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Crystallization and preliminary X-ray diffraction studies of a new crystal form of human secretory type IIA phospholipase A_2

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Human synovial type IIA phospholipase A_2 ($sPLA_2$-IIA) has been implicated in the pathogenesis of a number of inflammatory diseases and is a target for the development of therapeutically useful inhibitors. Biochemical evidence suggests a novel mechanism of inhibition for a series of peptide inhibitors originally derived from the primary sequence of the protein. On co-incubation with one of these inhibitors, single crystals of a hitherto unreported crystallographic form of sPLA2-IIA suitable for diffraction analysis were obtained. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters $a = 140.8$, $b = 38.9$, $c = 109.1$ Å, $\beta = 125.1^\circ$, and diffraction at 2.4 Å resolution has been observed.

1. Introduction

Human type IIA secretory phospholipase A_2 (EC 3.1.1.4; $sPLA_2$-IIA) is a member of a growing superfamily of 13–18 kDa calcium-dependent disulfide-linked α-helical proteins which hydrolyse the *sn*-2 fatty acyl ester bond of phosphoglycerides. Nine mammalian enzymes have been described to date and each is differentially expressed in a tissue-, species- and/or genotype-specific manner (Scott *et al.*, 1999). $sPLA_2$-IIA is pro-inflammatory *in vivo*, is associated with the onset and severity of rheumatoid arthritis (Lin *et al.*, 1996; Pruzanski *et al.*, 1988) and septic shock (Vadas, 1984) and is induced in the synovial tissue of patients with arthritis (Jamal *et al.*, 1998). Evidence also exists implicating $sPLA_2$-IIA in a number of other immune-mediated inflammatory conditions such as psoriasis, adult respiratory distress syndrome, asthma and pre-term labour (Anderson *et al.*, 1994; Vadas *et al.*, 1993). This enzyme is therefore a target for the development of inhibitors which may have therapeutic utility.

The crystal structure of $sPLA_2$-IIA has been determined (Scott *et al.*, 1991). Comparison of this structure with the structure of $sPLA_2$-IB (Dijkstra *et al.*, 1981), which is primarily secreted from the pancreas, shows marked structural conservation, particularly in the active site and hydrophobic channel in which substrate binds. The active site is lined by the invariant Leu2, Phe5 and Ile9 side chains of the N-terminal helix and is substantially conserved in all $sPLA_2$ structures described thus far. Inhibitors which bind directly to this site have been well characterized by X-ray crystallography (Scott *et al.*, 1991; Schevitz *et al.*, 1995; Oh, 1995; Cha *et al.*, 1996; Kitadokoro *et al.*, 1998). These inhibitors are largely non-selective for $sPLA_2$-IIA, showing inhibition of other subtypes (Bezzine *et al.*, 2000).

We have identified pentapeptide inhibitors derived from residues 70–74 of $sPLA_2$ which are selective for the enzyme subtype from which they are derived (Tseng *et al.*, 1996). Biochemical evidence implicates the N-terminal helix as a binding site for the peptide inhibitors. These data suggest a novel mode of inhibition of $sPLA_2$-IIA. Inhibition with the linear peptide compounds is weak ($IC_{50} \simeq 100$ mM) in a ^{3}H-labelled *Escherichia coli* membrane *in vitro* assay. In recent work, we have developed cyclic peptide inhibitors, cyclic Phe-Leu-Ser-Tyr-Arg and cyclic (2-napthylalanine)-Leu-Ser-(2-napthylalanine)-Arg, which are three and 70 times more potent than linear Phe-Leu-Ser-Tyr-Lys, respectively (Church *et al.*, 2000). The crystallographic structure of these compounds bound with $sPLA_2$-IIA would assist in establishing the mechanism of inhibition. Consequently, we have been co-crystallizing type IIA phospholipase A_2 with members of the series of inhibitors.

