Structure of Porcine Pancreatic Phospholipase A₂ at 2.6 Å Resolution and Comparison with Bovine Phospholipase A₂

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The previously published three-dimensional structure of porcine pancreatic pro phospholipase A₂ at 3 Å resolution was found to be incompatible with the structures of bovine phospholipase A₂ and bovine pro phospholipase A₂. This was unexpected because of the very homologous amino acid sequences of these enzymes. Therefore, the crystal structure of the porcine enzyme was redetermined using molecular replacement methods with bovine phospholipase as the parent model. The structure was crystallographically refined at 2.6 Å resolution by fast Fourier transform and restrained least-squares procedures to an R-factor of 0.241.

The crystals appeared to contain phospholipase A₂ and not pro phospholipase A₂. Apparently the protein is slowly converted under the crystallization conditions employed. Our investigation shows that, in contrast to the previous report, the three-dimensional structure of porcine phospholipase A₂ is very similar to that of bovine phospholipase A₂, including the active site. Smaller differences were observed in some residues involved in the binding of aggregated substrates. However, an appreciable conformational difference is in the loop 59 to 70, where a single substitution at position 63 (bovine Val → porcine Phe) causes a complete rearrangement of the peptide chain.

In addition to the calcium ion in the active site, a second calcium ion is present in the crystals; this is located on a crystallographic 2-fold axis and stabilizes the interaction between two neighbouring molecules.

1. Introduction

Phospholipases A₂ (EC 3.1.1.4) are ubiquitous lipid-degrading enzymes, which can be found both inside and outside the cell. A review of the occurrence and properties of intracellular phospholipases has been published by van den Bosch (1980). A detailed compilation of our present knowledge on the extracellular phospholipases A₂ has appeared recently (Verheij et al., 1981).

The extracellular phospholipases are abundant in pancreatic tissue and juice, and in snake venoms. They are rather small proteins (M, 14,000 for the monomeric

form) and require calcium for their catalytic activity. Their amino acid sequences are highly homologous. In the pancreas the enzyme is synthesized as a pro-enzyme. Upon secretion into the gastrointestinal tract this pro-enzyme is activated by trypsin, which splits off the seven N-terminal amino acid residues. The catalytic properties of precursor and enzyme, with respect to monomeric substrates, are quite similar. However, when substrate is present as an aggregate, such as micelles, there is a large increase in the enzymatic activity of the enzyme, but not of itszymogen (Pieterson et al., 1974).

In order to elucidate the structural basis of the catalytic mechanism of lipid degradation by phospholipase A$_2$, and in order to explain the remarkable differences between the active and precursor enzymes with respect to aggregated substrates, we initiated X-ray crystallographic studies of the pancreatic phospholipases. This has resulted so far in the highly refined three-dimensional structure of the bovine pancreatic phospholipase A$_2$ at 1.7 Å resolution (Dijkstra et al., 1981a) and the structure of the bovine pro-enzyme at 3.0 Å resolution (Dijkstra et al., 1982). This latter structure is being refined at 2.0 Å resolution. From the architecture of the active site of the enzyme we were able to propose a mechanism for catalysis by these enzymes (Dijkstra et al., 1981b).

Although the primary structures of all phospholipases A$_2$ from different sources (from both pancreas and snake venom) are highly homologous, there are many differences in the details of their catalytic properties. Even within the pancreatic enzymes differences have been reported in specific activities towards different substrates, affinity for lipid/water interfaces, and calcium binding properties (Dutilh et al., 1975; van Scharrenburg et al., 1981). In addition, the porcine enzyme has been reported to possess a second, low-affinity calcium binding site, which is not present in the bovine enzyme (Slotboom et al., 1978).

Although a three-dimensional model of the porcine pro-enzyme was proposed (Drenth et al., 1976) on the basis of an electron density map phased with a single heavy-atom derivative, including anomalous scattering differences, (SIRAS method) at 3.0 Å resolution, additional information cast doubt on the correctness of this structure (Dijkstra et al., 1978, 1981a). Therefore, a reinvestigation of the porcine structure clearly was imperative. Molecular replacement methods (rotation and translation functions; Rossmann, 1972) offer a fast and elegant way to solve the crystal structures of closely related proteins, without the need for heavy-atom derivatives. Our successful experience with these methods in solving the structure of the bovine pancreatic prophospholipase A$_2$ (Dijkstra et al., 1982), starting from the model of the active bovine enzyme, prompted us to use the same methods for the porcine enzyme.

