Fapy · dA Induces Nucleotide Misincorporation Translesionally by a DNA Polymerase**

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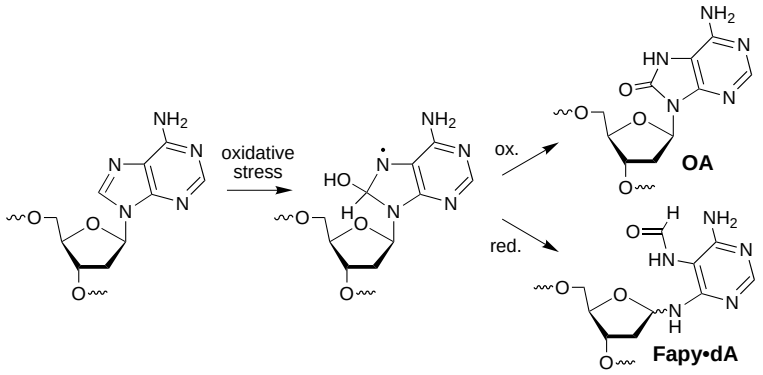
DNA is constantly exposed to endogenous and exogenous agents that modify its structure. DNA damage can give rise to mutations, which in turn can result in diseases such as cancer. Understanding the effects of individual lesions on the structure and function of DNA helps provide an understanding at the molecular level of the origin of genetically based diseases.[1] When deoxyadenosine in DNA is exposed to oxidative stress, the respective formamidopyrimidine (Fapy · dA) and 8-oxopurine (OA) lesions are produced from a common intermediate (Scheme 1).[2] Under O_2-deficient conditions, radiolysis of mammalian chromatin produces



Scheme 1. Formamidopyrimidine (Fapy · dA) and 8-oxopurine (OA) lesions produced from a common intermediate, upon exposure of deoxyadenosine in DNA to oxidative stress.

Fapy · dA in greater amounts than OA. The respective lesions derived from deoxyguanosine are formed in comparable relative yields.[2] A recent study on human tumor cells indicates that the purine lesions derived from deoxyguanosine are formed tenfold more readily than Fapy · dA and OA.[3] Very little is known about the mutagenicity of Fapy · dA, because biopolymers that contain Fapy · dA at defined sites were unavailable. Recently, we developed a method for the chemical synthesis of oligonucleotides that contain Fapy · dA and configurationally stable analogues at defined sites, and wish to provide the first report of the effect of these molecules on the fidelity of a DNA polymerase.[4]

Previous studies on the effects of Fapy · dA on polymerase activity relied on inferential evidence in which yields of randomly formed lesions were correlated with mutations that involved deoxyadenosine (dA).[5] Based on these studies, it was proposed that Fapy · dA induces misincorporation of dCTP opposite itself by adopting a *syn* conformation and thus presenting a deoxyguanosine (dG)-like hydrogen-bonding pattern. The N7-methylated analogue (MeFapy · dA) produced by methylation followed by hydrolytic ring opening cannot present a comparable dG-like hydrogen-bonding pattern, but was also proposed to direct the incorporation of dCTP. In contrast, in vitro and in vivo experiments on OA are unequivocal, thanks to the development of methods for synthesizing oligonucleotides that contain the lesion at defined sites.[6, 7] These studies revealed that dG was the most likely nucleotide to be incorporated opposite the lesion, other than thymidine (T).[7, 8] Structural studies suggested that the lesion forms a series of bifurcated hydrogen bonds to dG when it is present in the *syn* conformation.[9]

The kinetics for the insertion of the individual native nucleotides by Klenow exo$^-$ were measured opposite an equilibrium mixture of α- and β-Fapy · dA in a 30-mer template under single-hit conditions by using a standing start (Table 1).[10] For comparison, measurements were also made by using an otherwise identical template that contained dA instead of Fapy · dA. Incorporation (V_{max}/K_m) of thymidine (T) and deoxycytidine (dC) opposite Fapy · dA decreased almost fourfold and sixfold, respectively, relative to that observed when a template that contained dA was used. The latter observation is contrary to that anticipated based on conclusions reached in previous indirect studies of Fapy · dA.[5] In contrast, the frequency (F_{ins}) of misincorporation of dA and dG was almost 50 % greater when Fapy · dA replaced the native nucleotide in the template. The significance of these observations is magnified when the F_{ins} of dA and dG opposite Fapy · dA are compared to that of OA, which were reported previously.[7, 11] Misinsertion frequencies for the control templates that contain dA were remarkably similar in these independent experiments, despite differences in the local sequences of the templates, and utilization of Klenow in studies on OA. In contrast to the observations described with Fapy · dA (Table 1), the F_{ins} of dA, dC, and dG decreased when dA was

Table 1. Kinetic parameters of nucleotide insertion opposite Fapy · dA.

