Structural insight into dynamic bypass of the major cisplatin-DNA adduct by Y-family polymerase Dpo4

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Y-family DNA polymerases bypass Pt-GG, the cisplatin-DNA double-base lesion, contributing to the cisplatin resistance in tumour cells. To reveal the mechanism, we determined three structures of the Y-family DNA polymerase, Dpo4, in complex with Pt-GG DNA. The crystallographic snapshots show three stages of lesion bypass: the nucleotide insertions opposite the 3’G (first insertion) and 5’G (second insertion) of Pt-GG, and the primer extension beyond the lesion site. We observed a dynamic process, in which the lesion was converted from an open and angular conformation at the first insertion to a depressed and nearly parallel conformation at the subsequent reaction stages to fit into the active site of Dpo4. The DNA translocation-coupled conformational change may account for additional inhibition on the second insertion reaction. The structures illustrate that Pt-GG disturbs the replicating base pair in the active site, which reduces the catalytic efficiency and fidelity. The in vivo relevance of Dpo4-mediated Pt-GG bypass was addressed by a dpo-4 knockout strain of Sulfolobus solfataricus, which exhibits enhanced sensitivity to cisplatin and proteomic alterations consistent with genomic stress.

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Introduction

All living organisms are hindered by DNA damage that generates replication-blocking lesions in DNA. Therefore, there are DNA repair mechanisms to remove lesions and damage-tolerance mechanisms for replication through lesions. Translesion synthesis (TLS) is a DNA damage-tolerance mechanism that uses specialized DNA polymerases (e.g. members of the Y-family) to replicate damaged DNA (Friedberg et al, 2002; Lehmann, 2002). TLS has an important function in cell survival of DNA damage and is highly conserved from bacteria to humans (Goodman, 2002). cis-Diamminedichloroplatinum (II) (cisplatin) is a widely used chemotherapeutic agent that covalently modifies DNA to block DNA replication in rapidly dividing tumour cells (Davidson et al, 1975; Lippert, 1999). Cisplatin reacts with the N7 atoms of adjacent guanine (G) bases to create a 1,2-intrastrand covalent linkage, cis-Pt-1,2-d(GpG) (Pt-GG) (Supplementary Figure S1), which is the major cisplatin adduct, representing ~65% of the cisplatin-DNA adducts in cells (Pinto and Lippard, 1985; Sherman et al, 1985; Coll et al, 1990; Takahara et al, 1995; Jamieson and Lippard, 1999). By inhibiting DNA synthesis, cisplatin-DNA adducts slow cell division, possibly activating programmed cell death or apoptosis (Eastman, 1990; Cohen and Lippard, 2001). Thus, cisplatin is an effective anti-cancer drug, especially for cancers of the testis, ovary, bladder, head, neck, and lung. TLS and DNA repair mechanisms have contributed to acquired and intrinsic cisplatin resistance, thereby limiting the drug’s application and contributing to cancer recurrence (Siddik, 2003; Zorbos and Keppler, 2005). Cells have been shown to have the ability to replicate past cisplatin-DNA adducts (Gibbons et al, 1991; Mamenta et al, 1994; Vaisman et al, 1998). Some translesional DNA polymerases can bypass cisplatin-DNA adducts (Hoffmann et al, 1995, 1996; Vaisman and Chaney, 2000; Vaisman et al, 2000; Albertella et al, 2005) and are overexpressed in tumour cells (Canitrot et al, 1998; Bergoglio et al, 2001). In addition, TLS is increased in the drug-resistant cells (Gibbons et al, 1991; Mamenta et al, 1994), strongly suggesting that TLS is correlated with resistance to cisplatin (Richon et al, 1987; Gibbons et al, 1991; Roush et al, 1998). Thus, TLS is considered to be one of the main mechanisms of cisplatin resistance in cancer treatment (Vaisman et al, 1998; Suo et al, 1999).

Most of the specialized translesion DNA polymerases are members of the Y-family. These DNA polymerases have a spacious active site that can accommodate bulky and distorted DNA lesions (Yang and Woodgate, 2007). However, a result of the expansion of these enzymes’ functional repertoire and substrate specificity is that Y-family DNA polymerases replicate DNA with a higher error frequency (McCulloch and Kunkel, 2008). Hence, although Y-family DNA polymerases promote damage tolerance and cell survival, they also contribute to enhanced cellular mutagenesis. Human Y-family DNA polymerase η bypasses Pt-GG in a relatively efficient and error-free manner (Vaisman et al, 2000; Albertella et al, 2005). Recently, a structural study on yeast DNA polymerase η (yPolη) in complex with Pt-GG DNA provides the first glimpse of Pt-GG lesion bypass during...
the base insertion stages (Alt et al., 2007). The canonical dCTP is the preferred nucleotide inserted by yPol1 opposite both the 3' and 5'G base of a Pt-GG cross-link (Alt et al., 2007). However, the structures do not show dCTP insertion opposite the 5'G base (Alt et al., 2007). Also notable, the yPol1 structures do not have the whole double-base Pt-GG adduct accommodated within the active site. Further study is essential to answer the key question of how the bulky/angular-shaped Pt-GG adduct is translocated through the active site of a DNA polymerase during the lesion bypass.