2. Experimental Methods

Porcine prophospholipase A$_2$ was generously supplied by Professor G. H. de Haas and co-workers at the Biochemical Laboratory, University of Utrecht, The Netherlands. Previous reports on the crystal structure of porcine pancreatic phospholipase A$_2$ have referred to the molecular species as prophospholipase. However, the present study indicates that the crystals contain phospholipase and not prophospholipase. Thus in this paper we refer to the crystallized porcine enzyme (Drenth et al., 1976) as phospholipase A$_2$. 
(a) Crystallization and data collection

Crystallization was accomplished as follows (Drenth et al., 1976): the enzyme was dissolved in 0·05 M-Tris-maleate buffer (pH 7·2), with 5 mM-CaCl$_2$. To 50 µl of this solution was added 10 to 15 µl of methanol. After 1 week at room temperature crystals had grown to sizes up to 0·5 mm x 0·5 mm x 0·5 mm. The space group is $P3_121$ with $a = b = 69·82$ Å and $c = 67·66$ Å, with one monomeric molecule per asymmetric unit. This procedure was equally successful using porcine prophospholipase and porcine phospholipase as the starting material.

The old data set to 2·8 Å resolution was expanded by collecting data from an additional 5 crystals to 2·6 Å. This is about the maximum resolution obtainable with the present crystal form. The measuring time per reflection was 6 to 8 min. For details on the data collection and processing, see Dijkstra et al. (1981a). The new data were merged into the existing 2·8 Å data set, using $1/\sigma^2$ ($\sigma$ being the standard deviation derived from the counting statistics of each reflection) as weighting factors for the averaging. An $R_{\text{merge}}$ term was calculated for 103 reflections present in both the old and new data sets:

$$R_{\text{merge}} = \frac{\sum_{hkl} |(F_{\text{old}} - F_{\text{new}})|}{\sum_{hkl} \frac{1}{2} \times (F_{\text{old}} + F_{\text{new}})} \times 100\%.$$ 

This $R_{\text{merge}}$ value was 5·8%. Altogether 4603 reflections, out of the possible 6144 to 2·6 Å, were included in the final data set (74·9%).

(b) Rotation function calculations

As the parent model we used the refined bovine phospholipase (Dijkstra et al., 1981a) with the solvent atoms and the calcium ion omitted. This model was positioned in a rectangular unit cell with cell dimensions $a = 60·0$ Å, $b = 67·0$ Å, $c = 68·0$ Å. These cell dimensions are such that no intermolecular vectors shorter than 20 Å occur, because a radius of integration of 19·2 Å was used in the computations. A structure factor calculation with an overall temperature factor of 20 Å$^2$ was carried out with a maximum resolution of 3·0 Å. These calculated structure factors were, together with the observed structure amplitudes of porcine phospholipase $A_2$, used as input for Crowther's (1972) fast rotation function program. For further details see Dijkstra et al. (1982).

(c) Translation function studies

The parent model was centered on the origin of a cell with the precise dimensions of the porcine phospholipase cell and rotated according to the result of the rotation function. Structure factors for the model were calculated out to a resolution of 3·0 Å with an overall temperature factor of 20 Å$^2$. These structure factors, together with the observed structure factors of porcine phospholipase $A_2$, were used as input for the translation function program (Crowther & Blow, 1967). A detailed account of the application of this program in space group $P3_121$ has been published by Dijkstra et al. (1982).

(d) Refinement

The parent model, properly oriented and positioned in the porcine phospholipase cell according to the results of rotation and translation functions, was refined using Agarwal's (1978) fast Fourier transform algorithm, with regularization of the structure at frequent intervals using the model-fitting program of Dodson et al. (1976). In the later stages of the refinement we applied the restrained least-squares procedures of Konnert & Hendrickson (1980).
3. Results and Discussion

(a) Rotation function studies

The sequences of the bovine and porcine enzymes are the most homologous in all known pancreatic phospholipase $A_2$ primary structures (about 85% identity), except for some isoenzymes. Compared to the bovine enzyme the porcine phospholipase has one insertion (at position 121) and 19 substitutions (see Fig. 1). Because of this high degree of homology it is reasonable to expect that their three-dimensional structures are also similar. From circular dichroism measurements (Jirgensons & de Haas, 1977), a similar folding could also be predicted. Therefore, use of the molecular replacement method seemed legitimate.

