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Preliminary crystallographic studies of glycogen synthase from *Agrobacterium tumefaciens*

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Crystals of the glycogen synthase (GS) from *Agrobacterium tumefaciens* have been grown that diffract to 2.6 Å resolution. The enzyme, which is homologous to the starch synthases of plants, catalyzes the last reaction step in the biosynthesis of glycogen. It is a α-retaining glucosyltransferase that uses ADP-glucose to incorporate additional glucose monomers onto the growing glycogen polymer. Its homology with mammalian GSs is marginal, but several regions shown to be important in catalysis are strictly conserved. Knowledge of the crystal structure of GS will be a major advance in the understanding of glycogen/starch metabolism and its regulation. A rational approach in enzyme engineering can subsequently be envisaged. The multiwavelength anomalous diffraction approach will be used to solve the phase problem.

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1. Introduction

Glycogen and starch are the major carbon- and energy-storage compounds in nearly all living organisms. Animal and bacterial cells store glucose in the form of glycogen, which is a very large branched glucose polymer containing about 90% α-1,4-glucosidic linkages and 10% α-1,6-linkages. In the case of plants the nutritional reservoir is starch, of which there are two forms: amylose, the unbranched type, consisting of glucose residues with α-1,4 linkages, and amylopectin, the branched form, which has about one α-1,6 linkage per 30 α-1,4 linkages and is therefore similar to glycogen except for its lower degree of branching. Glycogen accumulates inside cells depending on the nutritional content of the growth media, eventually reaching >50% of the dry weight in bacteria.

The bulk biosynthesis of glycogen involves chain elongation *via* the formation of linear α-1,4-glycosidic linkages by glycogen synthase (GS; EC 2.4.1.21) and the introduction of branch-points through α-1,6 linkages by glycogen-branching enzyme (BE; EC 2.4.1.28).

Glycogen synthases from bacteria and higher plants (starch synthases) are α-retaining glucosyl transferases similar in amino-acid sequence [family 5; for the definition and classification of glycosyltransferases, see Henrissat & Bairoch (1996) and http://afmb.cnrs-mrs.fr/CAZY/] that use ADP-glucose as a sugar donor and have molecular weights of around 50 kDa. Mammalian and yeast glycogen synthases are larger enzymes ($\sim$80 kDa) that use UDP-glucose instead of ADP-glucose and belong to glycosyltransferase family 3. However, there is no detectable sequence similarity between the ADP-glucose- and UDP-glucose-requiring enzymes, hence their different family classification.

Two enzymatic reactions lead to the formation of the α-1,4-linkage backbone in glycogen or starch: (i) the transfer of the first glucose moiety into an acceptor to form an appropriate primer (initiation reaction) and (ii) the reaction that adds the rest of the residues to form the high-molecular-weight linear α-1,4 polymer (elongation reaction). For both reactions the specificity for ADP-glucose or UDP-glucose is strictly respected according to the sequence family. Nonetheless, despite using different sugar donors, mammalian/yeast and plant GSs share a common mechanism for initiation consisting in the formation of an α-1,4-primer of 6–7 glucose residues covalently bound by the reducing end to a tyrosine residue of an auto-glucosylating enzyme named glycogenin. Once the primer is formed, the processive activity of GS adds additional glucose residues at the non-reducing end (elongation reaction). In bacteria, the mechanism of initiation is not yet understood since no glycogenin or equivalent protein has yet been detected.

The tertiary structure of GS is not known, therefore functional knowledge has been derived *via* biochemical approaches. Study of the active site of GS has revealed that although bacterial and mammalian enzymes differ in primary structure and glucosyl donor, a lysine residue (Lys15) in the context Lys-*X*-Gly-Gly is conserved (Furukawa *et al.*, 1993). Using site-directed mutagenesis, it was demonstrated that Lys15 is involved in binding to the phosphate moiety adjacent to the glycosidic linkage of ADP-glucose, presumably by ionic inter-

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action. On the other hand, residues Gly17 and Gly18 modulate catalysis. Other studies have revealed that there is a further Lys residue (Lys277) that is also required for enzymatic activity (Furukawa *et al.*, 1994). Lys277 is also conserved in maize GS, an enzyme that has only 30% amino-acid identity to the *Escherichia coli* enzyme. Site-directed mutagenesis has indicated that Lys277 is involved in catalysis rather than binding to ADP-glucose. Multiple alignment of eukaryotic GSs revealed the presence of an invariant conserved 17-amino-acid stretch with the E-X_7-E motif. This motif is also conserved in four other families of retaining glycosyltranferases and may be part of the active site of eukaryotic glycogen synthases, in which both glutamic acids are required. In bacterial GSs, the E-X_7-E motif is partially conserved, although the second glutamic acid is replaced by a Tyr (Cid *et al.*, 2000).

We have observed that *Agrobacterium tumefaciens* glycogen synthase (AtGS) does not require any primer for the synthesis of glycogen (manuscript in preparation). Purification of AtGS revealed that glycogen synthesis occurs without the addition of exogenous α1–4 primer, thus suggesting that bacterial GSs could have both enzymatic activities: initiation (assigned to eukaryotic glycogenin) and elongation.

The determination of the atomic structure of AtGS will help to understand the molecular reaction mechanism and the regulation of glycogen synthesis.

