Crystallization and preliminary X-ray analysis of the glycogen synthase from *Pyrococcus abyssi*

Cristina Horcajada, Emili Cid, Joan J. Guinovart, Nuria Verdaguer and Juan C. Ferrera*

*Department de Bioquímica i Biologia Molecular, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1, E-08028 Barcelona, Spain, and Institut de Recerca Biomèdica de Barcelona, Parc Científic de Barcelona, Universitat de Barcelona, Josep Samitier 4-5, E-08028 Barcelona, Spain, and Institut de Biologia Molecular de Barcelona CSIC, Jordi Girona 18-26, E-08014 Barcelona, Spain

Correspondence e-mail: ferrer@bq.ub.es

Glycogen synthase catalyzes the transfer of glucosyl residues from ADP- or UDP-glucose to the non-reducing end of a growing α,1,4-glucan chain. To date, no crystallographic structure of an animal/fungal glycogen synthase (family 3 of the glycosyl transferases) or a bacterial/plant glycogen/starch synthase (family 5) has been reported. This paper describes the recombinant expression, crystallization and preliminary X-ray analysis of the glycogen synthase from the hyperthermophilic archaean *Pyrococcus abyssi*, the smallest enzyme of the members of families 3 and 5 of the glycosyl transferases. Crystals from this protein and from its selenomethionyl variant were grown in 100 mM sodium citrate pH 5.6 containing 20% PEG and 20% dioxane by the hanging-drop vapour-diffusion method at 293 K. The crystals, which grew as thin needles, diffracted to 3.5 Å resolution and belong to space group C2, with unit-cell parameters a = 202, b = 73, c = 149 Å, β = 131°. The crystallographic and biochemical data are consistent with either a dimer or a tetramer in the crystal asymmetric unit and a volume solvent content of 70 or 39%, respectively.

1. Introduction

The enzymatic formation of glycosidic bonds is by far the most frequent biochemical transformation on earth. In addition, the complex carbohydrates arising from these reactions are crucial for a large number of biological functions, which range from structural and energy-reserve roles to those related to signalling and molecular recognition. Despite its great relevance, little structural and mechanistic information is available for the enzymes that catalyze such reactions, glycosyl transferases (GTs). GTs catalyze the transfer of glycosyl residues from activated donors, such as di- and polysaccharides, sugar phosphates or, more frequently, glycosyl diphasmonucleotides, to oligosaccharides, proteins, lipids, DNA or several small molecules which act as specific acceptors. Essentially according to sequence similarities, GTs have been grouped into more than 60 families (Campbell et al., 1997; Coutinho & Henrissat, 1999). GTs are also classified as retaining or inverting on the basis of the relative anomeric stereochemistry of the substrate and the product in the reaction catalyzed.

Compared with glycosyl hydrolases, the structural information available for GTs is recent and rather limited. Until 1999, only the structure of an inverting β-glucosyl transferase from the T4 phage was known (Vrielink et al., 1994). Since then, a further six structures of GTs that also operate with inversion of the anomeric configuration of the transferred sugar have been solved (Gastinel et al., 1999; Charnock & Davies, 1999; Ha et al., 2000; Pedersen et al., 2000; Ünlügil et al., 2000; Mulchak et al., 2001). The structural knowledge of retaining GTs is restricted to six examples and has been obtained in the last 2 years. Two of these enzymes, LgtC from the bacterium *Neisseria meningitidis* (Persson et al., 2001) and rabbit-muscle glycogenin (Gibbons et al., 2002), belong to family 8 of the GTs. A bovine α,1,3-galactosyltransferase (Gastinel et al., 2001) and the human blood-group GTs GTA and GTB (Patenaude et al., 2002) are classified in family 6. Finally, OtsA from *Mycobacterium tuberculosis*, the structure of which has been solved recently (Gibson et al., 2002), is classified in family 20. Preliminary crystallographic studies have also been reported on the glycogen synthase from *Agrobacterium tumefaiciens* (Guerin et al., 2003), an enzyme that belongs to family 5 of the GTs.

