Structural Evidence of a Passive Base-flipping Mechanism for β-Glucosyltransferase*

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β-Glucosyltransferase (BGT) is a DNA-modifying enzyme and a glycosyltransferase. This inverting enzyme transfers glucose from UDP-glucose to the 5-hydroxymethyl cytosine bases of T4 phage DNA. From previous structural analyses we showed that Asp-100 and Asn-70 were, respectively, the catalytic base and the key residue for specific DNA recognition (Larivi`ere, L., Gueguen-Chaignon, V., and Moréra, S. (2003) J. Mol. Biol. 330, 1077–1086). Here, we supply biochemical evidence supporting their essential roles in catalysis. We have also shown previously that BGT uses a base-flipping mechanism to access 5-hydroxymethyl cytosine (Larivi`ere, L., and Moréra, S. (2002) J. Mol. Biol. 324, 483–490). Whether it is an active or a passive process remains unclear, as is the case for all DNA cleaving and modifying enzymes. Here, we report two crystal structures: (i) BGT in complex with a 13-mer DNA containing an A:G mismatch and (ii) BGT in a ternary complex with UDP and an oligonucleotide containing a single central G:C base pair. The binary structure reveals a specific complex with the flipped-out, mismatched adenine exposed to the active site. Unexpectedly, the other structure shows the non-productive binding of an intermediate flipped-out base. Our structural analysis provides clear evidence for a passive process.

The base-flipping mechanism has been widely studied and largely discussed in several reviews with the publication of the co-crystal structures of DNA methyltransferases, DNA base excision repair glycosylases, and endonucleases (1–4). Rotating the sugar-phosphate backbone around the flipped-out target base (or abasic site) allows the deoxyribose and the base being carried along to enter into the enzyme’s active site before the chemistry step is performed. Whether rotation is initiated by an active process in which the enzyme rotates the sugar-phosphate backbone or a passive one in which the enzyme binds to a spontaneously flipped-out base in a transient conformation remains unclear. An active mechanism such as “push-pull” (5), “pull-push” (6), or DNA compression imposed by a pinching action (7) were initially proposed and finally proven incorrect (1, 8–11). The T4 bacteriophage β-glucosyltransferase (BGT) uses a base-flipping mechanism to glucosylate the 5-hydroxymethyl cytosines of T4 phage DNA. Indeed, a previous structure of BGT in the presence of UDP and a 13-mer DNA containing an abasic site at the target base revealed that the deoxyribose moiety was flipped-out to an extrahelical position in the active site cavity (11). To obtain further information on the base-flipping mechanism, we determined the crystal structure of a binary complex, i.e. BGT in the presence of the same 13-mer oligonucleotide but with an adenine added to the abasic deoxyribose, leading to an A:G mispairing. As expected, the extrahelical mismatched adenine is located in the active site pocket. We also solved the structure of a ternary complex of BGT with UDP and a 13-mer DNA containing a single central C:G base pair. Surprisingly, the structure reveals a non-productive DNA complex. The structural analysis of both complexes indicates a passive role of BGT.

These structures also bring new insight into catalytic aspects. The BGT-DNA complex completes our structural study. Indeed, the catalytic mechanism of BGT was outlined by x-ray structures of the enzyme in native forms (12, 13), substrate UDP-glucose complexes (14), product UDP complexes (13, 15), and ternary UDP-DNA complexes (11). Now, it is clear that the inverting BGT possesses a random substrate binding. BGT belongs to the GT-B fold superfamily of the glycosyltransferases, identified as two flexible domains separated by a deep central cleft where the binding substrates are UDP-glucose and the DNA for BGT (16, 17). UDP-glucose binding induces a large conformational change that brings the two domains together. We have shown previously that BGT transfers the glucose through an in-line mechanism, with Asp-100 being the catalytic base that activates the attacking hydroxymethyl group of the flipped HMC (11, 14). We can now provide kinetic validation of our structural results using synthetic oligonucleotides containing hydroxymethyl uracil (HMU) bases that are substrates for BGT and thus can be glucosylated (18). We therefore investigated the role of two key residues as suggested by the x-ray structures.