We report three crystal structures of a model Y-family DNA polymerase, DNA polymerase IV (Dpo4) from Sulfolobus solfataricus, in complex with Pt-GG DNA at 2.9, 1.9, and 2.0 Å resolution, respectively. During the TLS process, DNA polymerases perform two fundamental steps: nucleotide incorporation opposite the damaged DNA base(s) and extension past the lesion. Our three ternary structures encompassed these pertinent steps of TLS: dCTP insertion opposite the 3' and 5'G bases of the Pt-GG adduct and correct dATP insertion for the extension step immediately downstream of the lesion. To support the structural results, we have investigated the bypass of Pt-GG catalysed by Dpo4 in solution. In addition, the in vivo function of Dpo4 was addressed for the first time through the construction and analysis of a S. solfataricus cell line harbouring a loss of function mutation leading to deficiency of this organism’s only lesion bypass polymerase.

Results

Overall structures of the Dpo4–DNA–dNTP ternary complexes

We have co-crystallized Dpo4 with template DNA containing Pt-GG with dNTPs against the 3'G (GG1), the 5'G (GG2) of Pt-GG, and the T base 5' to the lesion (GG3), respectively (Figure 1). All three of our Dpo4–Pt-GG–DNA–dNTP ternary structures (GG1, GG2, and GG3) share a common crystal form, with one complex per asymmetric unit (Table I). The overall ternary complexes are essentially identical to the type I complex and other active Dpo4 structures (Ling et al., 2001, 2003, 2004a, b). The type I structure is the first Dpo4–DNA–dNTP ternary complex structure with normal nucleotide incorporation and represents a common active form of Dpo4, as well as most other Y-family DNA polymerases (Supplementary Figure S2). In this common active form, the finger domain is in a closed conformation packing against the catalytic palm domain and contacts the replicating base pair in the active site. Structural superposition of the three complex structures and the type I shows that the Dpo4 remains an identical conformation with root mean square deviations (r.m.s.d.) <0.5 Å on all Ca atoms, pair wisely. The closed finger conformation has been observed in other Dpo4 structures, which were solved in different crystal forms (Ling et al., 2004a; Rechkoblit et al., 2006; Wong et al., 2008). Therefore, the closed finger conformation in our current structures is independent of crystal packing.

All three GG complexes have the same 18-mer DNA template that contains a site-specific Pt-GG adduct at the 14th and 15th positions from the DNA's 3' end (Figure 2). Three different 13-mer primers were designed to generate DNA substrates for nucleotide insertions opposite or extension beyond the Pt-GG adduct in the template strand (Figure 2). Deoxyox-terminated primers (ddC or ddG) were used to trap the ternary complexes in the desired reaction stages during TLS of Pt-GG. The single-stranded template residues 5' to the adduct are completely disordered in both insertion structures GG1 and GG2. The incoming nucleotides in the three GG complexes are superimposed well with the dNTP from the ternary complexes of Dpo4 and undamaged DNA (Figure 1D and E) (Ling et al., 2004a; Vaisman et al., 2005), showing that Dpo4 maintains the incoming dNTP in a similar position and orientation in the active site, regardless of the DNA template position. Thus, the structural differences are localized at the site of the DNA adduct in the template strand. The Pt-GG adduct conformation is varied in the three structures, which deviates from the low-energy Pt-GG conformation as described by the ideal torsion angles α and β (Supplementary Table S1). The torsion angle α defines the degree of Pt out-of-plane bending (C8-N7-C5-Pt, an ideal value of 180° for in-plane conformation), and β represents the torsion angle between the G base planes and the Pt-coordination plane (C8-N7-Pt-csN, an ideal value of 90°) (Yao et al., 1994). The torsion angles in our three structures deviate up to 10° beyond the range established from the single cis-Pt-GG adduct crystal structures (Sherman et al., 1985) and the protein-free Pt-GG adducted DNA structures (Takahara et al., 1995), and thus are further off from the ideal low-energy state (Yao et al., 1994). The DNA helices bound by Dpo4 are in a straight form with bending angles <18° over the 12-bp helical region, which is in contrast to the curved protein-free Pt-GG adducted DNA (Takahara et al., 1995) (Figure 3). It seems that the helical DNA geometry and the protein binding place additional strains to distort the Pt-GG adduct from its low-energy conformations. The distances between the Pt atoms of the incoming nucleotides and the C3' atoms at the 3' end of the primer strands are ~5 Å, a catalytically competent distance, in the presence of two catalytic divalent metal ions in all three structures (Figure 1).

