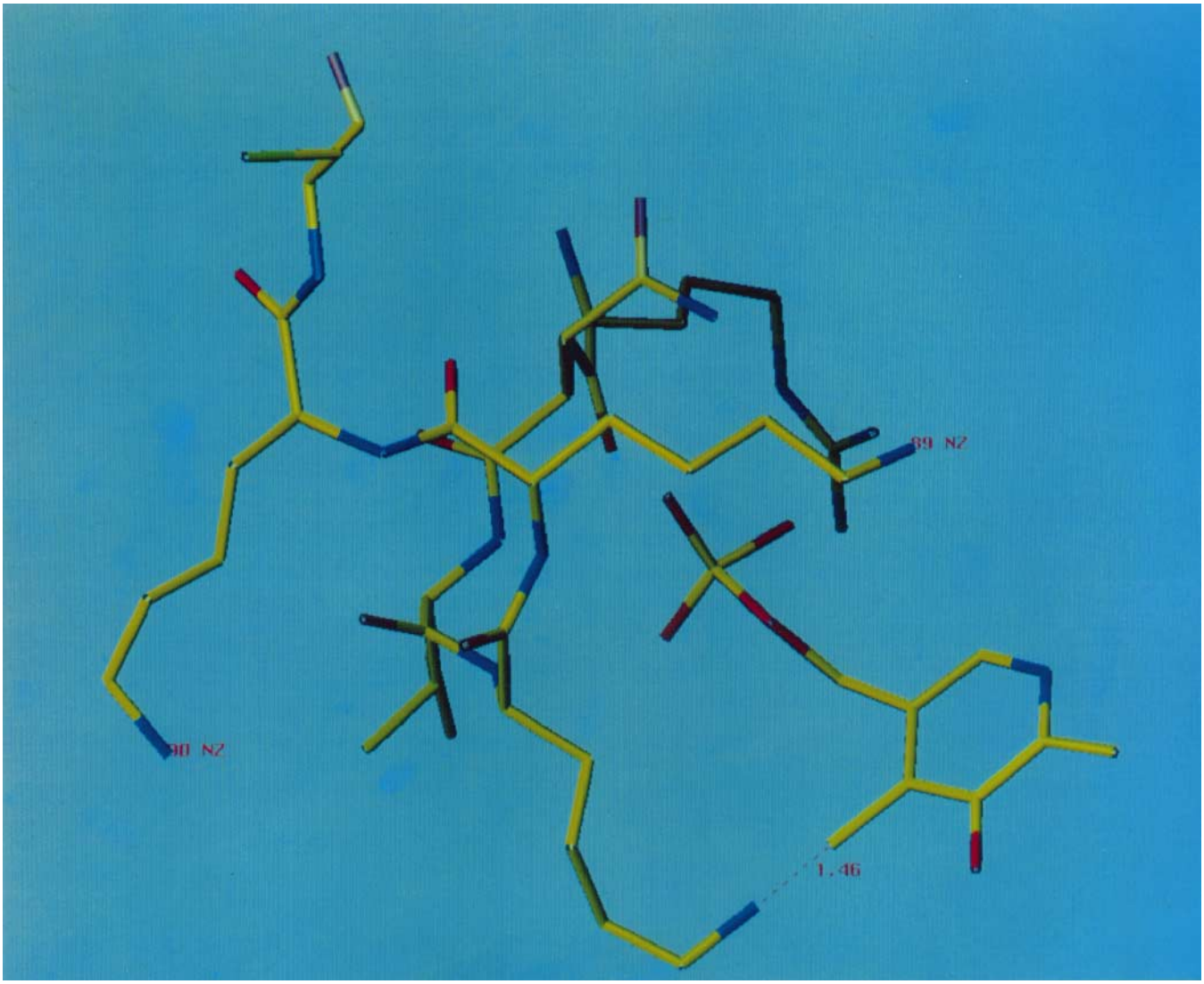

Fig. 1. A model of the pyridoxal phosphate fitting into the P-loop of notexin showing the binding distance between the ζ-amino group of Lys-88 and the carbon of the pyridoxal phosphate group.

