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Crystallization and preliminary crystallographic study of a ternary complex between the T4 phage β-glucosyltransferase, uridine diphosphoglucose and a DNA fragment containing an abasic site

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A base-flipping phenomenon has been established for DNA methyltransferases and for DNA base-excision repair glycosylases and is likely to prove general for enzymes that need access to DNA bases to undergo chemical reaction. T4 phage β-glucosyltransferase (BGT) is a good candidate for this novel mechanism. In order to confirm this, BGT was crystallized with an abasic site-containing DNA and uridine diphosphoglucose (UDP-glucose). The crystallization strategy is described. A complete data set was collected at 1.8 Å resolution on a Cu $K\alpha$ rotating-anode X-ray source. Molecular replacement was performed and the initial electron-density maps clearly show bound DNA.

1. Introduction

The *Escherichia coli* T4 bacteriophage β-glucosyltransferase (BGT) belongs to none of the 52 families of glycosyltransferases (GTs; Campbell *et al.*, 1997, 1998; Coutinho & Henrissat, 1999). However, BGT (Vrielink *et al.*, 1994; Moréra *et al.*, 1999), the core domain of glycogen phosphorylase (family 35; Artymiuk *et al.*, 1995; Holm & Sander, 1995), *E. coli* MurG (family 28; Ha *et al.*, 2000) and UDP-*N*-acetylglucosamine 2-epimerase (family 1; Campbell *et al.*, 2000) share a similar fold. These four enzymes form a 'clan' or superfamily of glycosyltransferases (Ünligil & Rini, 2000) and adopt the GT-B fold (Bourne & Henrissat, 2001). BGT is a monomer of 351 amino acids and consists of two domains with a similar Rossmann-like fold, separated by a deep central cleft where UDP-glucose binds.

BGT transfers the glucose moiety of uridine diphosphoglucose (UDP-glucose) to the modified cytosine bases of phage T4 double-stranded DNA, producing β-glucosyl-5-hydroxymethyl cytosine (Volkin, 1954; Jesaitis, 1956; Josse & Kornberg, 1962). BGT shows little DNA-sequence specificity, but only glucosylates 5-hydroxymethyl cytosines (5-HMC). The BGT mechanism is believed to require a flipped-out base. To prove this and to understand how BGT binds its DNA substrate and accesses the 5-HMC bases, we initiated the crystallographic study of BGT in complex with a DNA fragment and UDP-glucose.

The choice of DNA fragments and the stability of protein–DNA complexes are crucial for crystallization. A critical variable is the length of the DNA molecule. The length of our DNA fragments was inferred from a detailed analysis of a structural model of BGT–UDP bound to a modelled DNA (Moréra *et al.*, 1999). For DNA targets, we used two 13-mer double-stranded blunt-ended oligonucleotides. One of them contained an abasic (apurinic/apyrimidinic or AP) site.

Here, we describe purification, crystallization and preliminary X-ray diffraction studies that allowed us to obtain crystals of BGT in a ternary complex that diffracts to 1.8 Å resolution.

2. Material and methods

2.1. β-Glucosyltransferase purification and DNA preparation

The purification of BGT was simplified from that described in Tomaschewski *et al.* (1985). The BGT-encoding gene was inserted into the pBSK vector (Stratagen) and the recombinant plasmid was used to transform BL21(DE3) cells (Novagen). LB medium containing 150 μg ml^{-1} ampicillin was inoculated with an overnight pre-culture of BL21(DE3) transformant cells and BGT production was induced with 0.5 m*M* IPTG. Bacteria were then lysed by sonication and the lysate was ultracentrifugated. The soluble fraction was dialyzed in 10 m*M* potassium phosphate pH 7.5, 2 m*M* EDTA and 50 m*M* NaCl buffer and was applied to an SP-Sepharose (Pharmacia) cation-exchange column equilibrated in the same buffer. BGT-containing fractions were then loaded onto a Superose S-100 (Pharmacia) gel-filtration column equilibrated with 50 m*M* Tris–HCl pH 7.5, 20 m*M* $MgCl_2$. SDS–PAGE revealed a high degree of purity of BGT. BGT was concentrated to 6.5 mg ml^{-1} and stored frozen.

