Interlocking activities of DNA polymerase β in the base excision repair pathway

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Base excision repair (BER) is a major cellular pathway for DNA damage repair. During BER, DNA polymerase β (Polβ) is hypothesized to first perform gap-filling DNA synthesis by its polymerase activity and then cleave a 5′-deoxyribosyl-5-phosphate (dRP) moiety by its dRP lyase activity. Through gel electrophoresis and kinetic analysis of partial BER reconstitution, we demonstrated that gap-filling DNA synthesis by the polymerase activity likely occurred after Schiff base formation but before β-elimination, the two chemical reactions catalyzed by the dRP lyase activity. The Schiff base formation and β-elimination intermediates were trapped by sodium borohydride reduction and identified by mass spectrometry and X-ray crystallography. Presteady-state kinetic analysis revealed that cross-linked Polβ (i.e., reduced Schiff base) exhibited a 17-fold higher polymerase efficiency than uncross-linked Polβ. Conventional and time-resolved X-ray crystallography of cross-linked Polβ visualized important intermediates for its dRP lyase and polymerase activities, leading to a modified chemical mechanism for the dRP lyase activity. The observed interlocking enzymatic activities of Polβ allow us to propose an altered mechanism for the BER pathway, at least under the conditions employed. Plausibly, the temporally coordinated activities at the two Polβ active sites may well be the reason why Polβ has both active sites embedded in a single polypeptide chain. This proposed pathway suggests a corrected facet of BER in DNA repair, and may enable alternative chemical strategies for therapeutic intervention, as Polβ dysfunction is a key element common to several disorders.

Significance

Base excision repair (BER) is one of the major DNA repair pathways used to fix a myriad of cellular DNA lesions. The enzymes involved in BER, including DNA polymerase β (Polβ), have been identified and characterized, but how they act together to efficiently perform BER has not been fully understood. Through gel electrophoresis, mass spectrometry, and kinetic analysis, we discovered that the two enzymatic activities of Polβ can be interlocked, rather than functioning independently from each other, when processing DNA intermediates formed in BER. The finding prompted us to hypothesize a modified BER pathway. Through conventional and time-resolved X-ray crystallography, we solved 11 high-resolution crystal structures of cross-linked Polβ complexes and proposed a detailed chemical mechanism for Polβ’s 5′-deoxyribosyl-5-phosphate lyase activity.

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Biochemical studies of the processing of dRP moieties in yeast cell-free extract (17), steady-state kinetic studies of fully reconstituted human BER (4), and investigation of the numbers of endogenous AP sites in genomic DNA of rats and human tissue (5) all suggest that dRP cleavage is the rate-limiting step of the entire BER pathway. However, there is no experimental evidence to indicate that all potential steps associated with dRP cleavage by the lyase activity of Polβ (Scheme 2) occur after gap-filling DNA synthesis catalyzed by the polymerase activity. For example, if facile Schiff base formation occurs before and faster than nucleotide incorporation, the covalently linked Polβ–DNA intermediate, rather than the noncovalent binary complex Polβ•DNA, may catalyze gap-filling DNA synthesis. This possibility has never been

Scheme 1. The BER pathway. (A) The BER pathway in the literature as cited in the Introduction. (B) Our proposed BER pathway.

Scheme 2. Proposed chemical mechanism for the dRP lyase activity of hPolβ. Specific water molecules are denoted as X, Y, and Z.
investigated, and all previously published in vitro studies have used DNA substrates like either DNA2 (SI Appendix, Fig. S2A, i) (18–24) or a gapped DNA substrate containing a dRP mimic (SI Appendix, Fig. S2 A, ii) (11, 25).

Here, we generated a natural dRP moiety by using either UDG to process a nicked DNA substrate containing a 2′-deoxyuridine or UDG and apurinic/apyrimidinic endonuclease 1 (APE1) to initiate BER on a double-stranded DNA substrate containing a 2′-deoxyuridine. Addition of hPolβ, correct deoxyinosine triphosphate (dNTP), and then, sodium borohydride (NaBH₄) to the dRP-containing DNA products allowed for the capture of a reduced Schiff base and a β-elimination intermediate produced via hPolβ-catalyzed dRP cleavage (Scheme 2). Through X-ray crystallographic, kinetic, and mass spectrometric (MS) analysis of these cross-linked hPolβ complexes, we envisioned a detailed chemical mechanism for the dRP lyase activity of hPolβ.

In addition, we utilized presteady-state kinetic methods to evaluate the impact of the reduced Schiff base intermediate on the efficiency and fidelity of gap-filling DNA synthesis by the polymerase activity of hPolβ. Finally, we employed time-resolved X-ray crystallography to structurally characterize intermediates of gap-filling DNA synthesis by cross-linked hPolβ. Based on several lines of experimental evidence, we proposed a modified BER pathway (Scheme 1B), which posits an interlocking mechanism in which gap-filling DNA synthesis by the polymerase activity occurs between Schiff base formation and β-elimination, the two steps catalyzed by the lyase activity.

**Results**

**Investigating the Order of Enzymatic Reactions Catalyzed by hPolβ during BER.** To investigate if both Schiff base formation and β-elimination associated with dRP cleavage by the lyase activity of Polβ occur after gap-filling DNA synthesis catalyzed by the polymerase activity of Polβ during BER, we treated a doubly [32P]-labeled 2′-deoxyguanine (dG) or a dRP mimic attached to hPolβ (SI Appendix, Fig. S4A) (18–24) or a gapped DNA substrate containing a dRP mimic (SI Appendix, Fig. S2A, iii), a smaller version of DNA1 dRP (Fig. S1), and then, estimated the 5′-[32P]–19-mer consumption rate (0.31 ± 0.05 s⁻¹) through the early reaction time points (Fig. 1 E, F, and H). Interestingly, the rates for 5′-[32P]–19-mer consumption and Schiff base formation are comparable, and this seems reasonable because the Schiff base was formed from 19-mer (see above). In comparison, dCTP at its in vivo concentration (100 μM) was incorporated at a rate of 0.8 ± 0.3 s⁻¹ (Fig. 1G). Thus, Schiff base formation (4.5 s⁻¹) was determined to be 5.6-fold faster than polymerase-catalyzed gap-filling DNA synthesis (0.8 s⁻¹), which was found to be 2.6-fold faster than β-elimination (0.31 s⁻¹). The rate order was consistent with the observed initial reaction rates of hPolβ (K27)–[32P]–dRP–18-mer (0.032 s⁻¹), [32P]–13-mer (0.064 s⁻¹), and hPolβ (K27)–[32P]–HPP (0.256 s⁻¹) (Fig. 1 B and C). Together, these results suggested that hPolβ catalyzes Schiff base formation prior to the gap-filling DNA synthesis followed by β-elimination during BER.