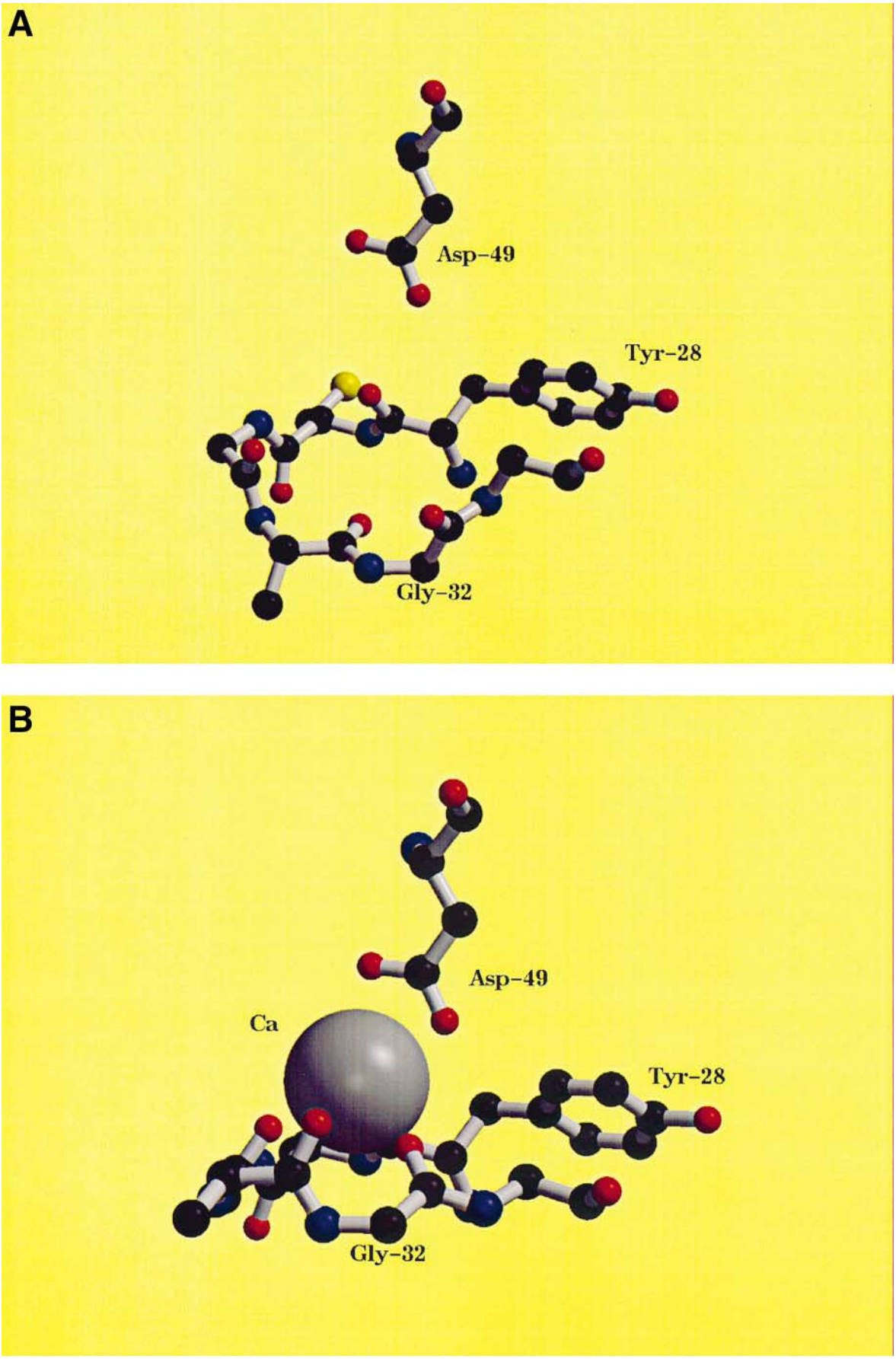

Fig. 2. Comparison of the notexin structure in the absence (A) and presence (B) of calcium ion.