Table 1
Crystallographic data.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	83.0
b (Å)	55.9
c (Å)	99.5
α (°)	90
β (°)	91.1
γ (°)	90
Resolution (Å)	1.8
Measured intensities	902604
Unique reflections	83161
Completeness (%)	98.1
$I/\sigma(I)$	19.1
R_{sym} (%)†	6.8

† $R_{sym} = \sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of a reflection and $\langle I\rangle$ is the average intensity of that reflection.

The two synthetic self-complementary highly purified oligonucleotides were purchased from MGW Biotech (Germany). The oligonucleotide (5′-GATACT*X*AGATAG-3′, with *X* representing an abasic site) and the oligonucleotide (5′-AAAAAACTTTTTT-3′) were annealed with their respective complementary strands (5′-CTATCTGAGTATC-3′) and (5′-AAAAAAGTTTTTT-3′) to produce DNA duplexes 1 and 2. DNA duplex 1 contains an AP site.

2.2. Gel-shift assay

BGT was mixed with DNA duplexes 1 and 2 in the presence of UDP-glucose in 100 m*M* Tris–HCl pH 7.5 and 25 m*M* $MgCl_2$ and incubated for 15 min at room temperature. The binding reaction was monitored by gel electrophoresis in TBE buffer (45 m*M* Tris–HCl pH 8.0, 45 m*M* boric acid, 0.5 m*M* EDTA) on non-denaturing 15% polyacrylamide gels. After electrophoresis, DNA was visualized with ethidium bromide staining under UV light.

For each duplex mixed with BGT in a stoichiometry of 2:1 and 1:1.5 in the presence of UDP-glucose, we identified in the gel a clearly retarded band for both stoichiometries, proving the formation of a stable non-covalent protein–DNA complex. However, the intensity of the shifted bands was greater for the DNA fragment with an abasic site (Fig. 1*a*). The influence of UDP or UDP-glucose on BGT–DNA binding using duplex 1 at a stoichiometry of 1:1 is very similar (Fig. 1*b*). We observed a higher affinity for DNA in the presence of the UDP product or the UDP-glucose substrate (Fig. 1*b*). Considering these observations, we decided to use the DNA duplex 1 containing an AP site and UDP-glucose to screen the initial crystallization conditions for a BGT–DNA complex.

2.3. Crystallization study

The DNA duplex 1 was mixed with BGT and 0.5 m*M* UDP-glucose in a 1:1 stoichiometry. Using the sparse-matrix crystallization screening kit (Hampton Research, USA and Jena BioScience GmbH, Germany), crystallization was performed with the hanging-drop vapour-diffusion method at a temperature of 291 K. Small crystals appeared within two to three weeks from only one condition (JBScreen 7, condition D5). The crystal solution contained 35 m*M* DNA and BGT, 0.5 m*M* UDP-glucose, 19% 2-methyl-2,4-pentanediol (MPD) and 0.8% *t*-butanol over pits containing 47% MPD and 2% *t*-butanol. The first generation of crystals (Fig. 2*a*) was improved by optimizing these conditions by varying the percentage of MPD, *t*-butanol and the initial concentration of the DNA–BGT complex. The crystals grew best in hanging drops containing 50 m*M* DNA and BGT, 0.5 m*M* UDP-glucose, 22% MPD and 2% *t*-butanol over wells containing 44% MPD and 4% *t*-butanol (Fig. 2*b*).

An electrophoresis mobility shift assay of the content of carefully washed and dissolved crystals demonstrated the presence of the protein–DNA complex (data not shown).

