Incorporation and Replication of 8-Oxo-deoxyguanosine by the Human Mitochondrial DNA Polymerase*

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To assess the role of oxidative stress on the replication of mitochondrial DNA, we examined the kinetics of incorporation of 8-oxo-7,8-dihydroguanosine (8-oxodG) triphosphate catalyzed by the human mitochondrial DNA polymerase. Using transient state kinetic methods, we quantified the kinetics of incorporation, excision, and extension beyond a base pair containing 8-oxodG. The 8-oxodGTP was incorporated opposite dC in the template with a specificity constant of 0.005 μM⁻¹ s⁻¹, a value ~10,000-fold lower than that for dGTP. Once incorporated, 96% of the time 8-oxodGMP was extended by continued polymerization rather than being excised by the proofreading exonuclease. The specificity constant for incorporation of 8-oxodGTP opposite a template dA was 0.2 μM⁻¹ s⁻¹, a value 13-fold higher than incorporation opposite a template dC. The 8-oxodG:dA mispair was extended rather than excised at least 70% of the time. Examination of the kinetics of polymerization with 8-oxodG in the template strand also revealed relatively low fidelity in that dCTP would be incorporated only 90% of the time.

Although many theories exist regarding the underlying molecular mechanisms behind aging in mammals, it is clear that mitochondrial integrity plays a major role (1–3). According to the free radical theory of aging, electrons from the electron transport chain are able to reduce molecular oxygen to form superoxide anions (O2⁻) during aerobic respiration. These reactive radicals go on to produce other reactive oxygen species (ROS)² (3). ROS can be generated at a few cellular sites, but in healthy tissues the majority are a result of aerobic metabolism, and consequently, they are always present during normal cellular activity. ROS attack a variety of different cellular macromolecules, including proteins, lipids, and DNA. However, damage to mitochondrial DNA (mtDNA) has been implicated as important in regard to aging, especially in postmitotic cells, such as neurons (4). A cycle is created in the mitochondria in which a continued state of oxidative stress leads to further damage to electron transport chain components, ultimately causing an energy decline, carcinogenesis, and many age-related diseases (5).

One of the most common products of oxidative DNA damage is 8-oxo-7,8-dihydroguanosine (8-oxodG), which is reported to be highly mutagenic and is commonly used as biomarker for oxidative stress. Basal levels of 8-oxodG in mtDNA between different species correlate negatively with longevity in many mammals and birds, which is not the case with nuclear DNA (6). Moreover, because of the proximity of mtDNA to the electron transport chain, the levels of oxidative damage are significantly higher than in the nucleus in all tissues of mammals and birds examined (7).

The repair of 8-oxodG in the mitochondria is carried out by the base excision repair pathway, and its efficiency can be evaluated by 8-oxodG glycosylase activity, which increases over the life span of rodents but does not seem to stop the accumulation of 8-oxodG during aging (8, 9). In addition to the direct damage of mtDNA by ROS, free nucleotides can be damaged and may compete with undamaged nucleotides during replication to increase the rate of mutation (for review, see Ref. 10). Humans encode a triphosphatase termed MutT homolog-1 that is able to alleviate the mutagenic effects of 8-oxodGTP by hydrolyzing it to 8-oxodGMP, thus reducing the occurrence of A to C transversions (11). This enzyme has been shown to be present in the cytosol and the mitochondria (12, 13), although in general, the mitochondrial DNA damage repair systems are relatively simple and limited compared with those in the nucleus (14).

The concern surrounding 8-oxodG stems from its mutagenic coding potential when copied by an assortment of mammalian and prokaryotic polymerases (10, 15–18). For all studies done with DNA polymerases, dCMP and dAMP were...
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FIGURE 1. Base-pairing conformations of 8-oxodG. Shown on the left is a normal Watson-Crick hydrogen bonding arrangement between 8-oxodGMP and dCMP in which both 8-oxodG and dCMP are in the anti conformation. Shown on the right is the Hoogsteen base pair of 8-oxodG and dAMP in which 8-oxodG is rotated to the syn conformation. The was adapted from Krahn et al. (20).

inserted across from 8-oxodGMP with comparable but varying efficiencies, which potentially results in a G to T transversion mutation. The molecular mechanism for the lack of specificity afforded by 8-oxodGMP is thought to be due to its ability to form either a normal Watson-Crick hydrogen bonding arrangement with dCMP or a Hoogsteen base pair with dAMP by flipping to the syn conformation as depicted in Fig. 1 (19, 20).

