Observing Translesion Synthesis of an Aromatic Amine DNA Adduct by a High-fidelity DNA Polymerase*

Received for publication, August 11, 2004, and in revised form, September 20, 2004
Published, JBC Papers in Press, September 22, 2004, DOI 10.1074/jbc.M409224200

Gerald W. Hsu‡, James R. Kiefer‡‡, Dominique Burnouf§, Olivier J. Bechere¶, Robert P. P. Fuchs¶¶, and Lorena S. Beese***

From the ‡Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 and
§Cancerogenese et Mutagenese Moleculaire et Structurale, Unité Propre de Recherche 9003 du Centre National de la
Recherche Scientifique, 67400 Strasbourg, France

Aromatic amines have been studied for more than a half-century as model carcinogens representing a class of chemicals that form bulky adducts to the C8 position of guanine in DNA. Among these guanine adducts, the N-(2′-deoxyguanosin-8-yl)-aminofluorene (G-AF) and N-2-(2′-deoxyguanosin-8-yl)-acetylaminofluorene (G-AAF) derivatives are the best studied. Although G-AF and G-AAF differ by only an acetyl group, they exert different effects on DNA replication by replicative and high-fidelity DNA polymerases. Translesion synthesis of G-AF is achieved with high-fidelity polymerases, whereas replication of G-AAF requires specialized by-pass polymerases. Here we have presented structures of G-AF as it undergoes one round of accurate replication by a high-fidelity DNA polymerase. Nucleotide incorporation opposite G-AF is achieved in solution and in the crystal, revealing how the polymerase accommodates and replicates past G-AF, but not G-AAF. Like an unmodified guanine, G-AF adopts a conformation that allows it to form Watson-Crick hydrogen bonds with an opposing cytosine that results in protrusion of the bulky fluorene moiety into the major groove. Although incorporation opposite G-AF is observed, the G-AF base pair induces distortions to the polymerase active site that slow translesion synthesis.

Metabolic activation of aromatic amines ultimately generates electrophilic aryl nitrenium ions that attack DNA to yield guanine adducts that impede replication and induce mutations that underlie tumorigenesis (Fig. 1) (for reviews, see Refs. 1, 2). These aromatic amine adducts, like other types of DNA damage induced by radiation or exposure to chemical carcinogens, fall into one of two classes with respect to DNA replication by replicative polymerases: lesions that block replication and lesions that slow replication. The N-2′-(2′-deoxyguanosin-8-yl)-acetylaminofluorene (G-AF)1 adduct belongs to the class of lesions that blocks replication (3–7) and requires that replicative polymerases be transiently replaced by specialized polymerases, most of which belong to the Y family of DNA polymerases, to achieve translesion synthesis (8). Bypass of blocking lesions thus involves the concerted action of replicative and specialized DNA polymerases, a process described by the so-called “polymerase switch model” (3, 9–11). By contrast, N-2′-(2′-deoxyguanosin-8-yl)-aminofluorene (G-AF) is bypassed by high-fidelity polymerases (4), although less rapidly than an unmodified template base (7, 12–14).

The different effects of G-AAF and G-AF on replication have been postulated to depend upon the conformation these adducts adopt (for a review, see Ref. 2). In duplex DNA, G-AF induces subtle structural alterations to preferentially adopt a stable “outside binding” anti conformation, where the guanine retains cognate Watson-Crick bonds with an opposing cytosine as the fluorene ring projects into the major groove (15–19). G-AAF is restricted from adopting this anti conformation by its N-acetyl group. As a consequence, G-AAF adducts impose a major destabilization of the helix by adopting an “inserted” syn conformation where the fluorene ring intercalates into the DNA, displacing the bases to partially denature the helix (17, 20–29). Although the conformations of aminofluorene adducts in DNA duplexes in the absence of protein have been studied by NMR and molecular dynamics simulations (16, 19, 30–34), there is no a priori expectation that these conformations are adopted in the context of a polymerase active site, because it is not known what effect the polymerase structure has on the allowed conformations of G-AF and G-AAF.