atoms. All refinement was done using the program X-plor [17], The Engh and Huber [21] parameters were used for refinement. A map calculated at this stage indicated that three regions in the structure were different; around residues 88 and 65 and around the calcium-binding region (residues 28–32). These regions were omitted and a round of refinement was performed (positional refinement, simulated annealing followed by temperature factor refinement).

The main chains of two of these regions were visible in the resulting electron density map. However, residues 87–89 did not show up. Further refinement was continued with all reflections in the range of 15–2.3 Å resolution.

The final R factor is 20.4% for all reflections, including 80 water molecules. Residues 87–89 continue to have high B factors and there seems to be no obvious density for these residues. The Ramachandran plot has 91% of the residues in the most favoured region and the remaining 9% in the additional allowed regions. The structure has a good stereochemistry with bond length deviation of 0.01 Å and bond angle deviation of 1.4°. The co-ordinates and structure factors have been deposited in the PDB.

3. Results

3.1. Titration with phosphate

From the uncorrected fluorescence spectrum, the photomultiplier output at 345 nm was plotted vs. phosphate concentration. This gave a B_{max} of 76 μM ($\sigma = 4$) and a K_d of 17 μM ($\sigma = 4$). The data were not consistent with two binding sites. The emission peaks were well gathered around 345 nm without any blue shift. The increase in intensity was approx. 20% upon titration.

3.2. Modification with pyridoxal phosphate

Chemical modification is one approach to deduce the function of a protein and pyridoxal phosphate, vitamin B6, is one of the reagents used for lysine modification [22]. Pyridoxal phosphate is a coenzyme participating in a variety of reactions, e.g. transamination, decarboxylation and modification of amino acid side chains. The function of pyridoxal phosphate is the same in all known reactions. It reacts with amino groups to form a reversible, planar Schiff base intermediate. This Schiff base can be reduced with sodium borohydride to form a stable compound.

The modified protein was purified on a SP-Sephadex 25 column and three peaks could be detected. The 280/325 nm absorption ratio for the first peak was 1.8 and for the second 3.0 which is close to the theoretical value for a mono-derivative. Reverse-phase chromatography following digestion of the second peak with *Staphylococcus* V-8 protease showed 5 peaks when detected at 280 nm, only one of which absorbed at 325 nm. Sequencing of the peak containing pyridoxal-lysine showed that the signals for Lys-88 and Lys-89 were weakened but not completely absent.

3.3. Pyridoxal phosphate labelling

The modelling of pyridoxal phosphate in the major binding site in the notexin structure was readily carried out with a minimum of changes of any side chain. There were three possible ways to superimpose the phosphate of pyridoxal

