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Crystallization and Initial X-ray Crystallographic Characterization of Recombinant Bovine Inositol Polyphosphate 1-Phosphatase Produced in *Spodoptera frugiperda* Cells

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Bovine inositol polyphosphate 1-phosphatase, a monomeric protein with a molecular mass of 44,000 Da, hydrolyzes the 1-position phosphate from inositol 1,3,4-trisphosphate and inositol 1,4-bisphosphate. The low abundance of inositol polyphosphate 1-phosphatase in tissues has precluded structural studies requiring large quantities of enzyme. We used recombinant Baculovirus harboring the cDNA of bovine inositol polyphosphate 1-phosphatase to infect *Spodoptera frugiperda* (Sf9) insect cells. Recombinant protein (25 mg per 1×10^9 cells) was purified to homogeneity. The enzyme produced in Sf9 cells was similar to the native purified protein as determined by immunoblotting catalytic properties, and inhibition by lithium ions. Crystals of the purified recombinant enzyme were grown by vapor diffusion. Precession photography was used to determine the parameters of inositol polyphosphate 1-phosphatase crystals. The tetragonal crystals belong to the space group $P4_1$ or $P4_3$, have unit cell dimensions of $a=b=51{\cdot}6$ Å, $c=143{\cdot}3$ Å, $\alpha=\beta=\gamma=90°$, and contain one molecule per asymmetric unit. We have collected a complete diffraction data set extending to 2·3 Å and are currently attempting to solve the three-dimensional structure of bovine inositol polyphosphate 1-phosphatase using a multiple isomorphous replacement strategy.

Keywords: crystallization; inositol phosphate; lithium; baculovirus

1. Introduction

Agonist induced phosphatidylinositol turnover is mediated by phospholipase C hydrolysis of phospholipids to yield diacylglycerol, inositol phosphates and cyclic inositol phosphates (Majerus, 1992; Berridge & Irvine, 1989). Roles in intracellular signaling have been well documented for diacylglycerol (Nishizuka, 1986) and inositol, 1,4,5-trisposphate (Berrridge & Irvine, 1984). The action of inositol phosphate phosphatases and kinases give rise to a plethora of inositol polyphosphates (Majerus *et al.*, 1988) that may also serve as signaling or regulatory molecules. Roles for other inositol phosphates and cyclic inositol phosphates have also been proposed (Majerus, 1992; Bansal & Majerus, 1990), however the function of most inositol polyphosphates remain unknown.

Inositol polyphosphate 1-phosphatase removes the 1-position phosphate from inositol 1,4-bisphosphate ($Ins(1,4)P_2$†) and inositol 1,3,4-trisphosphate ($Ins(1,3,4)P_3$) yielding inositol 4-phosphate and inositol 3,4-bisphosphate, respectively (Inhorn *et al.*, 1987; Gee *et al.*, 1988). Another enzyme involved in inositol phosphate metabolism, inositol monophosphate phosphatase, removes the phosphate from inositol 1-phosphate, inositol 3-phosphate or inositol 4-phosphate to generate inositol (Ackermann *et al.*, 1987; Hallcher & Sherman, 1980). Interestingly, these two enzymes share several properties including a requirement for magnesium ions for catalytic activity and both are inhibited by calcium and lithium ions (Gee *et al.*, 1988; Hallcher & Sherman, 1980; Inhorn & Majerus, 1987, 1988). Complementary DNA molecules

† Abbreviations used: $Ins(1,4)P_2$, inositol 1,4-bisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; p.f.u., plaque-forming units; PEG, polyethylene glycol.

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encoding inositol polyphosphate 1-phosphatase and inositol monophosphate phosphatase have been cloned from bovine sources (York & Majerus, 1990; Diehl *et al.*, 1990). Alignment of the amino acid sequences shows no overall similarity, however, two motifs are common to both enzymes and several bacterial and fungal proteins (York & Majerus, 1990; Neuwald *et al.*, 1991) implying that these regions are involved in enzyme function. The importance of these conserved sequences has been confirmed by the recent determination of the three-dimensional structure of human inositol monophosphate phosphatase (Bone *et al.*, 1992). These motifs are directly involved in metal binding and are likely to play a role in catalysis.