MATERIALS AND METHODS

DNA Preparation—The sequence of the 13-mer DNA containing an A:G mismatch site is 5’-GATACTAGTAGT-3’/5’-CTATCTGAGTATC-3’. The sequence of the 13-mer DNA containing a single central C:G base pair is 5’-AAAAAGTTTTTT-3’/5’-AAAAACTTTTTT-3’. These oligonucleotides were purchased from MWG-Biotech and hybridized by mixing equal concentrations of 100 μM in purified water, heating to 95 °C (5 min), and cooling to room temperature overnight.

Crystallization and Structure Refinement of BGT in Complex with the A:G Mismatch 13-Mer DNA—BGT was purified as described (19). Initial crystallization conditions were determined with the Yeast Structural Genomics Project roboticized crystallization platform (Oryx, hydroxymethyl cytosine; HMU, hydroxymethyl uracil; Mss, 4-morpholineethanesulfonic acid; SMUG1, single-strand nonfunctional uracil-DNA glycosylase.)

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Table I
Crystallographic data

|                          | BGT, UDP, and a 13-mer DNA containing a single central C-G base pair | BGT and a 13-mer DNA containing an A-G mispair |
|--------------------------|---------------------------------------------------------------------|-----------------------------------------------|
| Space group              | P 2 1, 2, 2                                                         | P 2 1                                         |
| Cell parameters          | a (Å) 87.4                                                         | 72.7                                          |
|                          | b (Å) 172.9                                                        | 70.2                                          |
|                          | c (Å) 60.8                                                         | 96.6                                          |
|                          | β (%) 90.6                                                        | 90.6                                          |
| Resolution range (Å)     | 20–2.5 (2.63–2.5)                                                 | 20–1.8 (1.86–1.80)                            |
| Unique reflections       | 31,422 (4364)                                                     | 90,030 (8946)                                 |
| Completeness (%)         | 98.3 (94.2)                                                       | 100 (100)                                     |
| I/σ                      | 16.36 (2.77)                                                      | 15.5 (2.64)                                   |
| Rsym (%)                 | 7.5 (34.8)                                                        | 5.9 (33.8)                                    |
| Rcryst (%)               | 21.1                                                               | 18.3                                          |
| Rfree (%)                | 27.2                                                               | 21.2                                          |
| Root mean square deviation | 0.00667                                                          | 0.00502                                       |
| Bond length (Å)          | 1.24                                                               | 1.18                                          |
| Average B-values (Å²)    | 45.4                                                               | 16.7                                          |
| Protein                  | Molecule A (Ner-Cter)                                            | 55.9 (39.9–72.4)                              |
|                          | Molecule B                                                        | 34.9                                          |
| DNA                      | 52.8                                                               | 32.1                                          |
| Flipped adenosine        | 82                                                                 | 12                                            |
| UDP                      | 43.7                                                               | 30.4                                          |
| Solvent                  |                                                                    |                                               |

*a The values for the last resolution shell are indicated in parentheses.
*b Rsym = \( \frac{2I}{I} - \frac{I}{2I} \) where \( I \) is the intensity of a reflection and \( J \) is the average intensity of that reflection.
*c Rcryst = \( \frac{2\langle F_{\text{obs}} \rangle \rangle - \langle F_{\text{obs}} \rangle}{\langle F_{\text{obs}} \rangle} \).
*d Rfree = \( \frac{2\langle F_{\text{obs}} \rangle \rangle - \langle F_{\text{obs}} \rangle}{\langle F_{\text{obs}} \rangle} \).
*e 5% of the data were set aside for free R-factor calculation.
*f N-terminal/C-terminal domains.