Arguably, the most important polymerase in regard to the replication of oxidatively damaged DNA is the human mitochondrial polymerase γ (Pol γ), which is solely responsible for the propagation of the mitochondrial genome. Mice expressing a homozygous knock-in mutant of a proofreading-deficient version of Pol γ prematurely developed numerous signs of aging (21). A more recent study using a similar mouse model also showed that the integrity of the mitochondrial genome was important in the development of premature signs of aging. However, this study found no increase in the levels of ROS as the amount of mtDNA mutations increased (22). Interestingly, a different approach aimed at directly assessing the role of ROS on aging in another transgenic mouse model was undertaken in which human catalase was expressed and localized to the mitochondria and shown to extend maximum life span (23).

Taken together, the available information pertaining to the molecular mechanisms of aging seems to implicate the integrity of the mitochondrial genome as an important factor. Specifically, it appears that the accumulation of mutations in the mtDNA appears to have a deleterious effect on metabolism and maximum life span. In addition, the toxic side effects of nucleoside analogs used to treat viral infections, although due primarily to inhibition of mitochondrial DNA replication (24), are compounded by the resulting, accumulative oxidative stress (25). We, therefore, sought to clearly define the role of 8-oxodG in mitochondrial DNA replication by means of transient state kinetic methods using recombinant human Pol γ holoenzyme and defined oligonucleotide primer/template combinations. Most strikingly, our results show that the free damaged nucleotide is incorporated efficiently opposite dA, and the polymerase efficiently extends the primer to bury the 8-oxodG:dA mismatch. Once in the template strand, 8-oxodG is a potent mutagen.

MATERIALS AND METHODS

Nucleotides and Oligonucleotides—The nucleotide, 8-oxodGTP, was purchased from TriLink BioTechnologies (San Diego, CA), and unmodified dNTPs were purchased from Sigma-Aldrich. Oligonucleotides containing 8-oxodGMP (except for a primer 3′-terminated with 8-oxodGMP) were purchased from The Midland Certified Reagent Co. (Midland, TX). All other oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Pol γ Expression and Purification—Overexpression and purification of recombinant human Pol γ were previously described (26, 27). Holoenzyme was reconstituted at a 1:5 ratio of catalytic subunit to accessory subunit. Forward polymerization studies were conducted using an exonuclease-deficient mutant (E200A).

Preparation of DNA—Studies were carried out using synthetic oligonucleotide primers and templates. The primers varied in length from 24 to 27 nucleotides, whereas the template was always 45 bases long. The general 25-mer primer sequence was 5′-GCCTGCGACGGCCTCAACCAACTCA-3′, and the 45-mer template sequence was 5′-GGACGGCATTTGACGACXTAGTTGTTGACCGCTGCGAGCC-3′, where X is either a primer or template sequence given above detailed under “Results” and in the figures. For example, replacement of template base X is designated as 45C-mer (Fig. 4). The primers were 5′-32P labeled using T4 polynucleotide kinase, according to the manufacturer’s instructions (Invitrogen). The reaction was terminated by incubation at 95 °C for 5 min, and excess nucleotide was removed using a Bio-Spin 6 column (Bio-Rad). The primer was annealed to 45-mer template by combining at an equimolar ratio, heating to 95 °C, and slowly cooling to room temperature. Primers containing 3′-terminal 8-oxodGMP were enzymatically synthesized as follows. A single nucleotide incorporation reaction was performed using 1 μM Pol γ holoenzyme (E200A), 100 μM duplex DNA (containing dC as the template base opposite 26th primer position), and 150 μM 8-oxodGTP in 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl2. The reaction mixture was incubated at 37 °C for 30 min, and then the product was gel-purified to obtain the 8-oxodGMP-terminated primer. Primer was labeled with 32P on the 5′-end, and DNA duplex was annealed as described above.

