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Crystallization, optimization and preliminary X-ray characterization of a metal-dependent PI-PLC from *Streptomyces antibioticus*

A recombinant metal-dependent phosphatidylinositol-specific phospholipase C (PI-PLC) from *Streptomyces antibioticus* has been crystallized by the hanging-drop method with and without heavy metals. The native crystals belonged to the orthorhombic space group *P*222, with unit-cell parameters $a = 41.26$, $b = 51.86$, $c = 154.78$ Å. The X-ray diffraction results showed significant differences in the crystal quality of samples soaked with heavy atoms. Additionally, drop pinning, which increases the surface area of the drops, was also used to improve crystal growth and quality. The combination of heavy-metal soaks and drop pinning was found to be critical for producing high-quality crystals that diffracted to 1.23 Å resolution.

1. Introduction

Phosphatidylinositol-specific phospholipases C (PI-PLCs; EC 3.4.1.11) catalyze the hydrolysis of phosphatidylinositol (PI) to inositol phosphates and the second messenger diacylglycerol (DAG; Rhee *et al.*, 1989). This is a key step in transmembrane signaling found in a wide range of organisms. The larger molecular-weight mammalian enzymes, which are all Ca^{2+} -dependent, were thought to be the only metal-dependent PI-PLCs. This was based on a significant number of X-ray structures of mammalian PI-PLC enzymes showing a conserved metal-binding motif (Essen, Perisic, Katan *et al.*, 1997; Essen, Perisic, Lynch *et al.*, 1997; Waldo *et al.*, 2010). Bacterial PI-PLCs, which are also represented by a number of X-ray structures (Goldstein *et al.*, 2012; Gässler *et al.*, 1997; Heinz *et al.*, 1996), have an active-site arginine that appears to play a similar role to the Ca^{2+} in the mammalian enzymes (Apiyo *et al.*, 2005).

Sequencing results from a strain of Actinomycetales, *Streptomyces antibioticus*, demonstrated that two PI-PLCs were present in the organism. One PI-PLC had notable sequence homology to the Ca^{2+} -binding domain of the larger mammalian enzyme (Iwasaki *et al.*, 1994). This enzyme, reported as PLC1 (and hereafter referred to as SaPLC1), was dependent on Ca^{2+} to generate *myo*-inositol-1-phosphate and *myo*-inositol-1,2-cyclic phosphate from PI simultaneously (Iwasaki *et al.*, 1998). The second PLC from the same organism, reported as PLC2, had GPI-anchor-degrading activity, was not Ca^{2+} -dependent and was reported to produce *myo*-inositol-1-phosphate and *myo*-inositol-1,2-cyclic phosphate from PI in a sequential manner. This demonstrated distinct functional roles for each PLC in the organism, with SaPLC1 having no detectable GPI-anchor-degrading activity and each enzyme having a distinct affinity for *myo*-inositol-1,2-cyclic phosphate (Iwasaki *et al.*, 1998).

Sequence alignment of the two PLCs from *Streptomyces* showed very weak homology (~15% homology depending on the alignment method used and the gap-penalty settings) to known mammalian enzymes and moderate homology to those from bacteria. Two histidines were identified in both PLCs that appeared to align with the histidines of the mammalian and bacterial enzymes, as shown in the original paper describing the cloning (Iwasaki *et al.*, 1998). The putative metal-binding site was identified using only three amino acids: (i) an asparagine located next to the first catalytic histidine, (ii)

a glutamate and (iii) an aspartate located in a position where an arginine residue would normally be found in the bacterial sequence. Interestingly, this is the same position that was altered from arginine to aspartate to generate an engineered metal-dependent PI-PLC (Apiyo *et al.*, 2005).

Functional and mechanistic investigations of SaPLC1 verified the Ca^{2+} dependence and showed that the mechanism of SaPLC1 was quite different from those of other PI-PLCs studied to date (Bai *et al.*, 2010). The enzyme showed unusually high stereoselectivity for isomers with sulfur at various positions near the phosphorus (Zhao *et al.*, 2003) and produced *myo*-inositol-1,6-cyclic phosphate instead of the usual *myo*-inositol-1,2-cyclic phosphate (Bai *et al.*, 2010). Details of this mechanism are shown in Fig. 1.

These novel structural and mechanistic properties prompted us to initiate crystal studies with the goal of determining the three-dimensional structure of this enzyme. Our expectations were to discover how a low-molecular-weight PI-PLC would function in a metal-dependent manner. Previous studies from our laboratory showed a collapsed active site when the conserved arginine was replaced by an aspartate in the bacterial enzyme (Apiyo *et al.*, 2005). This collapse was owing to the metal ion, which interacted directly with the carboxylate group of the aspartate residue, causing additional conformational changes in areas outside the active site that we did not anticipate. Because the enzyme from *Streptomyces* is naturally Ca^{2+} -dependent, we will be able to better understand the mechanistic

details without the need to modify the enzyme. Additionally, the spatial relationship of the hydroxyl groups of the inositol ring relative to the general acid/base amino acids in the active site of other PI-PLCs is likely to differ, as shown in Fig. 1. These diagrams show that the C2 OH group would be deprotonated prior to nucleophilic attack in the upper path, which is common to all known PI-PLCs, and that the C6 OH group would be deprotonated to drive catalysis in the lower pathway, which is found in SaPLC1 (Bai *et al.*, 2010).