3'-d(T A C C C T G C A C G A C A X TCA CGT GCA ACT TGC)
5'-d(A T G G G A C G T G C T G T

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3'-d(T A C C C T G C A C G A C A X TCA CGT GCA ACT TGC)
5'-d(A T G G G A C G T G C T G T N

X	dNTP	K_m [μM]	V_{max} [% min^{-1}]	F_{ins}[a]	F_{rel}[b]
A	T	0.13	4.9	1.0	–
A	A	147.7	18.7	2.9×10^{-3}	–
A	G	231.1	9.9	9.9×10^{-4}	–
A	C	76.3	8.1	2.4×10^{-3}	–
Fapy · dA	T	0.34	3.8	0.28	1.0
Fapy · dA	A	86.7	14.7	3.9×10^{-3}	1.4×10^{-2}
Fapy · dA	G	16.3	1.4	1.5×10^{-3}	5.4×10^{-3}
Fapy · dA	C	75.2	1.0	4.3×10^{-4}	1.5×10^{-3}

[a] $F_{ins} = (V_{max}/K_m,\ X = A,\ dNTP = T)/(V_{max}/K_m,\ X = A$ or Fapy · dA, dNTP = T, A, G, or C). [b] $F_{rel} = (V_{max}/K_m,\ X =$ Fapy · dA, dNTP = T)/(V_{max}/K_m, X = Fapy · dA, dNTP = T, A, G, or C).

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[**] We are grateful for support of this research by the National Institutes of Health (CA-74954).

Supporting information for this article is available on the WWW under http://www.angewandte.com or from the author.

  1433-7851/02/4105-0771 $ 17.50+.50/0

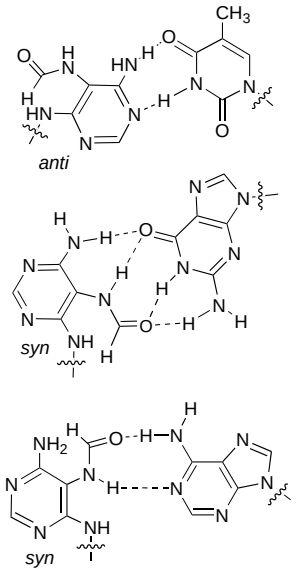
replaced in the template by OA.[7, 11] Comparing the F_{ins} for the independent experiments reveals that Fapy·dA was almost eight times more likely to induce the Klenow enzyme to incorporate dA and three times more likely to incorporate dG opposite itself than was OA. Furthermore, comparison of the K_m values (Table 1) indicate that the above preferences result from specific interactions between Fapy·dA and the incoming nucleotide. The implied preferences for Fapy·dA binding to dA and dG are supported by UV–melting studies on dodecamers (Table 2). Duplexes that contain Fapy·dA base paired to dA and dG are stabilized relative to the respective complexes that contain dA in the opposing strand.

Table 2. Comparison of thermodynamic properties of duplex melting between dA and Fapy·dA.

5'-d(T G C A C T X A C A G C)
3'-d(A C G T G A Y T G T C G)

X:Y	ΔG°_{298} [kcal mol^{-1}]	X:Y	ΔG°_{298} [kcal mol^{-1}]
A:T	14.61	Fapy·dA:T	12.97
A:A	10.40	Fapy·dA:A	11.46
A:G	12.60	Fapy·dA:G	13.01
A:C	10.54	Fapy·dA:C	9.82