Polymerization and Exonuclease Reaction Conditions—For reactions too fast to measure by manual mixing and quenching, a quench-flow apparatus (RFQ-3) from KinTek Corp. was used. All incorporation assays were performed at 37 °C in buffer containing 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 2.5 mM...
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\% \text{ Buried} = \frac{k_{\text{pol}}}{k_{\text{pol}} + k_{\text{exo}}} \times 100
\]  

(Eq. 5)

Simulation—The program KinTekSim (KinTek Corp.) was used for kinetic simulation of experimental data. The simulation was refined by an iterative process until a “best fit” was obtained according to the mechanism described under “Results.” The simulated curves were then exported from KinTekSim and plotted with the raw data in the program Grafit 5.

RESULTS

“Correct” Incorporation of 8-OxodGTP—To assess the physiological relevance of 8-oxodGTP on the overall fidelity of mitochondrial DNA replication, we inspected its incorporation by reconstituted exonuclease-deficient human Pol γ holoenzyme using a defined primer/template combination of 25 and 45 bases, respectively (see under “Materials and Methods”). The template was modified at the position across from the incoming nucleotide to be either dCMP or dAMP. The terminology used in this report will refer to 8-oxodGTP incorporation across from a template dCMP as correct and across from a template dAMP as “incorrect.” Only 8-oxodGTP was present during the incorporation reaction so that the primer would be extended by only one nucleotide to simplify data analysis and interpretation. The primer extension reactions were performed under single turnover conditions (enzyme > [DNA]) to circumvent the complications that arise from multiple turnovers with processive enzymes. A series of experiments was performed at various 8-oxodGTP concentrations to obtain the concentration dependence of the rate and amplitude of incorporation. For typical correct incorporation reactions under these conditions the concentration dependence of the rate of incorporation defines the ground state nucleotide dissociation constant or \( K_d \) and the maximum rate of incorporation or \( k_{\text{pol}} \). Additionally, because nucleotide binding rapidly comes to equilibrium (relative to the rate of incorporation) and is followed by a single rate-limiting step, the \( k_{\text{pol}} \) and \( K_d \) are equal to the \( k_{\text{cat}} \) and \( K_m \), respectively. As a consequence, the ratio, \( k_{\text{pol}}/K_d \) defines the specificity constant \( (k_{\text{cat}}/K_m) \) for incorporation.

In this context we refer to the theoretical values of \( k_{\text{cat}} \) and \( K_m \) that might be derived during processive DNA synthesis and not the artificially low and inaccurate values that are experimentally obtained in steady state measurements of single nucleotide incorporation, limited by the release of DNA from the enzyme. As discussed in the accompanying paper (30) and elsewhere (28, 31, 32), measurements of \( k_{\text{pol}} \) and \( K_d \) in rapid quench experiments provide the best estimate for nucleotide selectivity.

Incorporation reactions were initiated by rapidly mixing the preformed holoenzyme-DNA complex with 8-oxodGTP and Mg\(^{2+}\). Then, after various times the reactions were quenched with 0.5 M EDTA. The time dependence of the correct incorporation of 8-oxodGTP onto a template dCMP is shown in Fig. 2A. Unexpectedly, the extension reaction proceeded in a biphasic fashion, and therefore, the data were fit by nonlinear regression to a double exponential equation (Equation 2). The most obvious nucleotide concentration dependence of incorporation was the amplitude of the fast phase of the reaction and is shown.
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Figure 2. Incorporation of 8-oxodGTP onto dCMP. A, 100 nM exonuclease-deficient holoenzyme was preincubated with 90 nM 25/45-mer (G incorporation) DNA duplex and then rapidly mixed with Mg2+ and various concentrations of 8-oxodGTP (10 (▲), 25 (△), 50 (■), 100 (○), 200 (□), and 400 (○) μM). Each data set was fit by nonlinear regression to a double exponential equation (Equation 2) to obtain the rates and amplitudes of incorporation. B, the amplitudes of the fast phase were plotted as a function of 8-oxodGTP concentration. A fit of the data to a hyperbola (Equation 4) yields an apparent $K_d = 200 ± 30$ μM and an $A_{max}$ (maximum amplitude) = 62 ± 4 nM. C, the rate from the fast phase of each reaction was plotted as a function of 8-oxodGTP concentration to show that the rate of the initial fast phase is not clearly dependent upon the concentration of 8-oxodGTP. D, the rate from the slow phase of each reaction was plotted as a function of 8-oxodGTP concentration and fit to a hyperbola (Equation 3) to yield an apparent $K_d = 83 ± 7$ μM and a $k_{max} = 0.4 ± 0.02 s^{-1}$.