Furthermore, we investigated if the dRP lyase-catalyzed reactions were coupled with the polymerase-catalyzed dNTP incorporation during BER by performing the same assay in Fig. 1 but either in the presence of 1 μM dCTP or in the absence of any dNTPs (SI Appendix, Fig. S4). With 1 μM dCTP, the initial appearance times for hPolβ (K27)–[32P]–dRP–18-mer, [32P]–13-mer, and hPolβ (K27)–[32P]–HPP were 0.032, 0.064, and 0.256 s, respectively (SI Appendix, Fig. S4 H–J).
while the rates of Schiff base formation, gap-filling DNA synthesis, and β-elimination were estimated to be 4.9 ± 0.9, 0.35 ± 0.04, and 0.20 ± 0.05 s⁻¹, respectively (SI Appendix, Fig. S4 L–N). The 5'-[32P]-19-mer consumption rate was also estimated to be 3 ± 2 s⁻¹ (SI Appendix, Fig. S4K). All of those values are either the same or comparable with those corresponding values with 100 μM dCTP except the 2.3-fold-lower dCTP incorporation rate, which was due to the 100-fold-lower dCTP concentration. These data further suggest that the pattern of Schiff base formation prior to gap-filling DNA synthesis followed by β-elimination during BER was not altered by the change in dCTP concentration. Similarly, in the absence of dNTPs, the bands of hPolβ(K72)–[32P]–dRP–18-mer and hPolβ(K72)–[32P]–HPP initially appeared at 0.032 and 0.256 s, respectively (SI Appendix, Fig. S4 A–D), while Schiff base formation and β-elimination occurred at rates of 6 ± 1 and

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**Fig. 1.** Two hPolβ–DNA cross-linked intermediates formed during gap-filling DNA synthesis. DNA1N (10 nM) was processed by UDG to form DNA1dRP containing dRP–18-mer (A). Each circled “P” denotes a 32P-labeled phosphate, while the identical base sequences in DNA1dRP and DNA1dRP are shown in green. The newly formed 5'-[32P]-DNA1dRP was mixed with hPolβ (200 nM) and dCTP (100 μM) to form both cross-linked hPolβ–DNA complexes and 13-mer at different time intervals (Materials and Methods). After NaBH₄ reduction and trapping, equal volumes of the reaction mixtures were loaded and analyzed by both urea-based PAGE (B) and SDS-PAGE (C). In B, lanes C1, C2, and C3 denote 5'-[32P]-DNA1N after the NaBH₄ reduction treatment, 5'-[32P]-DNA1N after being treated with UDG and then NaBH₄ reduction, and 5'-[32P]-DNA1N after being treated with hPolβ and then NaBH₄ reduction, respectively. In C, free probes represent [32P]-labeled species (19-mer, reduced dRP–18-mer, 13-mer, 12-mer, and HPP). The percentages of the band intensities of [32P]-19-mer (green), hPolβ(K72)–[32P]–dRP–18-mer (salmon), [32P]–13-mer (gold), and hPolβ(K72)–[32P]–HPP (blue) relative to the intensities of their corresponding most-intense bands were individually plotted against time in a semilog fashion (D). The concentrations of [32P]-19-mer, total Schiff base formation products (hPolβ(K72)–[32P]–dRP–18-mer and hPolβ(K72)–[32P]–HPP), [32P]–13-mer, and hPolβ(K72)–[32P]–HPP are plotted against time from their early reaction time points in E–H, respectively. The plots were fit to [product] = Aexp(−kobs) to obtain the observed 19-mer consumption rate kobs (4 ± 1 s⁻¹; E), or [product] = A(1 − exp(−kobs) t) to yield the observed rate kobs for gap-filling DNA synthesis (0.8 ± 0.3 s⁻¹; G), Schiff base formation (4.5 ± 0.4 s⁻¹; F), or β-elimination (0.31 ± 0.05 s⁻¹; H). A indicates the reaction amplitude.
0.49 ± 0.04 s⁻¹, respectively (SI Appendix, Fig. S4 F and G). In the meantime, the upstream primer 12-mer was not elongated at all (SI Appendix, Fig. S4B), while the downstream primer 19-mer was consumed at a rate of 4.5 ± 0.8 s⁻¹ (SI Appendix, Fig. S4E). Considering that these kinetic data were comparable with those corresponding values obtained with either 100 or 1 μM dCTP (SI Appendix, Fig. S4O), we suggest that the dRP lyase activity of hPolβ acts similarly in the presence or absence of a correct dNTP and is thus independent of the polymerase activity. However, the polymerase activity may be impacted by rapid and preceding Schiff base formation catalyzed by the dRP lyase activity. This possibility was kinetically investigated.

Nucleotide Incorporation Efficiency and Fidelity with the Cross-Linked hPolβ (K72)–DNAΔRPR Complex. While the kinetic and structural mechanisms of DNA synthesis catalyzed by uncross-linked hPolβ have been extensively studied with model DNA substrates like DNAΔ (SI Appendix, Fig. S2 A, i) (24, 28–31), the effect of a natural dRP (SI Appendix, Fig. S2 A, iii) and a Schiff base intermediate (step 1 in Scheme 2) on the kinetics of nucleotide incorporation and the polymerase active site structure has yet to be investigated. To fill this void, we performed another partial BER reconstitution by treating a 2-deoxouridine-containing double-stranded DNA substrate with UDQ, APE1, and hPolβ followed by quenching and NaBH₄ reduction (additional methods are in SI Appendix). After purification, the cross-linked and reduced hPolβ–DNAΔRPR complex was used to determine the kinetic effect of the Schiff base intermediate (Scheme 2) on gap-filling DNA synthesis. For comparison, we performed presteady-state kinetic assays to determine kinetic parameters for correct or incorrect dNTP incorporation onto either 5’-[32P]–DNAΔRPR (SI Appendix, Fig. S2 A, iii) cross-linked to hPolβ (K72) or 5’-[32P]–DNAΔ (SI Appendix, Fig. S2 A, i) uncross-linked to hPolβ (Table 1 and SI Appendix, Fig. S5). Notably, DNAΔRPR, a shorter version of DNAΔRPR (Fig. 1A), has the identical base sequences as DNAΔ. The kinetic results of correct dCTP incorporation onto the hPolβ–[32P]–DNAΔRPR and unlabeled hPolβ–[32P]–DNAΔRPR were further confirmed through assays with α-[32P]–dCTP and unlabeled hPolβ–DNAΔRPR (SI Appendix, Fig. S5 I and R). The maximal correct dCTP incorporation rate (kₚ) for hPolβ–DNAΔRPR (0.72 s⁻¹) was expected to be close to the above estimated rate of 0.8 s⁻¹ (Fig. 1G) under the saturating dCTP concentration (100 μM vs. Kᵣ of 0.38 μM in Table 1) but was fourfold lower than the kᵣ with uncross-linked hPolβ–DNAΔ (2.9 s⁻¹), suggesting that the cross-link slowed dCTP incorporation. Although maximal misincorporation rates varied for the cross-linked and uncross-linked hPolβ complexes, misincorporation was uniformly slower than correct incorporation (Table 1). Interestingly, the apparent binding affinity (1/Kᵣ) for correct dCTP was increased by 68-fold for cross-linked (Kᵣ = 0.38 μM) relative to uncross-linked hPolβ (Kᵣ = 26 μM). Similarly, the cross-link also increased the affinity for incorrect dNTPs by three- to ninefold (Table 1). To confirm the tight binding affinity of correct dCTP, we performed microscale thermophoresis assays (32) with cross-linked hPolβ–DNAΔRPR and increasing concentrations of dCTP, which yielded a comparable Kᵣ value of 0.5 ± 0.2 μM for dCTP binding (SI Appendix, Fig. S5S). Strikingly, the polymerase efficiency (kᵣ/Kᵣ) of dCTP incorporation was increased by 17-fold due to the cross-link (Table 1). Importantly, the 17-fold enhancement in the gap-filling DNA synthesis efficiency for hPolβ–DNAΔRPR relative to hPolβ–DNAΔ resulted in an insignificant change of polymerase fidelity (10⁻⁵ to 10⁻⁶) (Table 1). Together, these kinetic data indicated that preceding Schiff base formation significantly enhances the gap-filling polymerase activity of hPolβ in the system that we employed.