GG1: dCTP insertion opposite the 3'G of Pt-GG (first insertion)

The GG1 structure represents the first insertion stage of Pt-GG lesion bypass. The incoming dCTP is poised for insertion opposite the 3'G base of the Pt-GG adduct at the active site. The position of the platinum atom is identified by an anomalous difference peak, which is shifted into the DNA major groove (Figure 1A). Moreover, the finger domain in the closed conformation wedges between two Pt-coordinated G bases and blocks the 5'G from entering the active site completely (Figure 2A). Thus, the 5' base of Pt-GG is in close contact with the finger domain and is positioned outside of the active site. As the 3'G and 5'G are covalently linked through cisplatin, the 3'G base is dragged into the major groove by the 5'G, thereby preventing the 3'G from resting in the optimal template position of an undamaged DNA template (Figure 1). Compared with an undamaged DNA template, the 3'G is shifted 4.0 Å towards the major groove (Figure 1A, D, and E). The DNA helix in GG1 keeps a straight form, and the adduct is not completely included into the helical structure yet. In the first insertion structure (GG1), Pt-GG possesses a large roll angle of 135° between the 5'G and 3'G bases due to its close contacts with the finger domain (Figure 2A). In this open angular conformation, the Pt atom is out of plane of the bases with the α angles deviated by 8–37°, and the Pt-
coordination plane off the ideal β torsion by 22–60° (Supplementary Table S1). The conformation of Pt-GG in GG1 are dramatically distorted due to the position of the finger domain wedging between the two G bases. In contrast, the Pt-GG adducts in the open ypol Z structure are very close to the ideal model with roll angles of $\beta$90°, α angles of $\sim$5° from ideal 180°, and β angles within 30° from ideal 90° (Alt et al., 2007).

The Pt-GG adduct outside of the active site is not stabilized by base stacking and Watson–Crick (WC) base pairing, leading to a disturbed template (poor density, Figure 1A) with high B-factors. The 3′G base of the Pt-GG adduct shifted into the major groove loses base stacking with Watson-Crick (WC) base pairing.

Figure 1 The structures of the GG1 (A), GG2 (B), and GG3 (C) Dpo4–DNA–dNTP ternary complexes. The finger, palm, thumb, and little finger domains distinguished by their respective colours in (B). The Pt-GG lesioned template is represented in magenta, with the platinum atom shown as a cyan ball. The zoomed in boxes of the active site are covered with the Pt-anomalous maps (5 σ) in orange. The catalytic residues are shown in the middle panel of Figure 1C, which present invariantly in all three structures. There are two base conformers of Pt-GG in (B), with each Pt atom at 0.5 occupancy. The top views of the replicating base pairs are covered by a blue 2Fo-Fc maps contoured to 1.0 σ at 2.9, 1.9, and 2.0 Å resolution, respectively. The A-T pair in green sticks superimposed with GG1 (A) is taken from a Dpo4–DNA–dNTP complex structure (PDB: 1S0O), which depicts the regular position of an undamaged purine-pyrimidine base pair in the Dpo4 active site. The grey nucleotide in (A, middle box) is a ghost model for the 3′ primer base that is disordered in GG1. The green spheres are Ca$^{2+}$ ions. (D) A top view comparison of the GG1 (blue), GG2 (red), and GG3 (cyan) replicating base pairs with an undamaged pyrimidinid-purine (T-A) replicating base pair (2AGQ, black). (E) A top view comparison of the Dpo4 GG1 base pair with the yeast pol η GG1 (2R8J, beige) and an undamaged purine-pyrimidine (A-T) (1S0O, green) replicating base pairs.
The structural perturbation within the DNA template end of the primer strand at the template-primer junction. The template C base upstream (3') to the Pt-GG lesion loses the base stacking interaction with the 3'G and is over wound by 12° (Figure 2A). Consequently, the 3'G base of the primer as a base pairing partner for the over wound template C base is disordered as its 3'end (Supplementary Figure S3). However, base stacking interactions were lost between the incoming nucleotide and the disordered primer base, which makes the ternary complex less stable than the structures with undamaged DNA. The lesion template-induced disordering of 3' terminal primer end would contribute to the impaired catalytic capability of Dpo4 at this stage (see In vitro Studies).

## Table I Data collection and refinement statistics

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*Values in parentheses are for highest-resolution shell.

NH₂ groups from the 3'G base (N2) of Pt-GG and the base (N4) of dCTP (Figure 1A). Noticeably, the H-bond is a symmetrical H-bond that is of greater-than-normal strength (Cleland et al., 1998; Kraut et al., 2006; Lone et al., 2007). The symmetric H-bond is formed between donor and acceptor atoms from identical NH₂ groups that have equal pKₐ values (Supplementary Figure S1). The unique H-bond provides a structural basis for the preference of dCTP insertion opposite the lesion in the primer extension assays (see In vitro Studies). Other bases, such as A, T, and G, do not have such a NH₂ group as found in C, to form a symmetrical and stronger H-bond with the major groove-shifted 3'G of Pt-GG in the GG1 structure. Therefore, dCTP is the preferred nucleotide incorporated at the 3'G of Pt-GG, despite the WC base pair being disrupted by the covalent modification of the DNA template base.

