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Preliminary crystallographic analysis of sugar cane phosphoribosylpyrophosphate synthase

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Phosphoribosylpyrophosphate synthases (PRS; EC 2.7.6.1) are enzymes that are of central importance in several metabolic pathways in all cells. The sugar cane PRS enzyme contains 328 amino acids with a molecular weight of 36.6 kDa and represents the first plant PRS to be crystallized, as well as the first phosphate-independent PRS to be studied in molecular detail. Sugar cane PRS was overexpressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. Using X-ray diffraction experiments it was determined that the crystals belong to the orthorhombic system, with space group $P2_12_12$ and unit-cell parameters a = 213.2, b = 152.6, c = 149.3 Å. The crystals diffract to a maximum resolution of 3.3 Å and a complete data set to 3.5 Å resolution was collected and analysed.

1. Introduction

Phosphoribosylpyrophosphate synthases (PRS; EC 2.7.6.1) are an enzyme family that is of central importance for cellular metabolism, catalyzing the reaction of ribose-5-phosphate and ATP, with the formation of 5-phosphoribosyl-α-1-pyrophosphate (PRPP) and AMP. The reaction product PRPP is used in eight different biochemical pathways including the pentose-phosphate pathway, the *de novo* and salvage purine and pyrimidine nucleotide pathways and the biosynthesis of nucleotide coenzymes and the amino acids histidine and tryptophan. Molecular characterization of PRS enzymes has mostly been performed on bacterial proteins, including the structural determination at 2.2 Å resolution of the *Bacillus subtilis* (Eriksen *et al.*, 2000) phosphate-dependent PRS. Most of the current knowledge of this central enzyme, including the only structure resolved at atomic resolution, is derived from work performed on phosphate-dependent PRS enzymes. Another related class of enzymes, the phosphate-independent PRS enzymes, are known and have been investigated less. Therefore, very little is known about the structural determinants responsible for the phosphate-dependence/independence of this class of enzymes.

Sugar cane is one of the world's most important cultivated crops. The cultivated varieties are the consequence of interspecific hybridizations, resulting in a very complex genome structure with a variable number of chromosomes in the range $2n$ = 70–120 and a large DNA content (Arruda, 2001). Despite the significant economic impact and the biological importance of this plant, very little was known about its molecular biology, resulting in a very poor ability to develop genetically engineered plants with useful characteristics. To correct this lack of molecular information, a recently concluded Brazilian genome program called SUCEST (sugarcane expressed sequence tag) laid the foundation for many functional studies of this organism (Vettore *et al.*, 2001). The SUCEST strategy was based on the random sequencing of EST (expressed sequence tag) fragments obtained from different plant tissues and allows investigation of the expressed genes of a complex genome. Based on the knowledge generated from the SUCEST project, several important genes have been identified, including representatives of the purine-salvage and *de novo* synthesis pathways (Jancso *et al.*, 2001). In the present report, we describe the purification, crystallization and preliminary crystallographic analysis of the sugar cane PRPP synthase enzyme as

Table 1
Crystallographic data for PRS.

Values in parentheses refer to data in the highest resolution shell (3.69–3.50 Å).

Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 213.2, b = 152.6, c = 149.3
Resolution (Å)	40–3.5
Rotation per exposure (°)	1.0
Time per image (s)	300
Temperature (K)	115
Measured reflections	850219
Independent reflections	69287
Mosaicity (°)	0.36
Completeness (%)	99.7 (99.7)
Multiplicity	5.3 (5.3)
Mean $I/\sigma(I)$	3.9 (1.6)
R_{merge} (%)	10.3 (43.2)

part of a continuing study of *de novo* and salvage pathway enzymes in several organisms.

2. Experimental

2.1. Expression and purification

The *prs* open reading frame (ORF) was amplified from cDNA clones identified from the SUCEST library by a PCR reaction as described (manuscript in preparation). The *prs*-containing PCR fragment was cloned into the pCR T7 NT TOPO (Invitrogen) vector and overexpressed in *Escherichia coli* BL21(DE3)pLysS cells. The expression and purification will be described elsewhere (manuscript in preparation). Briefly, a cell culture was grown at 310 K and 250 rev min^{-1} in LB medium containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol until OD_{600} reached 0.5. The culture was induced for 5 h with 1.0 m*M* IPTG under the same conditions. The induced cells were harvested by centrifugation at 8000 rev min^{-1} in a GSA rotor (Sorval) for 15 min at 277 K. The cell pellet was frozen at 193 K prior to the extraction and purification procedures.