Binary Crystal Structures of hPolβ Cross-Linked with DNA via a Natural dRP Moiety. To gain structural insight into the dRP cleavage mechanism, the above cross-linked and reduced hPolβ–DNAΔRPR complex was crystallized, and two structures, (hPolβ–DNAΔRPR)₁ and (hPolβ–DNAΔRPR)₂, were solved at 1.39- and 1.84-A resolution, respectively, through molecular replacement (Fig. 2 and SI Appendix, Fig. S1 A and B and Table S1). Notably, both hPolβ–DNAΔRPR structures display clear electron density for the reduced dRP in the ring-opened form and cross-linked to K72 (Fig. 2 A and B). These structures firmly corroborate the Schiff base formation product hPolβ (K72)–dRP–18-mer as inferred from the above MS/MS analysis. Both (hPolβ–DNAΔRPR)₁ and (hPolβ–DNAΔRPR)₂ superimposed well with each other (rmsd of 0.40 Å) (SI Appendix, Fig. S1E) and with the uncross-linked hPolβ–DNAΔ (rmsds of 0.63 and 0.73 Å, respectively) (16) and hPolβ–DNAΔHFF (rmsds of 0.67 and 0.74 Å, respectively) (11) structures, confirming that the cross-link did not adversely affect the overall protein structure (SI Appendix, Fig. S1E). Interestingly, (hPolβ–DNAΔRPR)₁ and (hPolβ–DNAΔHFF) exhibit different binding conformations of the cross-linked and reduced dRP moiety (Fig. 2C). This suggested that the dRP remains mobile in the dRP lyase active site despite being covalently attached to hPolβ. In contrast, the uncross-linked hPolβ–DNAΔHFF showed that the dRP-mimic moiety (SI Appendix, Fig. S2 A, ii) is bound at a single nonproductive docking site within the dRP lyase active site that would require ~120° rotation about the C5'-O-5'-phosphate bond for Schiff base formation (SI Appendix, Fig. S2C) (11).

The dRP lyase active site of (hPolβ–DNAΔRPR)₁ was superimposed well with those of uncross-linked hPolβ–DNAΔ and hPolβ–DNAΔHFF (SI Appendix, Fig. S2D). For example, the side chain of K35 was observed in the same position in all structures and likely functions to anchor the downstream primer to the active site through its interaction with the 3'-phosphate covalently connected to the dRP in the downstream primer (SI Appendix, Fig. S2 C–F). K72 was also observed in similar

Table 1. Kinetic parameters for gap-filling DNA synthesis catalyzed by cross-linked or uncross-linked hPolβ at 25°C

| Nucleotide | kᵣ (s⁻¹) | Kᵣ (μM) | kᵣ/Kᵣ (μM·s⁻¹) | Fidelity* |
|------------|----------|---------|-----------------|-----------|
| Cross-linked hPolβ–DNAΔRPR complex (DNAΔRPR, see SI Appendix, Fig. S2 A, iii) | | | | |
| dCTP       | 0.72 ± 0.05 | 0.38 ± 0.08 | 1.9 | — |
| dATP       | (4.5 ± 0.2) × 10⁻³ | 56 ± 10 | 8.0 × 10⁻⁵ | 4.2 × 10⁻⁵ |
| dGTP       | (5.7 ± 0.1) × 10⁻⁴ | 166 ± 12 | 3.4 × 10⁻⁶ | 1.8 × 10⁻⁶ |
| dTTP       | 0.020 ± 0.001 | 169 ± 34 | 1.2 × 10⁻⁴ | 6.3 × 10⁻⁵ |
| Uncross-linked hPolβ and DNAΔ (SI Appendix, Fig. S2 A, i) | | | | |
| dCTP       | 2.9 ± 0.2 | 26 ± 4 | 0.11 | — |
| dATP       | (1.0 ± 0.2) × 10⁻³ | 510 ± 267 | 2.0 × 10⁻⁶ | 1.8 × 10⁻⁵ |
| dGTP       | (3.7 ± 0.6) × 10⁻³ | 494 ± 171 | 7.5 × 10⁻⁶ | 6.8 × 10⁻⁵ |
| dTTP       | (1.0 ± 0.1) × 10⁻³ | 859 ± 274 | 1.2 × 10⁻⁶ | 1.1 × 10⁻⁵ |

*Defined as (kᵣ/Kᵣ)incorrect × (Kᵣ/Kᵣ)incorrect + (kᵣ/Kᵣ)correct.

dATP, 2'-deoxyadenosine 5'-triphosphate; dGTP, 2'-deoxyguanosine 5'-triphosphate; dTTP, 2'-deoxythymidine 5'-triphosphate.
positions among the binary structures, suggesting that dRP repositioning, rather than a protein conformational change, placed the dRP moiety for cleavage in the dRP lyase active site (SI Appendix, Fig. S2D). Interestingly, the K84 residue of the (hPolβ–DNA\textsubscript{dRP})\textsubscript{A} and (hPolβ–DNA\textsubscript{dRH})\textsubscript{B} structures exhibited different rotameric configurations with K84 of (hPolβ–DNA\textsubscript{dRH})\textsubscript{B} (Fig. 2F) adopting a similar position as in hPolβ DNA\textsubscript{A} and hPolβDNA\textsubscript{ATP} (SI Appendix, Fig. S2D). The alternative conformation of K84 in the (hPolβ–DNA\textsubscript{dRP})\textsubscript{A} structure was accompanied by a slight repositioning (1.5 Å) of the 5'-phosphate of the dRP relative to the dRP lyase active site (Fig. 2). Moreover, in both structures, K84 interacted with the 5'-phosphate of the dRP through charge–charge interactions and likely acts to capture and stabilize the dRP after it rotates into the dRP lyase active site. (Fig. 2 C and F).