Mechanisms of high-fidelity replication that are shared in large part by all polymerases, have been revealed by structural studies of DNA polymerase complexes (35–39) in combination with extensive enzyme kinetic studies (40–45) and are well represented by our model system, the thermophilic Bacillus DNA polymerase I fragment (BF) that is capable of catalyzing DNA replication in a crystal (46, 47). During the replicative cycle, the high-fidelity polymerase subjects bases and base pairs to conformational and geometric scrutiny through a series of sites to achieve accurate replication (Fig. 1B). Prior to incorporation, a template base is sequenced at the preinsertion site, a pocket with steric restrictions imposed by the surrounding O and O1 helices. Upon a conformational change of these helices from an “open” to a “closed” conformation, the template base transitions to the insertion site where it must

* This work was supported by Human Frontiers Science Program Grants RG0351/1998-M (to R. P. P. F. and L. S. B.) and NCI, National Institutes of Health Grant PO1 CA92584 (to L. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (codes 1UA0 and 1UA1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ Present address: Pfizer Inc., St. Louis, MO 63198.

‡‡ Present address: Radiation Biology and Oncology Laboratory, Queensland Institute of Medical Research, The Bancroft Centre, Royal Brisbane Hospital Past Office, Brisbane 4029, Queensland, Australia.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Box 3711, Duke University Medical Center, Durham, NC 27710. Tel.: 919-681-5267; Fax: 919-684-8855; E-mail: lsb@biochem.duke.edu.

1 The abbreviations used are: G-AAF, N-2′(2′-deoxyguanosin-8-yl)-acetylaminofluorene; G-AF, N-2′(2′-deoxyguanosin-8-yl)-aminofluorene; BF, Bacillus DNA polymerase I fragment; MES, 4-morpholinethanesulfonic acid; IS, insertion site.
adopt a \textit{anti} conformation to form a Watson-Crick base pair with the incoming dNTP. At the insertion site, base pairs that exhibit the shape and geometry of Watson-Crick base pairs are selected for, whereas base pairs that do not are selected against. Following formation of the new phosphodiester bond, the polymerase readopts the open conformation and the newly formed base pair translocates to the postinsertion site where it can pair with an incoming dCTP. By contrast, G-AAF cannot maneuver this transition as it is restricted from adopting an \textit{anti} conformation because of steric hindrance imposed by its N-acetyl group. Consistent with both structural and biochemical data, incorporation of dCTP opposite G-AAF, but not G-AF, was observed.

Following incorporation of dCTP in the crystal, G-AF adopts an \textit{anti} conformation at the postinsertion site, forming Watson-Crick hydrogen bonds with the opposing cytosine as the fluorene moiety projects into the solvent-exposed major groove. Structures of G-AF prior to and following dCTP incorporation reveal distortions to the polymerase active site that resemble distortions induced by DNA mismatches and lesions that reduce the efficiency of DNA replication.

**EXPERIMENTAL PROCEDURES**

**Preparation of AF-modified DNA Duplexes—**Chemical modification of an oligonucleotide (5'-ACTGCACCACTCC) to yield an oligonucleotide containing a single guanine residue with N-acetoxy-2-acetylamidofluorene to yield an oligonucleotide containing a single G-AAF adduct was performed as previously described (48). The G-AAF-containing nucleotide was converted to G-AF by alkaline deacetylation in the presence of 2-mercaptoethanol to prevent further oxidation (4, 49, 50). Both G-AAF- and G-AAF-containing oligonucleotides were purified by reverse phase high performance liquid chromatography (48), lyophilized, and then dissolved in a solution containing 10 mM cacodylate buffer (pH 7.5), 10 mM MgSO4, 50 mM NaCl, 0.5 mM EDTA, and 2 mM 2-mercaptoethanol.

**In Vitro Nucleotide Incorporation Opposite AF and AAF by BF—**AF- or AAF-modified template oligonucleotides and an unmodified template identical in sequence were annealed to a 5' 32P-labeled complementary 10-mer oligonucleotide positioning G-AF, G-AAF, or an unmodified guanine as the n template base. The primer was extended (standard reaction conditions 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 4% glycerol, 200 μM dNTPs, 20 °C) with 0.5 and 1.0 μM BF for nucleotide incorporation opposite unmodified guanine and G-AF or G-AAF, respectively. At each time point, 10 μl of the reaction mix was withdrawn and quenched with 30 mM EDTA (final concentration). Primers and products were separated by electrophoresis (15% polyacrylamide/8 M urea) and visualized (Amersham Biosciences 4450 Phosphorimage).