Although the enzymes of families 6 and 8 present no significant sequence homology, they show some common characteristics, such as the presence of a divalent cation essential for catalysis in the active site and coordinated to the side-chain carboxylates of two aspartate residues of the DXD motif. They also exhibit great structural similarity in the subdomain responsible for binding the glycosyl donor and in the overall fold (Pedersen et al., 2002; Ünlügil & Rini, 2000). This protein fold has been
named GT-A (Bourne & Henrissat, 2001) and is prototypically represented by the structure of SpsA (Charnock & Davies, 1999). In contrast, OtsA displays the twin Rossmann fold characteristic of the ‘fold family’ GT-B, which is represented by the inverting β-glucosyl transferase from the T4 phage (Vrielink et al., 1994).

Family 3 of the GTs, which comprises fungal and animal glycogen synthases (GSs), and family 5, which includes bacterial GSs and plant starch synthases (SSs), also operate with retention of configuration but possess characteristics that clearly differentiate them from families 6 and 8 and make them similar to family 20. Firstly, GSs and SSs do not require a divalent cation for activity and therefore do not possess the distinctive cation-binding DXD motif. Secondly, although no structural information is available for any member of these two families, structure-prediction studies have shown that members of families 3 and 5 probably possess a common catalytic mechanism and a similar overall fold (Cid et al., 2000, 2002). This structural similarity has been extended to other GT families, which presumably also belong to the GT-B folding superfamily (Wrabl & Grishin, 2001; Bourne & Henrissat, 2001).

Here, we report the recombinant expression, crystallization and preliminary structural characterization of the GS from Pyrococcus abyssi (PaGS). This archaeal protein, which has been classified into family 5 of the GTs, is the smallest member of families 3 and 5 (Cid et al., 2002) and therefore represents the minimum catalytic unit of the α-1,4-glucan synthase superfamily.

2. Experimental results
2.1. Protein expression and characterization

The open reading frame of a hypothetical PaGS (PA2292; accession No. NC_008868) was amplified by PCR from a sample of P. abyssi genomic DNA, a generous gift from Dr R. Guerrero. The PCR-amplified fragment was cloned into pGFPCCR (Cormack & Somssich, 1997) and fully sequenced. The cDNA was then subcloned into pET28a(+) (Novagen), an expression vector that adds an amino-terminal hexa-His tag to the expressed protein. A culture of Escherichia coli BL21-CodonPlus (DE3)-RIL (Strategene) transformed with the PaGS-encoding plasmid was grown at 310 K in LB medium supplemented with 34 μg ml⁻¹ chloramphenicol and 10 μg ml⁻¹ kanamycin to an A₆₀₀ of 0.6, induced with 0.4 mM IPTG and then grown for an additional 24 h. Following harvesting, the cells were lysed by sonication and PaGS was obtained mainly in the form of insoluble aggregates. The inclusion bodies were washed with a buffer containing 500 mM NaCl, 2% Triton X-100 in 20 mM Tris–HCl pH 8.0 and dissolved in 20 mM Tris–HCl pH 8.0 plus 500 mM NaCl, 5 mM imidazole, 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol. The solubilized unfolded protein was loaded onto a HiTrap Nickel-chelating column (Amersham Biosciences) and then washed with the same buffer in which 6 M guanidine hydrochloride was replaced by 6 M urea. The in-column refolding of the bound protein was performed by the use of a linear 6–0 M urea gradient. The refolded recombinant protein was finally eluted with a linear gradient of 5–500 mM imidazole in 20 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 mM 2-mercaptoethanol. Fractions containing PaGS were concentrated, loaded onto a Superdex 200 HR10/30 gel-filtration column (Amersham Biosciences) and eluted with 20 mM Tris±HCl buffer pH 7.4 containing 500 mM NaCl, 2% Triton X-100, 0.1 mM CaCl₂ and 0.1 mM MgSO₄. The purified protein ran on a denaturing SDS polyacrylamide gel as a single band of approximately 100 kDa. The purified protein was subjected to the classical assay of GS activity (Thomas et al., 1968) and was shown to catalyze the transfer of glucosyl units from ADP-glucose or UDP-glucose to a growing chain of glycogen. Furthermore, this activity was resistant to prolonged periods of incubation at high temperature (data not shown). Other GSs of archaeal origin use UDP-glucose (Cardona et al., 2001) or ADP-glucose and UDP-glucose (Gruyer et al., 2002) as glucosyl donors.