Precatalytic Ternary Structures of hPolβ Cross-Linked with DNA in the Presence of a Correct Nucleotide and Noncatalytic Divalent Metal Ions. To provide a structural basis for significantly higher dCTP binding affinity and incorporation efficiency with cross-linked over uncross-linked hPolβ (Table 1), we crystallized and determined two ternary structures of hPolβ–DNA\textsubscript{dRP}•dCTP ([hPolβ–DNA\textsubscript{dRP}•dNTP]) and (hPolβ–DNA\textsubscript{dRH}•dNTP]) in the presence of noncatalytic Ca\textsuperscript{2+}, rather than catalytic Mg\textsuperscript{2+} (SI Appendix, Fig. S1 C and D and Table S1). Both (hPolβ–DNA\textsubscript{dRP}•dNTP)\textsubscript{1} and (hPolβ–DNA\textsubscript{dRH}•dNTP)\textsubscript{2} superimpose well with the uncross-linked ternary structure of hPolβ DNA\textsubscript{A}•dCTP with Ca\textsuperscript{2+} (18), with rmsd values of 0.806 and 0.763 Å, respectively (SI Appendix, Fig. S1F). Relative to the hPolβ–DNA\textsubscript{dRP} binary structures, both (hPolβ–DNA\textsubscript{dRP}•dNTP)\textsubscript{1} and (hPolβ–DNA\textsubscript{dRH}•dNTP)\textsubscript{2} contained a closed protein conformation with the typical thumb subdomain closure (SI Appendix, Fig. S1G) induced by nucleotide binding (33). Notably, the hPolβ conformational closure did not significantly alter the dRP lyase domain structure (SI Appendix, Fig. S1G) as the cross-linked dRP moiety has well defined electron density (Fig. 2 D and E) and was observed in a similar position as within the hPolβ–DNA\textsubscript{dRP} binary structures, except for the relatively free movement of the 5'-phosphate of the dRP moiety (Fig. 2F). For instance, water molecule Y that bridges E71 and the C2' atom of the dRP was in a nearly identical position in the binary and ternary structures (Fig. 2F). However, K84 in the hPolβ–DNA\textsubscript{dRP}•dCTP structures aligns well with the same residue in (hPolβ–DNA\textsubscript{dRP})\textsubscript{B}, not in (hPolβ–DNA\textsubscript{dRH})\textsubscript{A} (Fig. 2F).

Comparison of the polymerase active site in the hPolβ–DNA\textsubscript{A}•dCTP structures and in hPolβDNA\textsubscript{A}•dCTP revealed minor positioning changes of active site residues, dCTP, and metal ions (Fig. 3 A–D). For example, several hydrogen bonds between the side chains of active site residues and the bound dCTP were reduced by 0.1 to 0.3 Å, while the hydrogen bonds in the nascent base pair were shortened by 0.3 to 0.5 Å due to dCTP movement toward the templating dG in the hPolβ–DNA\textsubscript{dRP}•dCTP structures (Fig. 3 A–C). Notably, the two 3'-terminal nucleotides of the upstream primer in (hPolβ–DNA\textsubscript{dRP}•dNTP)\textsubscript{1} displayed C1'-exo rather than C3'-endo sugar puckering as observed for the nucleotides in the analogous positions in (hPolβ–DNA\textsubscript{dRP}•dNTP)\textsubscript{2} and hPolβDNA\textsubscript{A}•dCTP (16, 18, 20, 23). Such an altered sugar puckering in

Fig. 2. hPolβ dRP lyase active site comparison. (A and B) dRP lyase active sites of the (hPolβ–DNA\textsubscript{dRP})\textsubscript{A} (A; gray) and (hPolβ–DNA\textsubscript{dRH})\textsubscript{B} (B; orange) complexes. (C) Superposition of (hPolβ–DNA\textsubscript{dRP})\textsubscript{A} (gray) and (hPolβ–DNA\textsubscript{dRH})\textsubscript{B} (orange). Water molecules are depicted as spheres in the respective colors of the overlapped complexes. (D and E) dRP lyase active sites of the (hPolβ–DNA\textsubscript{dRP}•dNTP)\textsubscript{1} (D; blue) and (hPolβ–DNA\textsubscript{dRH}•dNTP)\textsubscript{2} (E; yellow) complexes. In A, B, D, and E, water molecules are depicted as red spheres. (F) Overlay of dRP lyase active sites in (hPolβ–DNA\textsubscript{dRP})\textsubscript{A} (green), (hPolβ–DNA\textsubscript{dRH})\textsubscript{B} (red), (hPolβ–DNA\textsubscript{dRH}•dNTP)\textsubscript{1} (blue), and (hPolβ–DNA\textsubscript{dRH}•dNTP)\textsubscript{2} (yellow) for 20 s (magenta), 30 s (cyan), 40 s (orange), 60 s (wheat), 20 min (gray), and 60 min (black) of Ca\textsuperscript{2+} to Mg\textsuperscript{2+} exchange and 60 s of Ca\textsuperscript{2+} to Mn\textsuperscript{2+} exchange (pink). Water molecules X, Y, and Z are shown as spheres colored as in their corresponding structures. Electron densities Fo–Fc, omit maps (green; 3σ), and 2Fo–Fc maps are shown as red and blue mesh, respectively.
Fig. 3. Comparison of the hPolβ polymerase active site in various structures. (A–C) Interactions between polymerase active site residues and dCTP are shown for (A) hPolβ–dNTP (green; Protein Data Bank [PDB] ID code 4KLD), (B) hPolβ–dNTP (blue), and (C) hPolβ–dNTP (yellow). (D) Superposition of the zoomed polymerase active sites in hPolβ–dNTP (green), hPolβ–dNTP (blue), and hPolβ–dNTP (yellow). Water molecules are shown as red spheres. Calcium and sodium ions are displayed as yellow and purple spheres, respectively. (E) Zoomed views of the upstream primer 3'-OH, dCTP, selected active site residues, and metal ions in hPolβ–dNTP (green; PDB ID code 4KLD), hPolβ–dNTP (blue), and hPolβ–dNTP (yellow). The electron density (purple; 5σ) depicts the Fo–Fc omit maps for each Mg2+ ion. The water molecules are displayed as red spheres. The Mg2+ ions are shown as green spheres. (F) Zoomed active site showing the primer 3'-terminal deoxycytidine (dC), the incorporated dC, pyrophosphate, aspartate residues, and divalent metal ions after 60 s of Ca2+ to Mg2+ exchange in crystallo. The metal ion binding sites A, B, and C are designated using a, b, and c, respectively. The electron density (purple; 5σ) depicts the Fo–Fc omit maps for each Mg2+ ion. The water molecules are displayed as red spheres. The Mg2+ ions are shown as green spheres. (G) Overlap of the polymerase active sites in the 40 s (purple) and 60 s (yellow) of Ca2+ to Mg2+ exchange structures. Each Mg2+ is shown as a green sphere. Mg2+ at the C site moved by at least 0.6 Å. Important interactions in all panels are shown as dashed lines with distances in angstroms.
Postcatalytic Binary Structures of hPolβ Cross-Linked with a Nicked DNA Product. After 60 s of Ca\(^{2+}\) to Mg\(^{2+}\) exchange, the in crystallo reaction was 100% complete, although hPolβ was still in the closed conformation with pyrophosphate bound (Fig. 4A, f). To examine if the cross-linked hPolβ can open its conformation after phosphodiester bond formation, in order to release pyrophosphate, we performed the Ca\(^{2+}\) to Mg\(^{2+}\) exchange for 20 and 60 min, and their crystal structures were also solved with resolutions of 2.90 and 2.96 Å, respectively (Fig. 4A, g and h and SI Appendix, Table S1). Interestingly, the cross-linked hPolβ, like uncross-linked hPolβ (18, 20, 23), opened its protein conformation as indicated by the movement of helix N away from the newly incorporated dCMP, the repositioning of active site residues (Y271, F272, D192, D190, and D256), and release of both pyrophosphate and three Mg\(^{2+}\) ions (Fig. 4A, g and h). Surprisingly, the incorporated dCMP in both the 20- and 60-min structures had poor electron density and projected freely rather than base paired with the templating nucleotide dG. The dynamic motion of the incorporated dCMP suggests that the cross-linked hPolβ–nicked DNA product complex is not suitable for further DNA synthesis, therefore requiring β-elimination to occur to complete dRP cleavage and finish the role of hPolβ in BER.