Table 1 gives a summary of the results obtained with the rotation function as a function of resolution range. In the present study it appeared essential, in order to obtain the correct result, to include data extending to 3·0 Å. When the highest resolution was 4 Å or worse, noise peaks became higher than the correct solution. The solution of the rotation function is $\alpha = 40^\circ$, $\beta = 48^\circ$, $\gamma = 355^\circ$. From the solutions obtained in the different resolution ranges we estimate the error in these values to be about 4°.

(b) Translation function studies

In order to determine the position of the enzyme in the unit cell we calculated translation functions at different resolution ranges.

Looking for the vectors between the model molecule in its starting orientation and the molecule rotated about the 31 axis, the highest peak occurred in the section $z = 1/3$ at position $x = 0·855$, $y = 0·81$. This confirms that the space group of the porcine enzyme is $P3_{1}2_{1}$ and not its enantiomorph $P3_{2}2_{1}$. Table 1 summarizes the results obtained with the translation function in section $z = 1/3$.

Table 1

<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
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<td>(a) Ala-Leu-Trp-Gln-Phe-Asn-Gly-Met-Ile-Lys-Cys-Lys-Ile-Pro-Ser-Ser-Glu-Pro-Leu-Leu-Asp-Phe-Asn-Asn-</td>
<td>Arg Ser Ala Gly His Met</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(b)</td>
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</tr>
<tr>
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<td>100</td>
<td>105</td>
<td>110</td>
<td>115</td>
<td>120</td>
</tr>
</tbody>
</table>

Fig. 1. Comparison of the amino acid sequences of: (a) bovine pancreatic phospholipase $A_2$ (Fleer et al., 1978); and (b) porcine pancreatic phospholipase $A_2$ (de Haas et al., 1970; Puijk et al., 1977).
STRUCTURE OF PORCINE PHOSPHOLIPASE A₂

Table 1
Summary of results obtained with the rotation and translation functions

<table>
<thead>
<tr>
<th>Resolution range (Å)</th>
<th>3-5</th>
<th>3-6</th>
<th>3-7</th>
<th>3-8</th>
<th>3-9</th>
<th>3-10</th>
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<tr>
<td>No. of reflections (F_{obs})</td>
<td>5042</td>
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<td>6041</td>
<td>6222</td>
<td>6349</td>
<td>6432</td>
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<tr>
<td>No. of reflections (F_{calc})</td>
<td>4115</td>
<td>5141</td>
<td>5620</td>
<td>5907</td>
<td>6139</td>
<td>6326</td>
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<tr>
<td>Rotation function result†</td>
<td>1·1</td>
<td>1·2</td>
<td>1·3</td>
<td>1·3</td>
<td>1·7</td>
<td>1·7</td>
</tr>
<tr>
<td>Translation function result†</td>
<td>2·1</td>
<td>2·5</td>
<td>2·5</td>
<td>2·6</td>
<td>2·5</td>
<td>1·4</td>
</tr>
</tbody>
</table>

† Indicated in this Table is the ratio (peak height of correct solution)/(height of highest noise peak).

The results are not very dependent on the resolution, except that lower-resolution data seem to impair the result. This translation function calculation does not provide information on the z coordinate of the molecular centre. The z coordinate can, however, be determined by a translation function search for the vectors between the model molecule and the model rotated about one of the 2-fold axes perpendicular to the 3₁ axis. Using the 2-fold axis parallel to the [110] direction, which lies in the plane z = 0, we observed a peak at 0·415, 0·585, 0·615. Solving the appropriate equations for the translation function yields as the approximate position of the molecular centre: x = 0·37, y = -0·22, z = -0·31. The positioning of the molecule in its appropriate orientation in the P₃₁₂₁ cell of porcine phospholipase A₂ yielded a crystallographic R-factor of 0·50 for 3879 unique reflections between 7·1 and 2·8 Å. Individual temperature factors as present in the parent model (Dijkstra et al., 1981a) were used. The rotation and translation parameters were refined by carrying out rigid body movements with concomitant R-factor calculations. The final result was a reliability factor of 0·396, indicating the close similarity between the porcine and bovine enzymes. The corresponding rotation and translation parameters are: α = 42·9°, β = 48·2°, γ = 353·0°, x = 0·37, y = -0·22, z = -0·30. This means that, although the rotation function results deviated 2 to 3° from the correct value in both α and γ, this did not prevent a very accurate solution of the translation function. A (2Fₒ - Fₑ) Fourier map calculated at this stage confirmed our solution of the rotation and translation functions. Most of the map was clear and unambiguous. The calcium ion could be placed without difficulty. Near the N terminus of the parent model (Ala₁) we looked for electron density representing the seven extra residues, still assuming this to be the structure of the prophyrophospholipase. There was some density that might accommodate about three of the extra residues, but the connection with Ala₁ was not clear. Also some parts of the loop 63 to 77, which is near the N terminus, were clearly not in an area of density. In order to assess the differences in three-dimensional structure more reliably, we decided to refine the structure in spite of the relatively low resolution of the observed data.