This report discusses crystal-growth optimization and heavy-atom soaks for the purpose of determining the structure of this enzyme. While the heavy-atom soaks that were intended to provide phasing information were not successful, we did find specific metals that resulted in significantly improved crystal quality in a number of our samples. Addition of these metals, along with minimization of the effective surface area of the drop, were found to be important parameters for producing high-quality crystals and collecting high-resolution data for this enzyme.

2. Materials and methods

2.1. Expression and purification of the native enzyme

Expression of SaPLC1 proceeded in *Escherichia coli* BL21 (DE3) pLysS cells (EMD4 Biosciences) as described previously (Zhao *et al.*, 2003) with minor modifications to the induction procedure. The

Figure 1
Mechanism of PI-PLCs. The upper path is common to all known PI-PLCs. The lower path is found in SaPLC1.

construct used for expression included the full-length SaPLC1 gene cloned downstream of the *Nde*I restriction site in the pET-28a expression vector (Novagen) with kanamycin resistance on the expression vector and chloramphenicol resistance on the pLysS vector [carried in the *E. coli* BL21 (DE3) pLysS cells]. The *Bam*HI site (afforded by the parent vector) was used as the 3' cloning site, with a stop codon introduced prior to the *Bam*HI site using sequence-specific primers. This produced a 339-amino-acid protein (including the six-histidine tag) of 36 675 Da with an isoelectric point (pI) of 6.07. The full-length sequence of the protein (including the signal sequence) is available in GenBank under accession No. AB439135.1. The construct that was used for all crystallization trials included a tag, MGSSHHHHHHSSGLVPRGSHM-E(27)-SaPLC1, in which E(27)

marks the first amino acid of the SaPLC1 protein in the GenBank entry given above and is the 27th amino acid in the SaPLC1 sequence in this entry.

Autoinduction (in lieu of IPTG induction) was completed in modified LB medium that included 50 mM Na_2HPO_4 , 50 mM KH_2PO_4 , 25 mM ammonium sulfate, 2 mM MgSO_4 , 0.5% (w/v) glycerol, 0.05% (w/v) glucose and 0.2% (w/v) lactose. All chemicals (other than crystallization screens) were purchased from Sigma-Aldrich. Cells were initially grown at 310 K with appropriate antibiotics and shaking at 225 rev min^{-1} until the OD_{600} reached 0.6, at which point the temperature was lowered to 298 K and the shaking speed was decreased to 150 rev min^{-1} . The cells were grown for an additional 12 h from this point before harvesting.

Cells were harvested and the cytoplasmic extract was prepared as described by Zhao *et al.* (2003). The cleared lysate was applied onto a 5 ml HiTrap chelating column (GE Healthcare) charged with Ni^{2+} and equilibrated with 50 mM Tris-HCl pH 8, 100 mM NaCl, 20 mM imidazole, and the protein was eluted with the above buffer containing 250 mM imidazole. The eluted protein was then desalted using a G-25 column (GE Healthcare) followed by gradient elution from a Q-Sepharose column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer pH 8.0. The protein typically eluted from the gradient in 210 mM NaCl. The purity of SaPLC was assessed as a single-molecular-weight band around 38 kDa using SDS-PAGE (12%). A gel showing the purity of the enzyme following elution from the Q-Sepharose column is shown in Fig. 2. The enzyme was then concentrated to 55 mg ml^{-1} for initial crystallization trials using Vivaspinn concentrators with a 10 kDa molecular-weight cutoff (Vivaproducts Inc.). Thus, the final condition for initial crystallization trials was 210 mM NaCl, 20 mM Tris-HCl pH 8.0. Subsequent optimization steps included overnight dialysis into 5 mM HEPES buffer pH 7.0 (with no salt) prior to concentration. Typically, 2–3 ml of protein at $\sim 1 \text{ mg ml}^{-1}$ was dialyzed in 4 l of buffer during each purification cycle prior to the concentration step. The protein was stable in this buffer at up to 55 mg ml^{-1} and these conditions (5 mM

Figure 2
SDS-PAGE gel of purified SaPLC1. Lane 1, molecular-weight markers (labeled in kDa). Lanes 2–9 are representative fractions that were concentrated for crystallization (see §2). Lane 10 is the total soluble lysate prior to purification. The largest band running near the 35 kDa molecular-weight marker protein is SaPLC1.