As a first step toward determining the structure of bovine inositol polyphosphate 1-phosphatase we have used recombinant baculovirus to produce recombinant enzyme, thus overcoming the problem of its relative low abundance in tissues. We have grown crystals of the recombinant enzyme and used them to determine the X-ray crystallographic parameters and to collect a complete diffraction data set to 2·3 Å resolution.

2. Materials and Methods

(a) *Materials*

pJVP10Z expression plasmid was kindly provided by Christopher Richardson (Toronto, Canada). *Spodoptera frugiperda* (Sf9) insect cells and AcMNPV baculovirus DNA were purchased from Invitrogen. Bio-gel Phenyl-5-PW and DEAE-5-PW HPLC columns were purchased from Biorad. Vapor diffusion trays and 0·7 mm quartz capillary tubes were purchased from Charles Supper Co. Restriction enzymes, G-25 sephadex spin columns, random hexamer radiolabeling kit and salmon sperm DNA were purchased from Boehringer-Mannheim. [^{3}H]inositol 1,4-bisphosphate and [^{3}H]inositol 1,3,4-trisphosphate were purchased from NEN. Inositol 1,4-bisphosphate was purchased from Sigma. Inositol 1,3,4-trisphosphate was purchased from Calbiochem. Serum-free media (Sf900), Grace's powdered media, yeastolate, lactalbumin hydrosylate, gentamycin, and heat-inactivated fetal bovine serum were purchased from Gibco. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer. Other reagents were purchased from Sigma.

(b) *Contruction of recombinant baculovirus*

The cDNA of bovine inositol polyphosphate 1-phosphatase contained in the bacterial expression plasmid, pTrp1ptase (York & Majerus, 1990) was modified to contain 5′ and 3′ *Nhe*I restriction enzyme sites as follows. The 5′ *Nhe*I site was engineered by cloning the complementary oligonucleotides 5′-AATTCGCTAGCAAAAAAA-CCTATAAA-3′ and 5′-TATTTATAGGTTTTTTTGCTA-GCCG-3′ into the *Eco*RI and *Nde*I sites of pTrp1ptase to yield pBac1pt–A. All cloning steps were performed as described (Sambrook *et al.*, 1989). The 3′ *Nhe*I site was made by cloning the complementary oligonucleotides 5′-CAAGCTTGCTAGCAGCT-3′ and 5′-GCTAGCAAGCT-TGAGCT-3′ into pBac1pt–A at the *Sac*I site to yield pBac1pt–B. The 1·2 kb inositol polyphosphate 1-phosphatase cDNA fragment was released from pBac1pt–B by digestion with *Nhe*I, gel purified and subcloned into the *Nhe*I site of the transfer plasmid, pJVP10Z, to yield pJVP10Z1pt. Sf9 cells (2×10^6) were co-transfected with 2 μg of pJVP10Z1pt and 1 μg of AcMNPV baculovirus DNA as described (Summers & Smith, 1988). Recombinant viral particles were purified using a blue color agarose plaque assay (Summers & Smith, 1988). Partially purified recombinant viral stocks were used to infect Sf9 cells and four days after infection DNA was isolated and probed with radiolabeled inositol polyphosphate 1-phosphatase cDNA by southern slot blot (Sambrook *et al.*, 1989). Purified recombinant viral particules were amplified in media containing 1% (v/v) fetal bovine serum as described (Summers & Smith, 1988).