Discussion

Defining a Modified DNA BER Pathway. Previous biochemical, biological, and partial and full in vitro reconstitution studies have determined the sequential reactions catalyzed by four enzymes during BER (4, 5, 17) but assumed that all catalytic actions by the dRP lyase activity of Polβ occur after gap-filling DNA synthesis catalyzed by its polymerase activity (Scheme L4) (4, 12). To investigate whether the two enzymatic activities of hPolβ actually are coupled during BER, the nicked DNA substrate DNA\(^{1}\)-N\(^{2}\)-5′-dG \(\rightarrow\) 3′-OH-PNAdRP (Fig. 1E), and their crystal structure was also solved. In the resulting 2.21-Å structure (SI Appendix, Fig. S6G), the F6\(-\)E7 electron density maps clearly show 100% product formation, two hexacoordinated Mn\(^{2+}\) at the A and B sites, and one Mn\(^{2+}\) at the C site (Fig. 4A, i and SI Appendix, Fig. S6H). The C-site Mn\(^{2+}\) is coordinated by three water molecules and nonbridging oxygen atoms of the newly formed phosphodiester bond and pyrophosphate (SI Appendix, Fig. S6H). Additionally, overlaying the 60 s of Ca\(^{2+}\) to Mg\(^{2+}\) exchange and the 60 s of Ca\(^{2+}\) to Mn\(^{2+}\) exchange product-state structures shows that the three divalent metal ions at each site were nearly superimposable (SI Appendix, Fig. S6I).

(hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_1\) caused the upstream primer 3′-OH to flip away from the α-phosphate (P\(_α\)) of dCTP and increased the distance between them by 1.7 Å (Fig. 3E). Interestingly, the flipped 3′-OH cannot serve as a coordination ligand, resulting in Na\(^+\) bound at the divalent metal ion binding A site in (hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_1\), rather than Ca\(^{2+}\) as in the (hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_2\) and hPolβ–DNA\(^{eDCTP}\) structures (Fig. 3E). The A site in (hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_2\) also lacks a coordinating water molecule, which is present in both (hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_1\) and hPolβ–DNA\(^{eDCTP}\) (Fig. 3E). As in hPolβ–DNA\(^{eDCTP}\), the divalent metal ion binding B site was occupied by Ca\(^{2+}\) in both (hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_1\) and (hPolβ–DNA\(^{AR\&R}\)–dNTP)\(_2\) (Fig. 3E).

Snapshots of Nucleotide Incorporation by the Cross-Linked hPolβ–DNA\(^{AR\&R}\) Complex. To investigate if cross-linked hPolβ, like uncross-linked hPolβ (18, 20, 23), can incorporate a correct dNTP in crystallo, we performed Ca\(^{2+}\) to Mg\(^{2+}\) exchange for 20, 30, 40, and 60 s before flash freezing the crystals in liquid nitrogen (SI Appendix). The crystals diffracted to 1.91 to 2.20 Å for the dCTP incorporation intermediates (SI Appendix, Table S1), and the structures were solved by molecular replacement (Fig. 4). For 20, 30, and 40 s of Ca\(^{2+}\) to Mg\(^{2+}\) exchange (corresponding to 30, 50, and 70% of dCTP incorporation, respectively), partial occupancies of the reactants (the upstream primer 3′-nucleotide and dCTP) and products (pyrophosphate and 2′-deoxyctydine-5′-monophosphate [dCMP]) were modeled to account for the simultaneous electron density gain caused by phosphodiester bond formation between the primer 3′-OH and the P\(_α\) of dCTP and loss due to bond breakage between the P\(_α\) and β-phosphate of the dCTP (Fig. 4A, c–e). These features along with the steric inversion of the P\(_α\) geometry are consistent with an S\(_\beta\)2 reaction (18, 23, 34). Overall, the time-resolved reaction-state structures are almost superimposable, contain a closed protein conformation, and possess similar dRP lyase and polymerase active sites (Fig. 4 and SI Appendix, Fig. S6 A, C, and E). Strikingly, in addition to the two canonical hexacoordinated Mg\(^{2+}\) ions at the A site and B site observed in the precatalytic ternary structures (Fig. 3E), a third Mg\(^{2+}\) at the C site was captured in later reaction stages with an occupancy of 0.7 and 1.0 in structures with 70 and 100% product formation and the appearance of the C-site Mg\(^{2+}\) was captured in later reaction stages with an occupancy of 0.7 and 1.0 in structures with 70 and 100% product formation, respectively (Figs. 3F and 4A, e and f). This is similar to previously reported time-resolved structures for dNTP incorporation onto DNA by uncross-linked hPolβ, where ongoing product formation and the appearance of the C-site Mg\(^{2+}\) were concurrent events (18, 20–23, 35). From 70 to 100% dCTP incorporation, the C-site Mg\(^{2+}\), coordinated by water molecules and the nonbridging oxygen atoms of P\(_α\) and P\(_β\) of dCTP (or nonbridging oxygen atoms of the newly formed phosphodiester bond and pyrophosphate) (Fig. 3F), moved 0.6 Å, while the Mg\(^{2+}\) ions at the A and B sites did not change their positions (Fig. 3G).