in Fig. 2B. The amplitude appeared to saturate in a hyperbolic fashion as a function of 8-oxodGTP concentration and was fit by nonlinear regression to a hyperbola (Equation 4) defining a maximum amplitude ($A_{max}$) of 62 ± 4 nM and an apparent $K_d$ of 200 ± 30 μM. A plot of the rate of incorporation for the same phase (Fig. 2C) reveals that there is no clear dependence on the concentration of nucleotide. If one simply looks at the concentration dependence of the fast phase of the reaction, it appears that, at ground state nucleotide binding is reversibly linked to bond formation between 8-oxodGMP and the primer strand. However, one further complication is illustrated in Fig. 2D, where the rate of the slow phase is plotted as a function of 8-oxodGTP concentration. It also saturates with high concentrations of 8-oxodGTP. The data were fit to a hyperbola (Equation 3) defining a maximum rate of 0.4 ± 0.02 s⁻¹ and an apparent $K_d$ of 83 ± 7 μM. These data suggest that the chemistry step is reversibly linked to nucleotide binding and that the release of pyrophosphate from the active site is relatively slow versus what is observed in the isomerization of the polymerase after primer extension. The possibility that the enzyme remains in a closed conformation after the chemistry step would explain the fact that the amplitude of the fast phase is dependent on the concentration of 8-oxodGTP in solution because the active site resides responsible for catalysis would still be in alignment to perform the reverse reaction. But once the enzyme undergoes a conformational change and releases pyrophosphate, the reaction

would precede to a common end point as a function of the rate of that isomerization step. To illustrate this we performed a kinetic simulation using the program KinTekSim (Fig. 3A) using a simple three-step mechanism (Fig. 3B). The curves that are superimposed with the raw data were generated by an iterative process using the kinetic parameters obtained by nonlinear regression as an initial guide. The final best-fit rate constants are shown with the mechanism used. An initial collision of 8-oxodGTP with the E-DNA complex fits best with a true $K_d$ of about 135 μM. Next, the chemistry step follows at a rate of about 2 s⁻¹ ($k_{forward}$) and is reversible at a rate of about 0.7 s⁻¹ ($k_{reverse}$). It is not known if a conformational change limits the rate of the chemistry step. After the chemistry step, the release of pyrophosphate (~0.4 s⁻¹) limits the net rate of incorporation. It is reasonable to suppose that a conformational change defines the rate of pyrophosphate dissociation, but we have no evidence to address this issue. Pyrophosphate release is essentially irreversible because of the relatively low concentration of

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The concentration of substrate 26-mer remaining versus time. The data were fit by nonlinear regression to a single exponential equation (Equation 1) to obtain a $k_{\text{exo}}$ of 0.075 ± 0.002 s$^{-1}$.