(c) *Expression and purification*

Sf9 cells were maintained in spinner flasks at 28°C in Grace's media at pH 6·1 supplemented with 3·3 g/l yeastolate, 130 mg/l lactalbumin hydrosylate, 50 μg gentamycin/ml, and 10% heat inactivated fetal bovine serum until cell densities reached 1×10^6 cells/ml (log phase). Viral infections were performed by incubating cells (1×10^9) with 100 ml of viral particles (10^9 p.f.u.) to achieve a multiplicity of infection equal to one for 1 h at 25°C. Cells were then diluted 1 : 10 to 1×10^6 cells/ml with serum-free growth media, SF-900 (the final serum concentration was 0·1% as the viral stocks were in 1% serum) and grown until levels of inositol polyphosphate 1-phosphatase reached greater than 25 mg/l of medium (4 to 6 days). Cellular enzyme levels also reached greater than 25 mg per 10^9 cells, however this enzyme underwent proteolysis upon cell lysis despite the use of various protease inhibitors. In contrast, enzyme released from cells upon viral lysis was intact. Media was harvested by pelleting cellular debris at 7500 ***g*** for 15 min followed by filtration through a 0·45 micron cellulose acetate filter, and concentration to 50 ml using an Amicon hollow-fiber concentrator. After dialysis in 20 mM Hepes, 3 mM $MgCl_2$ (pH 7·5), (buffer A) the sample was loaded onto a Biorad DEAE HPLC column (0·75 cm × 7·5 cm), washed with 10 column volumes of buffer A, and eluted using a 15 column volume linear gradient from to 0 300 mM NaCl in buffer A. Fractions were assayed for enzyme activity (Inhorn & Majerus, 1987), peak fractions were pooled, adjusted to 33% ammonium sulfate saturation and loaded onto a Biorad Phenyl HPLC column (0·75 cm × 7·5 cm) equilibrated in 33% of ammonium sulfate saturation in buffer A (pH 7·5), (buffer B). The column was then washed with 10 column volumes of buffer B (pH 7·5), and eluted with a 15 column volume reverse linear gradient from 33% to 6·6% ammonium sulfate saturation in buffer A (pH 7·5). In some preparations, a second phenyl column was run as described above in order to obtain homogeneous protein. Purified protein was analyzed by SDS/PAGE (Laemmli, 1970) and stained using Coomassie blue dye.

(d) *Characterization of recombinant enzyme*

Purified recombinant inositol polyphosphate 1-phosphatase was used for all studies and was judged to be greater than 95% pure by SDS/PAGE analysis (Fig. 1A). Enzyme was assayed utilizing [^{3}H]Ins(1,3,4)P_3 as substrate as described; after incubation at 37°C reaction mixtures were loaded onto 0·2 ml Dowex-formate column, equilibrated with 0·425 M NH_4COOH, 0·1 M HCOOH and the product [^{3}H]Ins(3,4)P_2 was eluted with 20 column volumes of buffer A. Radioactivity was measured by liquid scintillation counting. Enzyme reactions utilizing