2. Materials and methods

2.1. Protein expression and purification

Conditioned media were obtained from fermentation of the stably transfected CHO cell line 2B1 containing the $sPLA_2$-IIA cDNA under the control of the human metallothionein promoter and were stored at 377 K in 1 M NaCl. 20 l of media contains approximately 4 mg of the protein as determined by ELISA (Tseng *et al.*, 1996). 2 l batches were then centrifuged at 7000*g* (Sorvall RC5Cplus, Sorvall Instruments) for 30 min to reduce solid matter. $sPLA_2$-IIA was purified to homo-

geneity by a one-step affinity chromatography procedure (Äkta Explorer, Pharmacia Biotech, Sweden) as follows. Supernatant was loaded on to an affinity column coupled with the $sPLA_2$-specific monoclonal antibody 10B2. The column was then washed extensively (0.1 *M* Tris–HCl pH 7.4, 1 *M* NaCl), eluted with 0.1 *M* glycine pH 2.7 and fractions pooled for the crystallization experiments. Concentration of the protein and buffer exchange was performed by filtration using Plus-20 (Amicon) and Ultrafree-MC (Millipore, USA) concentrators in benchtop centrifuges at 15 000*g* (Beckman GS-6R, Beckman Instruments; Labofuge 400R, Heraeus Instruments). The enzyme was assayed for activity and was within the range of specific activity as previously reported (Tseng *et al.*, 1996). The identity was verified by N-terminal sequence analysis and purity was confirmed by SDS–PAGE analysis followed by silver staining. Major losses of protein occurred if freshly siliconized glassware and regenerated cellulose membranes were not used. This procedure routinely resulted in 60% recovery of $sPLA_2$-IIA for use in crystallization setups.

2.2. Peptide synthesis

Cyclic peptides were assembled using f-moc solid-phase chemistry without removal of side-chain protection groups prior to cleavage from the resin. Peptides were cyclized by amide-bond formation using standard peptide-synthesis activation and coupling chemistry prior to deprotection (Auspep, Australia). All peptides were purified by reverse-phase HPLC as described in Tseng *et al.* (1996). Peptide purity and identity was confirmed by mass spectrometry and amino-acid analysis.

2.3. Crystallization

Crystallization conditions were surveyed by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates (Linbro, ICN Biomedicals, Ohio). Cover slips were purchased pre-siliconized from Hampton Research (Laguna Niguel, CA, USA). 4 µl drops were used in the presence of the inhibitor at approximately threefold molar excess over protein. Solutions and tissue-culture plates were all stored at crystal growth temperature (293 K) in a Sanyo MIR-2530 incubator (Sanyo Electric, Japan) unless otherwise stated. Macroseeding was performed by transferring microcrystals into fresh hanging drops with the assistance of nylon loops made specifically for the purpose.

Diffraction data were collected on a DIP-2030b system with Cu $K\alpha$ radiation at 45 kV and 100 mA generated on a Nonius rotating-anode generator with focusing mirrors unless otherwise stated. A nitrogen-gas Cryostream Cooler (Oxford Cryosystems, England) was used on the system with a temperature of 100 K. All crystals were protected for flash-cooling using mother liquor with protectant solvent. The Advanced Photon Source (APS; Argonne National Laboratory, IL, USA) beamline 14BMC was used in conjunction with the Quantum 4 detector (ADSC, Poway, CA, USA) and the Molecular Structure Corporation crystal cooler (MSC, Woodlands, TX, USA). Data were processed using *DENZO* (Otwinowski & Minor, 1997); maps were calculated with the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) and were examined using *O* (Jones *et al.*, 1991). Data collected at APS were processed the with *DPS*/*MOSFLM* 5.5 package (Nielson *et al.*, 1998; Leslie, 1992).

