On the mechanism of preferential incorporation of dAMP at abasic sites in translesional DNA synthesis. Role of proofreading activity of DNA polymerase and thermodynamic characterization of model template-primers containing an abasic site

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Received September 21, 1994; Revised and Accepted November 25, 1994

ABSTRACT
DNA polymerases preferentially incorporate dAMP opposite abasic sites (A-rule). The mechanism of the A-rule can be studied by analyzing three dissected stages of the reaction including (i) initial nucleotide insertion, (ii) proofreading excision of the inserted nucleotide and (iii) extension of the nascent primer terminus. To assess the role of the stage (ii) in the A-rule, kinetic parameters of the proofreading excision of primer terminus nucleotides opposite abasic sites were determined using E.coli DNA polymerase I Klenow fragment. The relative efficiency of the excision ($V_{max}/K_m$) revealed that removal of A was the least favored of the four nucleotides, but the differences in the efficiencies between excision of A and the other nucleotides was less than 2-fold. In addition, in an attempt to reconcile kinetic data associated with the stages (i) or (ii), the differences in free energy changes ($\Delta\Delta G^\circ$) for the formation of model template-primers containing XN pairs (X = abasic site, N = A, G, C or T) were determined by temperature dependent UV-melting measurements. The order of $\Delta\Delta G^\circ$ was $XG > XA = XC > XT$, with $\Delta\Delta G^\circ$ being 0.5 kcal/mol for the most stable $XG$ and the least stable $XT$. Based on these data, the role of the stage (ii) and energetic aspects of the A-rule are discussed.

INTRODUCTION
DNA is replicated by DNA polymerases with extremely high fidelity. The average error rate during replication is estimated as $10^{-9}$ per nucleotide incorporated (1,2). However, a number of exogenous and endogenous agents that generate DNA damages reduce the fidelity of DNA replication by altering the coding properties of the DNA template. Abasic sites are the most ubiquitous of these DNA lesions and it is estimated that 2000–10 000 abasic sites are produced per cell per day by spontaneous hydrolysis of the N-glycosidic bond (3). These lesions are also formed by chemical modification of bases by carcinogen or alkylating agents (4—6), and ionizing radiation (7). In addition, they also appear transiently as intermediates of base excision repair by DNA N-glycosylases (8).

It is well established that abasic sites remaining after DNA repair block DNA synthesis, constituting lethal lesions (9,10). Blocking DNA synthesis is not an exclusive consequence and a small fraction of DNA polymerases encountering the lesion continue synthesis past the lesion, resulting in translesional DNA synthesis. The translesional synthesis at abasic sites is error-prone and mutagenic since no coding information is available at this site. Although there are several exceptions in eukaryotic cells (11—14), it has been shown that during the translesional synthesis, dAMP is most frequently incorporated at abasic sites in vitro (15—20) and E.coli (21,22), which is referred as an A-rule (23). There are three possible mechanisms to account for the preferential incorporation of dAMP at abasic sites (23,24). These include (i) preferential insertion of A by DNA polymerases, (ii) less efficient removal of A than the other nucleotides by a 3'-5' exonuclease associated with DNA polymerases and (iii) preferential extension of a primer terminus containing A opposite abasic sites. The first mechanism has substantial supporting evidence (15—24). Although available data are limited, the third mechanism appears to be operating for human immunodeficiency virus (HIV)-1 reverse transcriptase, but not avian myeloblastosis virus (AMV) reverse transcriptase (24). However, to our knowledge, studies to elucidate the role of the second mechanism have not been reported so far.

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In this study, oligonucleotide primers with different 3'-terminal nucleotides were annealed to a template containing a model abasic site (X) to construct all four XN pairs at the primer terminus (N = A, G, C or T). Kinetic parameters for the proofreading excision of the terminal nucleotide N opposite the abasic site by a 3'-5' exonuclease were determined using *Escherichia coli* DNA polymerase I Klenow fragment (Pol I). To further delineate the mechanism of the A-rule, the difference in free energy changes ($\Delta\Delta G^\circ$) of model template-primers having different XN pairs at primer termini were determined by UV-melting measurements. Based on the enzymatic and thermodynamic data obtained, the role of proofreading excision in the A-rule and energetic aspects associated with primer termini at abasic sites are discussed.

**MATERIALS AND METHODS**

**Materials**

T4 polynucleotide kinase and *E.coli* DNA polymerase Klenow fragment (Pol I) were purchased from Toyobo. Nuclease P1 and alkaline phosphatase from USB. [γ-32P]ATP (110 TBq/mmol) was obtained from Amersham.