France (20). Crystals were grown in hanging drops containing 71.5 μM BGT and DNA, 6% (v/v) polyethylene glycol 20,000, and 50 mM Mes, pH 6.5, over pits containing 12% (v/v) polyethylene glycol 20,000 and 100 mM NaCl, pH 6.5. The crystals were transferred to a cryo-protectant solution containing the mother liquor and 35% (v/v) glycerol and flash-frozen in liquid nitrogen. Data were collected at 100 K by a MAR Research CCD detector on beamline BM30A at the European Synchrotron Radiation Facility (Grenoble, France). Diffracted intensities were evaluated using MOSFLM (21) and further processed with the CCP4 program suite (22). Overall statistics are given in Table I. The structure was solved by molecular replacement with AmoRe (23) using the coordinates of apo BGT (Protein Data Bank code 1JEJ) as a search model. The first 2F – F electron density maps examined using TURBOFRODO (24) showed clear density for both monomers in the asymmetric unit and a bound DNA fragment that we built. Only minor modifications of the protein structure were required. Water molecules were gradually added during further conjugate gradient refinement with CNS (25). Molecular graphics images were generated using PyMOL (26).

Crystallography and Structure Refinement of BGT in Complex with UDP and a 13-Mer DNA Containing a Single C-G Base Pair—BGT was purified as described (19). Initial crystallization conditions were determined with the Yeast Structural Genomics Project robotized crystallization platform (20). Crystals were grown in hanging drops containing 71.5 μM BGT and DNA, 0.5 mM UDP, 11% (v/v) polyethylene glycol 5000 monomethyl ether, 75 mM lithium sulfate, and 50 mM Tris, pH 8.5. The crystals were transferred to a cryo-protectant solution containing the mother liquor and 20% (v/v) glycerol and flash-frozen in liquid nitrogen. Data were collected at 100 K on an ADSC Quantum Q4 detector on beamline ID14-H1 at the European Synchrotron Radiation Facility. Diffracted intensities were evaluated using the programs DENZO and SCALEPACK (27) and further processed using the CCP4 program suite (22). Overall statistics are given in Table I. The structure was solved by molecular replacement with AmoRe (23) using the coordinates of BGT-UDP (Protein Data Bank code 1JG6) as a search model. The first 2F – F electron density maps examined using TURBOFRODO (24) showed clear density for both the protein and UDP and revealed a bound DNA fragment that we built. Only minor modifications of the protein structure were required. Water molecules were gradually added during further conjugate gradient refinement with CNS (25). Molecular graphics images were generated using PyMOL (26).

Site-directed Mutagenesis via PCR—The plasmid pBSK-BGT encoding the wild type BGT protein was used as a template to generate single mutations in the DNA sequence with the QuikChange site-directed mutagenesis kit (Stratagene). This kit utilizes the double-stranded DNA vector with an insert of interest and a two-primer system to generate site-specific mutations. The sequence of the mutagenic oligonucleotide N70A is 5′-GTTAATTCCTTCTATGGCCTTTTTTTTGGC-3′.

The construction was sequenced (Génôme Express, Grenoble, France) to check the presence of the mutation of interest. The mutagenized plasmid, designated pBSK-BGT-N70A, carried DNA inserts encoding the mutated BGT protein and was used to transform BL21(DE3) cells (Novagen). The mutant was expressed and purified according to the same procedures as for the wild type protein.

Kinetic Assays—The enzymatic activity was measured with a 13-mer DNA containing a single central HMGU base pair as a substrate. The glucosylation of 5-hydroxymethyl cytosine was measured via the transfer of [14C]glucose. The incubation mixture (final volume of 20 μl at 30 °C) contained 50 mM Hepes (pH 7.9), 25 mM MgCl₂, 10–20 μM DNA, 150 μM UDP-Glc (Sigma), 15 μM [14C]UDP-glucose, and 0.05 μM wild type BGT or a D100A or N70A mutant. The reaction was stopped at different incubation times up to 3 h. Samples were spotted onto DE-81 filters. Following three washes with 0.5 M NaH₂PO₄, the filters were dried and quantitated by liquid scintillation counting.