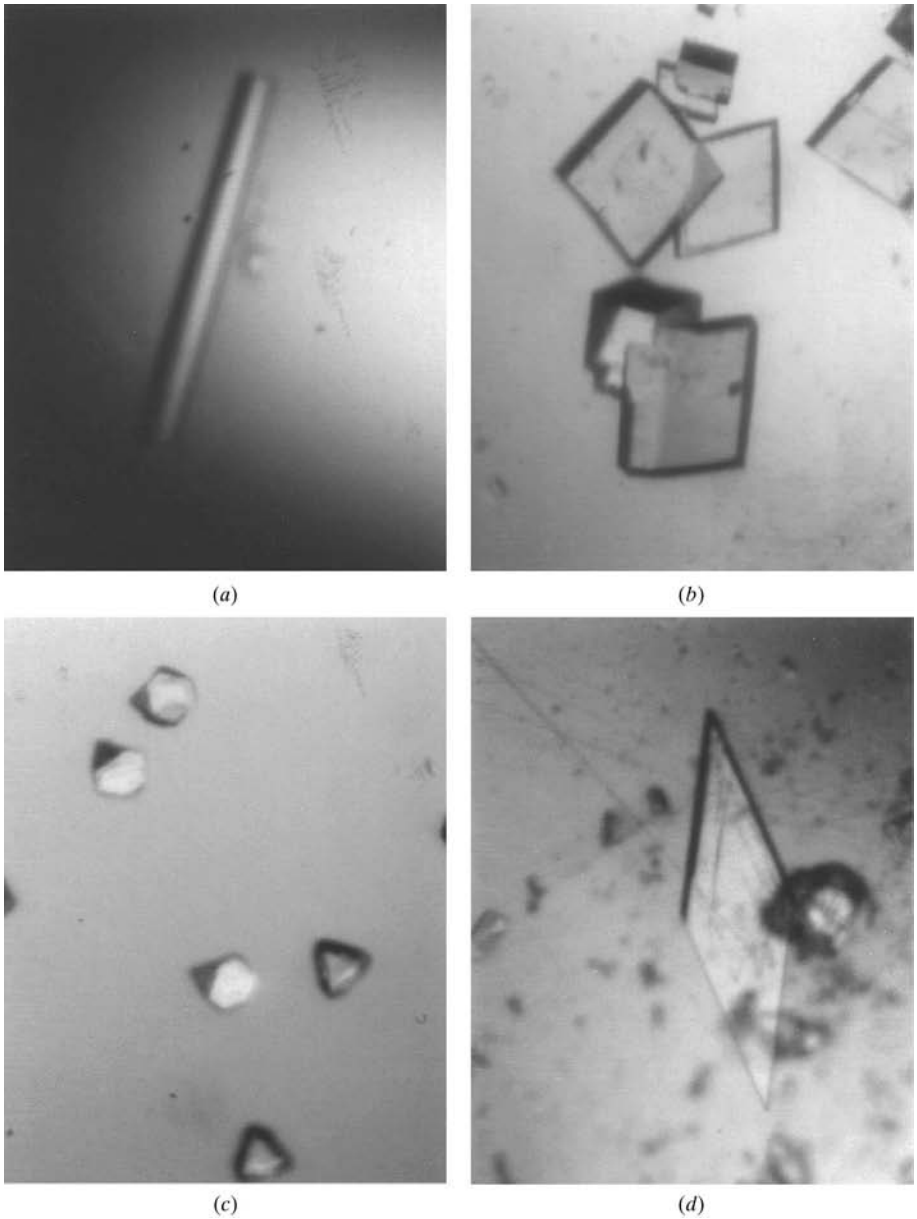

Figure 1
Crystals of $sPLA_2$-IIA: (*a*) hexagonal, (*b*) type II, (*c*) type III, (*d*) type IV, which were suitable for crystallographic analysis.