(c) The refinement

Because of the great computational advantages of Agarwal's fast Fourier refinement procedure (speed and convergence radius; Agarwal, 1978) and because
of the good experience with this method in our laboratory (Dijkstra et al., 1981a),
we started the refinement with this program. The starting model for the
refinement was the rotated and translated bovine phospholipase A2 model, after
removal of the side-chain atoms beyond Cβ of those residues that are different in
the two enzymes (Fig. 1). Ala1 and residues 120 to 123 were also left out, because
we expected the structures to be rather different in these regions. Neither solvent
atoms nor the calcium ion were included. Table 2 gives a summary of the progress
of the refinement. After each series of refinement cycles a \((2F_{\text{obs}} - F_{\text{calc}})\) Fourier
map was calculated, the model was examined and, when necessary, rebuilt on an
Evans & Sutherland Picture System II, using the program GUIDE (Brandenburg
et al., 1981).

After the first series of refinement cycles all the side-chains of the amino acid
residues that differ in the porcine and bovine enzymes could be incorporated. Also
residues 120 to 123 showed up clearly, and the calcium ion was added. It was
obvious that the loop from 60 to 70 was wrongly placed. Only those residues in
this loop that could readily be placed in areas of density were added to the model.
Several interpretations of this part of the molecule were tested during the next
three series of refinement cycles: none of them was quite satisfactory.
Reinterpretation of the density at this stage indicated that the protein in the
crystals probably was active phospholipase A2 and not the pro-enzyme. It then
became possible to assign density to all residues in the loop from 60 to 70. A
second calcium ion was also included in the new model.

At this stage we switched to the restrained least-squares refinement program of
Konnert & Hendrickson (1980). Table 3 gives a summary of the weights used and
the improvement in the geometry of the model. van der Waals' distances and
hydrogen bonds were included in the refinement. In particular, the chiral
volumes, the non-bonded contacts and the temperature factors needed restraints.
The root-mean-square (r.m.s.) shift in the co-ordinates of all atoms in the model

\[ R\text{-factor} = \frac{\sum |F_{\text{obs}} - kF_{\text{calc}}|}{\sum F_{\text{obs}}}. \]

\( \sum |F_{\text{obs}}| \) and \( 2\sigma \) A; from series VII onwards between 7-1 and 2-8 A.
### Table 3

*Summary of input parameters and results obtained with the Restrained Least Squares Refinement procedure*

<table>
<thead>
<tr>
<th></th>
<th>Input s.d.†</th>
<th>Refinement series VI</th>
<th>Refinement series VII</th>
<th>Refinement series VIII</th>
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<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>1-2 distances (Å)</td>
<td>0.015</td>
<td>0.048</td>
<td>0.007</td>
<td>0.007</td>
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<td>1-3 distances (Å)</td>
<td>0.050</td>
<td>0.087</td>
<td>0.049</td>
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<td>1-4 distances (Å)</td>
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<td>0.120</td>
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<td>Plane distances (Å)</td>
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<td>0.0440</td>
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<td>Chiral volumes (Å³)</td>
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<td>Single torsion contacts (Å)</td>
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<td>S—S bonds (Å)</td>
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<td><strong>Temperature factors:</strong></td>
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<tr>
<td>Main-chain bond (Å²)</td>
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<td>Main-chain angle (Å²)</td>
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<td>3.56</td>
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<td>Side-chain bond (Å²)</td>
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<td>Side-chain angle (Å²)</td>
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<td>3.78</td>
<td>3.69</td>
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<td><strong>Structure factors:</strong></td>
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<td>81.4</td>
<td>78.6</td>
<td>79.6</td>
</tr>
<tr>
<td>r.m.s. shifts in co-ordinates between subsequent refinement series (Å)</td>
<td>—</td>
<td>—</td>
<td>0.412</td>
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</table>

† s.d., standard deviation.
during this series (VI) of refinement cycles was 0.412 Å. For the main chain N, Ca and C atoms the r.m.s. shift was 0.218 Å. The average temperature factor (B) decreased from 22.8 to 21.3 Å² for all atoms, but for the main-chain N, Ca and C atoms it increased from 15.9 to 20.2 Å². The r.m.s. B difference between the input and output models of refinement series VI was 19.4 Å² (for the main chain N, Ca and C atoms 18.3 Å²). Because of the limited resolution the refinement of truly individual temperature factors is not feasible and therefore we gave high weights on the restraints of the temperature factors. This is reflected in the close similarity of the average temperature factors of main-chain and side-chain atoms.