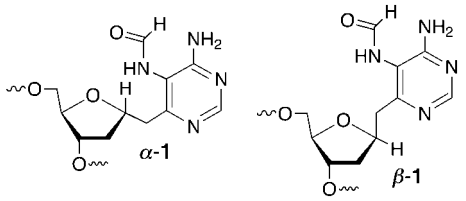
The preference exhibited by oligonucleotides that contain Fapy·dA to interact with dA and dG in kinetic and thermodynamic experiments can be rationalized by hydrogen-bonding patterns that involve the *syn* conformation of the β-isomer, which differ with respect to the orientation of the formamide group (Scheme 2). The thymidine-like hydrogen-bonding pattern relieves possible steric interactions between



Scheme 2. Oligonucleotides that contain Fapy·dA interact preferentially with dA and dG in kinetic and thermodynamic experiments. This can be rationalized by hydrogen-bonding patterns of the *syn* conformation of the β-isomer.

the carbonyl group and the glycoside nitrogen. Association between Fapy·dA and dG can be ascribed to a series of interactions similar to those characterized in a duplex that contains OA:dG base pairs.[9] There are more base-pairing schemes for Fapy·dA than for OA because of the greater degrees of freedom that can be attributed to the cleavage of the imidazole ring, as well as the existence of the lesion as an anomeric mixture.[12]

Oligonucleotides that contain single diastereomers of non-epimerizable analogues of Fapy·dA, α-**1**, β-**1** were prepared in order to probe the role of individual anomers in the above observations.[4] Molecular mechanics calculations with the



Cerius2 program indicate that replacement of the glycoside amine with a methylene group does not significantly alter the minimum energy structure (data not shown). The incorporation of thymidine opposite α-**1** and β-**1** is impaired approximately tenfold relative to templates that contain Fapy·dA (Table 3). However, internal comparisons of the frequency of insertion (F_{rel}) of the four native nucleotides opposite β-**1** reveal a remarkably similar pattern to that observed with the template that contains Fapy·dA (Table 3). In contrast, α-**1** exhibits a much higher preference (F_{rel}) for incorporating dA, dG, and especially dC relative to T. These data suggest that β-**1** is a more realistic mimic of Fapy·dA and that the glycoside nitrogen atom in the lesion is not involved in specific interactions.

In conclusion, experiments on oligonucleotides that contain Fapy·dA and configurationally stable analogues incorporated at defined sites enabled us to determine that this DNA lesion directs the misincorporation of dA translesionally. The conclusions drawn from these studies are different to those

Table 3. Kinetic parameters of nucleotide insertion opposite α-**1** and β-**1**.

3'-d(T G G T A C C C T G C A T G C T G X GTC TCG GCA ACT TGC GGA)
5'-d(A C C A T G G G A C G T A C G A C

Klenow exo -
dNTP

3'-d(T G G T A C C C T G C A T G C T G X GTC TCG GCA ACT TGC GGA)
5'-d(A C C A T G G G A C G T A C G A C N

X	dNTP	K_m [(μM]	V_{max} [% min^{-1}]	F_{ins}[a]	F_{rel}[b]
A	T	0.04	8.2	1.0	–
A	A	125.8	49.9	1.8×10^{-3}	–
A	G	58.8	11.6	1.0×10^{-3}	–
A	C	56.3	2.6	2.7×10^{-3}	–
β-**1**	T	0.90	5.5	2.7×10^{-2}	1.0
β-**1**	A	321.8	55.0	7.8×10^{-4}	2.9×10^{-2}
β-**1**	G	74.9	2.6	1.5×10^{-4}	5.6×10^{-3}
β-**1**	C	96.0	1.7	7.8×10^{-5}	2.9×10^{-3}
α-**1**	T	6.3	29.3	2.0×10^{-2}	1.0
α-**1**	A	83.5	17.1	9.1×10^{-4}	4.6×10^{-2}
α-**1**	G	39.9	2.7	3.7×10^{-4}	1.9×10^{-2}
α-**1**	C	29.6	21.9	3.3×10^{-3}	0.17

[a] $F_{ins} = (V_{max}/K_m, X = A, dNTP = T)/(V_{max}/K_m, X = A, \alpha$-**1**, or β-**1**, dNTP = T, A, G, or C). [b] $F_{rel} = (V_{max}/K_m, X = \alpha$-**1** or β-**1**, dNTP = T)/(V_{max}/K_m, X = α-**1** or β-**1**, dNTP = T, A, G, or C).