**Crystallography—**Crystallization of BF with AF-modified DNA Duplexes and Catalysis in the Crystal—Purification of BF and crystallization with DNA substrates were performed as described previously (46, 47) with AF-modified DNA duplexes used in place of unmodified DNA duplexes. Incorporation of dCTP opposite G-AF was performed by transferring BF-DNA co-crystals into solutions containing 30 mM dCTP as described previously for accurate DNA synthesis in the BF-DNA crystal (47).

**Data Collection, Structure Determination, and Superposition—**Crystals were transferred to cryoprotectant solution (60% saturated ammonium sulfate, 24% sucrose, 100 mM MES, pH 5.8) prior to being flash-frozen in liquid nitrogen. Data were collected at 98 K using a RAXIS-II or RAXIS-IV detector (Molecular Structure) on a Rigaku rotating anode x-ray generator operated at 50 mA and 100 kV with λ (CuKα) = 1.542 Å and processed using DENZO and SCALEPACK (51). Diffraction resolution was limited by the edge of the detector. Data for the BF co-crystal structure with C-G-AF at the post-1S was 77% complete at 2.0 Å. The co-crystals belong to spacegroup P2$_1$2$_1$2$_1$. and contain one molecule/asymmetric unit. Phases were calculated using molecular replacement (PDB accession code 2BDP as the probe) and models refined using crystallography NMR software (52). Force field and topology parameters allowing multiple sugar pucker conformations were used for the DNA and were modified to include parameters for the G-AF residue generated using XPLO2D (53). The structures were visualized and modified with the program O (54). Quality of the final models was assessed using PROCHECK (55) and REDUCE (56). Superpositions of the Cu atoms of BF residues 646–655, 823–838, and 863–869 were done using LSQMAN (57).
RESULTS

Replication of G-AF and G-AAF in Solution and in BF Crystals—AF- and AAF-modified DNA duplexes were used to determine the ability of BF to incorporate nucleotide opposite G-AF and G-AAF in solution and in the crystal. Incorporation of dCTP opposite G-AF is observed in solution but occurs less efficiently than dCTP incorporation opposite unmodified guanine (Fig. 2A). Replication fidelity is retained as extended primers were only observed with dCTP and not other dNTPs. By contrast, G-AAF blocks DNA replication by BF as incorporation of dCTP was not observed (Fig. 2B).

The ability to observe DNA synthesis in BF polymerase crystals allowed us to determine structures of G-AF before (Fig. 3) and after (Fig. 4) dCTP incorporation. These structures, solved to 2.1 and 2.0 Å resolution, respectively (Table I), show G-AF positioned at two sites: the template preinsertion site (n) and the postinsertion site (n-1). The latter structure was indistinguishable from a co-crystal structure of BF bound to a DNA duplex that contained a C:G-AF (primer:template) base pair at the postinsertion site (Table I).

G-AF at the Template Preinsertion Site—Positioning of the G-AF template base in the preinsertion site results in localized structural perturbations (Fig. 3). The G-AF template base (n) adopts a syn conformation and is accommodated by the preinsertion site, but with a disordered O1 helix. The fluorene moiety makes extensive van der Waals contacts with the preinsertion site surface and is essentially buried from solvent exposure, whereas the base of the G-AF residue forms a stacking interaction with the sugar of the n-1 template base (Fig. 3B). Despite this interaction and the disorder of the O1 helix, the O helix remains ordered and in an open conformation well positioned for transfer of the n template base from the template preinsertion site to the insertion site.

The remainder of the polymerase active site is unperturbed by the presence of G-AF at the template preinsertion site (Fig. 3A). Hydrogen bonds between protein residues that read the DNA minor groove (Gln-797 and Arg-615) and the n-1 base pair are retained. Both the primer and template bases adopt conformations identical to those of cognate bases at this site. Consequently, there is no distortion of the DNA duplex region, and positioning of the 3'-hydroxyl and assembly of the catalytic site remain intact.

G-AF at the Postinsertion Site—A nucleotide was incorporated opposite G-AF in the BF crystal resulting in the formation of a C:G-AF base pair positioned at the postinsertion site (Fig. 4). The C:G-AF base pair results in significant distortions to all active site regions (postinsertion site, insertion site, template preinsertion site, DNA duplex region) except for the catalytic site (Fig. 4). These distortions are reminiscent of distortions induced when a G:T mismatch occupies the polymerase active site (58).