RESULTS

Structure of the BGT-A-G Mismatch DNA Complex—Details of the crystallographic data and structure refinement at 2.5-Å resolution are shown in Table I. A DNA duplex is bound to three monomers, namely molecule A, molecule B, and a crystal symmetric of molecule B (Fig. 1). A structural comparison of molecules A and B with an unligated protein (Protein Data Bank codes 1JEJ and 2BGT) and the UDP-glucose/UDP and UDP-DNA complexes (Protein Data Bank codes 1JG6 and 1M5R, respectively) shows that they are similar to the substrate-free forms. The root mean square deviations for all Ca atoms are 0.7 and 1.1 Å, respectively. In contrast to UDP-glucose binding, DNA binding has no effect on domain closure. The oligonucleotide is well defined in the electron density map, whereas the opposite end contacts the other side of the sym...
metric molecule, forming two nonspecific complexes (Fig. 1). Each end of the DNA lies mainly against the C-terminal domain for molecule B and against the N-terminal domain for the symmetric molecule (Fig. 1). The loop regions (14–18, 214–220, and 235–237) of the N- and C-terminal domains of molecule B establish Van der Waals contacts and polar interactions via Asn-14, Asn-215, Arg-217, and Arg-237 with the DNA backbone, the terminal 13:14 and 12:15 base pairs, and the bases from 16 to 19. Surprisingly, these loop regions contribute to a rather large protein-DNA interface of 1203 Å². In contrast, the other complex buries an interface of 677 Å², an area two times smaller than that observed in the specific complex (11) (calculated using the program ASA from A. Lesk, Cambridge, UK, and a 1.4-Å probe). The loop region 101–104 and helix H9251 (140–150) of the N-terminal domain and only four residues (225, 226, 259, and 260) of the C-terminal domain make Van der Waals interactions with the DNA.

The middle part of the DNA interacts with molecule A, making a specific binary complex (Fig. 1). The mismatched adenine A7 is well defined in the electron density map (Fig. 2A). As expected, it is flipped out of the DNA helix and penetrates into the active site pocket. As proposed, the pyrimidine wedged between Val-18 and Leu-103 makes hydrophobic contact with Asn-10, Pro-19, Glu-22, Thr-99, and Asp-100 (11). The B-factor of 82 Å² indicates mobility facilitated by a rotation of the sugar-phosphate backbone around the target base. The N-terminal domain only binds to the DNA (Fig. 3) and forms a large BGT-DNA interface of 1441 Å² (calculated using the program ASA from A. Lesk, Cambridge, UK, and a 1.4 Å probe). This value, in the range of other protein-DNA interfaces (28), was higher in the ternary specific complex because of the contribution of the C-terminal domain (11). The structural observations for base flipping in the binary complex are identical to those in the ternary complex. The DNA possesses the same large distortion around the flipped adenine that induces a bend of 40° (calculated with CURVES) (29) with a marked widening of the major groove. It also makes few identical hydrogen bonds with the protein groups from the N-terminal domain (Figs. 4, A and B). Notably, DNA interactions are mainly van der Waals contacts (Figs. 4, A and B). The loop 70–74 penetrates into the DNA duplex through the major groove. The side chain of Asn-70 occupies the space left by the flipped adenine, packs against the orphan guanine G20, and provides hydrogen bonds to both the guanine and the opposite strand backbone with the O4⁺ sugar atom and an oxygen phosphate of base A8 (Fig. 2).
as defined in the legend to Fig. 1. The DNA is in gold and protein molecules are colored brown. Flipped-out deoxyribose/adenosine in substrate. Mutant N70A gives the same result with an HMU:G DNA nine base with an adenine base abolishes BGT activity, and the nine opposite the target flipped-out base. Replacing the guanine group of Arg-115 interacts with O6 and N7 (Fig. 2). These interactions, which are only possible in the absence of a base-pairing partner, are absolutely specific for guanine. Similarly, the mismatch-specific uracil-DNA glycosylase (MUG) (30) and the MutM DNA glycosylase (also known as Fpg) (10, 31, 32) make specific contacts with the Watson-Crick face of the guanine opposite the target flipped-out base. Replacing the guanine base with an adenine base abolishes BGT activity, and the mutant N70A gives the same result with an HMU:G DNA substrate.