**Probability of Extension**—The probability of extension to bury a misincorporated or damaged base pair depends on the relative rate of removal versus the rate of incorporation of the next correct base pair. Therefore, we examined kinetic parameters for the incorporation of dTTP (next correct base pair after an 8-oxodG:dC base pair). The experiment was performed in a manner identical to that of 8-oxodGTP, and the results are shown in Fig. 5A. The time dependence of incorporation followed a single exponential rise, and the rates of incorporation saturated hyperbolically to define a $k_{\text{pol}}$ of 1.8 ± 0.1 s$^{-1}$ and a $K_d$ of 9.3 ± 1.0 μM. Comparing the $k_{\text{pol}}$ of 1.8 s$^{-1}$ to the $k_{\text{exo}}$ of 0.075 s$^{-1}$ for 8-oxodGMP predicts that the probability of burial is about 96% (Equation 5). However, if the physiological concentrations of dTTP in the mitochondria are as low as 1 μM, as has been estimated (33), then the probability of extension versus excision would be 70%. Although the physiological concentrations of dTTP in the mitochondria are not known with certainty, it appears that 8-oxodGMP, once incorporated, is most often buried rather than excised.
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**FIGURE 6. Incorporation of 8-oxodGTP onto dAMP.** A, exonuclease-deficient holoenzyme (100 nM) was preincubated with 90 nM 25/45CA-mer DNA duplex and then rapidly mixed with Mg²⁺ and various concentrations of 8-oxodGTP (1 (●), 5 (□), 10 (●), 20 (△), and 40 (▲) µM). Each data set was fit by nonlinear regression to a single exponential equation to obtain the rate of incorporation. B, the observed rates were plotted as a function of 8-oxodGTP concentration and fit to the hyperbola to yield a $K_d = 3.3 \pm 1.0 \mu M$ and a $k_{pol} = 0.62 \pm 0.05 \text{s}^{-1}$.

**Incorrect Incorporation of 8-OxodGTP**—Because of the possibility that the formation a Hoogstein base pair between an incoming 8-oxodGTP and a template dAMP might lead to relatively efficient incorporation, we inspected the kinetic parameters governing this process. Shown in Fig. 6A is the time dependence of primer extension performed at several concentrations of 8-oxodGTP. The data were best fit to a single exponential equation, and the rate of incorporation saturated in a hyperbolic fashion as a function of concentration (Fig. 6B) defining a $k_{pol}$ of 0.62 ± 0.05 s⁻¹ and a $K_d$ of 3.3 ± 1.0 µM to yield an overall specificity constant of 0.187 ± 0.0586 µM⁻¹ s⁻¹. To our surprise, 8-oxodGTP is a 12.5-fold better substrate for incorporation across from dAMP compared with dCMP. Because of this result, it was important to define the probability of burial of this mutagenic base pair by measuring the rate of removal and the kinetic parameters for the next correct incorporation (results not shown). All the results examining polymerization with regard to the incorporation of 8-oxodGTP (correct and incorrect) are shown in Fig. 7 as will be detailed under “Discussion.”

**Fidelity of Polymerization with 8-OxodGMP in the Template Strand**—Because no detailed studies have been reported for the fidelity of the replication of oxidatively damaged DNA using the human mitochondrial polymerase, we performed a series of single turnover experiments examining the incorporation and removal of nucleotides before, onto, and beyond an 8-oxodGMP in the template strand. In all cases the incorporation data were easily explained by a rapid equilibrium binding model, where the observed rate of polymerization saturated hyperbolically, defining the kinetic parameters for incorporation. The removal of each nucleotide was also inspected so that the probability of extension versus excision could be calculated as summarized in Fig. 8. The data for the two most probable replication routes are depicted as a side-by-side comparison and are referred to as correct and incorrect pathways. The correct pathway refers to the incorporation of dCTP onto a template 8-oxodGMP, and the incorrect pathway refers to dATP incorporation. Table 1 was constructed to compare our results for the incorporation of all four nucleotides onto a template 8-oxodGMP.

It is clear from the data summarized in Fig. 8 that once dCTP or dATP is incorporated opposite 8-oxodG, the polymerization reaction continues to “bury” either 8-oxodG:dNTP base pair. Although the specificity constants for the subsequent incorporations of dTTP and dGTP are reduced somewhat from those seen with a normal primer/template, the rates of excision by the 3’-5’ exonuclease are not sufficiently fast relative to the rates of extension to contribute to a fidelity. Thus, there is no proofreading of errors opposite 8-oxodG.

**DISCUSSION**

One of the most important results of this report is the significant preference for 8-oxodGTP incorporation onto a template dAMP rather than dCMP. To quantify the likelihood of misincorporation, the discrimination against 8-oxodGTP versus either dGTP or dTTP must be compared (Fig. 7). Discrimination is the ratio of the specificity constant ($k_{pol}/K_d$ or $k_{cat}/K_m$) for the correct incorporation over that of the misincorporation. The resulting dimensionless number represents the magnitude of the preference for the correct nucleotide. A discrimination of 10,000 against 8-oxodGTP with respect to the direct competition with dGTP is lower than for most misincorporation events. However, the overall frequency of incorporating 8-oxodG can be reduced further by lowering the concentration of 8-oxodGTP in the mitochondria relative to that of dGTP. The concentrations of 8-oxodGTP in the mitochondria are not known, but the high concentrations of dGTP may lead to significant rates of oxidation to form 8-oxodGTP (33). This points to an important role for the triphosphatase, MutT homolog-1, in reducing the levels of 8-oxodGTP and thereby reducing rates of incorporation.