Oligodeoxyribonucleotides with or without a modification for templates, primers, and those with partially self complementary sequences for UV-melting measurements were synthesized by standard solid-phase phosphoramidite chemistry on a Milligen/Biosearch Cyclone Plus DNA synthesizer. The phosphoramidite monomer of a model abasic site (1,4-anhydro-2-deoxy-α-ribofuranose) was synthesized following the reported method (25) except that the protection of phosphate was achieved by a 3'-cyanoethyl group. Characterization of the monomer has been reported previously (26). The synthesized oligonucleotides were purified to homogeneity by HPLC as described (27). The nucleotide sequence and absorption coefficient calculated based on the reported method (28). The molecular absorption coefficients of the oligonucleotides were determined by the absorbance at 260 nm using the molecular weight of the oligonucleotides synthesized, and the band intensity was analyzed on a Beckman DU68 spectrophotometer equipped with a gel scan unit and gel scan area program. The band intensity that appeared in the autoradiogram was a linear function of the radioactivity of the band under these conditions. In order to ensure linear conversion of substrates with incubation time, template-primers were incubated with Pol I with varying incubation periods for 1–30 min in a preliminary experiment. The conversion of the substrate was virtually linear with incubation time up to 5 min in most cases. Thus, all incubations were performed for 5 min in the experiments to obtain kinetic parameters. The initial velocity ($V$, nM/min) for the excision of a 3'-primer terminus nucleotide opposite an abasic site was determined based on equation (1),

$$V = \frac{\text{[S]} \cdot I_0(I_0 + I_1)}{3}$$

where [S] is the concentration of substrate (template-primers) and $I_0$ and $I_1$ are band intensities of the original primer (17mer) and the excised product (16mer) after Pol I reaction, respectively.

**UV-melting measurement**

Absorbance vs. temperature melting curves were measured at 260 nm on a Hitachi U-3200 or U-3120 spectrometer equipped with a Hitachi SPR-10 temperature controller. The heating rate was 0.5 or 1.0°C/min, and the buffer for measurement was 0.1 M NaCl, 10 mM Na$_2$HPO$_4$, 0.1 mM EDTA, pH 7.0. For each oligonucleotide, 9–10 absorbance vs. temperature profiles were obtained for a 100-fold range in strand concentration, and melting curves were analyzed with a two-state model as described previously (33,34). The experimental errors of $\Delta\Delta G^\circ$ were ±0.1 kcal/mol under the present conditions.

**RESULTS**

**Kinetic parameters for the excision of a 3'-terminal nucleotide opposite an abasic site**

Figure 1 shows nucleotide sequences of oligonucleotides used for a 3'-5' exonuclease assay with Pol I (A) as well as those for UV-melting measurements (B). To obtain kinetic parameters for proofreading excision of primer terminus nucleotides opposite an abasic site, the duplexes (Fig. 1A) were constructed by annealing templates (26mer) and primers (17mer), and treated with Pol I. Products were analyzed by denaturing polyacrylamide gel electrophoresis as described in Materials and Methods. Figure 2 shows the
The original primer was too slow to be quantitated accurately under conditions of Michaelis-Menten kinetics. The other duplexes also exhibited similar hyperbolic relationships (data not shown). The S-V plot for the duplex CG could not be obtained since the conversion of the resultant 16mers was negligible so that bands containing a C A mismatch at the primer terminus were not detected within the detection limit of the present densitometry (ca. 1% intensity of the original primer band). On the contrary, with the duplex CG containing a matched primer terminus, a band arising from excision of the primer terminus nucleotide was not detected under these conditions (data not shown).