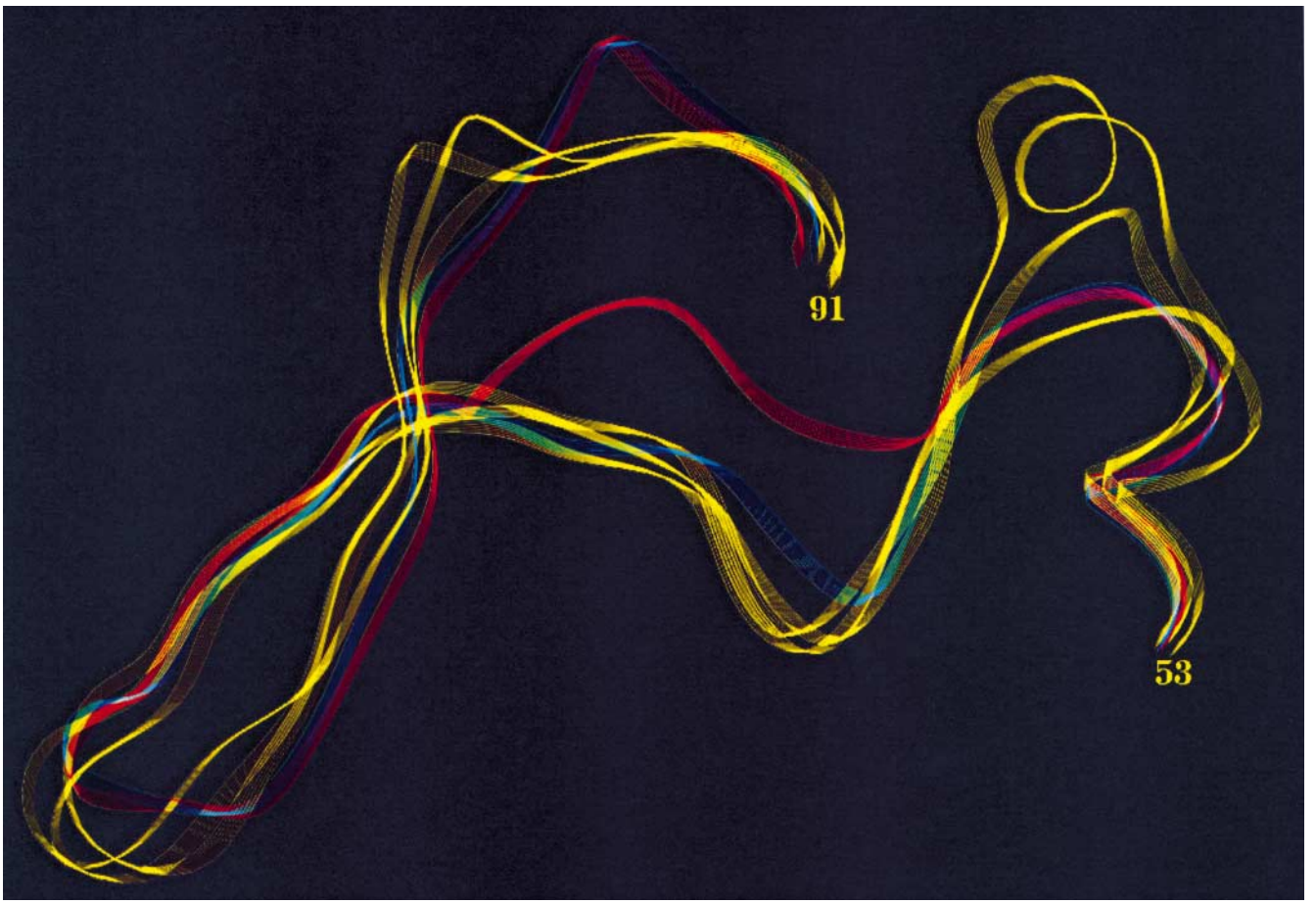

Fig. 3. Superposition of residues 53–91 of different phospholipase A_2 structures. Notexin in the presence of phosphate (red) and absence (blue) and three other class I phospholipases in yellow.

Table 1
Deviation of main chain nitrogen (N), main chain carbon (Cα), main chain carbon (CO), carbonyl oxygen (O) and the gamma carbon (Cγ) in notexin in the presence and absence of calcium ion

Residue no.	N (Å)	Cα (Å)	CO (Å)	O (Å)	Cγ
28	0.6	0.6	0.6	0.6	–
29	0.5	0.5	<0.5	0.5	–
30	0.8	1.0	1.6	1.9	–
31	3.8	4.2	4.5	5.7	–
32	3.4	3.6	2.4	3.3	–
33	1.7	0.9	0.8	0.7	–
34	0.9	0.7	0.8	1.0	–
49	<0.3	<0.3	<0.3	<0.3	0.4

phosphate on the tetrahedral sulphate ion in the notexin structure. Two of these alternatives required considerable distortion of the pyridoxal phosphate molecule and were less probable. The third alternative gave an almost relieved conformation of the pyridoxal phosphate molecule and required a minimum of rebuilding of the neighboring side chains (Fig. 1).