To provide additional evidence for the presence of the C-site metal ion, the precatalytic crystals of hPolβ–DNA\(^{AR\&R}\)–dCTP with Ca\(^{2+}\) were soaked with Mn\(^{2+}\) for 60 s (SI Appendix, Like Mg\(^{2+}\), Mn\(^{2+}\) supported dCTP incorporation by hPolβ–DNA\(^{AR\&R}\) (SI Appendix, Fig. S6G). In the resulting 2.21-Å structure (SI Appendix, Table S1), the F6\(-\)F7 electron density maps clearly show 100% product formation, two hexacoordinated Mn\(^{2+}\) at the A and B sites, and one Mn\(^{2+}\) at the C site (Fig. 4A, i and SI Appendix, Fig. S6H). The C-site Mn\(^{2+}\) is coordinated by three water molecules and nonbridging oxygen atoms of the newly formed phosphodiester bond and pyrophosphate (SI Appendix, Fig. S6H). Additionally, overlaying the 60 s of Ca\(^{2+}\) to Mg\(^{2+}\) exchange and the 60 s of Ca\(^{2+}\) to Mn\(^{2+}\) exchange product-state structures shows that the three divalent metal ions at each site were nearly superimposable (SI Appendix, Fig. S6I).

Materials and Methods. We used the Na\(_2\)B\(_4\)H\(_4\) reduction and trapping approach, which has been previously employed for biochemical and structural characterization of DNA glycosylases (36, 37). Notably, APE1 was not included in our first partial BER reconstitution assay (Materials and Methods) because it, like Polβ, can cross-link with a natural abasic site to form a similarly sized protein–DNA cross-linked product (38, 39) and complicate our investigation. As expected, hPolβ incorporated dCTP and extended the upstream primer 12-mer into 13-mer in a time-dependent manner (Fig. 1B). In the meantime, the downstream primer 19-mer was gradually consumed, leading to the formation of two hPolβ–DNA cross-linked intermediates (Fig. 1B–D). Our MS/MS analysis (SI Appendix, Fig. S3) and 11 solved crystal structures (Fig. 4B) collectively and consistently identified the cross-linked intermediates as hPolβ (K72)–dRP–18-mer and hPolβ (K72)–HPP. Notably, this is the first time that K72 was structurally identified as the primary nucleophile for Schiff base formation, and the unstable β-elimination intermediate was isolated and identified. Based on the initial appearance times for hPolβ (K72)–dRP–18-mer (0.032 s), 13-mer (0.064 s), and hPolβ (K72)–HPP (0.256 s) in the gels (Fig. 1B and C) and their initial formation rates of 4.5, 0.8, and 0.31 s\(^{-1}\), respectively, in the presence of 100 μM dCTP (SI Appendix, Fig. S4O), we propose that Schiff base formation with K72 of hPolβ occurred first followed by gap-filling DNA synthesis and finally, cleavage of the dRP moiety through β-elimination during BER. The reaction order of hPolβ was not affected by dCTP concentration considering that the same initial product formation times in the gels and comparable k\(_{obs}\) values were determined under 1 and 100 μM dCTP (SI Appendix, Fig. S4O). Taken together, we propose a

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modified BER pathway by incorporating the order of the reactions catalyzed by hPolβ (Scheme 1B). The interlocking sequence of the two enzymatic activities of hPolβ in the modified BER pathway is further supported by the fact that correct dCTP was incorporated by the polymerase activity with a 17-fold higher efficiency with cross-linked (1.9 μM⁻¹ s⁻¹) than uncross-linked (0.11 μM⁻¹ s⁻¹) hPolβ (Table 1). The enhanced polymerase efficiency and similarly high fidelity (10⁻⁵ to 10⁻⁶) (Table 1) indicate that it is advantageous to have gap-filling DNA synthesis occurring after Schiff base formation during BER. The modified reaction order is also consistent with the presence of two enzymatic activities within a single polypeptide chain of Polβ, which enables the interlocking mechanism to function optimally. If the reaction order was reversed, the uncross-linked DNA repairing intermediate
would likely dissociate from hPolβ based on a fast DNA dissociation rate of 2.8 s⁻¹ (40) and be subsequently damaged by cellular nucleases, leading to genomic instability. Furthermore, the analysis of the full reconstitution of human BER has shown that dRP cleavage by hPolβ is slower than gap-filling DNA synthesis and is actually rate limiting for the entire BER pathway (4). Based on our estimated rates of Schiff base formation (4.5 s⁻¹) and β-elimination (0.31 s⁻¹), we conclude that β-elimination limits overall dRP cleavage by hPolβ and thus, BER. Structurally, the 5'-phosphate of the cross-linked dRP moiety shifted its bound position within the dRP lyase active site over time (SI Appendix, Fig. S6 E and F). In addition, the relatively large distance (6.2 Å) between the C2' atom and the side chain of E71 (SI Appendix, Fig. S2F) would require E71 to abstract the C2' proton via the adjacent water molecule Y (Fig. 4B), rather than through direct extraction (see the discussion below). Taking into consideration of the dRP positioning dynamics following Schiff base formation, the requirement for water-mediated C2' proton abstraction, and the breakage of a strong C-O bond, it is reasonable to conclude that β-elimination has a relatively large energy barrier and is a slow step. In contrast, Schiff base formation is known to be rapid. For example, Schiff base formation in the catalytic cycles of Thermoplasma acidophilum transaldolase and fructose-6-phosphate aldolase occurs with rates of 50 to 70 s⁻¹ at 30°C (41). In comparison, Schiff base formation catalyzed by hPolβ is slower and still about 20-fold faster than β-elimination in the presence or absence of gap-filling DNA synthesis (SI Appendix, Fig. S4O). Interestingly, the dRP lyase and polymerase activities of hPolβ can be functionally uncoupled in vitro since one of them was active, while the other was not (SI Appendix, Figs. S4 and S5). Although the polymerase activity of hPolβ was 17-fold more efficient when it was interlocked with the dRP lyase activity (see above), the latter was insignificantly affected by the former as the dRP lyase displayed comparable activities in the presence or absence of dCTP incorporation (SI Appendix, Fig. S4O). We acknowledge that our experiments were carried out under specific experimental conditions and cannot exclude results carried out under all possible conditions.