The structural perturbation within the DNA template strand is propagated to the 3' end of the primer strand at the template-primer junction. The template C base upstream (3') to the Pt-GG lesion loses the base stacking interaction with the 3'G and is over wound by 12° (Figure 2A). Consequently, the 3'G base of the primer as a base pairing partner for the over wound template C base is disordered as the electron density for the G base completely disappears (Figure 1A; Supplementary Figure S3). The disordered primer base, which makes the ternary complex less stable than the structures with undamaged DNA. The lesion template-induced disordering of 3' terminal primer end would contribute to the impaired catalytic capability of Dpo4 at this stage (see In vitro Studies).

## GG2: dCTP incorporation against the 5'G of Pt-GG (second insertion)

An incoming dCTP is inserted opposite the 5'G of Pt-GG in the GG2 ternary complex that captures the second insertion stage. For the first time, this structure shows the two G bases of Pt-GG entering a polymerase active site simultaneously (Figure 1B). The adduct is in two alternate conformations (GG2a and GG2b) that are tilted about 10° with respect to each other (Figures 2B, C, and 3A). In the anomalous difference map, two discrete peaks show the alternate positions of the Pt atom (Figure 1B). The presence of the alternate conformers agrees with the bulged electron density map around the Pt-GG adduct (Figure 3A). The DNA helix keeps a straight form as observed in other Dpo4–DNA complex structures (Figure 3B). As it translocated into the active site (Figure 2E), the adduct adopts depressed roll (ρ) angles as low as 22° between the cross-linked bases, substantially less than ~90° roll angles observed in a single Pt-GG (Sherman et al., 1985). In other words, the roll angle is converted from 135° in GG1 to 22° in GG2 to fit Pt-GG into the active site (Figure 2E). Accordingly, the α and β torsion angles of Pt-GG deviate up to 60° from the low-energy conformation (Supplementary Table S2). A reduction in the roll angles and use of alternate conformations make the cross-linked Pt-GG more compatible with the base stacking system of a straight helix (Figure 3B). This set-up
reduces the perturbation caused by an angular adduct on the DNA helical structure and helps the lesion fit into the active site.

The 5′G of Pt-GG forms a WC base pair with the incoming dCTP in the active site (Figure 1B). The replicating base pair is in the regular position and superimposed well with the undamaged DNA replicating base pairs (Figure 1D). The finger domain is in contact with the replicating base pair (Figure 2B and C), which makes the 5′G of Pt-GG less tilted from the base stacking system than the 3′G base (Figure 3A and B). The χ-phosphate of dCTP was 4.5 Å from the C3′ atom of ddC at the 3′ end of the primer strand, indicating that the incoming nucleotide is poised for catalysis. The WC base paired replicating base pair explains the preference of dCTP over other dNTPs in the second insertion step (see In Vitro Studies). However, the alternate conformations and the geometric strain on the angular Pt-GG bases reflect a high energetic state for the GG2 complex, which contributes to the reduced nucleotide incorporation in the second insertion (Brown et al., 2008). Overall, the energetically unfavourable adduct conformation and the Pt-GG conformational conversion associated entropy cost may account for the lowest efficiency of nucleotide incorporation in the second insertion during the Pt-GG bypass (Brown et al., 2008).

**Figure 2** Pt-GG adducts in the active sites of Dpo4 and ypolZ. All panels are close-up views of the enzyme active sites where the finger domain is in cyan. The platinum (Pt) atom is shown as a cyan ball. The DNA is shown as stick-balls, with the template strand in orange and the primer strand in yellow. The sequences of the template/primer DNA in the complexes are shown on the tops of panels (A–D), cross-linked G bases are in red. The arrows indicate the incoming dNTP positions. (A) GG1, where dCTP (pink) is paired opposite the 3′G of Pt-GG (dark pink). (B, C) GG2, two alternate conformations of GG2 (translucent pink, GG2a in (B), GG2b in (C)), where dCTP (pink) is paired opposite the 5′G of Pt-GG. (D) GG3, where dATP is paired with the T base that is 5′ to the Pt-GG lesion. (E) Superposition of the GG1 (semi-transparent, left side) and GG2 (solid, right) structures of Dpo4, where DNA is shown as grey sticks with the 5′G of Pt-GG is in red with arrows, and the 3′G is in blue. The Pt-GG adduct is translocated in different positions and shows different conformations in the two structures. (F) Superposition of the same reaction stage complexes of ypolZ with the same colour scheme as (E). In ypolZ (F), the Pt-GG adduct remains in a similar position with the 5′G bases (red bases with an arrow) superimposed well in both reaction stages, no translocation of Pt-GG occurs.
GG3: nucleotide incorporation after the bypass of the Pt-GG adduct (extension step)
In the GG3 structure, the incoming dATP is base paired with the template T base 5’ to the lesion, illustrating primer extension after the Pt-GG lesion (Figure 1C). The Pt-GG adduct is in a depressed conformation with roll angle of 26° to fit into the straight DNA helix in GG3 (Figures 1C and 2D). As the Pt-GG adduct has moved out of the active site in GG3, the adduct is less strained than the adduct in GG2. The α/β torsion angles of Pt-GG in GG3 are a few degrees less distorted from the low-energy conformation than the lesion in GG2 (Supplementary Table S1). A single anomalous peak shows one position of the platinum atom with a well-defined lesion structure (Figure 1C). The replicating base pair exhibits normal WC base pairing in the active site and is superimposed well with the standard replicating base pair in Dpo4 (Figure 1D). The replicating base pair has the template T and dATP in plane, which is confined by the closed finger domain (Figure 2D). However, the base pair (5’G*:C) beneath the replicating base pair is significantly disrupted due to the tilted orientation of the 5’G base from the angular Pt-GG (Figure 3C). The G base buckles 15° and propels 18° from the base pair partner C base (Figures 1C and 3C). The disturbed base pair is not in the active site as a replicating base pair, so the structural distortion appears to be less disruptive to DNA replication as in GG1 and GG2. The crosslinked G base 18° tilting from the template T base in the active site, however, does disturb the replicating base pair as a poor stacking partner (Figure 3C), which corresponds to the nucleotide incorporation reduction observed in the extension stage (Brown et al., 2008). Overall, the GG3 complex structure was similar to that of Dpo4 with undamaged DNA (Ling et al., 2001, 2003, 2004a,b; Vaisman et al., 2005), which maintains an ideal WC base paring for the replicating base pair in the active site (Figure 1C). The DNA structural disturbance caused by Pt-GG in GG3 is mainly on the base pair which has moved out of the active site. Therefore, the Pt-GG lesion would impair catalysis less in the extension than that in the insertion stages (Brown et al., 2008).