3.4. Structure determination of sulphate/phosphate free notexin

Comparison of the notexin structure in the absence and presence of sulphate shows that the only significant difference is the region 60–75. In addition, there is also a small change in the calcium loop. In the absence of sulphate, residues 87–90 in the major sulphate/phosphate binding site lacks electron density and it is not possible to determine the location of these residues. The positions of the backbone atoms of residues 60–75 differ by approx. 2 Å.

3.5. Structure determination of notexin in the presence of Ca^{2+}

In the presence of Ca^{2+} ion, the calcium-binding loop is more compact and the involved residues are centred around the Ca^{2+} ion. The carbonyl oxygens of residues 28, 30 and 32 and the side chain of Asp-49 form a cage around the Ca^{2+} ion similar to the Ca^{2+} binding site in pancreatic phospholipases [10] (Fig. 2). Some of the Cα atoms of the involved residues differed as much as 4.2 Å as compared to the structure without Ca^{2+} ion (Table 1). Apart from these local differences at the Ca^{2+} binding loop, no other changes were detected.

4. Discussion

The only significant difference between the notexin structures with and without divalent anions is in the sequence of residues 60–75, which is obviously flexible. This may, at first glance seem trivial, but these changes occur in an interesting area. All known PLA_2 structures described so far are similar and the differences between them seem to be evenly spread. However, between residues 60 and 75 the different structures seem to coincide. This is also valid for notexin without a phosphate or sulphate ion. When the PLA_2 structures are compared with notexin containing sulphate, the structures are significantly different for residues 60–75 (Fig. 3). Since notexin is the only neurotoxic PLA_2 for which the structure has been solved, it is tempting to propose that the neurotoxicity is related to these differences.

The lack of density for the residues at the major anion binding site indicate a flexible structure of the involved residues. It is likely that the structure containing a quaternary ion is the physiological relevant molecule and that the ion will stabilise the structure of the binding loop and thereby alter the position of its neighbour structure, i.e. residue 60–75.

The sulphate-binding residues consist of Asn-86 and the three main-chain nitrogen atoms of residues 87, 88 and 89. This loop is short in comparison with the phosphate-binding loops (P-loops) of other proteins described earlier. In protein tyrosine phosphatases (PTP) the P-loop consists of 8 residues which are directly involved in the phosphate binding. The consensus sequence for the P-loops of different PTP is CXXXXXR [23]. In ATP- and GTP-binding proteins, e.g. elongation factors, adenylate kinases and phosphoglycerate kinases the consensus sequence of the P-loop motif is GXXXXGK(TS) [24].

We have known for many years that the reaction of notexin with pyridoxal phosphate only modified one or two lysines out of 11 and that the modified notexin retained all of the activities of the native protein (unpublished observation). Pyridoxal itself does not react with notexin under the same conditions because of the lack of the handle, the phosphate moiety, even at 10-fold higher concentration. Modelling of pyridoxal phosphate to the major anion site in notexin showed no steric hindrance (Fig. 1). Only minor displacements of the side chains were necessary to position the pyridoxal moiety suitable for reaction with the ζ-amino group of Lys-88 or Lys-89. These two alternatives could not be discriminated. This confirms the observation from the sequencing data which seemed to indicate partial modification of both lysines. The ease with which the PLP could be introduced into the structure without formation of any constraints explains why the modified notexin retains its activities.

A calcium ion is known to stabilize bovine PLA_2 and to protect it from degradation by trypsin and from modification by *p*-bromophenacyl bromide and this has been attributed to structural changes [25]. This observation is not simply explained just by comparing the structure of notexin in the presence and absence of calcium ions. The two structures have the same flexibility as judged from the B factors, and apart from the calcium loop the structure is intact. The change in conformation of the calcium loop does not seem to influence the residues or the structure in the vicinity or form any long-range interactions.