By adopting an anti conformation, G-AF forms Watson-Crick hydrogen bonds with the opposing cytosine as the AF moiety projects into the major groove. This conformation resembles the "external" conformation described in structures of AF in duplex
DNA determined by NMR (16). By protruding into the major groove, the AF moiety blocks the next template base, which is observed in a stacking interaction with the fluorene moiety, from occupying the template preinsertion site. A second block to the preinsertion site is presented by the loop between the O and O1 helices. The conformation of this loop is altered because of O and O1 helical distortion that causes Tyr-714 to shift by 2.8 Å from its cognate position.

Disruption of interactions between the template and polymerase are also observed at the postinsertion site. Here, the interaction between Gln-797 and the template minor groove is lost. The G-AF is rotated away from the polymerase surface and is displaced by 1.6 Å from its cognate position.

Although G-AF induces distortions to the template and its interactions with the polymerase active site, distortions to the primer strand and catalytic site are not observed. The newly incorporated primer base retains the minor groove interaction with Arg-615 and the 3'-OH is not displaced. Coordination of the 3'-OH by Asp-830 remains intact for the next 3'→5' nucleotidyl transfer reaction.

Structure Determination of BF Polymerase Complexes containing G-AAF-modified DNA Templates—To obtain structures of the G-AAF adduct placed at the preinsertion and postinsertion sites, experiments analogous to those done to capture G-AF at these sites were performed. The structure of a DNA duplex designed to position G-AAF at the preinsertion site was determined to 2.1 Å resolution. In this complex the protein adopts an open conformation and the DNA duplex region, catalytic site, and postinsertion site appear unperturbed. The preinsertion site, although well ordered, is empty. Instead of binding in the preinsertion site, the G-AAF template base is positioned over the site and is partially disordered. Attempts to incorporate dCTP opposite G-AAF in the BF crystal resulted in structures that are indistinguishable from the initial complex, suggesting that incorporation did not take place.
DISCUSSION

Anti Conformation of G-AF Promotes Accurate Replication—
Structures of G-AF as it undergoes one round of replication reveal that G-AF transitions from a syn conformation at the preinsertion site to an anti conformation at the postinsertion site. In so doing, two important features are observed: 1) ability to form cognate Watson-Crick hydrogen bonds with an opposing cytosine, and 2) protrusion of the AF moiety into the solvent-exposed major groove. These features promote accurate incorporation of dCTP over mutagenic incorporation of other dNTPs opposite G-AF and account for the low frequency of mutations (~10^{-5} G→T transversions) observed in vivo (59, 60).

Unlike G-AF, G-AAF is sterically restricted to a syn conformation by the N-2-acetyl group. Its transition to anti is severely impaired but not completely prohibited. The conformation of AAF in a DNA duplex determined in the absence of protein by biophysical techniques and NMR reveals a highly disruptive inserted conformation in which the fluorene ring stacks with helix base pairs, thereby displacing the attached guanine and sugar into the minor groove (insertion-denaturation model) (17, 20, 22, 28). It is unlikely that an inserted conformation of G-AAF would be accommodated by the BF active site in a manner amenable to replication. Correspondingly, the inability to incorporate any dCTP opposite G-AAF in solution and in the crystal reflects the blocking effect of G-AAF on replication by BF and other high-fidelity DNA polymerases. By contrast, bypass polymerases such as Pol V from Escherichia coli appear to be able to efficiently copy G-AAF-containing DNA templates (8). Bypass of G-AAF adducts by several specialized eukaryotic DNA polymerases has also been documented (see review Ref. 61).

Lesion Bypass of G-AF by BF—Despite the bulkiness of G-AF, nucleotide incorporation opposite the lesion is observed in solution and in the crystal. Primer extension assays demonstrate that nucleotide incorporation opposite G-AF proceeds, albeit less efficiently than replication of undamaged DNA, and dCTP is strongly preferred over all other dNTPs (Fig. 2A). Structures of G-AF at the pre- and postinsertion sites illustrate how nucleotide incorporation occurs despite distortions induced by G-AF to the template strand and polymerase.