The transformed *E. coli* cells produced very high levels of soluble sugar cane PRS enzyme (data not shown). The overexpressed PRS protein was purified to homogeneity by fractionation with ammonium sulfate followed by affinity chromatography on an Ni^{2+}–NTA column and elution with a continuous gradient of imidazole. The recombinant PRS protein migrates as a 42 kDa protein in a 15% SDS–PAGE (Laemmli, 1970). This molecular weight is that expected of the sugar cane PRS sequence with a hexahistidine tag at the N-terminus derived from the expression vector. The recombinant PRS protein was concentrated to 9 mg ml^{-1} by ultrafiltration on Centricon-30 membranes (Bradford, 1976).

2.2. Crystal growth, data collection and processing

Crystals were obtained by the hanging-drop vapour-diffusion method from drops containing 3 µl protein solution (9 mg ml^{-1}) and 3 µl well solution suspended over 500 µl well solution. The initial crystallization trials were carried out at 277 and 291 K using Crystal Screens I and II and Grid Screen Ammonium Sulfate from Hampton Research. Small crystals were obtained under condition No. 5 of Crystal Screen II [2.0 *M* ammonium sulfate, 5.0%(*v*/*v*) 2-propanol]. Further trials with the purpose of optimizing the initial crystallization conditions were performed by decreasing the concentration of ammonium sulfate to 1.8 *M* and maintaining the 2-propanol additive concentration at 5.0%(*v*/*v*). This condition at 291 K yielded the crystals that were used in the X-ray diffraction studies (Fig. 1*a*).

X-ray diffraction data sets were collected from flash-frozen (115 K) crystals using a MAR Research CCD detector at the Protein Crystallography beamline (National Synchrotron Light Laboratory, LNLS). In order to avoid radiation damage, crystals were mounted in plastic loops and immersed in a cryoprotectant solution containing 25% glycerol and the crystal mother liquor. Diffraction data were collected over 139 frames of 1.0° oscillation with the crystal-to-detector distance set to 165.00 mm. The intensities were indexed and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). The self-rotation function was calculated using the programs *AMoRe* and *POLARRFN* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The space group has been assigned as $P2_12_12$ based on inspection of the Bravais lattice, Laue symmetry and systematic absences (Dauter, 1997). The data are 99.7% complete to 3.5 Å resolution, with a total of 69 287 reflections. R_{merge} for the entire data set collected from a single crystal is 10.3%. Fig. 1(*b*) shows an oscillation photograph obtained from a crystal of sugar cane PRS. The unit-cell parameters were determined to be a = 213.2 (3), b = 152.6 (1), c = 149.4 (1) Å with e.s.d.s estimated from the fitting of 1825 reflections in a 1.0° oscillation photograph and calculated using *DENZO* (Otwinowski & Minor, 1997). The calculated unit-cell volume is 4.867 (3) × 10^6 $Å^3$. Although observable reflections extend to a limit of 3.3 Å, the usual criteria of acceptable noise level [50% of the reflections with $I > 2\sigma(I)$] and accuracy ($R_{merge} < 0.25$) indicate that this initial data set is better described as extending to 3.5 Å resolution. The statistics for data collection are summarized in Table 1. Sugar cane PRS has been shown to be active as a homohexameric form from size-exclusion chromatography and dynamic light-scattering (DLS) experiments (data not shown). There could be one, two or three PRS hexamer assemblies per asymmetric unit according to the calculated Matthews

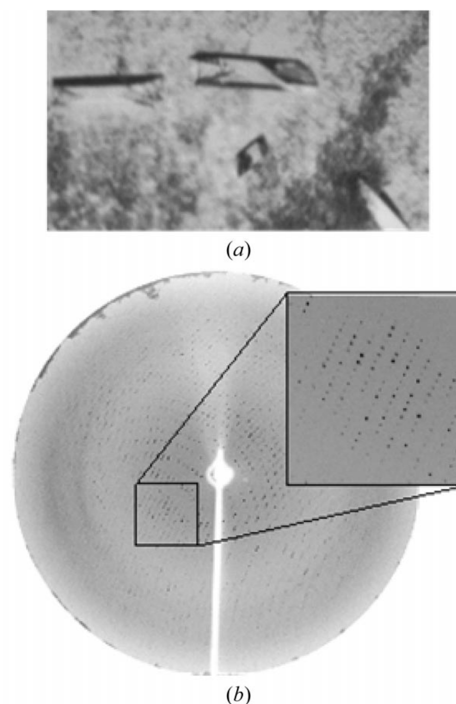

Figure 1
(*a*) Crystals of sugar cane PRS grown by the hanging-drop method. The average dimensions of these crystals were 250 × 80 × 60 µ*M*. (*b*). An X-ray diffraction pattern recorded at 115 K on a large MAR Research CCD at the LNLS.

Table 2
Molecular-replacement studies for sugar cane PRS showing the rotation-function parameters including their translation and rigid-body refinement.