The improvement in the geometry of the model and restraining the temperature factors increased the R-factor from 0.223 at the end of refinement series V to 0.239. In conjunction with a Ramachandran plot of the main-chain dihedral φ and ψ angles (Ramakrishnan & Ramachandran, 1965), a (2Fo −Fc) difference Fourier suggested a few changes in the model. At this stage the newly measured data became available and we expanded the resolution of our data set to 2.6 Å. With this expanded data set nine cycles of restrained least-squares refinement (series VII) were performed. A difference Fourier revealed the positions of five water molecules, but no changes in the model were apparent. The five water molecules were added to the model and the refinement was completed with another nine cycles of restrained least-squares refinement (series VIII). The difference Fourier calculated after this series was featureless. The final crystallographic R-factor is 0.241 for 4295 reflections between 7.1 and 2.6 Å. A smaller discrepancy between the observed and calculated structure factors might have been achieved by relaxing the restraints on distances, angles and temperature factors. However, we did not feel this to be warranted, taking into account the resolution of the observed data. In accordance with other structures refined at a similar resolution (e.g. see Deisenhofer, 1981), we estimate the r.m.s. error in the co-ordinates to be about 0.25 to 0.30 Å for the well-defined atoms. For side-chains of several residues at the molecular surface, the electron density is very weak and the present interpretation should be considered tentative. These uncertainties comprise residues Leu19 (including main-chain atoms), Met20 (including main-chain atoms), Asn24, Asp59, Asn79, Glu81, Lys113 and Lys121. The refined co-ordinates of porcine phospholipase A₂ have been deposited with the Brookhaven/Cambridge Protein Data Bank.

(d) Description of the three-dimensional structure of porcine phospholipase A₂

(i) Prophospholipase or phospholipase?

From the electron density maps it appeared that we had solved the structure of the porcine phospholipase and not of the prophospholipase. No density protrudes from Ala1 to accommodate the extra N-terminal residues of the pro-enzyme. Moreover, the packing of the molecules in the crystals is such that no room is available for extra residues. Furthermore, the N-terminal amino acid in dissolved crystals was determined by Edman degradation: it appeared to be Ala, which is
the N-terminal amino acid of active phospholipase, and not Glu or pyro-Glu, which is the N-terminus in the prophospholipase. Finally, in contrast to previous experience, the porcine phospholipase itself could be crystallized directly under identical conditions as described in Experimental Methods. Precession photographs obtained from these crystals and from the crystals used in the present study were indistinguishable. Because the starting material used for this present study had been proved to be prophospholipase, the prophospholipase was apparently slowly activated under the crystallization conditions used, and it was the active form that crystallized.

(ii) Folding of the peptide chain and the secondary structure

In accordance with the deductions from its amino acid sequence, the structure of porcine phospholipase is very similar to that of the bovine enzyme. Figure 2 is a stereoscopic pair showing the backbones of the two enzymes, after optimal superimposition, using a method similar to the one described by Rao & Rossmann (1973). A comparison of 112 equivalent Cα positions in the porcine and bovine phospholipases gives a root-mean-square difference of 0.47 Å (the carbon atoms of residues 59 to 70 and 120 to 121 were not included).

Three regions in the bovine and porcine enzymes are clearly different: residues 31 and 33, the loop from 59 to 70 and the C-terminal region around residues 120 and 121.

(1) By far the largest, and also by far the most unexpected difference occurs in the loop from 59 to 70. In the bovine enzyme the first part of this loop is a short α-helix (residues 59 to 66) and the second part forms a surface loop (from 67 to 70). In the porcine enzyme one single substitution has occurred at position 63: a Val in the bovine enzyme has been replaced by a Phe. Whereas in the bovine enzyme the side-chain of Val63 is at the surface of the molecule, the Phe63 side-chain is in the molecule's interior, probably because of its more hydrophobic character. The

Fig. 2. Stereo diagram of the superimposition of the Cα positions of porcine and bovine phospholipase A₂. The bovine enzyme is indicated by broken lines. All stereo diagrams in this paper were made using an interactive graphics system and the program GUIDE (Brandenburg et al., 1981).
short α-helix in the bovine enzyme has disappeared in the porcine enzyme, and instead, residues 67 to 71 in the porcine enzyme form one and a half turns of 3₁₀ helix. The r.m.s. deviation between the 12 residues from 59 to 70 in both enzymes is 4·7 Å for the Cα atoms. This result is extremely hard to predict as both side-chains are hydrophobic in character.