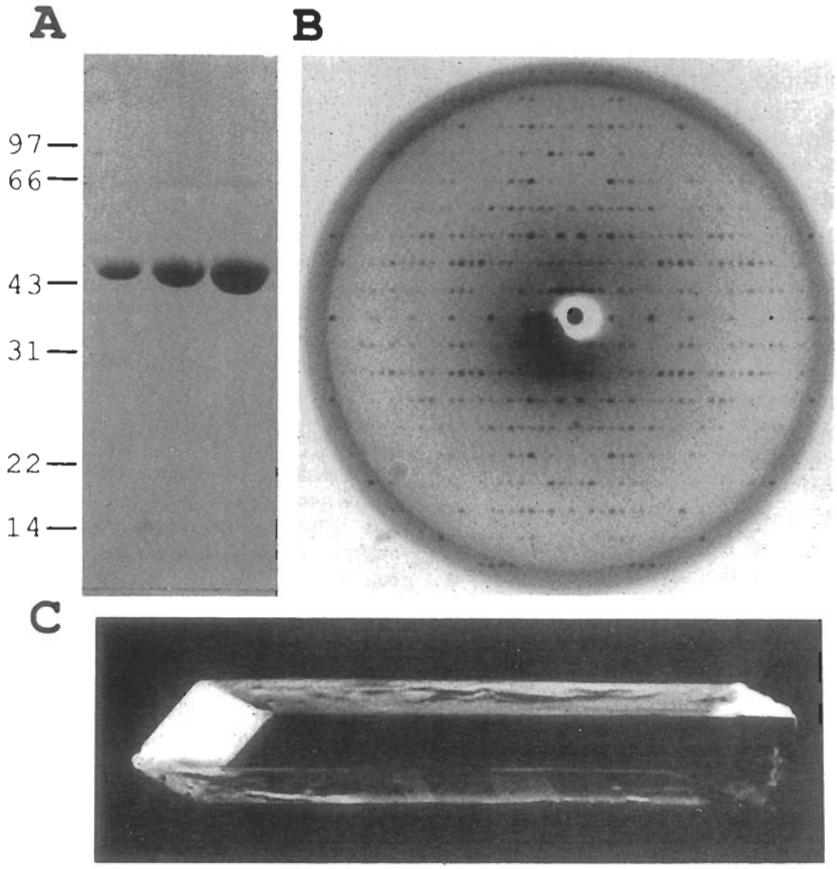

Figure 1. Purification, crystallization and precession photography of recombinant inositol polyphosphate 1-phosphatase. A, 4 and 8 μg of purified recombinant enzyme were separated on SDS/PAGE and stained with Coomassie brilliant blue dye; standards (kDa) are indicated to the left. B, 8° precession photograph of *h0l* layer (or 0*kl*, as they are indistinguishable) exposed for 16 h (*l* layer is horizontal). Crystal to film distance is 75 mm and outer rim is approximately 5·5 Å resolution. C, Crystal of recombinant enzyme with dimensions of 0·25 mm × 0·25 mm × 1·0 mm. Crystallization conditions are 13 mg/ml protein, 13% PEG-8000, 200 mM Li_2SO_4, 100 mM Tris (pH 6·3), 3 mM $MgCl_2$. The space group was determined to be either $P4_1$ or $P4_122$, or their respective enantiomorphs, with unit cell dimensions of $a=b=51{\cdot}6$ Å, $c=143{\cdot}3$ Å and $\alpha=\beta=\gamma=90°$.

[^{3}H]Ins(1,4)P_2 as a substrate were loaded onto 0·2 ml Dowex-formate columns equilibrated with 0·05 M NH_4COOH, 0·2 M HCOOH and the product [^{3}H]Ins(4)P was eluted with the same buffer.

(e) *Crystallization*

Initially, crystals were grown by sitting drop-vapor diffusion (McPherson, 1990) using an incomplete factorial method (Carter & Carter, 1979). Mother liquor (0·5 ml) containing the precipating agent, ions and buffer was placed in the reservoir of a 24 well crystallization tray. Three microliters of mother liquor and 3 μl of 10 mg inositol polyphosphate 1-phosphatase/ml were added to the cup, then the well was sealed and allowed to equilibrate for several weeks at 20°C or at 4°C. Conditions yielding crystals were systematically optimized by alteration of the pH, the precipitant concentration and the protein concentration as described (McPherson, 1990). We found that larger and more uniform crystals grew using hanging-drop vapor diffusion on silanized cover slips.

(f) *X-ray diffraction*

Crystals suitable for diffraction were mounted in 0·7 mm quartz capillaries and diffraction patterns were recorded using a Buerger precession camera (Charles Supper) with a crystal to film distance of 75 mm. Monochromatic nickel filtered CuK_α X-rays were produced from a Norelco generator at 40 kV and 20 mA.

Diffraction was measured at 4°C by ω scans on dual Xuong-Hamlin (Hamlin, 1985) Mark II multiwire area detectors (San Diego Multiwire System) equipped with helium boxes. CuK_α X-rays were produced by a Rigaku RU200 rotating anode operating at 50 kV and 150 mA equipped with a Supper graphite monochromator. High and low resolution data (θ_c values were $\pm 30°$ and $\pm 13°$, respectively) were collected at 4°C using a strategy devised by Xuong and colleagues (Xuong *et al.*, 1985) in which ω was incremented in 0·1 deg. frames at a rate of 25 s/frame (15 s/frame for low resolution data) with left and right detector to crystal distances of 880 and 835 mm, respectively. Intensity data reduction, merging and scaling was performed (Howard *et al.*, 1985).