In vitro studies of Pt-GG bypass by Dpo4
To examine Dpo4-mediated bypass of Pt-GG under conditions similar to crystal formation, we performed a running start assay with a short heteroduplex 10/18-mer at 23°C (Supplementary Figure S4). The template was modified at positions 14 and 15 with cisplatin, identical to the one used for crystallographic studies. There was significant accumulation of intermediate products (13- and 14-mers) corresponding to nucleotide incorporation opposite the two G bases of the Pt-GG adduct (13-mer is pre-first insertion, 14-mer is pre-second insertion), identical to the observation for the assays with longer substrates at 37°C (Brown et al., 2008). Thus, these results support our structural observations that the Pt-GG disturbs the replicating base pair in the active site in GG1 and GG2, which causes Dpo4 to pause during nucleotide incorporation opposite the Pt-GG adduct. Interestingly, the full product is 1 nt shorter in the lesion DNA than that of the undamaged DNA in the control panel (Supplementary Figure S4). The template was modified at positions 14 and 15 with cisplatin, identical to the one used for crystalllographic studies. There was significant accumulation of intermediate products (13- and 14-mers) corresponding to nucleotide incorporation opposite the two G bases of the Pt-GG adduct (13-mer is pre-first insertion, 14-mer is pre-second insertion), identical to the observation for the assays with longer substrates at 37°C (Brown et al., 2008). Thus, these results support our structural observations that the Pt-GG disturbs the replicating base pair in the active site in GG1 and GG2, which causes Dpo4 to pause during nucleotide incorporation opposite the Pt-GG adduct. Interestingly, the full product is 1 nt shorter in the lesion DNA than that of the undamaged DNA in the control panel (Supplementary Figure S4). The shorter product is likely caused by a frameshift mutation due to template-primer misalignment. The misalignment has been observed in the Dpo4 structures in which template-primer junctions are disturbed in the active site (Ling et al., 2001, 2004a; Bauer et al., 2007). The single deletion observed in our running start assay supports the template-primer junction instability caused by the Pt-GG adduct, such as the disordered 3′ end primer end in GG1. The frameshift mutations have been also observed in Pt-GG replication by human DNA polymerase η in vitro (Bassett et al., 2002), which may be a common mechanism in TLS of Pt-GG.

To confirm that WC base paring in the active site is disturbed by the Pt-GG lesion in solution, we performed the nucleotide incorporation assays using individual dNTP nucleotides (Figure 4). Less product formation was observed.
when Dpo4 was replicating cisplatin-DNA compared with undamaged DNA (Figure 4), supporting the structural disturbance of the replicating base pairs in the GG1, GG2, and GG3 structures. During Pt-GG lesion bypass, Dpo4 preferentially inserted a canonical dCTP opposite the Pt-GG lesion in GG1, GG2, and GG3 (extension) reaction stages with a dNTP (A, C, G, or T). M represents the marker for the primer strand. In each panel, nucleotide incorporation assays were terminated after 10 min for undamaged DNA (left) and after 60 min for Pt-GG DNA (right).

Figure 4 Specificity of Dpo4-mediated nucleotide incorporation was tested for the (A) GG1 (first insertion), (B) GG2 (second insertion), and (C) GG3 (extension) reaction stages with a dNTP (A, C, G, or T). M represents the marker for the primer strand. In each panel, nucleotide incorporation assays were terminated after 10 min for undamaged DNA (left) and after 60 min for Pt-GG DNA (right).

