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Crystallization and preliminary X-ray analysis of the glycerol-3-phosphate 1-acyltransferase from squash (*Cucurbita moschata*)

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Glycerol-3-phosphate 1-acyltransferase (E.C. 2.3.1.15; G3PAT) catalyses the incorporation of an acyl group from either acyl-acyl carrier proteins (acylACPs) or acylCoAs into the *sn*-1 position of glycerol 3-phosphate to yield 1-acylglycerol 3-phosphate. Crystals of squash G3PAT have been obtained by the hanging-drop method of vapour diffusion using PEG 4000 as the precipitant. These crystals are most likely to belong to space group $P2_12_12_1$, with approximate unit-cell parameters $a = 61.1$, $b = 65.1$, $c = 103.3$ Å, $\alpha = \beta = \gamma = 90^\circ$ and a monomer in the asymmetric unit. X-ray diffraction data to 1.9 Å resolution have been collected in-house using a MAR 345 imaging-plate system.

1. Introduction

Glycerol-3-phosphate 1-acyltransferase (E.C. 2.3.1.15; G3PAT) catalyses the incorporation of an acyl group from either acyl-acyl carrier proteins (acylACPs) or acylCoAs into the *sn*-1 position of glycerol 3-phosphate to yield 1-acylglycerol 3-phosphate (Murata & Tasaka, 1997). Three types of G3PAT are present in plants, localized either in plastids (including chloroplasts), the cytoplasm or mitochondria. The G3PAT in chloroplasts is soluble, whereas the cytoplasmic and mitochondrial localized forms are bound to the endoplasmic reticulum and the outer membrane, respectively.

Analyses of the lipid composition of chilling-sensitive *versus* chilling-resistant plants reveals a major difference in the acyl composition of phosphatidyl glycerol (PG) which is not found in other complex lipids (Murata, 1983). The nature of the fatty acid at the *sn*-1 position of the glycerol moiety of PG in the chloroplast enzyme is closely correlated with the sensitivity of plants to chilling temperatures (Murata *et al.*, 1982). For example, chilling-resistant plants such as spinach and *Arabidopsis* prefer the *cis*-unsaturated fatty acid oleate [C18:1; $CH_3-(CH_2)_7-CH{=}CH-(CH_2)_7-CO_2^-$] at the *sn*-1 position and discriminate against the non-fluid palmitate [C16:0; $CH_3-(CH_2)_{14}-CO_2^-$], whereas chilling-sensitive plants such as squash accept both fatty acids equally (Murata & Tasaka, 1997). Semi-chilling-sensitive tobacco plants (*Nicotiana tabacum*) transformed with the genes for the G3PAT from either squash (chilling sensitive) or *A. thaliana* (chilling resistant) display a dramatic alteration in their sensitivity to chilling temperatures. The plants transformed with the squash enzyme show an elevated level of saturated fatty acids in PG and are dramatically more chilling sensitive, whereas the plants transformed with the *A. thaliana* gene contain higher levels of unsaturated fatty acids in PG and are more chilling resistant (Murata *et al.*, 1992). Hence, the substrate specificity of G3PAT is clearly of major importance in chilling sensitivity in plants and represents a clear example where a single gene transfer has had major effects on the chilling resistance.

The chloroplast-specific G3PATs from squash (*C. moschata*; Ishizaki *et al.*, 1988), pea (*Pisum sativum*; Weber *et al.*, 1991), cucumber (*Cucumis sativum*; Johnson *et al.*, 1992), *A. thaliana* (Nishida *et al.*, 1993), kidney bean (*Phaseolus vulgaris*; Fritz *et al.*, 1995) and spinach (*Spinacia oleracea*; Frentzen *et al.*, 1983) have been cloned and sequenced. The putative mature squash protein comprises 368 amino-acid residues (preceded by a leader sequence of 28 amino acids) with a molecular weight of approximately 41 kDa (Ishizaki *et al.*, 1988). Other plant G3PATs are predicted to be of similar length and molecular weight, although the precise processing site at which the leader sequence is cleaved is currently unknown. There is a considerable level of sequence identity between the G3PATs from a number of different species that present different traits of chilling resistance or sensitivity. For example, *Arabidopsis* (Nishida *et al.*, 1993) and squash (Ishizaki *et al.*, 1988) G3PATs show 65.7% identity over the entire sequence. Therefore, differential substrate specificity must be achieved solely by small structural differences between these enzymes in their substrate-binding pockets. Furthermore, sequence analysis of a broad array of