3. Results

(a) *Recombinant baculovirus construction and expression*

We have constructed recombinant baculovirus harboring the cDNA encoding inositol polyphos-

phate 1-phosphatase and a β-galactosidase transcriptional unit. The cDNA was first inserted into the transfer plasmid, pJVP10Z, under the control of the baculovirus polyhedrin promoter. This plasmid also contained the β-galactosidase transcriptional unit under the control of the P10 very late promoter. Co-transfection of the transfer plasmid and wild-type baculovirus DNA into Sf9 cells allowed homologous recombination between virus and transfer plasmid at a frequency estimated to be 0·1% (data not shown). Seven days after transfection the media containing recombinant and wild-type viral particles was harvested. We serially diluted the media and performed a colorometric agarose plaque assay to identify and purify the recombinant baculovirus. Recombinant virus generate blue colored, occlusion negative, (polyhedrin-less) plaques within three days.

We used seven partially purified recombinant virus samples to infect Sf9 cells to verify that the inositol polyphosphate 1-phosphatase cDNA was transferred into the baculovirus genome. Four days later, total cellular DNA was prepared and probed with radiolabeled cDNA encoding bovine inositol polyphosphate 1-phosphatase by Southern slot blot (data not shown). Three of the seven samples tested positive and were subsequently purified using repeated rounds of the plaque assay until no wild-type, occlusion positive, virus infected cells were present. We then amplified two of these viral samples to titers of approximately 1×10^7 plaque forming units (p.f.u.) per ml and used them for enzyme production.

Recombinant protein production in Sf9 cells was optimized by infecting log phase cultures of Sf9 cells with recombinant virus at multiplicities of infection of 0·1, 1·0 and 10·0/p.f.u. per Sf9 cell. Inositol polyphosphate 1-phosphatase activity was measured in cellular homogenates and media from 48 to 168 hours after infection (data not shown). We achieved maximal cytosolic enzyme levels, 30 mg of inositol polyphosphate 1-phosphatase per 1×10^9 cells, at a multiplicity of infection of 1·0, 84 hours after infection. However this enzyme was partially degraded despite the presence of protease inhibitors and multiple methods of cell breakage. Maximum enzyme was released into the media under these conditions 96 to 144 hours after infection. Interestingly, inositol polyphosphate 1-phosphatase released into the media was stable and not degraded despite the absence of added protease inhibitors.

(b) *Purification and characterization*

Recombinant inositol polyphosphate 1-phosphatase was purified to homogeneity from media of Sf9 cells infected with recombinant baculovirus using DEAE and Phenyl HPLC (Fig. 1A) as described in Materials and Methods. Recombinant protein levels released in the medium represented about 10% of the total protein with the major contaminant protein being bovine serum albumin. Purified recombinant inositol polyphosphate 1-phosphatase migrated on SDS/PAGE as a single band with a mass of 44,000 daltons consistent with the size of the native enzyme. Immunoblotting using rabbit polyclonal antisera raised against bovine inositol polyphosphate 1-phosphatase showed a single band free of lower molecular weight degradation fragments (data not shown).