Figure 3
Optimization of SaPLC1 crystals. The initial condition (see text) produced fine needles, as shown in (a) and (b). Subsequent drops set up around this condition varying the PEG concentration and PEG molecular weight produced some needles that were thicker than others in some trials, as shown in (c), (d) and (e). The larger crystals shown in (d) and (e) were almost always found near the drop edge. In (a), (b), (c) and (e) the crystals were photographed such that each ten units on the microscope ruler represent 100 μm (the smallest increment on the ruler is 10 μm).

HEPES pH 7.0 with no salt) became the standard for all subsequent crystal-optimization experiments.

2.2. Crystallization

The initial crystallization trials used standard hanging-drop vapor diffusion in 22 mm well VDX crystallization plates (Hampton Research). Trials initially used 1 μ l protein solution and 1 μ l well solution with a 1 ml reservoir volume from commercially available crystallization screens. A total of 292 conditions were initially screened from Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, Crystal Screen Cryo and Crystal Screen 2 Cryo (all from Hampton Research). Fine screens were set up around the only condition that gave results: 25.5% (w/v) polyethylene glycol (PEG) 4000, 170 mM ammonium acetate, 85 mM sodium acetate trihydrate pH 4.6, 15% (w/v) glycerol (condition No. 10 from Crystal Screen Cryo). These initial conditions produced fine needles, as shown in Figs. 3(a) and 3(b).

Our first steps in crystal optimization were to alter the PEG concentration and molecular weight. PEGs with molecular weights between 2000 and 20 000 were used at various concentrations while maintaining the same pH and salt concentrations. Crystal growth was most effective when using PEG 10 000 and this PEG became our standard for all further optimization steps. In subsequent trials in which the pH was altered, we found that crystals were able to form below pH 5.0 provided that the ammonium acetate concentration was around 170 mM. However, no crystals were observed under any conditions above pH 5.0 or when the ammonium acetate concentration was either too low or above 180 mM. This was tested at a number of different protein concentrations, and the drops were either clear or a heavy precipitate formed. During these optimization steps, we observed that larger plates started to develop in conditions that had initially produced fine needles. These plates became larger in all three dimensions when crystal growth occurred near the drop edge, as shown in Figs. 3(c) and 3(d). This optimal growth near the drop edge prompted us to minimize the ratio of surface area to drop volume using the following technique: after the protein had been applied to the cover slip, the drop was spread outwards from the center using a pipette tip until the final drop was 2–3 times the original diameter. Mother liquor was then applied to the center of this ‘flattened disc’ of protein sample with no additional mixing in a 1:1 ratio. We refer to this method as ‘drop pinning’, as described below. Fig. 3(e) shows a representative crystal from this type of drop-pinning experiment. To ensure that the protein that we had crystallized was indeed SaPLC1, we harvested 3–4 crystals and ran them on an SDS–PAGE gel next to SaPLC1 that had not been crystallized (Supplementary Fig. 1¹).

2.3. Drop pinning

As discussed above, crystals were observed growing near the drop edges, but in almost all cases no crystallization occurred in the middle of the drops. We reasoned that this was a consequence of faster supersaturation at the edge of the drops, so we attempted to minimize the total drop volume during crystallization using 0.5 μ l protein and 0.5 μ l mother liquor. This was challenging to set up manually and these trials resulted in rapidly dehydrated drops and no crystals. However, when the 1 $\times$ 1 μ l drops were spread out (as described in §2.2), we observed that crystals consistently formed on the edges but never in the center of the drops. We also noticed that these drops did not dehydrate as rapidly as the drops that were set up with a reduced

volume. It is also important to note that crystals never formed on cover slips that were not silanized.

The physical reasons for these crystal-growth properties are still not clear and we have not performed a systematic study to better understand this phenomenon. We reasoned that there is possibly a region in the drops where the surface of the silanized cover slip and the liquid–air boundary are optimal for nucleation under these chemical conditions. Owing to our use of PEG and the tendency of PEG to form a skin-like layer of material over the drop, which we consistently observed, we feel that the kinetics of nucleation and the kinetics of PEG separation (creating the PEG ‘skin’) were optimal only in certain regions of the drop. They appeared to be optimal where the silanized cover slip and the liquid–air boundary of the drop provided the appropriate volume of space under these chemical conditions. Otherwise, the drops would have formed at the interface of the cover slip or at the interface of the liquid–air boundary, which would have resulted in crystals being observed throughout the drop. The term ‘pinned’ is used here to represent the fixing of the position of the nucleation–crystallization site at a location in the drop between the air–liquid boundary and the surface of the cover slip.

After three to five weeks at 277 K, rectangular crystals were observed using this method (Figs. 4a and 4b). Some of these were

Figure 4

Further optimization of crystal growth using drop pinning and microseeding and macroseeding methods. (a) A rare case in which a crystal of nearly 160 μ m along the longest edge formed from the initial needles. (b) Typical crystal formation using the drop-pinning technique. (c) A microseeded crystal in a standard drop; the largest crystal shown is the seeded crystal, which was originally only the size of the many smaller crystals that formed after the drop had been set up. (d) A crystal cluster achieved by a series of macroseeding steps (two steps) using crystals such as those pictured in (c). In (a), (c) and (d) the crystals were photographed such that each ten units on the microscope ruler represent 100 μ m (the smallest increment in the ruler is 10 μ m).