Selenomethionyl PaGS was expressed in E. coli BL21-CodonPlus (DE3)-RIL in minimal media supplemented with 0.2% glucose, 2 μM MgSO₄, 0.1 μM CaCl₂ and a mix containing all essential amino acids at 40 mg ml⁻¹ except Gly, Ala, Pro, Asn, Cys and Met. When the culture reached an OD₆₀₀ of 0.6, selenomethionine (50 mg ml⁻¹) was added and at the same time the synthesis of Met was repressed by the addition of 100 mg ml⁻¹ Phe, Thr and Lys and 50 mg ml⁻¹ Leu, Ile, Val and Pro (Van Duyne et al., 1993). After an additional 15 min, expression of the recombinant protein was induced with 0.4 mM IPTG and the culture was grown for 24 h. Selenomethionyl PaGS was purified following the same protocol as used for the native PaGS.

2.2. Crystallization and X-ray analysis

Samples of the PaGS protein (8 mg ml⁻¹) in 20 mM Tris–HCl buffer pH 7.4 containing 500 mM NaCl and 1 mM DTT were used for crystallization tests with the hanging-drop vapour-diffusion method, which were carried out at 293 K in multiwell plates utilizing commercial kits from Hampton Research. Typically, 1 μl protein solution was mixed with an equal volume of reservoir solution. Small needle-like crystals were obtained with 100 mM sodium citrate pH 5.6 containing 20% PEG 4000 and 20% dioxane. Microseeding with these crystals at 16%, PEG 4000 and 20% dioxane produced larger needles (0.2 × 0.05 × 0.02 mm) (Fig. 1). The crystals were frozen in liquid
nitrogen, adding 15% glycerol to the crystallization buffer as a cryoprotectant. Crystals of selenomethionyl PaGS were obtained and processed following an identical protocol. Synchrotron radiation at the ESRF microfocus beamline ID13 (λ = 0.975 Å) was used to obtain two data sets (Table 1). For technical reasons, data collection was not pursued to completeness and consequently the multiplicity in both data sets is low. A multiwavelength anomalous diffraction experiment with the selenomethionyl derivative was not attempted, since beamline ID13 is not easily tunable. Images of both data sets were processed and scaled using the DENZOSCALEPACK package (Otwinowski & Minor, 1997) and TRUNCATE from the CCP4 program suite (Collaborative Computational Project, Number 4, 1994). Crystals proved to be monoclinic (C2), with a unit-cell volume of 1,655,000 Å³. These data are compatible with the presence of two to four protein molecules in the asymmetric unit and a solvent content ranging from 70 to 39%, respectively (Matthews, 1968). A self-rotation function calculated with the program MOLREP (Vagin & Teplyakov, 1997) revealed an NCS peak perpendicular to the crystallographic twofold axis that could correspond to the local twofold axis of a crystallographically independent dimer in the crystal unit cell (Fig. 2).

3. Conclusions

The open reading frame PA2292, which resulted from the complete sequencing of the P. abyssi genome and was annotated as a hypothetical GS, encodes a thermostable enzyme capable of catalyzing the transfer of glucosyl units from ADP- or UDP-glucose to a growing glycogen chain. The recombination expression of PaGS in E. coli yielded insoluble aggregated protein, which was purified, refolded and crystallized. The crystals diffracted to 3.5 Å using synchrotron radiation. Further improvement of the quality of the crystals and complete MAD collection should allow the solution of the crystal structure of PaGS. These experiments are under way.

We thank Rosa M. Pérez for valuable help with the microseeding experiments, the staff at beamline ID13, ESRF, Grenoble and Tanya Yates for assistance in preparing the English manuscript. This work was supported by grants BMC2002-00705 of DGI-MCYT to NV. Data were collected at ESRF, Grenoble within a block allocation group (BAG Barcelona). X-ray data collection was financially supported by the ESRF and by grant HPRI-CT-1999-00022 from the EU.

References