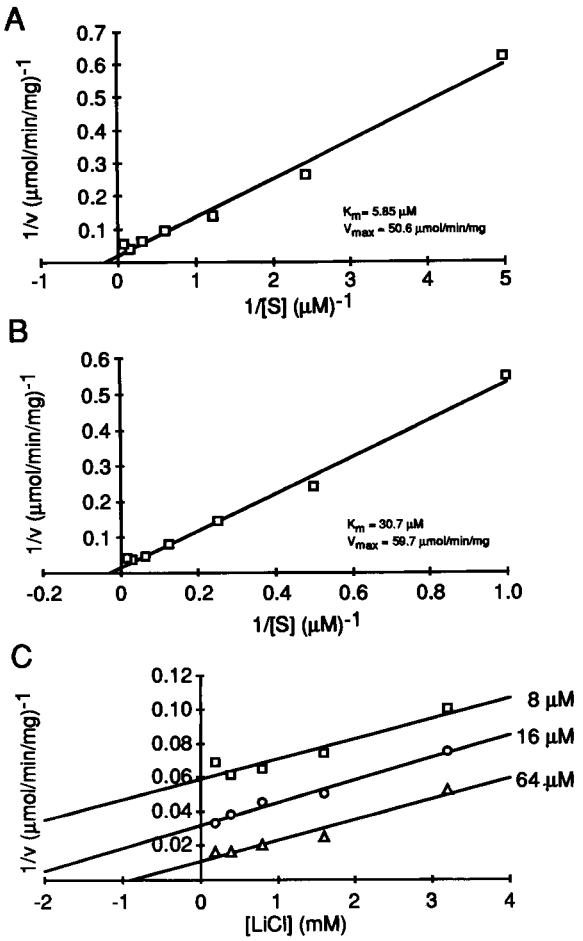

Figure 2. Kinetic parameters of recombinant inositol polyphosphate 1-phosphatase. A, Ins(1,4)P_2 hydrolysis. $7{\cdot}5 \times 10^{-8}$ mg of recombinant enzyme was incubated with varied amounts of Ins(1,4)P_2 (0·2, 0·4, 0·8, 1·6, 3·2, 6·5 and 13 μM) for 10 min. Product was eluted from column and counted (5000 c.p.m. total per assay). Velocity is expressed as a product formed per min per mg of enzyme. B, Ins(1,3,4)P_3 hydrolysis. Assays were performed as above, however, Ins (1,3,4)P_3 concentrations were 1, 2, 4, 8, 16, 32 and 64 μM and $3{\cdot}0 \times 10^{-7}$ mg of recombinant enzyme was used per assay. C, Inhibition of Ins(1,3,4)P_3 hydrolysis by lithium. Recombinant enzyme 4×10^{-7} mg) was incubated with either 8, 16 or 64 μM Ins(1,3,4)P_3 in the presence of 0·2, 0·4, 0·8, 1·6 or 3·2 mM LiCl for 10 min.

Kinetic parameters of recombinant inositol polyphosphate 1-phosphatase (Fig. 2) were determined to be similar to those for the native bovine enzyme (Inhorn & Majerus, 1987, 1988). The K_m values (Fig. 2A and B) for Ins(1,4)P_2 and Ins(1,3,4)P_3 were 5·9 μM and 30·7 μM, respectively as compared to the native enzyme values of 5·0 μm and 20·0 μM. Maximal velocities (Fig. 2A and B) for Ins(1,4)P_2

and Ins(1,3,4)P_3 hydrolysis were 50·6 μmol/min per mg protein and 59·7 μmol/min per mg protein, respectively compared to 50 μmol/min per mg for the native enzyme. Lithium uncompetively inhibited Ins(1,3,4)P_3 hydrolysis (Fig. 2C) with an apparent K_i for Ins(1,3,4)P_3 as compared to 0·3 mM for the native enzyme.

(c) *Crystallization*

Crystals of recombinant inositol polyphosphate 1-phosphatase were grown by sitting-drop vapor diffusion using a variety of conditions including polyethylene glycol, ammonium sulfate, high ionic strength, 2-methyl-2,4-pentanediol and isopropyl alcohol. One of the 30 initial conditions (10 mg protein/ml, 10% PEG-8000, 200 mM Li_2SO_4, 100 mM Tris (pH 7·0), 3 mM $MgCl_2$) resulted in clusters of needle-like crystals within one week and one additional experiment yielded small crystals after six months. Conditions were optimized by varying protein concentrations, pH and PEG-8000 concentrations. Tetragonal shaped crystals (Fig. 1C) suitable for diffraction with dimensions routinely exceeding 0·2 mm × 0·2 mm × 1·0 mm were obtained using 13 mg/ml protein, 13% PEG-8000, 200 mM Li_2SO_4, 100 mM Tris (pH 6·3), and 3 mM $MgCl_2$. Crystals reach maximum size after five to seven days and are stable for at least six months, however, we have been unable identify an artificial liquor suitable for storing crystals.