¹ Supplementary material has been deposited in the IUCr electronic archive (Reference: BO5112).

Table 1

Data-collection statistics using an in-house X-ray source.

Values in parentheses are for the highest resolution shell. SaPLC1 crystals were soaked in iridium and cadmium chloride salt solutions. Data were collected at a wavelength of 1.54178 Å with 1° oscillations: 180 frames were collected for the native crystal, 100 frames for the iridium-soaked crystal and 360 frames for the cadmium-soaked crystal. Images were generated on an R-Axis HTC detector (Rigaku) and were processed with *DENZO* and *SCALEPACK* in *HKL-2000*.

Data set	SaPLC1 (native)	SaPLC1 (iridium soak)	SaPLC1 (cadmium soak)
Space group	<i>P</i> 222	<i>P</i> 222	<i>P</i> 222
Unit-cell parameters (Å)	<i>a</i> = 41.26, <i>b</i> = 51.86, <i>c</i> = 154.78	<i>a</i> = 41.04, <i>b</i> = 51.48, <i>c</i> = 154.00	<i>a</i> = 41.04, <i>b</i> = 51.48, <i>c</i> = 154.32
No. of observations	31720 (3100)	28908 (2736)	32383 (2201)
Mosaicity (°)	0.54	0.50	0.67
Resolution (Å)	23.17–1.80 (1.86–1.80)	23.17–1.80 (1.86–1.80)	24.67–1.76 (1.82–1.76)
Completeness (%)	99.8 (99.9)	92.5 (88.9)	96.5 (66.6)
Multiplicity	5.3 (5.4)	3.1 (3.1)	6.6 (6.3)
Mean <i>I</i> /σ(<i>I</i>)	24 (12.6)	16.2 (11.7)	75.4 (25.9)
<i>R</i> _{merge} [†] (%)	4.2 (16.2)	3.0 (24.7)	3.8 (11.7)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observed intensity of reflection *hkl*.

larger single crystals, as shown in Fig. 4(*a*), and some were clusters, as shown in Fig. 4(*b*). Initially, fine brown-hued needle crystals formed and grew outwards from a central point in many (not all) of the drops. After this initial needle-crystal formation, which typically occurred after one to two weeks, small square microcrystals began to form in the drop near the needle crystals. Gradually, after 2–4 additional weeks of growth, a rectangular crystal form that typically grew as a cluster of plates developed, with subsequent disappearance of the needles. The crystal shown in Fig. 4(*a*) formed over a six-month period and transitioned from fine needles to a large rectangular single crystal. It was not common to observe single crystals owing to the tendency of the crystals to produce clusters of plates. In many cases the drop edges became too dry at the air–drop boundary and produced a layer of material on top of the crystals, like a ‘skin’, that could not be removed. In cases where the air–drop boundary was minimal, the regions just inside the air–drop boundary developed the best single crystals. Crystals used for initial data-collection experiments were typically 0.05 × 0.075 × 0.2 mm in size. The final crystal-growth conditions were 85 mM sodium acetate trihydrate pH 4.6–4.8, 170 mM ammonium acetate, 18–22% (*w/v*) PEG 10 000, 4–12% (*w/v*) glycerol at 277 K. Owing to the observation of initial brown needle crystals that subsequently developed into larger crystals, we decided to attempt seeding trials to improve the crystal size and quality.

2.4. Seeding

For our seeding trials, larger crystals such as that shown in Fig. 4(*c*) were grown up to two or three times their original dimensions using macroseeding and microseeding techniques (McRee, 1999). The largest crystal in Fig. 4(*c*) is from a seeded crystal, while the other smaller crystals in the same drop are new crystals that formed. Crystals for seeding were soaked briefly (30–200 s) in modified mother liquor (85 mM sodium acetate trihydrate pH 4.6–4.8, 170 mM ammonium acetate) as an etching step with 75% concentrations of the original PEG and glycerol prior to being added to the new protein/mother liquor mixture [85 mM sodium acetate trihydrate pH 4.6, 170 mM ammonium acetate, 13.5% (*w/v*) PEG 10 000, 7.5% (*w/v*) glycerol]. The protein concentration in the new drop for seeding was typically 30 mg ml^{−1}.

Microseeding occurred using a number of crystals harvested at the same time. These crystals ranged in size from 10 to 40 μm. These multiple crystals were placed in an Eppendorf tube, mixed, etched,

mixed again and set up with fresh protein as described above. Additionally, for macroseeding, a single plate was broken off from the edge of a cluster. These single crystals for macroseeding were typically 30–50 μm in size. This single crystal was etched for a varying amount of time (30–200 s) before being used in a new drop containing fresh mother liquor and protein. Fig. 4(*d*) shows a crystal that was grown in two seeding steps: microseeding followed by macroseeding. Interestingly, these crystals still tended to form clusters. However, the clusters could be broken apart and used for data collection or for additional seeding.