3. Results and discussion

The conditions for the crystallizations are 10 mg ml^{-1} protein with 4.5 *M* NaCl, 0.1 *M* Tris–HCl pH 7.4, 5 m*M* $CaCl_2$, 0.5 m*M* β-octyl-glucopyranoside as precipitant, which is similar to those reported previously

(Scott *et al.*, 1991). Inhibitors were at saturation in all the solutions added to the protein for the crystallizations, producing a threefold molar excess. Behaviour in the hanging drops was dependent on the time of incubation of the protein and inhibitor prior to setup. Four crystal types have been observed in the course of these experiments. Freshly prepared mother liquor containing 15% glucose cryo-protectant solution provided the most stable conditions for low-temperature data collection within the concentration range tested [10–20%(*w*/*v*) glucose].

In the absence of inhibitors, single hexagonal crystals with unit-cell parameters $a = b = 75.9$, $c = 89.4$ Å and space group $P6_122$ grew to $0.5 \times 0.1 \times 0.1$ mm in 5–6 d as previously reported (Scott *et al.*, 1991). When protein and the linear peptide inhibitor (Phe-Leu-Ser-Tyr-Lys) are mixed and immediately used for crystallization, single hexagonal crystals (type I) also grew within the same time frame (Fig. 1*a*). These conditions occasionally produced showers of crystals and some intergrowth and twinning. Crystals grown in the presence of Phe-Leu-Ser-Tyr-Lys became sensitive to manipulation and have not been observed to diffract much beyond 2.8 Å on the rotating-anode system. However, at the APS strong diffraction was observed and data were collected to 2.2 Å. A complete data set to 2.4 Å (98.3% complete, $R_{\rm merge}$ = 7.6%, fivefold redundancy) was processed. Evidence for the bound peptide was not found. The contacts formed between $sPLA_2$ molecules in this crystal form, particularly around the N-terminus, do not allow for the significant extra density expected from peptide binding to this site.

Following this result, hanging drops were set up using cyclic Phe-Leu-Ser-Tyr-Arg which had been preincubated with the protein in 2 *M* NaCl, 0.1 *M* Tris–HCl pH 7.4, 5 m*M* $CaCl_2$, 0.5 m*M* β-octyl-glucopyranoside for 2–3 d at 293 K. Microcrystals grew in a cold room at 277 K after two weeks and were used for macroseeding at 293 K. Further crystal forms have been grown, each type growing in different drops. Type II crystals (Fig. 1*b*) grew two months after macroseeding and showed the most regular prismatic morphology of those in this report ($0.25 \times 0.2 \times 0.1$ mm), with unit-cell parameters $a = 79.8$, $b = 101.7$, $c = 63.9$ Å, $\beta = 96.9^\circ$ and space group $C2$. This form is prone to twinning and the quality was variable. Type III crystals (Fig. 1*c*) grew to $0.15 \times 0.15 \times 0.15$ mm in three months. As these crystals are twinned, unambiguous analysis of this crystal cell has not been possible, but spacings suggest unit-cell dimensions $a \simeq b \simeq c \simeq 90$ Å, with unit-cell angles close to 90°.

Type IV crystals (Fig. 1*d*) also grew from the macroseeding over a three-month period. Crystal dimensions were $0.3 \times 0.1 \times 0.05$ mm. The crystals belonged to space group $C2$, with unit-cell parameters $a = 140.8$, $b = 38.9$, $c = 109.1$ Å, $\beta = 125.1^\circ$. A data set to 2.8 Å has now been collected and processed (79.4% complete, $R_{\rm merge}$ = 8.1%, 2.5-fold redundancy). Diffraction at 2.4 Å was observed. Assuming four molecules in the asymmetric unit, the Matthews coefficient $V_{\rm M}$ (Matthews, 1968) is 2.4 $Å^3\ Da^{-1}$, falling in the middle of the normal range. Molecular replacement has been successfully used to solve the structure from $sPLA_2$-IIA crystals containing six molecules in the asymmetric unit (Oh, 1995). This approach will be applicable to solve the structure of type IV crystals using the atomic coordinates of human $sPLA_2$-IIA (as deposited in the Protein Data Bank; Bernstein *et al.*, 1977; PDB code 1pod) as a probe molecule.

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