Structure of a Non-productive BGT-UDP-DNA Complex—BGT was co-crystallized with UDP and a standard 13-mer oligonucleotide containing a single central C:G base pair intended to provide a unique guanine recognition site. Unexpectedly, BGT-UDP is bound at the end of the double-stranded DNA. The structure was determined at 1.8-Å resolution and refined to an R-factor of 18.3% (R_{free} = 21.2%) with very good stereochemistry and a B-factor for both the protein and the DNA indicating a stable complex (Table I). Two identical ternary complexes occupy the asymmetric unit with an root mean square deviation value of 0.19 Å between all Ca atoms (Fig. 5). Because of UDP binding, BGT adopts the closed conformation that is found in all complexes with at least one bound UDP-glucose or UDP. The two DNA fragments are very well defined, with all of the bases being visible in the electron density map. They display a standard B-form, except at one end where the terminal base pair T13:A14 is completely disrupted with both bases in extrahelical conformations (Fig. 5). The extrahelical position of the terminal thymine brings the base to the entrance of the enzyme’s active site (Fig. 2C). The pyrimidine ring and one phosphate oxygen are held in a compact fold by polar interactions with Ser-68 and Ser-192, respectively. The partner adenine A14 prolongs one strand of the other oligonucleotide in the asymmetric unit by stacking onto the adenine A1 (Fig. 5) and interacts with the side chain hydroxyl of Tyr-119 that extends the opposite strand by stacking onto T26 (Fig. 4). As observed in the specific complexes, Phe-72 and Gly-73 form an additional base pair by stacking on the successive base pair T12 and A15 (Fig. 2C), thus inserting the DNA duplex via the major groove. The DNA-BGT interface of 1600 Å² requires the same protein regions. However, the loop 70–74 moves 2 Å toward the DNA helix, and the positions of the two partner terminal bases are completely different from those of the flipped-out adenine and the orphan guanine G20 (Fig. 2). Although the terminal thymine is extrahelical, it fails to bind properly in the active site pocket. This finding could be explained by the lack of interaction between its partner adenine and Asn-70. Rejection of the terminal adenine is fully consistent with the specificity of Asn-70 for guanine. The absence of constraints and the stabilization of Asn-70 on the DNA substrate leads to an intermediate flipped-out position of the target base, providing a non-productive complex. Strikingly, similar complexes have been reported for the uracil-DNA glycosylase SMUG1 (33). Two crystal structures called jilted and poised complexes by the authors show SMUG1 bound at the end of the DNA substrate with the partner terminal bases in extrahelical conformations (33). An extrahelical base is located either in the active site pocket or close to it, but with non-productive binding. Just as for BGT, a protein loop inserts into the DNA duplex.

