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Active site and catalytic mechanism of phospholipase A2

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Published in:
Nature

DOI:
[10.1038/289604a0](https://doi.org/10.1038/289604a0)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1981

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Dijkstra, B. W., Drenth, J., & Kalk, K. H. (1981). Active site and catalytic mechanism of phospholipase A2. *Nature*, 289(5798), 604-606. <https://doi.org/10.1038/289604a0>

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Table 2 20.1 MHz ^{13}C NMR data for compound A and model compounds

Compound A			Model compounds		
Carbon*	Position	Shielding (p.p.m. relative to $(\text{CH}_3)_4\text{Si}$)	Structure with relevant carbons indicated (<i>italic</i>)		Shielding (p.p.m. relative to $(\text{CH}_3)_4\text{Si}$)
Two CH_3 groups	6, 7	14.7, 15.1	$(\text{CH}_3)_2\text{AsO}_2\text{H}$		17.1
CH	1	108.2		C-1	108.0§
CH	2	74.8		C-2	74.3§
CH	3	76.2†		C-3	70.9§
CH	4	77.3†		C-4	83.0§
CH_2	5	36.5	$\text{HOCH}_2\text{CH}_2\text{AsO}_3\text{H}_2$		36.6
CH_2	1'	71.3‡	$\text{HOCH}_2\text{CHOHCH}_2\text{SO}_3\text{H}$		65.0
CH	2'	66.9‡	$\text{HOCH}_2\text{CHOHCH}_2\text{SO}_3\text{H}$		68.4
CH_2	3'	54.3	$\text{HOCH}_2\text{CHOHCH}_2\text{SO}_3\text{H}$		54.0

* CH_3 , CH_2 and CH groups were identified by off resonance decoupling.

† The differences in shieldings for C-3 and C-4 of compound A and the C-3 and C-4 of methyl β -ribofuranoside are accommodated by the 5-deoxy structure postulated for the former. It is assumed that the less electronegative substituent ($-\text{CH}_2\text{As}$) at the 5 position will produce effects on the shieldings of C-3 and C-4 analogous to (for example) the differences between the shieldings of C-4 and C-5 in glucose and 6-deoxyglucose¹⁶. Thus, differences in shielding of about 5 p.p.m. for C-3 and C-4 (in opposite directions) when compared with methyl β -ribofuranoside are accommodated.

‡ The differences in shieldings for C-1' and C-2' and the relevant carbons in the model compound are accounted for by the formation of the glycosidic linkage through $-\text{Cl}'\text{H}_2\text{O}-$.

§ Ref. 7.

Compounds A and B are quite similar to isofloridoside (2,3-dihydroxypropyl α -D-galactopyranoside), a constituent of some red algae⁸. Free 2,3-dihydroxy-1-propane sulphonic acid has been reported as a component of algae⁹. However, compounds A and B are apparently unrelated to the arsenophospholipids and related de-acylated compounds containing a trimethylarsonium lactate residue demonstrated by Cooney *et al.*¹⁰ to be present in unicellular marine algae exposed to radiolabelled arsenate. Such compounds were not detected in *Ecklonia*.

Evidently, *Ecklonia* is processing its arsenate uptake by conversion to the sugar derivatives described here. Benson has speculated¹¹ that algae have evolved the capacity to detoxify arsenate in marine waters low in phosphate. Western Australian coastal waters are notably deficient in phosphate¹². The two compounds reported here can readily be envisaged as being intermediates in the production of dimethylarsinic acid (shown by Andreae⁵ to be present in marine waters of the photic zone) and arsenobetaine. The latter would require further reduction and methylation of the arsenic atom and oxidation at the C-4 of the sugar residue. Such conversions might well be mediated by the microflora of the sediments contributed to by decaying *Ecklonia*, and arsenobetaine may enter the food chains, eventually reaching rock lobsters and whiting via detritivores. We are now studying these processes.

We thank Dr L. T. Byrne for ^{13}C NMR spectra, Dr A. J. Jones for 270-MHz ^1H NMR spectra and discussions, Drs D. A. Hancock and H. E. Jones for their interest, and Professor D. W. Cameron for much helpful discussion and encouragement. C, H microanalyses were performed by the Australian Microanalytical service.

Received 24 October; accepted 1 December 1980.

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Active site and catalytic mechanism of phospholipase A_2

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The esterolytic enzyme phospholipase A_2 specifically splits the 2-acyl linkage of phosphoglycerides¹ in a calcium-dependent reaction. In the pancreas the enzyme occurs as a zymogen which is activated on secretion into the duodenal tract by the removal of seven amino acid residues from the N terminus by trypsin^{2,3}. Having refined our X-ray analysis of the crystal structure of bovine pancreatic phospholipase A_2 from 2.4 Å (ref. 4) to 1.7 Å resolution⁵, we now describe how the structure of the molecule may account for the specificity of the enzyme and for the sudden and dramatic change in activity when the substrate concentration passes the critical micelle concentration⁶.