(Figure 4A and B). The pronounced C base mismatches are likely associated with the unstable replicating base pairs in the lesion DNA structures, which increases the probability of template-primer misalignment. Template-primer misalignment is sequence dependent (Ling et al., 2004a; Garcia-Diaz and Kunkel, 2006; Bauer et al., 2007), thus, a two G base lesion, such as Pt-GG, in the template may promote pronounced C misincorporation specifically.

Earlier kinetic studies have suggested that two distinct populations appear during nucleotide incorporation against the 3’G and 5’G of the Pt-GG lesion by Dpo4 (Brown et al., 2008). We confirmed that two distinct kinetic populations also existed with Dpo4 by performing similar kinetic assays with short primer-template DNA at room temperature (data not shown). Biphasic kinetics is consistent with the multiple conformations and mobility of the DNA substrate observed in the GG1 and GG2 structures.

In vivo studies of Pt-GG bypass

TLS by the human Y-family enzymes is a putative mechanism of intrinsic cisplatin resistance (Rabik and Dolan, 2007). However, the overlapping functions among the 16 human DNA polymerases complicate the contribution by Y-family DNA polymerases (Shachar et al., 2009). *S. solfataricus* is an ideal model organism, as it encodes only one Y-family DNA polymerase, Dpo4, which has TLS function *in vitro*. To better understand how a Y-family enzyme participates in the mechanism of drug resistance, we constructed a *dpo-4* knockout mutant of *S. solfataricus* by targeted gene disruption (Schelert et al., 2004) and characterized the consequence of Dpo4 deficiency. The *dpo-4* chromosomal mutation was confirmed using PCR to show that the mutant strain carried a disrupted *dpo-4* allele due to lacS gene insertion; and by western blot analysis to show the absence of detectable levels of Dpo4 in cell extracts (Supplementary Figures S5 and S6). Physiological studies that compared the *dpo-4* mutant with its otherwise isogenic wild-type parent were conducted and showed that Dpo4 was not essential for life as the growth rate, cell yield, efficiency of plating, and cell morphology were all normal in the *dpo-4* mutant strain (data not shown). These data also indicate that Dpo4 did not contribute substantially to replicative synthesis of the chromosome. However, chemical challenge using cisplatin reveals that Dpo4 deficiency elicited significant biological effects. To directly evaluate the *in vivo* function of Dpo4 towards cisplatin, a dose-response study was conducted using both the wild-type and *dpo-4* disruption mutant cell lines. The 50% lethal concentration (LC50) values of cisplatin were 23 μg/ml for the wild-type and 16 μg/ml for the *dpo-4* mutant (Supplementary Figure S7). The reduction in LC50 dose of cisplatin in the *dpo-4* strain shows that Dpo4 has a significant function in the tolerance of cisplatin.

Proteomic analysis revealed that Dpo4 deficiency perturbed the abundance of many proteins (Figure 5; Supplementary Table S2). For example, proteins with increased abundance in the *dpo-4* mutant include peptidyl-prolyl isomerase (SSO0758), fibrillarin (SSO0940), and single-stranded DNA binding protein (SSO2364). Together, increased abundance of these proteins suggests that Dpo4 deficiency elicits some form of genomic stress and is consistent with heightened sensitivity to cisplatin drug challenge.
Discussion

Structural snapshots capture unique Pt-GG conformational transitions in an active Y-family polymerase

Our structures provide the first complete picture of how a Y-family DNA polymerase traverses the two G bases of a Pt-GG cross-link and catalyses primer extension beyond the damaged site. The adduct in the template strand is shown in three distinct positions relative to the incoming nucleotide at the active site, clearly illustrating the translocation of the lesion template during bypass. Particularly, all three DNA-bound Dpo4 structures are in a common closed conformation in which the finger, thumb, and little finger domains grip DNA tightly (Ling et al., 2001; Nair et al., 2006; Lone et al., 2007; Kirouac and Ling, 2009). Interestingly, Pt-GG undergoes significant conformational changes, as it travels through the active site from GG1 to GG3. With the conformational change, the 5′G base of Pt-GG is able to enter into the active site in GG2 and GG3, which have the entire double-base adduct accommodated in a straight DNA helix. The unique conformational change of Pt-GG is not observed in the earlier ypol structures (Figure 2F) (Alt et al., 2007). The second insertion ypol structure with dATP does not translocate the 5′G into its active site and keeps the Pt-GG adduct in similar conformations and positions in both insertion stages (Figure 2F). In addition, our structures with an extension stage further highlight the importance of the depressed Pt-GG conformation to the DNA helix in the productive ternary complexes after the first insertion.