(2) The insertion at the C terminus in the porcine enzyme is clearly at position 121. Residues 118 and 119 are still remarkably well in place, while residue 120 is bending a little outwards. Residue 122 in the porcine enzyme has its Cα atom already more or less in the same position as 121 Cα in the bovine enzyme (the discrepancy is 0·62 Å). The result of this insertion is thus a more obtuse bend of the polypeptide chain in the porcine enzyme.

(3) The third region with conformational differences occurs around Leu31 and Gly33, which is part of the calcium binding loop. The Cα atoms of residues 31 in the two models are 1·06 Å apart (see section (iv) below and Fig. 4). No change in amino acid sequence, which might explain this difference, occurs in this region. Presumably, substitutions further away cause this difference.

The remaining parts of the enzymes are very similar in structure, including the hydrogen-bonding pattern in the regions of secondary structure.

(iii) The active site

The active site of phospholipase A₂, as inferred from the crystal structure of the bovine enzyme, is made up of several invariant residues: His48, Asp99, Phe5, Ile9, Phe22, Ala102, Ala103, Phe106 and the disulfide bridge between Cys29 and Cys45 (Dijkstra et al., 1981b). Also, the essential calcium ion is in this region. We compared the active sites of porcine and bovine phospholipase A₂. Figure 3 shows the result of this comparison after optimal superimposition of the active site residues. The r.m.s. discrepancy between 82 atom pairs is 0·35 Å. This discrepancy is of the order of the sum of the estimated errors in the structures of the porcine and bovine enzymes. The small tilt in the ring of Phe106 is not significant. Apart from the above residues, which were proposed to be involved in the binding and hydrolysis of monomeric substrate (Dijkstra et al., 1981b), the residues hydrogen-bonded to the Asp99 side-chain are also in identical positions. Thus, within the accuracy of the present models, the active sites of bovine and of porcine

![Fig. 3. A stereo drawing with a comparison of the active sites in the porcine and bovine phospholipases A₂. The heavy lines represent the porcine enzyme.](image-url)
phospholipases $A_2$ are identical. The same applies to the relative mobility of the active site residues with respect to the rest of the molecule: in the bovine enzyme, the active site residues have a low mobility and their average temperature factor is $59 \text{ A}^2$. This is about 55% of the average temperature factor of all protein atoms. In the porcine enzyme, where the temperature factors are about twice as high as in the bovine enzyme, this ratio is 60%. Finally, a water molecule is located near His48 Nδ1 in the porcine active site, in a position similar to that of the putative catalytic water molecule in the bovine active site. This close similarity between the active sites suggests similar catalytic properties for action on monomeric substrates. Unfortunately, reliable comparative kinetic constants pertinent to the hydrolysis of monomers are not available.

(iv) The calcium binding site in the active centre

The essential cofactor, calcium, is bound to porcine phospholipase $A_2$ with an affinity constant of about $4 \times 10^2 \text{ M}^{-1}$ at pH 6.0, whereas, under identical conditions, the bovine enzyme has a slightly lower affinity for the metal ion ($K_a = 1.25 \times 10^2 \text{ M}^{-1}$) (Meyer et al., 1979). In the three-dimensional structure of bovine phospholipase $A_2$ calcium was found to be co-ordinated by seven oxygen ligands: the carbonyl oxygens of Tyr28, Gly30 and Gly32, the carboxylate oxygens of Asp49 and two water molecules. Apart from these residues co-ordinating the calcium ion, several other highly conserved residues in the loop from 25 to 42 serve to stabilize the conformation of the calcium binding site. Figure 4 shows that the greater part of this region is identical in porcine and bovine phospholipases. However, differences are seen in the loop from residue 30 to 34. In particular, the conformation of Leu31 is quite different, both with respect to its side-chain and its main chain. Also, residue 33 is in a different position. The cause of these differences remains to be established. No amino acid substitution occurs in this region. Presumably, substitutions elsewhere in the

![Fig. 4. Stereo picture of the calcium binding loops in the porcine (heavy lines) and bovine phospholipases $A_2$. Residue names are those of the porcine enzyme.](image)
molecule affect the conformation of this part. There might be an intimate relation between this conformational difference and the difference in calcium affinity between the two enzymes.