The initial velocity of the proofreading excision (V) with various concentrations of the substrates (S) was determined using densitometric quantitation and equation (1) (see Materials and Methods). Figure 3 shows a typical S-V plot obtained for the duplex XC. The S-V plot was an approximately hyperbolic function characteristic of Michaelis-Menten kinetics. The other duplexes also exhibited similar hyperbolic relationships (data not shown). The S-V plot for the duplex CG could not be obtained since the conversion of the original primer was too slow to be quantitated accurately under these conditions. The kinetic parameters were obtained by plotting V/S vs. S (Hanes–Woof plot), where x and y intercepts of the linear least square fit correspond to Vmax/Km and Km/Vmax, respectively. Figure 4 shows examples of the Hanes–Woof plots obtained from a set of the reactions. The kinetic parameters were determined based on four independent experiments and summarized in Table 1. The apparent affinity (Km) of Pol I to the duplexes XN (N= A, G, C or T) exhibited a slight variation up to 3-fold depending on the primer terminus nucleotide opposite the abasic site, with G the highest (6.3 nM) and A the lowest (19.4 nM). The apparent affinity to the duplex CA containing a mismatched primer terminus was comparable with that of the duplexes XC or XT. The dependence of Vmax on the nucleotide opposite the abasic site was less evident than Km, and the maximum variation for the highest and the lowest G was only 1.8-fold. The relative efficiency (Vmax/Km) of the proofreading excision of A opposite an abasic site was the lowest of the four nucleotides, but the differences in the relative efficiencies between A and the other nucleotides was at most 1.7-fold, mainly due to near cancellation of Vmax and Km. Comparison of the relative efficiencies (Vmax/Km) indicates that for the four nucleotides opposite an abasic site, A is excised with the lowest efficiency by the 3'-5' exonuclease associated Pol I, but the differences in the efficiencies were very small. In addition, there was no clear systematic preference for particular bases, for example purines versus pyrimidines etc.

Figure 2. Polyacrylamide gel analysis of products obtained by Pol I 3'-5' exonuclease assay. Partial duplex substrates with different 3'-primer terminus nucleotides (duplexes XA, XG, XC, XT or CA) were prepared by annealing appropriate templates (26mer) and primers (17mer). The substrates were incubated with Pol I (0.02 U) at 25°C for 3 min. Products were separated on a 20% polyacrylamide gel as described in Materials and Methods. Concentrations of the substrate used are indicated at the top of the gels. The top band corresponds to the original primer (17mer) and the lower band (indicated as an excision product) to 16mer produced by 3'-5' exonuclease action of Pol I on a 3'-terminal nucleotide.

Figure 1. Nucleotide sequences of oligonucleotides used in this study. (A) Template-primers for Pol I 3'-5' exonuclease assays. X denotes an abasic site. (B) Oligonucleotides for UV-melting measurements. The oligonucleotides have a common self-complementary core sequence (underlined) without or with dangling nucleotides, where X and N denote an abasic site and a nucleotide (A, G, C or T), respectively. Duplex forms are shown in sequences.
Figure 3. Plot of the reaction velocity (V) for the excision of a 3'‐primer terminus nucleotide in duplex XC as a function of the substrate concentration (S). The duplex XC with various concentrations (5–50 μM) was incubated with Pol I (0.02 U) at 25°C for 3 min. Products were separated by 20% denaturing polyacrylamide gel electrophoresis. Conversion of the original primer (17mer) to a 16mer was analyzed by the densitometry of the gel autoradiogram. The reaction velocity for the proofreading excision (V) was calculated using equation (1) as described in Materials and Methods.

Table 1. Kinetic parameters for the proofreading excision of a primer terminus nucleotide opposite abasic sites by Pol I 3'-5' exonuclease

<table>
<thead>
<tr>
<th>Primer terminus</th>
<th>K_m (nM)</th>
<th>V_max (nM/min)</th>
<th>V_max/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>XA</td>
<td>19.4 (±4.0)</td>
<td>4.6 (±1.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>XG</td>
<td>6.3 (±2.5)</td>
<td>2.6 (±0.9)</td>
<td>1.7</td>
</tr>
<tr>
<td>XC</td>
<td>10.6 (±4.7)</td>
<td>3.8 (±1.0)</td>
<td>1.5</td>
</tr>
<tr>
<td>XT</td>
<td>10.6 (±5.3)</td>
<td>4.0 (±1.7)</td>
<td>1.6</td>
</tr>
<tr>
<td>CA</td>
<td>9.6 (±3.5)</td>
<td>2.5 (±0.8)</td>
<td>1.1</td>
</tr>
</tbody>
</table>

aAverage results of four independent experiments, with standard deviation in parentheses.
bX: abasic site.
cRelative value.