membrane-bound glycerolipid acyltransferases from bacterial, plant and animal sources has identified a ubiquitous H(X)$_4$D motif, also detected in the sequences of the soluble chloroplast G3PATs, which is thought to play a similar role in catalysis as the aspartate, histidine and serine catalytic triad of the serine proteases, with the hydroxyl group of the glycerol 3-phosphate acyl acceptor being analogous to the hydroxyl group of the catalytic serine (Heath & Rock, 1998). In order to contribute to an understanding of the differential substrate specificity of related 1-acyltransferases from chilling-sensitive and chilling-resistant plants, we have initiated a structural study of the squash enzyme.

2. Materials and methods

The cDNA for the putative squash G3PAT mature enzyme, modified by the replacement of the three N-terminal residues EPA by the sequence MAS, was cloned and expressed in *Escherichia coli* using the bacteriophage T7 promoter/T7 polymerase and IPTG induction using standard procedures (Studier *et al.*, 1990; Nishida *et al.*, 1993). The recombinant enzymes were soluble and were typically expressed at levels of up to 10% of the total soluble protein. For purification, the wet weight of cells obtained from a 1500 ml culture was defrosted, suspended in buffer *A* (40 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA) and disrupted by ultrasonication for 4 × 20 s at 16 µm amplitude. Cell debris was removed by centrifugation at 19 000 rev min^{-1} for 15 min at 277 K in a Beckman J2-21 refrigerated centrifuge. The supernatant fraction typically contained about 500 mg of protein in total and was applied to a 30 ml DEAE-Sepharose Fast Flow column (Pharmacia) equilibrated with buffer *A*. The proteins were eluted with a 300 ml linear gradient of NaCl from 0 to 0.4 *M* in buffer *A* at a flow rate of 4 ml min^{-1}. Fractions containing G3PAT were combined and 4 *M* ammonium sulfate solution was added to give a final concentration of 1.5 *M*. The resulting solution was clarified by centrifugation as described above and applied to a 20 ml phenyl-Toyopearl 650S column (Toyo-Soda) equilibrated with 1.5 *M* ammonium sulfate in buffer *A*. Elution was performed using a 300 ml reverse concentration gradient of ammonium sulfate from 1.5 to 0 *M* at a flow rate of 3 ml min^{-1}. Fractions were collected and analysed for protein concentration by the method of Bradford (1976) using the Bio-Rad Dye Reagent. G3PAT eluted from the column as the main protein peak at 0.9 *M* ammonium sulfate. Fractions with the highest protein concentration were combined and concentrated to a volume of 1–2 ml using a Viva-Spin concentrator (Viva Science) and applied to a 1.6 × 60 cm Hi-Load Superdex-200 gel-filtration column (Pharmacia) equilibrated with 0.1 *M* NaCl in buffer *A*. Gel filtration was performed at a flow rate of 1 ml min^{-1}. The G3PAT eluted from this column as a protein with an apparent molecular mass of approximately 40 kDa, indicating that it is a monomer in solution under these buffer conditions. The peak fractions were combined and concentrated using the Viva-Spin concentrator to a final protein concentration of between 20 and 25 mg ml^{-1}; this sample was used directly for crystallization experiments. The typical yield of protein was 8–13 mg from a 1500 ml culture, with a purity estimated by SDS–PAGE of between 80 and 90% depending upon the original level of G3PAT expression in each batch of cell paste.