(v) The second calcium binding site in porcine phospholipase A₂

In addition to the Ca²⁺ bound in the active site (see above), the presence of a second calcium binding site has been reported for porcine phospholipase (Slotboom et al., 1978; Andersson, 1981). This site is also specific for calcium, but has a much lower affinity for the metal ion \( K_a \approx 50 \, \text{m}^{-1} \) at pH 7.5. From nuclear magnetic resonance studies and ultraviolet light spectroscopy, it was concluded that this second Ca²⁺ binding site must be located close to the N terminus of the polypeptide chain (Slotboom et al., 1978).

Electron density comparable in height to the density of the active site calcium ion in a \((2F_o - F_c)\) map was found at 7.8 Å from the Ala₁ NH₃⁺ group. This density was distinctly higher than that of the water molecules found in the structure. Moreover, two carboxylates were in close contact with this area of density. Therefore it was assigned to a calcium ion. This second calcium ion is located on a crystallographic dyad and two symmetry-related molecules contribute to its coordination sphere. Thus there is only half a calcium ion per phospholipase molecule at this site in the crystal structure. The metal ion is co-ordinated by four ligands: two carbonyl oxygen atoms of Ser72 and two Oε atoms of Glu92. Space is available for two water molecules to serve as additional calcium ligands, but at this resolution no electron density is present to account for them. In the crystal structure the positive charge on the calcium ion is thus fully compensated by two carboxylates. This calcium ion stabilizes the interaction between two neighbouring molecules in the crystal structure.

It is uncertain whether this intermolecular calcium position in the crystal structure is identical to that of the second calcium ion as shown from solution studies. Evidence against a direct extrapolation of the crystal studies to solution studies is twofold:

1. In the crystals only 0.5 Ca²⁺ per phospholipase molecule is present at this site, whereas in the solution experiments a result of 1.0 Ca²⁺ per molecule was obtained for this second site (Slotboom et al., 1978).

2. In contrast to the crystallographic result, it was demonstrated from proton titration experiments that Glu71 is a ligand of this second calcium ion (Donné-Op den Kelder et al., 1983). In the crystal structure Glu71 is close to the second calcium ion, but its carboxyl group is turned away from the calcium. However, a simple rotation about the Cγ-Cδ bond would bring the Oε1 or Oε2 atom of Glu71 within liganding distance of the calcium.

Thus, in order to reconcile these conflicting results we propose that in solution, where porcine phospholipase is a monomeric enzyme, the second calcium ion is coordinated by the side-chains of Glu71 and Glu92, the carbonyl oxygen of residue 72 and by some water molecules. Because in the bovine enzyme Glu71 is replaced by an Asn, it is obvious that a similar site in the bovine enzyme cannot exist, as indeed has been observed (Donné-Op den Kelder et al., 1983).
(vi) The N terminus

The N-terminal region and, especially, the α-NH$_3^+$ group of Ala1 play an important role in the activity of the enzyme on aggregated substrates (Pieterson et al., 1974; van Dam-Mieras et al., 1975). In bovine phospholipase the α-NH$_3^+$ group of Ala1 was found to be hydrogen-bonded to the carbonyl oxygen of the side-chain of Gln4, to the main-chain carbonyl oxygen of Asn71 and to a water molecule, which in turn is hydrogen-bonded to the hydroxyl group of Tyr52, the carbonyl oxygen of Pro68 and to the O$_{δ1}$ of the catalytic site residue Asp99 (Dijkstra et al., 1981a). In the porcine enzyme the same hydrogen-bonding pattern is present, although the finer details of the structure in this region are different (Fig. 5). In the porcine phospholipase as in the bovine enzyme, an internal water
molecule is located between the N terminus and the active site. However, this water molecule does not form a hydrogen bond with the carbonyl oxygen of Pro68. In contrast, hydrogen bonds are present between the N of Leu2 and the carbonyl oxygen of Tyr69 and between the Oe1 of Gln4 and Gln73 N. These hydrogen bonds are absent in bovine phospholipase. In order to form the latter hydrogen bond in the porcine enzyme the Gln4 side-chain adopts a different conformation, and also the conformation of Ala1 is slightly changed. As a result, in the porcine enzyme the N terminus is somewhat more open to the solvent than it is in the bovine enzyme.