  1433-7851/02/4105-0772 $ 17.50+.50/0 *Angew. Chem. Int. Ed.* **2002**, *41*, No. 5

from previous experiments in which Fapy·dA was randomly produced in DNA.[5] Moreover, this investigation provides the first data that suggest that Fapy·dA may be a more potent premutagenic lesion than the chemically related OA. The significance of these in vitro experiments is magnified by the observation that Fapy·dA is produced in higher yield than OA.[2c]

Experimental Section

Enzyme Kinetics: The appropriate primer (25 pmol) was labeled at its 5′-terminus with ^{32}P. The labeled primer was annealed to the appropriate template (375 pmol) and unlabeled primer (225 pmol) in ST buffer (20 mM Tris, pH 7.5; 100 mM NaCl) for 5 min at 90 °C (60 °C for Fapy·dA and its control duplex), and slowly cooled to room temperature. The duplexes were purified from excess [γ-^{32}P]ATP by passing through a G-25 Sephadex spin column equilibrated with ST buffer. The final concentration of the duplex was 3.13 μM. Reactions were carried out by mixing a 2× duplex-enzyme cocktail (5 μL) to a 2× dNTP solution (5 μL). The reactions were run for a set period of time and quenched with 20 μL of 95% formamide loading buffer containing 20 mM EDTA. The samples were denatured (90 °C for 3 min and cooled immediately to 0 °C), subjected to 20% denaturing PAGE, and analyzed on a Storm 820 phosphorimager equipped with Imagequant version 3.3 software. Kinetic parameters were calculated by using a Hanes–Woolf plot. All values are the average of at least three independent experiments. The 2× duplex-enzyme cocktail consisted of duplex DNA (100 nM), Klenow exo$^-$ (20 nM), and BSA (0.2 μg μL^{-1}) in 2× Klenow buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 15 mM DTT). Klenow was pretreated with inorganic pyrophosphatase (0.2 U per unit of enzyme) at room temperature for 10 min before adding the duplex. The 2× duplex-enzyme cocktail was incubated for 5 min at room temperature. The 2× dNTP solutions were made by diluting 100 mM stock solutions with the appropriate volume of water and stored at 4 °C. The concentrations of dNTP and reaction times were chosen such that the total amount of extension did not exceed 20%.[10]

UV–Melting Experiments: Samples (total volume = 300 μL) contained a 1:1 ratio of complementary oligodeoxynucleotides. Duplex concentrations ranged from 1 to 10 μM. Solutions were prepared by the addition of appropriate volumes of stock solutions of the two complementary oligodeoxynucleotides to 150 μL of 2× PIPES (1,4-piperazine bis-(ethanesulfonate) sesquisodium salt) buffer (20 mM PIPES (pH 7.0), 20 mM $MgCl_2$, 200 mM NaCl), followed by dilution with distilled, deionized water to a volume of 300 μL. The complementary oligodeoxynucleotides were hybridized by heating the samples to 60 °C, followed by slow cooling over 2–3 h to room temperature. Melting studies were carried out in 1-cm pathlength quartz cells. Absorbance was monitored while the temperature was ramped at a rate of 0.5 °C min^{-1} from 25 to 75 °C. Melting temperatures were calculated by a computer fit of the first derivative of absorbance with respect to $1/T$.[13] All reported values are the average of at least four measurements. As a result of the lability of Fapy·dA to heat,[12] studies were carried out to ensure that significant decomposition (deglycosylation) did not occur during the repetitive hybridization and melting cycles required to obtain the above data. Following completion of UV–melting experiments 2 pmol of the recovered DNA (Fapy·dA and control containing dA) were radiolabeled by polynucleotide T4 kinase using [γ-^{32}P]ATP and precipitated to remove the residual nucleotide triphosphate by using standard methods. Labeled DNA (0.4 pmol) was resuspended in NaOH (0.1M, 10 μL) and heated at 55 °C for 20 min. The reaction was cooled in an ice bath and neutralized with aqueous HCl. The samples were taken up in 95% formamide loading buffer, and analyzed as described above. Decomposition was 5% relative to the control. **Supporting Information Available:** Direct comparison of kinetic experiments on Fapy·dA and OA.

Received: October 23, 2001 [Z18106]

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