After successfully optimizing crystals for diffraction analysis and collecting data on an in-house X-ray source, we attempted to find phase solutions using molecular-replacement (MR) methods. Details of these trials will be discussed in a later section of this report. Owing to difficulties in finding phase solutions using MR, we continued to attempt to grow crystals using selenomethionine-labeled protein and attempted heavy-metal soaks and cocrystallization with heavy metals.

2.5. Heavy-atom screens and native gel analysis

Heavy-atom soaks were performed with Heavy Atom Screen M1 (Hampton Research). Crystals were soaked in heavy-atom solutions at concentrations of up to 10 mM or at the maximum permissible solubility for 2–7 d and then back-soaked for at least 24 h. Many trials resulted in visible damage to the crystals (even at low concentrations of the heavy atom). These heavy atoms were not pursued further.

In addition, the SaPLC1 enzyme was added to the optimal crystallization condition with amounts of the metal ranging from 500 nM to 1 mM. This provided an array of SaPLC1 crystals with potentially integrated heavy-atom ions. During our screening, we found that CdCl₂ and IrCl₃ solutions were tolerated by our crystals at higher concentrations and for longer soaking times. Other complex salts of the same heavy atoms [*e.g.* K₃Ir(NO₂)₆ and (NH₄)₃IrCl₆] did not have the same properties and seemed to damage more crystals compared with the chloride salts. We screened the cadmium and iridium crystals using an in-house X-ray source and found modest improvement in the data quality, as shown in the statistics of our in-house data collection (Table 1).

To help select those crystals that might contain heavy metals, native PAGE gels were set up to observe any potential protein interaction with the metals. This type of analysis has been successful using Phastgels (Amersham Pharmacia Biotech) with no denaturants added (Boggon & Shapiro, 2000). One of the challenges for our gel-shift experiments was that the pH for our crystal-growth conditions was 4.6. While we could run standard native gels at standard pH values (typically above pH 8.0), we were concerned about the relevance of any data obtained that showed changes in migration under alkaline conditions when our crystals formed under acidic conditions. For this reason, we prepared the gels to run at the same pH as our crystal-growth buffer and reversed the polarity relative to a standard native gel. The native gel containing samples of enzyme with no metal added, CdCl₂ added, HgCl₂ added, TiCl₃ added and OsCl₃ added is shown in Fig. 5. Samples were prepared for gel analysis using each metal that showed promise during the initial heavy-atom screens. The iridium sample (run on a separate gel) looked similar to the CdCl₂, HgCl₂ and TiCl₃ samples and is not shown.

The osmium chloride lane showed slower (retarded) migration of protein relative to protein sample with no metal added. Results from the gel-migration analysis showed that at pH 4.6 the CdCl₂, HgCl₂ and TiCl₃ samples migrated more rapidly into the gel compared with the sample to which no heavy-atom salt was added. The OsCl₃ sample was the only sample that showed retarded mobility relative to the

sample with no heavy-atom salt added, as shown in Fig. 5 (lane 5). In fact, it appears as if very little protein entered the gel at all. Initially, we interpreted these gel results to indicate that iridium, mercury and thallium bound and cadmium did not. However, we had to reinterpret our gel analysis based on our diffraction results, as discussed later in this report. For completeness, all available crystals were screened using our in-house X-ray source regardless of the migration pattern on native PAGE gels. Unfortunately, no heavy-atom sites could be found for use in phase determination.

2.6. Methods to produce a phase solution

As mentioned above, our heavy-atom screens did not produce crystals with heavy atoms for use in determining phases. This is still an ongoing process. In the meantime, we initiated MR searches and attempted to grow selenomethionine-derivative crystals to determine the phases. Selenomethionine-labeled protein was successfully prepared in the same manner as the native protein. No solubility problems were detected during the purification steps or while concentrating the protein to 55 mg ml^{-1} for crystallization trials. However, no crystals were observed after 6–12 months using our standard crystallization conditions. Crystallization was attempted using standard drops and with drop pinning as described earlier in this report. The protein concentration, ammonium acetate concentration, PEG concentration and PEG molecular weight were all varied based on the standard conditions. Drops were either clear or contained precipitate, but no crystals were observed. We are screening our original conditions again to search for another crystallization condition, but have not yet been successful. We are also using our seeding methods (as described above) using native crystals to seed selenomethionine-derivative crystal growth. All of these methods are being employed and trials are still under way.