(vii) *The interface recognition site*

A major difference between porcine and bovine phospholipases is the lower affinity of the bovine enzyme for lipid/water interfaces. Moreover, the porcine enzyme can degrade more densely packed monolayers of lecithin than the bovine enzyme (Dutilh *et al.*, 1975; van Scharrenburg *et al.*, 1981). To explain the interaction of the phospholipase molecule with aggregated substrates like micelles, Pieterson *et al.* (1974) proposed that the enzyme possesses a special site, topographically distinct from the active site and called the Interface Recognition Site (IRS). Numerous kinetic and amino acid modification experiments combined with the three-dimensional structure of bovine phospholipase gave a clear picture of this site (for a review, see Volwerk & De Haas, 1982). On the basis of the X-ray structure of bovine phospholipase the following 20 residues were indicated as (possible) IRS residues (Dijkstra *et al.*, 1981b): 2, 3, 6, 17, 19, 20, 23, 24, 31, 56, 65, 67, 69, 70, 72, 116, 117, 119, 120 and 122 (numbering in Fig. 1). These residues are arranged in a broad ring around the entrance to the active site (Fig. 6). None of these residues is absolutely conserved in all the phospholipases sequenced so far. Between the bovine and porcine enzyme, five substitutions have occurred: at positions 6 (Asn in bovine → Arg in porcine), 17 (Glu → His), 20 (Leu → Met), 72

![Fig. 6. A stereo diagram showing a comparison between the residues involved in the binding of ordered substrates in porcine and bovine phospholipases A2. Broken lines represent the bovine enzyme.](image)
(Asn → Ser) and 120 (Lys → Thr). Moreover, in the porcine enzyme the insertion at position 121 is also part of the IRS, as well as Leu64, due to the different conformation of the loop 59 to 70 (see Fig. 6). Omitting these two latter residues, and 65 and 67 (which deviate more than 3.5 Å), the Ca atoms for the 18 other (possible) IRS residues differ by only 0.69 Å (r.m.s.), indicating the similarity in folding of the greater part of the main chain in the IRS region.

Most of the side-chains are also quite similar in conformation in both enzymes with the exception of Tyr69, which has a very different conformation (the Oη atoms differing by no less than 5.6 Å).

These conformational differences might play a role in explaining the different affinity of the bovine and porcine enzymes for aggregated substrates. On the other hand the substitutions at positions 6 (Asn → Arg) and 17 (Glu → His) make the IRS more basic in character in the porcine enzyme and this suggests a simple electrostatic explanation for its tighter binding to the polar surface layer of the substrate. This is supported by the following observation: a semi-synthetic bovine phospholipase A2, which has an Arg at position 6 instead of an Asn, has an affinity for aggregated substrates comparable to that of the porcine enzyme (van Scharrenburg et al., 1981). Therefore, differences in the binding properties for aggregated substrates seem to be due to differences in the side-chain characteristics, but the effect of a conformational difference cannot be excluded.

4. Conclusions

The present investigation has revealed the three-dimensional structure of porcine phospholipase. The previous structural report on this enzyme (Drenth et al., 1976) is clearly not correct. The diffraction data of the native enzyme, although of limited resolution and quality, contain the right information. Probably, the use of only one heavy-atom derivative and an over-optimistic view of its quality as an isomorphous derivative have misled the authors.

The discovery that the porcine phospholipase crystals contained the enzyme and not the pro-enzyme was a complete surprise. Although it has been known for a long time that solutions of prophospholipase are slowly converted to yield the phospholipase, this was not expected to occur in mixed solvents. Presumably, some proteolytic activity is still present in the prophospholipase preparation and this proteolytic activity is not inhibited by 20% methanol solutions. In the case of the bovine pro-enzyme, which is crystallized from 50% 2-methyl-2,4-pentanediol, we did obtain crystals of the prophospholipase. As was to be expected from the high degree of sequence homology between the bovine and porcine phospholipases, the three-dimensional structures of these two enzymes are very similar. The most remarkable difference in structure occurs in the region 59 to 70, where a single amino acid substitution (Val → Phe) causes a large and unexpected conformational rearrangement of the polypeptide chain. The active sites of the bovine and porcine enzymes appeared to be virtually identical. The Interface Recognition Site contains various differences between the two enzymes: in one part due to amino acid substitutions, in another due to a different conformation of the 59 to 70 loop.
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REFERENCES


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