This is the first instance in which the slow pyrophosphate product release rate was shown to reduce $k_{cat}/K_m$, defining the nucleotide specificity constant (Fig. 3B). By allowing for the reversal of chemistry, the slow product release rate effectively reduces $k_{cat}/K_m$ 3-fold according to the constants defined for 8-oxodG incorporation. Similarly, we have data showing that the chemistry step for AZT-triphosphate incorporation is
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**Overview of correct and incorrect incorporation of 8-oxodGTP kinetic parameters.** The kinetic parameters obtained for the incorporation of 8-oxo-dGTP onto either dCMP (Correct) or dAMP (Incorrect) are shown above. In addition, other kinetic parameters such as the rate of excision and the incorporation of the next correct nucleotide are illustrated to the side of the corresponding step in the pathway. Most notable is a comparison of the values for the discrimination against 8-oxo-dGTP incorporation across from either dCMP or dAMP, respectively. This comparison predicts that the probability of incorrect incorporation is about 15-fold greater than that of the correct incorporation. Furthermore, the slow excision of 8-oxo-dGMP irrespective of template base suggests it is stably incorporated rather than removed for greater than 90% of events. The maximum rate of incorporation, k_{pol}, was estimated from the rate of the fast phase of incorporation at the highest concentration of 8-oxo-dGTP used. The K_s was determined using the program KinTeKSim by global fitting to all the data for the incorporation of 8-oxo-dGTP onto dCMP simultaneously. A value of 3000 for the discrimination against 8-oxo-dGTP in the correct pathway was calculated as the ratio of correct dGTP incorporation determined previously (31) divided by the approximate specificity constant for 8-oxo-dGTP incorporation determined using the above values [(k_{pol}/K_s)_dGTP/(k_{pol}/K_s)_8-oxodGTP]. The percent buried was calculated using Equation 5. The discrimination against 8-oxo-dGTP onto dAMP was calculated as in the correct pathway except that the specificity constant of dATP incorporation was used (k_{pol}/K_s)_dATP/(k_{pol}/K_s)_8-oxodGTP).

FIGURE 7. Overview of correct and incorrect incorporation of 8-oxodGTP kinetic parameters. The kinetic parameters obtained for the incorporation of 8-oxo-dGTP onto either dCMP (Correct) or dAMP (Incorrect) are shown above. In addition, other kinetic parameters such as the rate of excision and the incorporation of the next correct nucleotide are illustrated to the side of the corresponding step in the pathway. Most notable is a comparison of the values for the discrimination against 8-oxo-dGTP incorporation across from either dCMP or dAMP, respectively. This comparison predicts that the probability of incorrect incorporation is about 15-fold greater than that of the correct incorporation. Furthermore, the slow excision of 8-oxo-dGMP irrespective of template base suggests it is stably incorporated rather than removed for greater than 90% of events. The maximum rate of incorporation, k_{pol}, was estimated from the rate of the fast phase of incorporation at the highest concentration of 8-oxo-dGTP used. The K_s was determined using the program KinTeKSim by global fitting to all the data for the incorporation of 8-oxo-dGTP onto dCMP simultaneously. A value of 3000 for the discrimination against 8-oxo-dGTP in the correct pathway was calculated as the ratio of correct dGTP incorporation determined previously (31) divided by the approximate specificity constant for 8-oxo-dGTP incorporation determined using the above values [(k_{pol}/K_s)_dGTP/(k_{pol}/K_s)_8-oxodGTP]. The percent buried was calculated using Equation 5. The discrimination against 8-oxo-dGTP onto dAMP was calculated as in the correct pathway except that the specificity constant of dATP incorporation was used (k_{pol}/K_s)_dATP/(k_{pol}/K_s)_8-oxodGTP).