2. Results and discussion

2.1. Expression and purification

Recombinant *A. tumefaciens* GS containing a histidine tag at the C-terminus (hGS) was constructed [spanning the entire ORF from Met1 to His480 (Uttaro & Ugalde, 1994), plus five extra histidines] and cloned in plasmid pBBRMCS-4 (Kovach *et al.*, 1995), rendering the plasmid pBG19. pBG19 was introduced in *A. tumefaciens* double mutant A5130 that lacks phosphoglucomutase (*pgm*) and glycogen phosphorylase (*glgP*) activities (Ugalde *et al.*, 1998). This double mutant is unable to synthesize glucose-1P, ADP-glucose or UDP-glucose and thus glycogen or maltodextrins. Recombinant hGS was produced with plasmid pBG19 in *A. tumefaciens* strain A5130 grown in AB medium with 1% glycerol. Cells were harvested, washed once with 50 m*M* Tris–HCl pH 8 and resuspended in 50 m*M* Tris–HCl pH 7.6, 20% sucrose, 5 m*M* EDTA, 1 m*M* PMSF, 200 µg ml^{-1} lysozyme. After incubating for 2 h at 277 K, cells were centrifuged, resuspended in 50 m*M* Tris–HCl pH 7.6, 5% sucrose, 3 m*M* β-mercaptoethanol (buffer *A*) and disrupted using a French press in the presence of protease inhibitors plus 20 µg ml^{-1} DNAse. The suspension was centrifuged for 30 min (15 000*g*) and the supernatant was ultracentrifuged for 120 min (100 000*g*). The clarified supernatants were salted out with $(NH_4)_2SO_4$ at 65% saturation and pellets were resuspended and dialyzed overnight against buffer *A*. Anion-exchange chromatography was performed using a DEAE-Sepharose column. The enzyme was eluted with a linear 0–0.6 *M* NaCl gradient and active fractions were pooled and thoroughly dialyzed against buffer *A* without β-mercaptoethanol (buffer *B*) at 277 K. This solution was subjected to chelating Sepharose chromatography. The column was washed with buffer *B* plus 0.5 *M* NaCl and eluted with buffer *B* plus 50 m*M* EDTA. After concentration and buffer exchange, the solution was applied to a Mono Q HR 5/5 column. Elution was achieved with a 0–0.5 *M* NaCl gradient in buffer *A*. The active fractions were then diluted tenfold in buffer *A* plus 1.5 *M* $(NH_4)_2SO_4$ and applied to a phenyl Superose HR 5/5 column. The enzyme was finally recovered with a linear gradient of 1.5–0 *M* $(NH_4)_2SO_4$.

2.2. Crystallization

Initial crystallogenesis conditions were searched for using the hanging-drop vapour-diffusion method in Linbro plates with Hampton Research Crystal Screen kits (Hampton Research, California, USA). AtGS was concentrated to 6 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.5. Crystals were obtained (Fig. 1) from several conditions including high-molecular-weight PEGs and with pH $\simeq$ 7.5. These crystals were small and grew to their final size in 1–2 h. Optimization was then achieved by fine screening around the initial conditions, always in the presence of 5% 2-propanol (which proved to be essential), also changing the concentrations of PEG 4000 and screening different buffers around pH 7.5. The best crystals were obtained by microseeding as follows: 4 µl drops of a 1:1 mixture of 6 mg ml^{-1} protein and 100 m*M* HEPES pH 7.5, 5% isopropanol, 10% PEG 4000 were equilibrated over 1 ml of the same buffer for 4–6 h. Microseeding was then performed by streaking previously crushed crystals with a cat whisker. Crystals appeared after 1–2 d and grew as plaques, reaching 0.1 × 0.1 × 0.03 mm after a few days. For the X-ray diffraction experiments, the crystals were frozen in liquid nitrogen using 100 m*M* HEPES pH 7.5, 5% 2-propanol, 15% PEG 4000, 30% glycerol as cryoprotectant.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 64.4, b = 87.1, c = 79.5, β = 90.32
Resolution (Å)	2.6 (2.74–2.6)
Unique reflections	27237
Completeness (%)	100 (100)
$R_{\rm sym}$† (%)	6.1 (19.2)
Multiplicity	3.9
$\langle I/\sigma(I)\rangle$	8.3 (3.8)

† $R_{\rm sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

2.3. Preliminary crystallographic characterization

X-ray diffraction data sets were collected from single crystals at 110 K at the ESRF synchrotron, Grenoble, France on beamline ID29 (λ = 0.9797 Å). Diffraction was isotropic to 2.6 Å. Data were processed (Table 1) using the programs *MOSFLM*, *SCALA* and *TRUNCATE* from the *CCP*4 program suite (Collaborative Computa-

Figure 1
Monoclinic crystals of the glycogen synthase of *A. tumefaciens*, which are plates of dimensions 0.1 × 0.1 × 0.03 mm.

tional Project, Number 4, 1994). Crystals proved to be monoclinic ($P2_1$), with a unit-cell volume of 446 608.4 Å^3. Two molecules are present per asymmetric unit, with a Matthews coefficient of 2.11 Å^3 Da^{-1}, resulting in 41.4% solvent. No significant non-origin peaks were detected in the native Patterson map. A self-rotation function, calculated with the program *AMoRe* (Navaza, 1994), revealed a single NCS peak (at 25% of the origin-peak height) perpendicular to the crystallographic twofold axis that could correspond to the local twofold axis of the crystallographically independent dimer.

Initial molecular-replacement calculations using low-homology models such as arginase (PDB code 1cev) proved unsuccessful.

2.4. Selenomethionine substitution

A double mutant *pgm*/*metA* was constructed in order to obtain a genetic background unable to synthesize glucose-1-phosphate and methionine (MetA catalyses the first step in the synthesis of methionine). The plasmid expressing the recombinant hGS was introduced into this strain and protein was purified from cells grown in a defined medium with all the amino acids except methionine, the four nucleosides, vitamins, glycerol as the carbon source and selenomethionine. Incorporation of SeMet was confirmed measuring the absorption edge using synchrotron radiation and progress is being made towards solving the phase problem by multiwavelength anomalous diffraction methods.

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