		α (°)	β (°)	γ (°)	x	y	z	CC	Rf	RH
SOLUTIONF1_1	1	52.97	87.30	276.71	0.0195	0.0125	0.1943	36.9	55.2	36.6
SOLUTIONF2_1	1	52.93	87.28	36.83	0.0196	0.0125	0.1946	37.0	55.2	36.6
SOLUTIONF3_1	1	52.97	87.29	156.74	0.0195	0.0125	0.1943	36.9	55.2	36.6
SOLUTIONF4_1	1	137.22	71.87	300.16	0.4206	0.4940	0.2524	35.7	55.3	37.2
SOLUTIONF5_1	1	137.26	71.93	180.15	0.4205	0.4938	0.2524	35.7	55.3	37.2
SOLUTIONF6_1	1	137.14	71.89	59.90	0.4204	0.4939	0.2524	35.7	55.2	37.2
SOLUTIONF7_1	1	126.28	90.09	216.26	0.4857	0.0256	0.2699	36.1	55.8	36.5
SOLUTIONF8_1	1	126.36	90.13	96.09	0.4853	0.0253	0.2694	36.2	55.8	36.7
SOLUTIONF9_1	1	126.29	90.09	336.09	0.4853	0.0253	0.2695	36.2	55.8	36.6

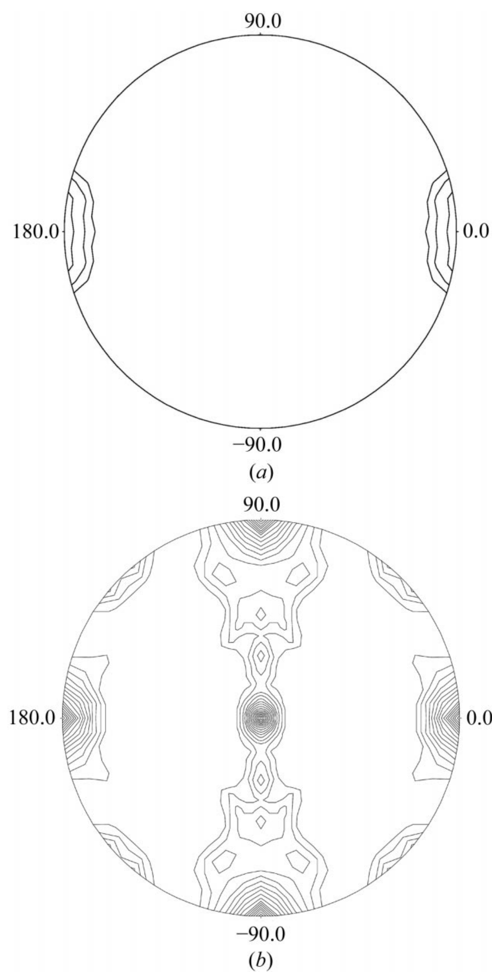

Figure 2
κ = 120° (*a*) and κ = 180° (*b*) sections of the self-rotation function calculated from sugar cane PRS diffraction data. Integration radii are 20 Å for κ = 120° and 30 Å for κ = 180°, using data between 15 and 3.5 Å.

coefficient (Matthews, 1968; Kantardjieff & Rupp, 2003). If only one hexamer is present in the asymmetric unit, the resulting solvent content should be near the upper Matthews coefficient boundary of 74.3%. If three hexamers are presented, the solvent content is near the lower Matthews coefficient boundary of 23.0%. According to recent Matthews statistic redistributions (Kantardjieff & Rupp, 2003), the best estimated solvent content is obtained when two hexamers are present per asymmetric unit, yielding a Matthews coefficient of 2.4 $Å^3$ Da^{-1} and a crystal solvent fraction of 48.7%.

Several self-rotation functions were calculated using different integration radii from 15 to 40 Å and including data between 15.0 and 4.5, 4.0 and 3.5 Å resolution. Evidence of peaks in the map at a height of 6σ at polar angles φ = 53, ω = 12.1° is observed using κ = 180° (Fig. 2). This self-Patterson map clearly revealed the presence of twofold non-crystallography symmetry (NCS) axes. Alignments have shown 24.2% sequence identity with the homologous *B. subtilis* phosphoribosylpyrophosphate synthetase (Eriksen *et al.*, 2000; PDB codes 1dkr and 1dku). This homologuous structure contains a quaternary arrangement, with the individual subunits related by a threefold axis with a perpendicular twofold axis forming 32 point symmetry (Eriksen *et al.*, 2000). As expected, molecular-replacement studies (Navaza, 1994) using the quaternary *B. subtilis* PRS as a model found a γ = 120° rotation connecting the three positions of every rotation-function parameter, as can be seen in Table 2. The results from size-exclusion chromatography and DLS are consistent with a hexameric arrangement and point to a homohexameric assembly of the sugar cane PRS. The successful crystallization of the sugar cane PRS enzyme to give crystals suitable for structure determination should allow us to answer many of the fundamental questions that remain unclear about the mechanism of catalysis and of the role of phosphate in the regulation of the catalytic mechanism.

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