MR searches have been completed using the two closest homologs (based on amino-acid sequence and protein function) in the Protein Data Bank (PDB). These are represented by PDB entries 1dix (Essen *et al.*, 1997) and 1ptg (Heinz *et al.*, 1996). The 1dix structure represents the first structure of a PI-PLC from a mammalian source (*Rattus norvegicus*) and the 1ptg structure represents a bacterial form from *Bacillus cereus*. Both of these proteins were used in MR searches; the sequence identity was very low (less than 15%) for both 1dix and

1ptg. The MR trials were completed using the *Phaser* program and combined rotational searches with translational searches. Typically, the top five rotational search results were used in the translational searches. Initially, the 1ptg structure was used as a search model with all amino acids represented. After unsuccessful trials using the entire protein, a polyalanine model was constructed to maintain the overall fold while removing the amino-acid side chains. This was also not successful and did not yield better statistics than the first search. We then removed the loop regions from the 1ptg protein model, leaving only the α -helices and β -strands, and performed searches with this model, but still without success. These deletions were also performed with the polyalanine model and did not give better statistics. We then moved on to the mammalian protein.

The 1dix structure searches proceeded in the same manner as the 1ptg MR searches. Because the mammalian protein contained multiple domains [PLC X-box, PLC Y-box, plekstrin homology (PH) domain, EF-hand domain and C2 domain], we removed all domains except for the PLC X-box and PLC Y-box domains, which matched the domains in SaPLC1. These domains represent two halves of a TIM-barrel structure that come together to form the active site. No searches containing the other domains were performed. The choice of the PLC X-box and PLC Y-box domains was based on the initial sequence alignment produced in the original paper describing the cloning of SaPLC1 and SaPLC2 (Iwasaki *et al.*, 1998). We used a number of different homology-detection programs to improve our protein-sequence alignments, but these programs could only identify the catalytic general acid–base histidines and the putative Ca^{2+} -binding site within the SaPLC1 protein and did not give a better alignment compared with the original. Secondary-structure prediction programs were also used to determine α -helical and β -strand sections of SaPLC1 in an effort to predict which secondary structures of 1dix and 1ptg to use in the search model. This added to the number of search models compared with the original searches performed with 1ptg.

To summarize, our MR searches included 1ptg and 1dix with the following parameters: (i) proteins from the PDB (no waters or other small molecules) with appropriate domains, (ii) polyalanine models, (iii) models with loop regions removed and (iv) models with regions of questionable secondary structure removed. These were prepared in a combinatorial manner and a queue was set up using the *Phaser* program (McCoy *et al.*, 2007) to take the top five translation searches and use these in rotation searches. These searches ran on two Linux computers for weeks, but produced no solutions with a Z-score greater than 6. A Z-score of 8 or greater is considered to be a definite solution. We are still attempting to find search models and refine these methods, but with such low sequence homology we are limited in our ability to predict which model to use (mammalian or bacterial) and which deletions (if any) need to be performed. These searches are ongoing as we continue to move this project forward.

3. Results and discussion

3.1. Preliminary X-ray analysis

All X-ray experiments were performed using a liquid-nitrogen stream (100 K), with each crystal being vitrified in the cryostream with no added cryoprotectant. Our crystallization conditions included glycerol, which appears to be adequate to protect our crystals from damage arising from cooling. Initial data collection and processing for the native crystals showed that the protein crystallized in the orthorhombic space group *P*222. The unit-cell parameters were $a = 41.26$, $b = 51.86$, $c = 154.78 \text{ \AA}$. The resolution was $1.80 \text{ \AA}$ in the highest

Figure 5

SaPLC1 native gel run with and without heavy metals. Samples were prepared by mixing a final concentration of protein at 3.8 mg ml^{-1} in 85 mM sodium acetate trihydrate pH 4.6, 170 mM ammonium acetate with the respective heavy-metal solution (metal salt dissolved primarily in H_2O) and were then mixed with a loading buffer (1:1) consisting of 25 mM sodium acetate pH 4.6, 10% sucrose and methylene blue dye. The samples were: lane 1, enzyme with no metal solution; lane 2, enzyme plus CdCl_2 solution; lane 3, enzyme plus HgCl_2 solution; lane 4, enzyme plus TiCl_3 solution; lane 5, enzyme plus OsCl_3 solution.

resolution bin at 99.9% completeness. Additional parameters are shown in Table 1, in which the best diffraction data for each type of crystal are given. In addition to the collection of native data sets, two successful derivative data sets were collected from crystals produced using iridium and cadmium soaks. These two derivatives were the only successful heavy-atom soaks that gave quality diffraction that might be adequate for heavy-atom-derived phasing. All other soaks either produced no diffraction or the diffraction quality was so poor (over 3.5 Å resolution) that complete data sets were not collected. Additionally, screening crystals that may have contained other heavy metals, either cocrystallized or soaked, showed epitaxial twinning in many cases and data collection from these crystals is not reported. The two successful data sets collected using iridium-soaked and cadmium-soaked crystals are shown in Table 1.