Thermodynamic stability of a primer terminus nucleotide paired with an abasic site

To elucidate whether there may be correlation between the difference in free energy changes (ΔΔG°) in the formation of XN pairs (N = A, G, C or T) at a primer terminus and the preference of nucleotides incorporated opposite abasic sites, a series of free energy differences were determined for paired and unpaired terminal nucleotides constituting the primer terminus regions. For this purpose, oligonucleotides were synthesized that consisted of a self complementary core sequence (oligo 1) and one or two dangling nucleotides (oligos 2N, 3N and 4N, see Fig. 1B). The oligonucleotides form duplexes by themselves using the self complementary core sequence and the dangling nucleotides mimic template and primer termini as shown in Figure 1B. Since the self duplexes formed by the oligos have 2-fold symmetry and contain two identical termini, thermodynamic parameters for an individual terminus can be evaluated by dividing the experimentally determined thermodynamic values by a factor of 2.

UV-melting curves were measured with varying the concentrations of the oligos 1–4 and thermodynamic parameters were obtained by the van’t Hoff method (35) using a two-state model and equations (2) and (3),

\[ T_m^{-1} = (2.3/\Delta H^\circ) \log(C_t) + (\Delta S^\circ/\Delta H^\circ) \]

\[ \Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \]

where C_t is the total concentration of the single strand and T_m is the melting temperature. The UV-melting profiles of the self duplexes were sigmoidal and exhibited melting cooperativity. Figures 5 and 6 show a typical UV-melting curve and van’t Hoff plots, respectively, for some of the duplexes studied. From the van’t Hoff plots, thermodynamic parameters (ΔH°, ΔS°, ΔG°) for the duplex formation were determined (data not shown). The free energy increments (ΔΔG°37) associated with the addition of terminal
nucleotides relative to the core sequence were obtained by subtracting the free energy change (ΔG°*) of the core duplex (oligo 1) from that of oligo 2N, 3N or 4N. These are summarized in Table 2. As can be seen for oligo 2N, the 3' dangling nucleotides tended to stabilize the core duplex up to 0.3 kcal/mol, which agrees with a previous report (30). Addition of an abasic site (X) opposite the 3' dangling nucleotides in oligo 3N lead to further stabilization of the duplexes when the nucleotides opposite X were purines, whereas it exhibited no or even destabilizing effects for pyrimidines opposite X. Further addition of a 5' dangling nucleotide (A) in oligo 4N exhibited different effects for purines; A opposite X was destabilized while G was stabilized. The addition of a 5' dangling nucleotide showed a very small (T) or negligible (C) effect on stability. These results suggest that the stability of a 3' terminal nucleotide paired with an abasic site decreases in the following order: XG > XA = XC > XT, but the difference in free energy changes for the most stable G and the least stable T was only 0.5 kcal/mol.

Table 2 Free energy increments (ΔAG°37) of terminal nucleotides relative to the core duplex (oligo 1)\(^a\)

<table>
<thead>
<tr>
<th>oligo</th>
<th>ΔAG°37 (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2N</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>CA</td>
<td>-0.1</td>
</tr>
<tr>
<td>-0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>-0.1</td>
<td></td>
</tr>
<tr>
<td>3N</td>
<td></td>
</tr>
<tr>
<td>GX</td>
<td>GX</td>
</tr>
<tr>
<td>CA</td>
<td>GX</td>
</tr>
<tr>
<td>-0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>4N</td>
<td></td>
</tr>
<tr>
<td>GXA</td>
<td>GXA</td>
</tr>
<tr>
<td>CA</td>
<td>GXA</td>
</tr>
<tr>
<td>-0.1</td>
<td>-0.5</td>
</tr>
<tr>
<td>-0.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)In 0.1 M NaCl, 10 mM phosphate buffer (pH 7.0) and 0.1 mM EDTA.