**Overview of correct and incorrect pathways of polymerization with 8-oxo-dGMP present in the template strand.** Shown in the scheme are the kinetic parameters that were obtained for each step of DNA synthesis for the two most likely routes (dCTP or dATP incorporation onto 8-oxo-dGMP). Depicted at the top of the scheme is the correct incorporation of dATP one base before 8-oxo-dGMP in the template strand. The branch point occurs when the polymerase either correctly incorporates dCTP (left-hand pathway) or incorrectly incorporates dATP (right-hand pathway). There is about a 90% probability that dCTP is chosen. The rates of excision are also shown for each of the steps, which allows for the calculation for the percent buried (next to the corresponding step). With respect to the probability of forward polymerization directly across from 8-oxo-dGTP, the proofreading exonuclease function is essentially negligible. Determined using the ratio of the specificity constant for dCTP incorporation over that of dATP incorporation (k_{pol}/K_s)_dCTP/(k_{pol}/K_s)_8-oxodGTP), the percent buried was calculated using Equation 5. In Fig. 8 we summarize the kinetics of incorporation, excision, and extension with 8-oxo-dGMP in the template. The first step shown in the pathway is dATP incorporation preceding the 8-oxo-dGMP in the template. This step is minimally affected when compared with the incorporation onto an undamaged DNA substrate (40 ± 7 versus 57 ± 6 μM−1 s−1, respectively). The most affected incorporation event is the insertion of a nucleotide directly across

Reversible and pyrophosphate release is slow. This represents a novel mechanism by which a polymerase can increase discrimination against an undesirable substrate. Pyrophosphate release is normally considered to be fast to account for fast processive synthesis. Examination of the kinetics processive synthesis reveals that each incorporation reaction proceeds at a rate approximately equal to the rate observed in a single turnover (28, 34). Therefore, there is no significant delay after the incorporation of one nucleotide and preceding the binding of the next. Steps involving pyrophosphate release and translocation are not well defined but must be faster than the rate of incorporation measured in a single turnover.

In the case of 8-oxo-dG and AZT, slow pyrophosphate release allows for the direct reversal of the chemistry step and reduces k_{cat} for incorporation. It is interesting to speculate that the mitochondrial polymerase evolved this method to reduce incorporation of 8-oxo-dG and that AZT may react in a similar way.

The vast majority of incorporation events go unnoticed by the proofreading exonuclease in that the probability of forward polymerization was much faster than the rates of excision for each base pair involving 8-oxo-dG. With respect to continuing replication, there is a surprisingly small effect resulting from the presence of 8-oxo-dGMP in the primer or template strand. Processive polymerization experiments were performed in the presence of a DNA trap to inspect the possibility that Pol γ may dissociate during replication if a damaged base is nearby. Pol γ efficiently copied 20 nucleotides beyond the damaged DNA site (to the end of the template strand) regardless of whether 8-oxo-dGMP was in the primer or template as well as correctly or incorrectly base paired (data not shown).

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from 8-oxodG. The specificity constant for dCMP incorporation across from 8-oxodG is reduced by about 470-fold from 8-oxo:dG. The specificity constant for dCMP incorporation (dCTP onto 8-oxodGMP) divided by that of each of the dNTPs (\(k_{\text{pol}}/K_{d}\))dNTP).