The iridium-soaked crystal showed fewer reflections, lower completeness and lower signal intensity relative to the native crystal. The mosaicity was slightly better than the native crystal, but the multiplicity was lower and R_{merge} in the highest resolution bin increased from 16.2% for the native crystal to 24.7% for the iridium-soaked crystal. Even though this soaked crystal produced adequate diffraction, no peaks were observed in the Patterson maps for this derivative. It is also interesting to note that there was no change in mobility in the native PAGE analysis for the protein soaked with this metal. This is discussed later in this report.

Continuing our in-house X-ray analysis, we collected data from a cadmium-soaked crystal, which gave data that were higher in quality than the iridium-soaked crystal. The number of observations was slightly greater than for the native crystal, but the mosaicity increased from 0.54° (native) to 0.67° (cadmium soak). The completeness was significantly lower in the highest resolution bin, but the signal intensity and multiplicity were higher than both the iridium-soaked crystal and the native crystal. The R_{merge} was also lower in the highest resolution bin compared with the other samples. Overall, this sample showed significant promise as a heavy-atom-phasing data set, but like the iridium-soaked crystal no peaks were found in the Patterson maps. This sample also showed no change in mobility in the native PAGE analysis. Both of these samples showed promising isomorphous χ^2 test values using *SCALEPACK* (Rossmann & van Beek, 1999). While these data sets were being collected, additional MR solutions were sought as described earlier, but without success. Synchrotron time became available and we were able to analyze our crystals with much greater beam intensity, as discussed below.

3.2. Synchrotron data collection

Native data sets collected at the Advanced Photon Source (APS) at Argonne National Laboratory (Argonne, Illinois, USA) showed a significant increase in data quality. The resolution in the highest bin improved from 1.8 Å at our home source to 1.6 Å at APS with a completeness of 97.7%. These data are shown in Table 2. The number of observations, signal intensity and mosaicity also improved. Overall, this data set represented a significant improvement over the data collected in-house. However, we still had no phase solutions from our previous two data sets after many MR trials. Owing to the change in the mobility of the osmium-soaked protein using the native PAGE analysis, we invested more time in growing these types of crystals. Initially, the osmium-soaked crystals were not available for in-house data collection owing to problems in optimizing the soaking conditions. These samples were also not available during our initial APS data-collection time slot where the first native crystal data sets were collected. We were able to prepare osmium-soaked samples in time for data collection at the Stanford Synchrotron Radiation Light-

Table 2

Data-collection statistics for native and soaked SaPLC1 at synchrotron sources.

Values in parentheses are for the highest resolution shell. SaPLC1 soaked in an osmium salt solution showed the best data quality. A total of 180° of data were collected as 360 frames with 0.5° oscillation for osmium-soaked SaPLC1. A total of 180° of data were collected as 180 frames with 1° oscillation for the native enzyme that was not soaked with metals. *MOSFLM* was used to process the data. All crystals were flashed-cooled in a liquid-nitrogen gas stream at 100 K with no added cryoprotectant.

Data set	SaPLC1 (osmium soak)	SaPLC1 (native)
Synchrotron beamline	BL9-2, SSRL	22-BM, APS
Spot size (mm)	0.6 × 0.21	0.08 × 0.04 FWHM
Detector	MAR Mosaic 325 CCD	MAR 225 CCD
Wavelength (Å)	0.82653	1.0089
Space group	<i>P</i> 222	<i>P</i> 222
Unit-cell parameters (Å)	<i>a</i> = 51.97, <i>b</i> = 154.82, <i>c</i> = 41.32	<i>a</i> = 52.06, <i>b</i> = 155.19, <i>c</i> = 41.54
Total observations	712443 (1000339)	312742 (45616)
Unique observations	97853 (14115)	44086 (6266)
Mosaicity (°)	0.21	0.46
Resolution (Å)	51.99–1.23 (1.30–1.23)	32.31–1.60 (1.69–1.60)
Completeness (%)	99.9 (100)	98.5 (97.7)
Multiplicity	7.3 (7.1)	7.1 (7.3)
Mean $I/\sigma(I)$	22.8 (6.4)	20.1 (11.0)
$R_{\text{merge}}^{\dagger}$ (%)	4.9 (25.6)	8.2 (25.5)

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the i th observed intensity of reflection hkl .

source (SSRL; Menlo Park, California, USA), where we found that the data from these samples were much higher in quality than those collected from native samples at APS.

As shown in Table 2, the osmium soak produced a significant increase in the resolution and data quality of the crystal. This was in contrast to the iridium-soaked and cadmium-soaked crystals, which showed only a modest improvement over the native crystals, as shown in Table 1. The diffraction limit for this sample was 1.23 Å. The mosaicity was also much lower than that of the data collected at APS; the number of observations (total and unique) was more than double that for the native crystal data set collected at APS. When reporting these statistics, it is important to point out the differences in detectors and spot size at the different beamlines. Although the beamline and detector quality were better at SSRL, we believe that the osmium-soaked crystal is still far superior to the native crystals or any of the derivatives tested prior to the osmium-soaked crystal. Data sets were collected from iridium-soaked and cadmium-soaked crystals at SSRL, but showed no improvement over those from the native and osmium-soaked crystals (and also showed some twinning). These statistics are not shown in the tables.