Acknowledgements: The authors thanks Dr. Ulla Uhlin and Enrique Carredano, Dept. of Molecular Biology, SLU, Uppsala, for help with the molecular modelling, Uno Skatt for making the pictures of Fig. 2 and Bo Ek, Dept. of Genetics, SLU, Uppsala, for sequencing peptide C.

References

[1] Karlsson, E., Eaker, D. and Rydén, L. (1972) Toxicon 10, 405–413.
[2] Halpert, J. and Eaker, D. (1975) J. Biol. Chem. 250, 6990–6997.
[3] Halpert, J. and Eaker, D. (1976) J. Biol. Chem. 251, 7343–7347.
[4] Harris, J.B., Karlsson, E. and Thesleff, S. (1973) Br. J. Pharmacol. 47, 141–146.
[5] Cull-Candy, S.G., Fohlman, J., Gustavsson, D., Lüllmann-Rauch, R. and Thesleff, S. (1976) Neuroscience 1, 175–180.
[6] Dowdall, M.J., Fohlman, J.P. and Watts, A. (1979) in: Presynaptic Action of Snake Venom Neurotoxins on Cholinergic Systems (Ceccarelli, B. and Clementi, F.) pp. 63–76, Raven Press, New York.
[7] Halpert, J., Eaker, D. and Karlsson, E. (1976) FEBS Lett. 61, 72–76.

[8] Westerlund, B., Nordlund, P., Uhlin, U., Eaker, D. and Eklund, H. (1992) FEBS Lett. 301, 159–164.
[9] Dijkstra, B.W., Drenth, J., Kalk, K.H. and Vandermaelen, P.J. (1978) J. Mol. Biol. 124, 53–60.
[10] Dijkstra, B.W., Kalk, K.H., Hol, W.G.J. And Drenth, J. (1981) J. Mol. Biol. 147, 97–123.
[11] Renetseder, R., Brunie, S., Dijkstra, B.W., Drenth, J. and Sigler, P.B. (1985) J. Biol. Chem. 260, 11627–11634.
[12] Freifelder, D. (1976) Physical Biochemistry; Applications to Biochemistry and Molecular Biology, pp. 411–443, Freeman, New York.
[13] Marquart, D.W. (1963) J. Soc. Ind. Appl. Math. 11, 431–441.
[14] Bevington, P. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York.
[15] Forray, A.W., Olsgaard, R.B., Nolan, C. and Fischer, E.H. (1971) Biochimie 53, 269–281.
[16] Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard, M. (1991) Acta Crystallogr. A47, 110–119.
[17] Brünger, T.A., Kuriyan, J. and Karplus, M. (1987) Science 235, 458–460.
[18] Otwinowski, Z. (1993) in: Proceedings of the CCP4 Study Weekend (Sawyer, L., Issacs, N. and Bailey, S. eds.) pp. 56–62, SERC Daresbury Laboratory, UK.
[19] Collaborative Computational Project, No. 4 (1994) Acta Crystallogr. D50, 760–763.
[20] Navaza, J (1994) Acta Crystallogr. A50, 157–163.
[21] Engh, R.A. and Huber, R. (1992) Acta Crystallogr. A47, 392–400.
[22] Benesch, R., Benesch R.E., Kwong, S., Acharya, A.S. and Manning, J.M. (1982) J. Biol. Chem. 257, 1320–1324.
[23] Su, X.D., Taddei, N., Stefani, M., Ramponi, G. and Nordlund, P. (1994) Nature 370, 575–578.
[24] Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) Trends Biochem Sci 15, 430–434.
[25] Pieterson, W.A., Volwerk, J.J. and De Haas, G.H. (1974) Biochemistry 13, 1439–1445.