**Discussion**

Catalytic Mechanism—The structure of the specific BGT-DNA complex reveals through the flipped adenine that BGT does not present binding specificity for the target bases. This observation is in agreement with biochemical data showing that BGT can glucosylate HMU bases (18). The structure also shows that BGT does not possess an ordered bi-bi mechanism. Each substrate can bind BGT randomly. Whereas the DNA binding site is independent of the C-terminal domain, UDP-glucose binding requires both domains. Indeed, the two domains form the glucose site. That is why, in contrast to DNA binding, UDP-glucose sugar donor binding leads to rigid body domain closure. Either BGT first binds DNA on its N-terminal domain only and then UDP-glucose brings the C-terminal domain with the loop 189–195 to form the complete active site of BGT, or BGT first binds UDP-glucose, leading to a complex pre-formed for DNA binding. It has been shown that UDP-glucose binding increases the affinity for DNA binding (18). However, an extensive study, using the gel mobility technique, of wild type and mutant BGT affinity for oligonucleotides differing in sequence and length was unsuccessful and led to artificial smearing and nonspecific complex formation. The reason for this failure appears obvious, considering our two struc-
tural examples of nonspecific DNA complexes. Similar results for mobility shift assays have been reported for HhaI methyltransferase (34). Domain closure is a common feature among GT-B enzymes and appears to be compulsory for glucose transfer. In BGT, an additional conformational change occurs in loop 189–195, induced by the Asp-100-Arg-191 salt bridge between the two domains. This brings critical residues (Arg-191, Arg-195, and Ser-192) in contact with both the pyrophosphate moiety of UDP-glucose and the DNA backbone of the sugar acceptor. Loop 189–195 accounts for 10% of the total buried protein surface in the BGT-DNA interface in the specific ternary complex (11). Ser-192 brings extra stabilization of the bent DNA in the vicinity of the flipped base. Mutating Asp-100 alters the architecture of the active site, as the loop does not undergo the expected conformational change (11). Asp-100 is essential for complete formation of the catalytic site by positioning and maintaining the 189–195 loop in a suitable conformation. This loop constrains the UDP-glucose and the flipped HMC to the correct position for catalysis.

In addition to its structural role, we showed from a co-crystal structure of D100A-UDP-glucose that Asp-100 was the catalytic base (14). Now, biochemical data validate the structural results.

FIG. 4. Schematic diagram of BGT-DNA contacts in the specific binary complex (A) and the specific ternary complex (B). The adenine or abasic site is in an extrahelical conformation. Polar and van der Waals contacts with the DNA backbone are shown with dashed and wavy lines, respectively. The water molecules are labeled W. Four inserted residues (Asn-70, Phe-72, Gly-73, and Gly-74) stabilize the distorted conformation of the DNA in the vicinity of the flipped adenine or abasic site through a series of van der Waals contacts (wavy lines). The figure was modified from the output of NUCPLOT (40).

FIG. 5. Asymmetric unit content of the nonspecific BGT-UDP-DNA complex. Two identical BGT-UDP-DNA complexes belong to the asymmetric unit. Protein molecules are shown in ribbon representation according to the domain colors defined in the legend to Fig. 1. UDP is shown in a Corey-Pauling-Koltun space-filling model representation and is in yellow. One DNA is in gold, the other in red. Tyr-119 (Y119) and Phe-72 (F72) side chains are labeled.
because the D100A mutant shows no detectable activity. Both the position and the orientation of the flipped mismatched adenine were conserved to model a 5-hydroxymethyl cytosine base that is in agreement with our previous model showing a geometry compatible with an S2,2 direct displacement mechanism (11, 14).

Base-flipping Pathway and Mechanism—The flipped adenine rotates ~127° from the major groove. This would be similar for an HMC base. The 5-glucosylhydroxymethyl group of an 5-methylcytosine base interacts with the space left by the everted base, specifically recognizes the intrahelical cytosine points toward the major groove. If this base rotates backwards via the minor groove after glucosylation, the group is too bulky to cross the DNA helix without a steric clash. Thus, there is no doubt that the return pathway is through the major groove. Because the glucose transfer reaction is reversible (35), the forward pathway is the same and appears to be the shortest. Because BGT penetrates into the DNA duplex via the same groove, the base-flipping step must precede the protein loop insertion step as proposed for UDG from a presteady-state study (6). Because the inserted loop is responsible for all DNA contacts in the vicinity of the extrahelical bases, specifically recognizes the position and the orientation of the flipped mismatched adenine.

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