### References

1. Loeb, L. A., Wallace, D. C., and Martin, G. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 18769–18770
2. Dufour, E., and Larsson, N. G. (2004) Biochim. Biophys. Acta 1658, 122–132
3. Shigenaga, M. K., Hagen, T. M., and Ames, B. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10771–10778
4. Barja, G. (2004) Trends Neurosci. 27, 595–600
5. Alexeyev, M. F., Ledoux, S. P., and Wilson, G. L. (2004) Clin. Sci. (Lond.) 107, 355–364
6. Gao, D., Wei, C., Chen, L., Huang, J., Yang, S., and Diehl, A. M. (2004) Am. J. Physiol. Gastrointest. Liver Physiol. 287, 1070–1077
7. Herrero, A., and Barja, G. (1999) Aging (Milano) 11, 294–300
8. Souza-Pinto, N. C., Hogue, B. A., and Bohr, V. A. (2001) Free Radic. Biol. Med. 30, 916–923
9. Stevnsner, T., Thorlsrud, T., Souza-Pinto, N. C., and Bohr, V. A. (2002) Exp. Gerontol. 37, 1189–1196
10. Kamiya, H. (2003) Nucleic Acids Res. 31, 517–531
11. Sakumi, K., Furuchi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., and Sekiguchi, M. (1993) J. Biol. Chem. 268, 23524–23530
12. Yoshimura, D., Sakumi, K., Ohno, M., Sakai, Y., Furuchi, M., Iwai, S., and Nakabeppu, Y. (2003) J. Biol. Chem. 278, 37965–37973
13. Kang, D., Nishida, J., Iyama, A., Nakabeppu, Y., Furuchi, M., Fujitawa, T., Sekiguchi, M., and Takeshige, K. (1995) J. Biol. Chem. 270, 14659–14665
14. Larsen, N. B., Rasmussen, M., and Rasmussen, L. J. (2005) Mitochondrion 5, 89–108
15. Einolf, H. J., Schnetz-Boutaud, N., and Guengerich, F. P. (1998) Biochemistry 37, 13300–13312
16. Einolf, H. J., and Guengerich, F. P. (2001) J. Biol. Chem. 276, 3764–3771
17. Pinz, K. G., Shibutani, S., and Bogenhagen, D. F. (1995) J. Biol. Chem. 270, 9202–9206
18. Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) Nature 349, 431–434
19. Gannett, P. M., and Sura, T. P. (1993) Chem. Res. Toxicol. 6, 690–700
20. Krahn, J. M., Beard, W. A., Miller, H., Grollman, A. P., and Wilson, S. H. (2003) Structure (Camb) 11, 121–127
21. Trifunovic, A., Wredenberg, A., Falkenberg, M., Spellbrink, J. N., Rovio, A. T., Bruder, C. E., Bohlooly, Y., Gidlof, S., Oldfors, A., Wibom, R., Tourneil, J., Jacobs, H. T., and Larsson, N. G. (2004) Nature 429, 417–423
22. Kujoth, G. C., Hiona, A., Pugh, T. D., Someya, S., Panzer, K., Wohlgemuth, S. E., Hofer, T., See, A. Y., Sullivan, R., Jobling, W. A., Morrow, J. D., Van Remmen, H., Sedivy, J. M., Yamasoba, T., Tanokura, M., Weindruch, R., Leeuwenburgh, C., and Prolla, T. A. (2005) Science 309, 481–484
23. Schriner, S. E., Linford, N. J., Martin, G. M., Treuting, P., Ogburn, C. E., Emond, M., Coskun, P. E., Ladiges, W., Wolf, N., Van Remmen, H., Wallace, D. C., and Rabinovitch, P. S. (2005) Science 308, 1909–1911
24. Lee, H., Hanes, J., and Johnson, K. A. (2003) Biochemistry 42, 14711–14719
25. Lewis, W., Copeland, W. C., and Day, B. J. (2001) Lab. Investig. 81, 777–790
26. Graves, S. W., Johnson, A. A., and Johnson, K. A. (1998) Biochemistry 37, 6050–6058
27. Johnson, A. A., Tsai, Y., Graves, S. W., and Johnson, K. A. (2000) Biochemistry 39, 1702–1708
28. Patel, S. S., Wong, I., and Johnson, K. A. (1991) Biochemistry 30, 511–525
29. Donlin, M. J., Patel, S. S., and Johnson, K. A. (1991) Biochemistry 30, 538–546
30. Lee, H. R., and Johnson, K. A. (2006) J. Biol. Chem. 281, 36236–36240
31. Johnson, A. A., and Johnson, K. A. (2001) J. Biol. Chem. 276, 38090–38096
32. Tsai, Y. C., and Johnson, K. A. (2006) Biochemistry 45, 9675–9687
33. Mathews, C. K. (2006) FEBS J. 270, 1300–1314
34. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) J. Biol. Chem. 267, 25988–25997