Revising the dRP Lyase Chemical Mechanism. While the ring-closed dRP is more populated at equilibrium compared with the more reactive ring-opened aldehyde form (Scheme 2) (42, 43), it has been hypothesized that either K35 or K72 could facilitate ring opening through protonation of the O4' atom (SI Appendix, Fig. S2 E and F) of the dRP (11). However, K35-mediated dRP ring opening seems unlikely due to the large distance (6.8 Å) between K35 and the O4' atom in both (hPolβ–DNAÅRR+iNTP) and (hPolβ–DNAÅRR+iNTP) (SI Appendix, Fig. S2 E and F), the structures of the cross-linked intermediate prepared from our second partial BER reconstitution (SI Appendix). In contrast, K72, protonated under physiological pH, catalyzed the dRP ring opening, resulting in a deprotonated K72 primed for nucleophilic attack on the aldehyde form of the dRP to yield a Schiff base. Schiff base formation will, in turn, shift the equilibrium from the ring-closed to the ring-opened dRP (44). Moreover, the proximity of Y39 to K72 (3.9 to 4.2 Å) (Fig. 4B) in the hPolβ–DNAÅRR+iNTP structures suggests that Y39 may stabilize the deprotonated form of K72 to a hydrogen bond. In addition, water molecule X (Fig. 4B) is bound near K72 and should facilitate proton transfer during Schiff base formation. These arguments in combination with the reduced Schiff base seen in the structures of (hPolβ–DNAÅRR+iNTP) and (hPolβ–DNAÅRR+iNTP) lead to steps 1 and 2 in the proposed dRP lyase chemical mechanism (Scheme 2).

Following Schiff base formation, the C2' proton (SI Appendix, Fig. S2 E and F) must be abstracted for β-elimination and release of the dRP cleavage product. Two basic residues in the dRP lyase active site, E26 and E71, have been proposed to perform this abstraction (45). Based on the model of the dRP mimic (SI Appendix, Fig. S2 A, ii) rotated into the dRP lyase active site of hPolβ–DNAÅRR+iNTP (SI Appendix, Fig. S2C), E26 was suggested to be the residue that abstracts the C2' proton through a bridging water molecule (11). However, both E26 and E71 in all of our cross-linked hPolβ structures (Figs. 2F and 4B) are far too removed from the C2' atom to abstract the proton. In addition, E26 does not form any water-mediated contact with C2' and therefore, likely does not participate in C2' proton abstraction. On the other hand, water molecule Y is observed bridging E71 and the C2' atom in all of the cross-linked hPolβ structures (Fig. 4B). Thus, we hypothesize that E71 catalyzes the β-elimination reaction through a water-assisted C2' proton abstraction (step 3 in Scheme 2). Interestingly, there is a water molecule (Z) bound near the 3'-phosphate covalently connected to the dRP moiety (Fig. 4B). In this position, water molecule Z can provide a proton and facilitate the β-elimination reaction (step 4 in Scheme 2). Lastly, water molecule Y deprotonated by E71 serves as the nucleophile to hydrolyze and release K72 of hPolβ and HPP (steps 5 and 6 in Scheme 2). Based on these 11 structures of cross-linked hPolβ (Fig. 4B), we proposed a detailed chemical mechanism for the dRP lyase activity of hPolβ, including the unexpected water-mediated β-elimination (Scheme 2).

Three Divalent Metal Ions Bound at the Polymerase Active Site of Cross-Linked hPolβ during Catalysis. The pre- and postcatalytic binary structures of cross-linked hPolβ do not possess divalent metal ions bound at the polymerase active site (SI Appendix, Fig. S6f). In contrast, there are two divalent metal ions at the A and B sites in the precatatytic ternary structure (hPolβ–DNAÅRR+iNTP) and three divalent metal ions at the A, B, and C sites in the late reaction-state structures (Fig. 4 A, e, f, and i). Unlike (hPolβ–DNAÅRR+iNTP)2, the precatatytic ternary structure (hPolβ–DNAÅRR+iNTP)3 has Na⁺ bound at the A site and Ca²⁺ at the B site (Fig. 4 A, a). The modeling of the A-site metal ion as Na⁺ is also reminiscent of postcatatytic structures, wherein the coordination ligand 3'-OH is lost and the Mg²⁺ is replaced with Na⁺ (18, 23). In the (hPolβ–DNAÅRR+iNTP) structure, the upstream primer terminus 3'-OH is facing away and far (5.4 Å) from the Pa of dCTP, and therefore, it is considered to be a nonproductive conformation; however, in (hPolβ–DNAÅRR+iNTP)3, the 3'-OH is pointing close to 3.7 Å of the Pa of dCTP, and the ternary structure is considered to be the productive conformation (Fig. 3E). When both Ca²⁺ ions at the A and B sites were replaced by Mg²⁺, the phosphodiester bond formation is initiated and accompanied by the appearance of Mg²⁺ at the C site (Fig. 4 A, i). Interestingly, only the C-site metal ion is dynamic based on its repositioning by at least 0.6 Å during phosphodiester bond formation (Fig. 3G). The dynamic nature of the C-site Mg²⁺ has also been observed with uncross-linked hPolβ during catalysis (23). As proposed previously (23, 34, 46, 47), the simultaneous appearance of the C-site Mg²⁺ with phosphodiester bond formation may imply that the C-site Mg²⁺ neutralizes the negative charge developed in the transition state and thereby, facilitates phosphodiester bond formation. The C-site Mg²⁺ may also act to stabilize the product state in order to prevent pyrophosphorylation (48, 49). Regardless, cross-linked hPolβ, as uncross-linked DNA polymerases (18, 20–23, 34, 35, 47), follows the “three–metal ion mechanism” rather than the “two–metal ion mechanism” (50) for catalysis.