The enzymatic mechanism of translesional DNA synthesis associated with abasic sites can be analyzed by studying three dissected stages involved in the processing of the encountered lesions by DNA polymerase: i.e. (i) initial nucleotide insertion, (ii) subsequent proofreading excision of the inserted nucleotide, and finally (iii) extension of the nascent primer terminus that escaped proofreading excision (23,24). Concerning stage (ii), previous studies have shown that turnover of dATP to dAMP catalyzed by *E.coli* Pol I (16) or Pol III holoenzyme (37) on templates containing abasic sites occurs with the highest rate of the four nucleotides. However, production of dAMP in these studies is a combined result of the insertion reaction during stage (i) and the excision reaction in stage (ii). Therefore, the results from the previous studies can not be simply compared with those presented here which deals with stage (ii) alone. The present study has shown that the difference in the relative efficiencies of proofreading excision of A and the other nucleotides was at most 1.7-fold for Pol I (Table 1). This result suggests that differential removal of primer terminal nucleotides by the 3'→5' exonuclease associated with Pol I plays only a minor role in the selection of nucleotides opposite abasic sites in translesional DNA synthesis. In contrast, the initial insertion in the stage (i) reaction exhibits a more pronounced preference for A over the other nucleotides compared to the stage (ii) reaction. The relative efficiencies of the insertion of A by DNA polymerases are roughly 20-50-fold greater than the least favored pyrimidines (18,19). This situation is similar to the fidelity mechanism of Pol I by which correct and incorrect nucleotides are discriminated. Kinetic studies using a presteady state method have shown that the selection of correct versus incorrect nucleotides derived from stages (i), (ii) and (iii) are estimated as 104–106, 4–60 and 6–340, respectively (38), where stage (i) makes a dominant contribution to the correct
nucleotide selection. Thus, we assume that in translesional synthesis associated abasic sites, differential proofreading excision of the nucleotide incorporated opposite the lesions makes a minor contribution to the preferential incorporation of A as compared to selection during stage (i). Concerning this conclusion, the study on the editing specificity of *E. coli* Pol III by Brenowitz *et al.* is noteworthy (39). They show that thermal instability of the primer terminus is the main determinant of the exonuclease activity of Pol III. All mismatched primer terminus are removed with virtually the same efficiency. These data appear to be consistent with what is observed opposite abasic sites in this study.

In this study, we have also determined the difference in free energy changes (ΔΔG°37) for XN pairs (N = A, G, C, T) incorporated at primer termini in an attempt to reconcile the free energy data with frequencies of nucleotide insertion in stage (i) or proofreading excision in stage (ii). With the blunt-ended oligo 3N having a terminal XN pair, purines opposite an abasic site were more stable than pyrimidines by 0.4 kcal/mol (Table 2). However, the addition of a 5’ dangling nucleotide next to the abasic site in template strands resulted in destabilization of A opposite the abasic site and stabilization of G. Stability of pyrimidines opposite the abasic site was barely affected by the 5’ dangling nucleotide. The ΔΔG°37 between the XN pairs was relatively small and at most 0.5 kcal/mol for the most stable XG and the least stable XT. Thus, the ratio of the equilibrium constants for the formation of XG and XT pairs at the primer termini [K(XG)/K(XT)] is only 2.3 at 37°C. Furthermore, the order of ΔΔG°37 (G > A = C ≥ T) also failed to account for the general order of the insertion frequencies in stage (i) (A > G > T = C) (18,19) or the difference in the excision efficiencies in stage (ii), although the latter is considerably smaller. Insensitivity of ΔΔG°37 to terminal modifications in duplex oligonucleotides, as demonstrated for the terminal nucleotides opposite abasic sites in this study, may be a general feature since a similar result has been observed for the terminal mismatches, where ΔΔG°37 are only 0.2–0.4 kcal/mol for terminal AT compared with GT, CT and TT mispairs (40). It is also noted that the impact of an abasic site on the stability of DNA appears to be quite different depending on the sites in where the lesion is embedded. When incorporated into the middle of duplexes, an abasic site dramatically reduces the duplex stability (41–43) and the ΔΔG°37 relative to the fully paired parental duplex is evaluated as 6.5 kcal/mol (42). This is in contrast to the present results that an abasic site incorporated in the terminal regions did not exert a significant influence on the stability of the core duplex.

The lack of correlation between ΔΔG° for XN pairs and the insertion or excision frequencies may arise from two reasons. First, as pointed by Goodman *et al.* (44), exclusion of water molecules from the active sites (i.e. polymerase site or 3’-5’ exonuclease site) of a polymerase–DNA complex may differentially increase the free energy changes (ΔG°). Second, the nucleotide insertion and excision by DNA polymerases are intrinsically non-equilibrium reactions. Nevertheless, the free energy changes derived from melting measurements are valid only when dealing with an equilibrium process, and they do not reveal any information about reaction rates. In general, enzymes accelerate particular reactions by stabilizing the transition state, not the final state. The former is best described by the free energy of activation (ΔG‡) and the latter by the free energy (ΔG°). Thus, to reconcile the kinetic data on DNA polymerase reactions with thermodynamic parameters, it may be appropriate to use the difference in the free energy of activation at the transition state (ΔΔG‡) instead of that in free energy at the final state (ΔΔG°). Both the first and second points need to be further assessed to fully account for the molecular mechanism of the A-rule including the thermodynamic aspect.

REFERENCES