Unique mechanism for double-base lesion bypass

The Pt-GG bypass differs from the only other double-base bypass in complex with Dpo4 for the cis-syn cyclobutane pyrimidine dimer lesion (CPD, TT dimer). For CPD lesion bypass, the entire TT dimer enters Dpo4’s active site during the two-stage bypass, without conformational changes occurring in the dimer (Ling et al., 2003). The rigid and compact nature of the CPD allows Dpo4 to accommodate the entire double-base lesion in the active site for both insertion stages. In Pt-GG bypass, Dpo4 has to alter the conformation of the adduct to accommodate the Pt-GG adduct in its active site. The structural differences reflect the varying catalytic efficiencies of Dpo4 bypassing different double-base lesions at the two insertion stages. Dpo4 bypasses the 5′T (second insertion) more efficiently than the 3′T (first insertion) because the double-base lesion does not fit the ‘closed off’ the active site well at the first insertion stage (Ling et al., 2003). In contrast, the insertion efficiency at the second insertion in Pt-GG bypass is much lower (180-fold) than the first insertion (Brown et al., 2008). The structural basis for lower dCTP incorporation against the 5′G at the second insertion would be strong geometric strains on the adduct when the angular adduct moves into the active site and the translocation-coupled conformational conversion, which is likely to be entropically costly. Thus, TLS for different double-base lesions catalysed by the same enzyme occurs through different bypass mechanisms, as revealed by the structural analyses.

Of the human Y-family enzymes, polβ bypasses cisplatin-DNA adducts error freely while polκ performs error-prone TLS across Pt-GG (Bassett et al., 2003; Shachar et al., 2009). Human polκ keeps a similar closed conformation on the finger domain as Dpo4 and other mammalian Y-family polymerases (Supplementary Figure S2). A polη structure is only available from the yeast homologue (Alt et al., 2007), in which the finger domain is 16° open from the common closed conformation. The open conformation of ypolη seems unable to translocate the Pt-GG adduct within the active site (Figure 1D). Moreover, based on steady-state kinetic data, the extension step for hpolη is more difficult than Dpo4 in the incorporation opposite the lesion (Bassett et al., 2003), which is different from Dpo4 that has lowest replication efficiency at the second insertion. This discrepancy may be caused by structural differences observed in the two polymerases. The open conformation of ypolη does not confine the entire adduct into the active site (Alt et al., 2007), which may affect the extension step. A recent study suggested that multiple polymerases participate in the bypass of Pt-GG in mammalian...
The mobile adducts destabilize the replicating base pair

Earlier biphasic kinetic data support that the Dpo4–DNA complex exists as at least two distinct populations (Brown et al., 2008). Pt-GG in different structural environments adopts different conformations with varied roll angles and α/β torsion angles (Sherman et al., 1985; Takahara et al., 1995; Ondorf et al., 1999). The two guanine bases in the cis-
Pt(NH3)2[d(pGpG)] (a single Pt-GG) crystal structure has a perpendicular conformation with a roll angle of ~90° and α/β angles differ from the ideal values in a narrow range of 5–30° (Sherman et al., 1985). When a Pt-GG adduct is incorpo-
rated in the middle of a duplex DNA, the roll angle is compressed to 30°, with the torsion angles α/β deviated from ideal values up to 50° in protein-free adducted DNA helix (Takahara et al., 1995). The distorted and angular Pt-GG causes the 12-base pair duplex bending to a 43° curve (Figure 3D; Takahara et al., 1995). In GG2 and GG3, the DNA helices are straight with bending angle <18° over a 12-bp helical region, which further compresses the Pt-GG adduct with roll angle as low as 22° (Figure 3B and C). The Pt-GG adduct is distorted with the α/β torsion angles up to 60° to fit into the active site and helical DNA, 10° more distortion than the ones observed in the Pt-GG adducts in protein-free DNA (Takahara et al., 1995). The α/β torsion angles in the Dpo4 complexes are energetically unfavourable based on the molecular simulation (Yao et al., 1994). Particularly, the additional depression on the roll angle is reinforced by the close contact of the finger domain on the replicating base pair in GG2 (Figure 2B and C). The depression, combined with alternate conformations, brings the adduct to nearly parallel conformations in GG2 (Figure 3B). The geometric strains from the distortion would bring the adduct to a high-energy state that takes alternate conformations in GG2. Overall, Pt-GG is highly mobile in the two insertion structures due to its solvent exposure (GG1) and geometric strains (GG2). A similar mobile Pt-GG adduct also exists in the cisplatinated nucleosome structure where the intrastrand platinum adducts are packed into helical DNA (Wu et al., 2008). At 3.4 Å resolution, elongated Pt-anomalous density indicates the inherent mobility of the platinum atom covalently bonded to the parallel purine bases, which contrasts with the spherical anomalous selenium peaks in the same structure (Wu et al., 2008).

In summary, destabilization of the adduct perturbs the DNA structure, so that catalytic efficiency and fidelity are reduced in Dpo4-mediated bypass of Pt-GG (Brown et al., 2008). The misaligned G*:dCTP in GG1 and alternate G*:dCTP base pair in GG2 make replicating base pairs unstable, which would increase the probability of mutations and decrease the populations of the productive complexes of Dpo4–DNA. The mobility is consistent with the following observations in solution: (1) the pronounced C base misincorporation in the primer extension assays (Figure 4); (2) 72- and 860-fold reduction in incorporation efficiency ($k_p/k_d$) at the first and second insertion steps, respectively, and six-fold reduction at the extension step, relative to control undamaged DNA (Brown et al., 2008); and (3) two orders of magnitude of fidelity reduction in Pt-GG adducted DNA replication compared with undamaged DNA replication by Dpo4 (Brown et al., 2008). Interestingly, the symmetric H-bonding in the misaligned G*:dCTP at the first insertion (GG1) and WC H-bonding in the second insertion (GG2) still provide specificity for selective dCTP incorporation against the Pt-GG lesion though enhanced misincorporation occurs in the disturbed base paring environment.