An initial crystallization screen was carried out using the Hampton Research Crystal Screen Kit Number 1 by the hanging-drop method of vapour diffusion in a room whose temperature was controlled at 290 K. Refinement of the preliminary conditions led to the growth of large crystals of squash G3PAT using 14–25% PEG 4000 as the precipitant in 100 m*M* citrate buffer pH 5.6 containing 100 m*M* ammonium acetate and 10%(*v*/*v*) 2-propanol (buffer *B*). These crystals had approximate dimensions 0.15 × 0.1 × 0.7 mm and a needle-like morphology. The optimum PEG 4000 concentration was 17%.

For data collection, a single crystal was stabilized in buffer *B* containing 30% PEG 4000 prior to flash-freezing at 100 K. Data were collected from a native crystal to 1.9 Å resolution (Fig. 1) using a MAR 345 detector with double-mirror-focused Cu $K\alpha$ X-rays produced by a Rigaku RU-200 rotating-anode generator. The 1° rotation images were processed and merged using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

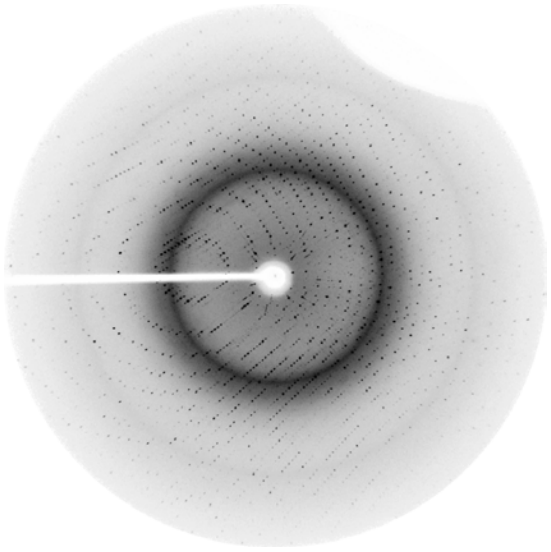

Figure 1
A representative diffraction image from a frozen (100 K) native squash G3PAT crystal. The maximum resolution at the edge of the image plate is 1.9 Å.

Table 1
X-ray data-collection statistics for the native squash G3PAT crystal.

Values in parentheses refer to the highest resolution shell (1.94–1.90 Å resolution).

Resolution range (Å)	17–1.90
Unique reflections	32652 (2085)
Completeness (%)	98.4 (95.9)
Multiplicity	3.3 (2.9)
$I/\sigma I$	12.4 (4.4)
$R_{\rm merge}$†	0.06 (0.25)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the integrated intensity of a given reflection.

3. Results and discussion

The data-collection statistics for the 1.9 Å resolution native data set are presented in Table 1. Analysis of the X-ray diffraction data using the autoindexing routine in *DENZO* indicated that the crystals belonged to the orthorhombic system point group 222, with unit-cell parameters a = 61.1, b = 65.1, c = 103.3 Å, $\alpha = \beta = \gamma = 90°$ and a unit-cell volume of 4.11 × 10^5 Å^3. The $V_{\rm M}$ value of 2.50 Å^3 Da^{-1} for a monomer in the asymmetric unit (assuming a subunit molecular mass of 41 kDa; Ishizaki *et al.*, 1988) lies within the range given by Matthews (1968) and is consistent with biochemical studies which suggest that this enzyme is a monomer in solution (Nishida *et al.*, 1987). Axial reflections along h00, 0k0 and 00l are systematically absent when h, k or $l = 2n + 1$, identifying the space group as most likely being $P2_12_12_1$. Currently, no three-dimensional structure is available for any homologous protein. Therefore, a search has been undertaken to find suitable heavy-atom derivatives to use in a multiple isomorphous replacement solution of the structure. The results should provide important insights into the mechanism by

which this class of enzymes discriminate between different fatty acyl substrates.

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