Before incorporation of dCTP, G-AF occupies the preinsertion site in a manner similar to an unmodified template base. Although there are localized distortions of the protein in the vicinity of G-AF, the presence of G-AF at the preinsertion site does not affect the catalytic site, the postinsertion site, or the O helix. The presence of an active site that remains largely unperturbed permits nucleotide incorporation opposite G-AF. In pairing with cytosine, G-AF adopts an anti conformation to form cognate Watson-Crick hydrogen bonds as the AF moiety protrudes into the major groove. The AF moiety is accommodated by the polymerase because the major groove is predominantly solvent-exposed, whereas the minor groove interacts extensively with the polymerase. The ability of G-AF to adopt conformations at the preinsertion and insertion sites that do not disrupt assembly of the catalytic site promotes nucleotide incorporation opposite G-AF.

Following dCTP incorporation opposite G-AF, the newly formed C-G-AF base pair induces distortions to the polymerase active site that affect subsequent DNA synthesis. The most significant of these distortions are to the preinsertion site that is blocked to the next template base by two structural features: the projection of the fluorene moiety over the preinsertion site and the distorted conformation of the O and O1 helices. Disruption of the preinsertion site is accompanied by disruptions to the minor groove interactions between the template and the polymerase. These interactions are disrupted as a consequence of template distortions that displace the n-1 template base. Similar distortions to the template and preinsertion site are observed when certain DNA mismatches or the non-bulky oxidative DNA lesion 8oxoguanine are bound at the polymerase active site (58, 62). These distortions likely account for observed reductions in rates of DNA synthesis past mismatches, 8oxoguanine, and G-AF by BF (62) and other related high-fidelity polymerases (12, 45, 63–67).

Biological Significance of Stalled or Blocked Replication—
Structures of G-AF determined during the course of one replication cycle show us that mechanisms of high-fidelity replication can accommodate non-cognate template bases and accurately synthesize DNA despite distortions that cause the polymerase to stall. Accommodation of lesion-induced template distortions by high-fidelity polymerases has also been observed for other types of DNA lesions that are less bulky than G-AF or G-AAF but also impede DNA replication. Structures of 8oxoguanine, cyclobutane pyrimidine dimers, or abasic lesions bound by T7 DNA polymerase (67, 68), RB69 polymerase (69, 70), or BF (62) reveal that template distortions induced by these lesions do not necessarily result in distortions to the polymerase active site. However, interactions between the lesioned duplex and the polymerase are lost, thereby impairing lesion bypass and promoting partitioning of the DNA duplex to the exonuclease domain in the case of RB69 (69, 70). Rather than bypass lesions using mechanisms of replication for unmodified DNA, high-fidelity polymerases employ non-templated incorporation of dATP (the “A” rule; Ref. 71) to replicate cyclobutane pyrimidine dimers (68) and abasic lesions (70) and alternate modes of base pairing to replicate 8oxoguanine (62, 67). Although these mechanisms of translesion replication may not be as efficient as replication of undamaged DNA, they demonstrate the ability of high-fidelity polymerases to synthesize DNA despite lesion-induced distortions to the template or polymerase active site.

Although bypass of G-AF and other types of DNA lesions is achieved by high-fidelity polymerases, G-AAF blocks DNA replication by these polymerases, induces the SOS response, and requires specialized DNA polymerases for in vivo bypass (72–76). For replication to occur past G-AAF and other blocking lesions, the replicative polymerase is replaced by a specialized lesion bypass polymerase, most commonly a Y family polymerase (the polymerase switch model) (11). Structures of Y family polymerases bound to DNA lesions reveal features that allow replication of lesion-modified DNA bases (77–79). Unlike BF, Y family polymerases do not possess the necessary architecture to form a preinsertion site. Instead, these enzymes have modified O and O1 helices or lack them altogether. Consequently, the DNA template assumes a more linear conformation as it enters the active site rather than adopting the sharp turn observed in the context of BF. Replication of bulky adducts is further facilitated by the relative openness of the Y family polymerase active site, which can accommodate mismatched DNA base pairs and DNA lesions (80). Such a site is less likely to restrict the conformation of large adducts. The syn conformation, to which G-AAF adducts are restricted, may be accommodated by the active site of the Y family polymerase (81), which is unique among DNA polymerases in that it preferentially drives the templating base to the syn conformation during replication (82). The structural features that differentiate polymerase and other Y polymerases from high-fidelity polymerases indicate how Y polymerases are suited for translesion replication of conformationally restricted bulky adducts in a way high-fidelity polymerases are not.

Acknowledgment—We thank Eric Wise for assistance with data collection.