We used the X-ray scanning capabilities at SSRL to obtain fluorescence scans of the osmium-soaked, iridium-soaked and cadmium-soaked crystals. The osmium-soaked crystal was the only crystal to show positive results during the scan. The other metal-soaked crystals did not produce any fluorescent signal and showed no improvement in diffraction data quality. Further analysis of the iridium-soaked or cadmium-soaked crystals, which showed no fluorescent peaks, was not performed. The fluorescence scan for the osmium-soaked crystal is shown as Supplementary Fig. 2. This sample showed promise for phase determination using MAD based on the observed X-ray fluorescence, although the peak intensity appears weak. Complete data sets were collected at peak, edge and high-remote wavelengths from this osmium-soaked crystal and will be reported in a later publication. We have not been able to find specific heavy-atom sites after a number of searches. We are continuing our searches using *SHELXD* (Sheldrick, 2008) and other packages from the *CCP4* suite (Winn *et al.*, 2011; Cowtan *et al.*, 2011), but no specific positions have been found. We are still working on the phasing using a combination

of methods involving all of our data sets and will report the phasing and structure determination when these are completed.

3.3. Heavy-atom screen and native-gel analysis

The study by Boggon & Shapiro (2000) demonstrating the use of native gels to screen heavy atoms for potential derivatives showed differences in band migration, which was slowed relative to protein with no heavy atom. This is a consequence of the positively charged metal altering the net charge/frictional coefficient (with the samples migrating from a negative pole to a positive pole in the gel). Because our crystallization conditions were at pH 4.6 and we wanted to preserve the same amino-acid ionization states of our protein in our gel analysis, we chose to run an acidic gel and reverse the polarity. This would allow a migration-pattern comparison of the protein with the metals being screened that is the reverse of that shown previously, resulting in enhanced migration relative to the native protein. In our study, the CdCl_2 , HgCl_2 and TiCl_3 samples appeared to run forward of the protein without metals, which would indicate a positive result for protein binding. In addition, the OsCl_3 sample, which showed less migration into the gel compared with the protein with no metal added, would indicate a negative result for protein binding. These results were not at all easy to interpret.

Our diffraction analysis did not show any peaks in the Patterson maps for any of the heavy-atom samples. However, we did observe (as discussed above) increases in resolution and crystal quality with specific metals. This was most notable with the OsCl_3 sample, where our crystals diffracted to 1.23 Å resolution. This demonstrates that our native-gel results are clearly not positive for finding a heavy-atom derivative. If this were true we would have found heavy-atom sites in the Patterson maps. Instead, we are presenting these gel results to show that altered migration of one sample relative to all other heavy atoms (in this case Os compared with Cd, Hg, Ir and Ti) may be an indication of differences that could improve diffraction quality.

Figure 6

Diffraction image from an osmium-soaked Sa-PLC1 crystal. The image color is inverted to better illuminate the data range indicated by the resolution rings.

Native-gel migration studies have consistently shown that solution conditions (including heavy-atom addition) can cause precipitation or aggregation of the protein. Protein that has undergone significant irreversible aggregation will not enter the gel owing to the size of the aggregate. When considering these processes and observing that the OsCl_3 sample had slightly less mobility compared with the protein with no added metal, we cannot rule out some type of increased protein–protein interaction that results in diffraction improvement. It is also important to note that the chloride salts of all of the heavy atoms screened were the most favorable in not damaging the crystals. Bound chloride ions have been observed in many protein crystals, which would also cause a shift in mobility of the protein under these experimental conditions. Thus, the combination of Os^{3+} interaction and/or Cl^- interaction could be a factor in the diffraction improvement. These ideas will hopefully become clearer once we have phases and have started the refinement process.

4. Conclusions

Optimization of conditions for our crystals is described in this report. Our results showed that heavy-atom soaks produced better quality diffraction compared with native crystals. Additionally, native-gel analysis at the same pH as our crystallization conditions resulted in altered migration that correlated with the heavy atom producing our diffraction improvement. We also found that crystals forming near the edge of sitting drops may indicate a need to optimize the way that the drops are set up. To facilitate this optimization, we utilized drop pinning to increase the surface area to volume ratio in the drop to improve crystal growth. The combination of these methods has been a breakthrough for this specific protein and may assist others when optimizing their crystal-growth conditions. The end result of these optimization steps is the diffraction image shown in Fig. 6. Despite these successes, no heavy-atom positions were found. Our difficulty in finding specific locations for heavy atoms could arise from a number of factors and we are continuing our search and preparing larger crystals to increase the possibility of finding the metal locations. We are also performing experiments to grow selenomethionine-derivatized crystals of SaPLC1 using various seeding techniques such as those discussed in this report. These have not yet been successful.

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