Effect of the Cross-Link between hPolβ and DNA on Nucleotide Binding and Incorporation Kinetics. Although the cross-link decreased k_p of correct dCTP by 4-fold, it lowered its K_p by 68-fold, leading to a 17-fold higher gap-filling DNA synthesis efficiency (Table 1). Relative to uncross-linked hPolβ, the cross-link has a larger kinetic effect on K_p over k_p (Table 1). Structurally, the cross-link stabilizes the templating dG by anchoring the entire DNA
substrate, leading to both stronger stacking of dCTP with the 3’-terminal nucleotide of the upstream primer and the shortened hydrogen bonds within the nascent base pair by 0.3 to 0.5 Å in the hPolβ–DNA1dRP–dCTP structures (Fig. 3 A–C). Additionally, several hydrogen bonds between polymerase active site residues and dCTP were also shortened by 0.1 to 0.3 Å in the hPolβ–DNA1dRP–dCTP structures relative to the uncross-linked hPolβ–DNA3–dNTP structure. Together, these factors structurally contribute to the tighter binding of correct dCTP to cross-linked over uncross-linked hPolβ. However, the higher active site stability in hPolβ–DNA1dRP–dNTP over hPolβ–DNA3–dNTP may slow down the rate-limiting protein conformational change step in a general kinetic mechanism for DNA polymerases (51, 52), leading to slower dNTP incorporation by the cross-linked hPolβ.

In summary, we utilized two partial BER reconstitution assays, X-ray crystallography, and presteady-state kinetics to thoroughly investigate the effect of the cross-link between hPolβ and DNA via Schiff base formation on the polymerase and dRP lyase activities. Transient imino complexes between hPolβ and DNA containing a natural 5’-dRP were captured by NaNb4 reduction and DNA1dRP–dNTP, which slowly isomerized to (hPolβ–DNA1dRP–dNTP)2, the overall rate of the gap-filling DNA synthesis by cross-linked hPolβ would be decreased. Together, the two aforementioned kinetic factors linked to the two to the four-fold-lower  κ value with respect to the cross-linked over uncross-linked hPolβ.

Materials and Methods

The First Partial BER Reconstitution Assay. The purified 12-mer (5’-GTGCT-GATG GCCG-3’) and 19-mer (5’-UGTGCA GCCGGCTTGGTC-3’; U: 2’-deoxyuridine) were individually 5 radiolabeled for 30 min at 25°C by Optilinkase in the presence of [γ-32P]-adenosine triphosphate. A nicked DNA substrate (DNA1) was formed by annealing the unlabeled template 31-mer (5’-GGACCA-CACGGCTTGGTCGCGGCGATGAC-3’) with the [32P]-labeled upstream primer 12-mer and the downstream 19-mer. The doubly [32P]-labeled DNA12 (10 nM) was treated with UDG (40 nM) for 30 min prior to reacting with hPolβ (200 nM) in the presence of dCTP (0, 1, or 100 μM) in the reaction buffer (50 mM tris(hydroxymethyl)ammonium methanethiol [Tris] HCl, pH 7.8, 5 mM MgCl2, 50 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, 0.1 mg/mL of bovine serum albumin) at 25°C. Notably, DNA12 was processed by UDG to generate DNA1dRP containing a freshly produced abasic site. The reaction between DNA1dRP and hPolβ with dCTP was carried out in a rapid chemical quench apparatus RQF-3 (Kintek Corp.) and quenched after various times by 0.3 M EDTA. The quenched reaction mixtures were collected in microcentrifuge tubes containing NaBH4 (100 mM), which reduced and trapped cross-linked hPolβ–DNA–dNTP complexes. The reaction mixtures, in identical volumes, were loaded and analyzed via both a urea-based polyacrylamide gel electrophoresis (PAGE) sequencing gel and an SDS-PAGE gel to resolve DNA polymerization and the cross-linked hPolβ–DNA products, respectively. The [32P]-labeled products were quantified using a Typhoon TRIO (GE Healthcare) and ImageQuant (Molecular Dynamics). The plots of each product or the remaining 19-mer concentrations vs. early reaction times were fit to different equations to obtain corresponding rate constants (SI Appendix).

Identification of Cross-Linked hPolβ–DNA Products by Using Liquid Chromatography with Tandem Mass Spectrometry. The 0.256- and 20-s reduced reaction mixtures were separated via SDS-PAGE, and the hPolβ–DNA gel bands were sliced. The sliced gel pieces were washed with buffer (50% acetonitrile [ACN] in 2 mg/mL NH4HCO3) thrice. The washed gel slices were dehydrated by adding 100% ACN and resuspended in 30 μL of 50 mM NH4HCO3. Tris(2-carboxyethyl)phosphine (5 mM) was added for reduction, and the mixture was incubated at 55°C for 20 min. The remaining reducing agent was removed, and the gel slices were alkylated by adding 10 mM iodoacetamide and incubated in the dark at room temperature for 20 min. The alkylated gel slices were dehydrated by adding 100% ACN. One microgram of trypsin in 30 μL of 50 mM NH4HCO3 and additional 50 μL of 0.1% formic acid was added and incubated at 600 rpm and 37°C for 3 h. Digested peptides were eluted from the gel slices by vigorous vortexing in 50% ACN. Eluate was freeze dried and resuspended in 20 μL of 0.1% formic acid for LC-MS/MS (liquid chromatography with tandem mass spectrometry) analysis.

For the LC-MS/MS analysis, a Thermo Q Exactive HF (high-resolution electrospray tandem mass spectrometer) was used in conjunction with the Dionex Ultimate 3000 RSLCNano trap (1 μL sample volume) with a nanoLC column (Thermo μ-Precision 5 mm with nanoViper tubing 30-μm inner dimension × 10 cm). The flow rate was 300 nL/min for separation on an analytical column (Acclaim PepMap RSLC 75 μm × 15 cm nanoviper), Mobile phase A was composed of 99.9% H2O (EMD Omni Solvent) and 0.1% formic acid, while mobile phase B had 99.9% ACN and 0.1% formic acid. A 90-min linear gradient from 3 to 45% B was performed. During the chromatographic separation, the Q Exactive HF was operated in a data-dependent mode and under direct control of the Thermo Excalibur 3.1.66. The MS data were acquired using the following parameters: 20 data-dependent collision-induced dissociation MS/ MS scans per full scan (350 to 1,700 m/z) at 60,000 resolution. MS2 (fragment ion spectra from a second stage of mass spectrometry in tandem mass spectrometry) was acquired in centroid mode at 15,000 resolution. Ions with single charge or charges more than seven as well as unassigned charge were excluded. A 15-s dynamic exclusion window was used.

For data analysis, resultant raw files were searched against the protein sequence of hPolβ by using the Mascot search engine (SwissProt database; restricted to Homo sapiens) in error-tolerant mode to identify the peptides and the modifications. No decoy database was used as that is incompatible with the Error-Tolerant search.

Data Availability. Atomic coordinates and structure factors for the reported crystal structures have been deposited in Protein Data Bank (ID codes 7RBE, 7RBF, 7RGG, 7RBB, 7RBI, 7R8B, 7R8K, 7RBL, 7RBM, 7RBN, and 7RBO). All other data are included in the manuscript and/or SI Appendix.

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