Analysis of the structure at 2.4 Å resolution⁴ showed that the active site is in a depression at the molecular surface. The essential residue, His 48, which is directly involved in catalysis⁷, is located in this region (Fig. 1). The ϵ -N atom of His 48 is in close contact with one of the carboxylate oxygens of Asp 99 (for residue numbering see ref. 8). The wall of the active site is covered with hydrophobic residues: Phe 5, Ile 9, Phe 22, Ala 102 and 103, Phe 106 and the disulphide bridge between Cys 29 and Cys 45. All these residues are invariant in the phospholipase A_2 sequences known so far, except for an occasional replacement of Ala 103 by a Val (ref. 9) and Phe 22 by a Tyr (refs 9-13). The essential calcium ion is also in this region, but near Asp 49, not Asp 99 as previously suggested⁴.

The interior of the active site mainly consists of invariant hydrophobic residues, whereas the entrance is composed of variable residues, hydrophobic and polar (Fig. 2).

Although Asp-His couples occur frequently in protein molecules, the presence of this couple in the active site of phospholipase A_2 suggests a similarity to the proton relay system in the proteolytic serine enzymes. In these proteases—as in all proteolytic enzymes^{14,15}—there are three main stages in the splitting of the peptide bond: (1) nucleophilic attack by the serine OH; (2) proton transfer by the histidine's imidazole ring; and (3) fixation and stabilization of the substrate's carbonyl oxygen by slightly positively charged NH groups. In deacylation a water molecule replaces the serine OH group as the nucleophile. Phospholipase A_2 has exactly the same configuration in its

Fig. 1 A stereo pair showing the essential active site residues. The black dot is the calcium ion.

active site but has a water molecule instead of a serine OH. This water molecule is in the plane of the imidazole ring 3.1 Å from His 48δ-N1. Thus, the ester hydrolysis by phospholipase A₂ seems to be identical to the deacylation step—which is also ester hydrolysis—in the serine proteases, the only difference being that in the latter the substrate is covalently bound to the enzyme. Fixation and stabilization of the carbonyl oxygen atom of the ester bond might occur in phospholipase by means of the NH of Gly 30 and possibly by the calcium ion. This ion is also available for binding the substrate's phosphate group, especially because it has only one carboxyl group (Asp 49) in its coordination sphere. Substrate binding studies are required to test this hypothesis experimentally.

Phospholipase A₂ has a much higher activity towards aggregated than single phospholipid molecules (up to 1,000, ref. 16). Some workers attribute this effect to an entropic factor—a preferred orientation of the enzyme with its active site directed to the substrate layer¹⁷, or a preferred substrate conformation¹⁶. Others explain the effect by a change in enzyme conformation at the active site as a result of the binding to the substrate layer^{6,18}. With only the enzyme's native structure available the conformational change cannot be verified. However, we do know the part of the enzyme's molecular surface which interacts with the micelle and also that a complementarity exists between the active site and the conformation of the substrate molecules in the aggregated state.

If the enzyme has its active site directed towards the micellar surface three distinct regions for interaction with the micelle can be discerned (Fig. 2). One region comprises residues in a ring surrounding the entrance to the active site, the other two regions are at the extremes of the molecule. Several residues in these regions—those at positions 3, 6, 19, 31 and 69 (ref. 19)—have been implicated in the interaction with micellar substrates. Although none of these regions shows residues conserved at homologous positions, the hydrophobic or polar character of these residues is maintained. A special feature is the excess of at least two basic over acid residues, suggesting salt bridge formation with the phosphate groups in the micelle which would thus orient the enzyme with respect to the micelle surface. This is in agreement with a proposal by Brockerhoff and Jensen¹⁷.

Crystal structure and NMR studies have shown that phospholipid molecules in micelles or pseudo-micelles have a unique conformation²⁰. Their main features are parallel and

extended fatty acid chains, with a kink in the second chain at the α-C atom. The shape of the phospholipase A₂ active site is such that a substrate molecule in this conformation can easily enter the active site and approach His 48 with its scissile bond. A conformational change in the substrate molecule is not required, and model building studies indicate that the kink is an absolute requirement for fitting. The calcium ion in that case is available for binding the phosphate group, and the active site holds between four and six CH₂ groups of each fatty acid chain. This shows—as previously suggested¹⁶—that besides the orientation of the enzyme molecule a second entropic advantage is the presence of the right substrate conformation in aggregates like micelles.

Our crystallographic studies have also analysed the structure of bovine pancreatic phospholipase A₂. This precursor does not show the rate increase towards aggregated substrate. Although the structure of porcine pancreatic phospholipase A₂ at 3 Å resolution has been reported¹⁵, it was later

Fig. 2 Schematic representation of the face of the molecule which is directed towards the phospholipid layer. The residues in boxes, in the bovine enzyme, have their side chain pointing to the substrate layer. Residue 1 is incorporated because of its free NH₂ which is essential for binding. One set of residues is in a ring surrounding the entrance to the active site. Two other sets are on the left and on the right.

found to have been based on an incorrect interpretation of the electron density map. Recently, high-quality crystals of bovine phospholipase A₂ have become available, and preliminary crystallographic results indicate differences with the enzyme itself at the N terminus due to an increased mobility in this region. This agrees with studies by Slotboom *et al.*²¹ showing that a precise architecture of this region is required for micelle binding.