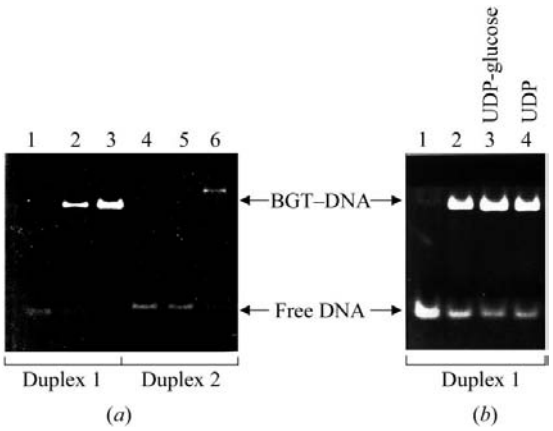

Figure 1
Polyacrylamide gel mobility shift assay of BGT–DNA complexes. (*a*) 10 µ*M* of the DNA duplex 1 (containing an AP site; lanes 1–3) and 10 µ*M* of the DNA duplex 2 (lanes 4–6) were incubated without the enzyme (lanes 1 and 4), with 5 µ*M* of BGT (lanes 2 and 5) or 15 µ*M* of BGT (lanes 3 and 6) in 100 m*M* Tris–HCl pH 7.5, 25 m*M* $MgCl_2$ and 200 µ*M* UDP-glucose. (*b*) Influence of UDP and UDP-glucose on BGT–DNA complex formation. 10 µ*M* of the DNA duplex 1 were incubated without the enzyme (lane 1) or with 10 µ*M* of BGT (lane 2) and 200 µ*M* UDP-glucose or UDP (lanes 3 and 4, respectively) in 100 m*M* Tris–HCl pH 7.5 and 25 m*M* $MgCl_2$.

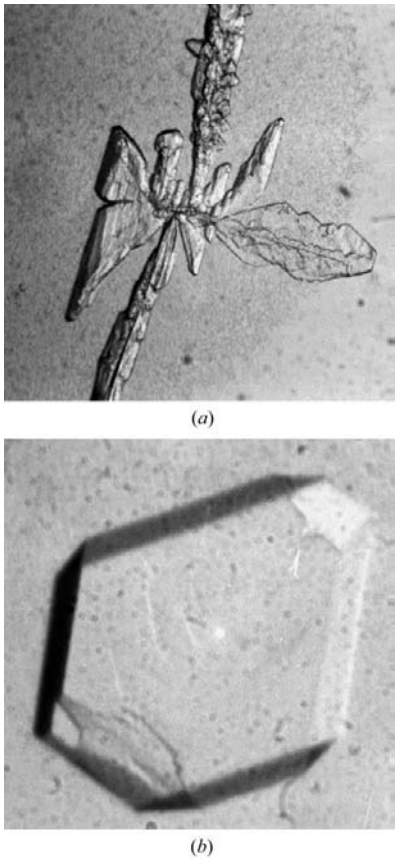

Figure 2
(*a*) The first crystals of the BGT–DNA–UDP-glucose complex obtained with a 13-mer double-stranded DNA fragment containing an abasic site. (*b*) The crystal (dimensions 0.65 × 0.55 × 0.1 mm) used for X-ray diffraction at 1.8 Å resolution after optimization of the crystallization conditions.

2.4. Diffraction data collection and processing

The best crystals were flash-frozen in nitrogen gas at 100 K (Oxford Cryosystems Cryostream Cooler). Data-collection experiments were carried out on a Rigaku rotating-anode RTP 300 RC X-ray generator operating at 50 kV and 100 mA, with the use of Osmic focusing optics. 250° of data were collected in 1° frames, with 5 min exposure per frame. Diffracted intensities were evaluated with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and were further processed using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Statistics are given in Table 1. Crystals belong to space group $P2_1$, with unit-cell parameters a = 83.0, b = 55.9, c = 99.5 Å, β = 91.1°.

3. Results and discussion

Our BGT–DNA–UDP model (Moréra *et al.*, 1999) helped us to determine a sensible length for the DNA target, considering that DNA has a strong preference for stacking end-to-end in the crystal, forming a pseudo-continuous helix. DNA fragments containing HMC bases are not available commercially. For that reason, we purchased two 13-mer double-stranded DNA analogues (duplexes 1 and 2; see §2.1). Duplex 1 carried an abasic site, while duplex 2 displayed only one C·G base pair in the centre of the fragment. The binding experiments indicated that both DNA fragments were long enough to form a stable complex with BGT and showed that BGT has a higher affinity for the duplex with an abasic site. This result reinforced the strong assumption that BGT uses a base-flipping mechanism.

Assuming that the asymmetric unit contains two complexes, the solvent content of the crystal is 49.4%. Molecular replacement was achieved using *AMoRe* (Navaza, 1994) in the resolution range 15–3.5 Å, using the crystal structure of BGT in complex with UDP as the search model (Moréra *et al.*, 2001; PDB code 1jg6). A solution giving two BGT molecules was found with a correlation factor of 60.1% and an initial R factor of 35.8%. The initial density maps show clear density for each BGT molecule and unambiguously show the presence of a bound DNA fragment. The structure is currently under refinement. This should give us the first crystal structure of a glucosyltransferase in a ternary complex. Furthermore, this structure will also be the first example of a base-flipping mechanism for an enzyme that is not a base-excision repair glycosylase or a DNA methyltransferase.

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