Conclusions

This work provides a new molecular model for TLS over DNA lesions. Our structures reveal that Pt-GG bypass is a unique, dynamic process in which the Pt-GG adduct undergoes conformational changes, as it is translocated through the ‘close-off’ active site of the Y-family polymerase. The angular double-base lesion adopts a depressed conformation to fit into the active site and the helical structure of DNA during the second insertion and extension stages. The incompatibility of angular adducts with helical DNA structure and the rigid active site, along with the stress caused by the depressed conformation, make the Pt-GG adduct in energetically unfavourable conformations. The disturbed and deformed DNA template leads to instability of the replicating base pair, resulting in low replication efficiency and reduced fidelity during Pt-GG bypass. Particularly, in the second insertion, the entropically costly conformational conversion may additionally impede nucleotide incorporation against the 5′G of Pt-
GG. The in vivo observations support that the Y-family polymerase Dpo4 has an important function in tolerance of cisplatin, implicating possible contributions of Y-family polymerases to cisplatin resistance. The knowledge of how platinum cross-linked lesions are replicated by a Y-family DNA polymerase may help the development of cross-linking platinum agents for cancer therapy.

Materials and methods

Protein purification, DNA preparation, and crystallization

Dpo4 was expressed and purified as described earlier (Liang et al., 2001). DNA oligonucleotide preparation was similar to that in the earlier reported work (Brown et al., 2008). A hanging drop vapor diffusion method was used to grow crystals of the Dpo4–DNA–dNTP ternary complex by using our earlier conditions with some modifications (Bauer et al., 2007). The detailed procedures are reported in the Supplementary data.

Data collection and structure determination

Diffraction data for the GG1, GG2, and GG3 crystals were collected at beamline 24-ID-C (Argonne National Laboratory, IL) and processed using DENZO and SCALExPACK (Otwinowski and Minor, 1997). All crystal structures were solved with molecular replacement program PHASER (McCoy et al., 2005), with the type I structure as search model (PDB: 1JX4). Iterative cycles of simulated annealing, positional refinement, and B-factor refinement were performed using CNS (Brünger et al., 1998), along with manual rebuilding of the model using the graphics program COOT (Emsley and Cowtan, 2004). The GG1, GG2, and GG3 structures were refined to 2.9, 1.9, and 2.0 A° resolution, respectively.

In vitro assays

All the in vitro assays were carried out as described earlier (Brown et al., 2008; Wong et al., 2008), with reaction temperature at 23°C. The detailed procedures are reported in the Supplementary data.
**Construction of the *S. solfataricus* dpo-4 mutant**

The dpo-4 knockout mutant of *S. solfataricus* was constructed as described earlier (Worthington et al., 2003; Schelert et al., 2004). The detailed procedures are reported in the Supplementary data.

**Molecular biology and proteomic methods**

DNA cloning, DNA sequencing, PCR, and plasmid transformation of *Escherichia coli* were performed as described (Rockabrand et al., 1998; Rolfsmeier et al., 1998; Haseltine et al., 1999). DNA concentrations were measured using a DuNa Quant 200 fluorometer (Hoefler). Protein concentrations were measured using the BCA Protein Assay Reagent Kit (Pierce). Chemiluminescent western blot analysis using anti-Dpo4 polyclonal sera was performed using the ECL system (Amersham Biosciences) as described (Rockabrand et al., 1998). Proteins were fractionated by two-dimensional SDS-PAGE as described (Hajduch et al., 2005). Protein samples were prepared using 50 ml of mid-exponential phase cultures collected by centrifugation at 3000g for 15 min at 26 C. Pelleted cells were extracted as described (Hajduch et al., 2005) and protein was precipitated for 1 h by exposure to five volumes of ice-cold 0.1 M ammonium acetate in 100% methanol at 22 C. Proteins were sequenced using tandem mass spectrometry (MS/MS) and peptides were identified by local BLAST against the *S. solfataricus* proteome as described (Worthington et al., 2003). Confirmed hits required a threshold peptide matching value (N) of at least five.

**In vivo cisplatin assay**

Cell lines were cultured at 80°C with aeration in a minimal salts medium (Allen, 1959), at pH 3.0 with tryptone 0.2% (wt/vol) as sole carbon and energy source. Growth was monitored at a wavelength of 540 nm using a Cary 50 spectrophotometer (Varian).

Cisplatin was prepared as fresh aqueous solutions (2 mg/ml) before use and was added at early logarithmic phase.

**Coordinates**

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, http://www.rcsb.org, with accession codes 3M9M, 3M9N, and 3M9O for the structures G1, G2, and G3, respectively.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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