We thank Professor G. H. de Haas and his coworkers for their cooperation, Dr W. G. J. Hol for stimulating discussions and Mrs C. A. Torfs-van Cotthem for her contributions. This work was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organisation for the Advancement of Pure Research.

Received 21 July; accepted 5 December 1980.

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Assignment of the disulphide bonds of leukocyte interferon

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Nucleotide sequencing of cloned cDNA can lead to rapid and precise prediction of the primary amino acid sequence of gene products, but it cannot establish such post-translational modifications as the existence and arrangement of disulphide bonds. For example, the complete sequences of at least one fibroblast¹ and seven^{2–4,12} leukocyte interferon genes are already known, but knowledge derived from analysis of the proteins is confined to information on the N-termini^{5–8} and some tryptic fragments⁹. The presence of at least one disulphide bond in leukocyte interferon is suggested by that molecule's sensitivity to reducing agents¹⁰. In addition, comparison of all leukocyte interferon gene sequences so far reported indicates four highly conserved cysteines. One of these genes has been engineered for efficient direct expression in *Escherichia coli*⁴ and we have purified the gene product, leukocyte interferon A (IFN- α A, Fig. 1) from bacterial extracts to a single species of molecular weight (MW) 19,400¹³. I report here the determination of the disulphide bonds of the purified protein by analysis of tryptic fragments. The results indicate that Cys 1 is bonded to Cys 98, and Cys 29 is bonded to Cys 138.

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      10      20      30      40      50      60      70      80      90     100
CYS ASP LEU PRO GLN THR HIS SER LEU GLY SER ARG ARG THR LEU MET LEU LEU ALA GLN MET ARG LYS ILE SER
      110     120     130     140     150
LEU PHE SER CYS LEU LYS ASP ARG HIS ASP PHE GLY PHE PRO GLN GLU GLU PHE GLY ASN GLN PHE GLN LYS ALA
      160
GLU THR ILE PRO VAL LEU HIS GLU MET ILE GLN GLN ILE PHE ASN LEU PHE SER THR LYS ASP SER SER ALA ALA
      170     180     190     200
TRP ASP GLU THR LEU LEU ASP LYS PHE TYR THR GLU LEU TYR GLN GLN LEU ASN ASP LEU GLU ALA CYS VAL ILE
      210     220     230     240     250
GLN GLY VAL GLY VAL THR GLU THR PRO LEU MET LYS GLU ASP SER ILE LEU ALA VAL ARG LYS TYR PHE GLN ARG
      260     270     280     290     300
ILE THR LEU TYR LEU LYS GLU LYS LYS TYR SER PRO CYS ALA TRP GLU VAL VAL ARG ALA GLU ILE MET ARG SER
      310
PHE SER LEU SER THR ASN LEU GLN GLU SER LEU ARG SER LYS GLU

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Fig. 1 The amino acid sequence of IFN- α A predicted from the nucleotide sequence of the gene in the plasmid directing its expression in *E. coli*⁴. The cysteine residues are underlined.

Digestion of native IFN- α A with trypsin produces a mixture of fragments which can be partially resolved by HPLC as shown in Fig. 2a. Treatment of part of the tryptic digest before HPLC analysis with dithiothreitol (DTT) yields the profile shown in Fig. 2b. At least five significant peaks are lost on DTT reduction, indicating the presence of a disulphide bond in each. Further experiments showed that peak 1 is associated with one of the disulphide bonds and peaks 2–5 are associated with the other.

At increasing trypsin/interferon ratios, peaks 2 and 3 increase in size relative to peaks 4 and 5 indicating that they are related by the presence of a resistant trypsin cleavage site. At a trypsin/interferon ratio of 1:10, peaks 4 and 5 almost disappear. The fact that the 2, 3 doublet and the 4, 5 doublet wax or wane in unison suggests that 2 is related to 3 and 4 is related to 5.

Samples of peaks 1, 4 and 5 (3–12 μ g) were subjected to amino acid analysis. The results were compared with calculated values for all possible disulphide-containing peptides predicted from the nucleotide sequence of the gene (Table 1). Also

Fig. 2 HPLC of a trypsin digest of IFN- α A. A solution of 500 μ g of native IFN- α A in 400 μ l of 50 mM sodium phosphate, pH 7.8 was incubated with 8 μ g of TPCK-trypsin (Worthington) for 24 h at room temperature. Analysis was on an Altex ODS-Ultasphere column with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid¹¹. a, One half of the digest was injected and individual peaks collected. b, 20 μ l of the digest were added to 20 μ l of 7 M urea, 50 mM DTT and incubated for 2 h at room temperature before injection on the same HPLC system.