(d) *Precession photography*

Crystals were mounted in quartz capillaries and aligned on a Buerger-type precession camera equipped with a nickel filter using still photographs. Eight degree precession photographs of *h0l* and *k0l* layers (Fig. 1B) were obtained after 16 hour exposure and were indistinguishable. The space group was assigned to either $P4_1$ or $P4_122$ with unit cell dimensions of $a=b=51·6$ Å, $c=143·3$ Å and $\alpha=\beta=\gamma=90°$. Due to the morphology of the crystals we were unable to obtain a *hk*0 precession photograph. We resolved the space group to $P4_1$ or its enantiomorph $P4_3$ by comparing the intensities of *hk*0 reflections measured during data collection (see below). Assuming one molecule per asymmetric unit, the calculated V_m value (Matthews, 1968) is 2·17 $Å^3$/Dalton.

(e) *Diffraction data*

Diffraction data from crystals was collected at high and low resolution as described in Materials and Methods. We found the stability of the crystals to be enhanced by cooling to 4 °C during data collection. A data set extending from 20 Å to 2·3 Å was collected from a single crystal over 36 hours which included 63,687 observations of 17,652 unique reflections. The data set was 99·3% complete with an average $I/\sigma(I)$ of 10·5 ($I/\sigma(I)$ for a narrow 2·3 Å shell was 3·0). The value of R_{sym} (as defined by $\Sigma|I(\text{ave-obs})|/\Sigma I(\text{ave})$) for all symmetry related reflections was 0·049.

4. Discussion

Inositol polyphosphate 1-phosphatase represents less than 0·006% of total cellular protein found in tissues, thereby precluding structural studies of native enzyme. We have inserted the cDNA for inpositol polyphosphate 1-phosphatase into the baculovirus genome under the control of the polyhedrin promoter to generate a recombinant baculovirus, Sf9 cells infected with recombinant baculovirus produce inositol polyphosphate 1-phosphatase as 10% of the total cellular protein. We have purified large quantities of recombinant enzyme and have shown that this enzyme is functionally similar to the native protein.

We have obtained crystals of recombinant inositol polyphosphate 1-phosphatase suitable for X-ray diffraction studies. We have collected a complete diffraction data set of the native recombinant enzyme extending to 2·3 Å. We are currently attempting to identify heavy metal derivatives to obtain phase information in order to solve the structure by multiple isomorphous replacement.

The structure determination of inositol polyphosphate 1-phosphatase will provide insight into identification of the residues involved in the substrate binding site. Enzymes that are involved in the phosphatidylinositol signalling pathway that have been cloned to date share no significant overall primary sequence homology. Interestingly, the two motifs common to inositol polyphosphate 1-phosphatase and inositol monophosphatase (York & Majerus, 1990; Neuwald *et al.*, 1991) have been shown to be involved in metal binding and potentially substrate catalysis (Bone *et al.*, 1992).

Additionally, the amino acid sequence of inositol polyphosphate 1-phosphatase has a putative bipartite nuclear localization signal (Dingwall & Laskey, 1991; Silver, 1991). Structure determination of a nuclear localization signal would be useful for identification of critical residues involved in targeting. Alignment of this putative motif, KKEGEKNKK, with proteins in the genbank data base showed significant homology with several heat shock proteins (HSP) including HSP90 thought to be involved in steroid hormone receptor modulation (Joab *et al.*, 1984; Catelli *et al.*, 1985; Samchez *et al.*, 1985). Localization of heat shock protein-90 has not been clearly determined. However, recent cellular immunolocalization experiments show that it is found in both the nucleus and cytosol of rabbit uterus cells even in the presence of steroid hormone (Sanchez *et al.*, 1985; Gasc *et al.*, 1990; Renoir *et al.*, 1990). We are currently attempting to determine the location of inositol polyphosphate 1-phosphatase in cells.

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