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Methylglyoxal, an endogenous aldehyde, crosslinks DNA polymerase and the substrate DNA

Naoko Murata-Kamiya and Hiroyuki Kamiya[1,*]

Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan and [1]Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

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ABSTRACT

Methylglyoxal, a known endogenous and environmental mutagen, is a reactive α-ketoaldehyde that can modify both DNA and proteins. To investigate the possibility that methylglyoxal induces a crosslink between DNA and DNA polymerase, we treated a 'primed template' DNA and the exonuclease-deficient Klenow fragment (KF^{exo-}) of DNA polymerase I with methylglyoxal *in vitro*. When the reaction mixtures were analyzed by SDS–PAGE, we found that methylglyoxal induced a DNA–KF^{exo-} crosslink. The specific binding complex of KF^{exo-} and 'primed template' DNA was necessary for formation of the DNA–KF^{exo-} crosslink. Methylglyoxal reacted with guanine residues in the single-stranded portion of the template DNA. When 2′-deoxyguanosine was incubated with *N*α-acetyllysine or *N*-acetylcysteine in the presence of methylglyoxal, a crosslinked product was formed. No other amino acid derivatives tested could generate a crosslinked product. These results suggest that methylglyoxal crosslinks a guanine residue of the substrate DNA and lysine and cysteine residues near the binding site of the DNA polymerase during DNA synthesis and that DNA replication is severely inhibited by the methylglyoxal-induced DNA–DNA polymerase crosslink.

INTRODUCTION

Methylglyoxal is a physiological metabolite formed by the fragmentation of triose phosphates, which are intermediates of glycolysis (1,2). Methylglyoxal is also formed by lipid peroxidation systems (3), by the metabolism of acetone (4) and aminoacetone (5) and by the degradation of DNA (6). In addition, methylglyoxal is ubiquitous in beverages and foods, such as coffee, toast and soy sauce (7–9), as well as in cigarette smoke (10).

Methylglyoxal is mutagenic in *Salmonella typhimurium*, *Escherichia coli* (7) and *Saccharomyces cerevisiae* (11). We previously reported that methylglyoxal induces mutations mainly at G:C base pairs in *E.coli* strains (12) and in simian kidney (COS-7) cells (13). Methylglyoxal is an α-ketoaldehyde that reacts with guanine (G) residues in DNA to form a tricyclic compound (14,15). Thus, methylglyoxal seems to induce mutations by reaction with G residues and by subsequent formation of mispairs.

Moreover, methylglyoxal reacts with free amino acids (16,17) and Arg and Lys residues in proteins under physiological conditions to form advanced glycation end products (18,19), which play an important role in the pathophysiology of aging and diabetic complications (20,21). It was also reported that the concentration of methylglyoxal in blood is elevated in diabetes mellitus patients (22–24). Thus, the reactions of methylglyoxal with proteins as well as DNA may cause many age-related human diseases.

We hypothesized that DNA–protein crosslinks could be induced by methylglyoxal, because it is a reactive α-ketoaldehyde that can modify both DNA and proteins. In particular, DNA replication may be severely inhibited when methylglyoxal-induced crosslinks are formed between DNA and DNA polymerases. In the present study we have treated a 'primed template' DNA and the 3′→5′ exonuclease-deficient Klenow fragment (KF^{exo-}) of *E.coli* DNA polymerase I with methylglyoxal *in vitro* to test this possibility. We found that methylglyoxal induced a DNA–KF^{exo-} crosslink. The reaction mechanism of DNA–KF^{exo-} crosslink formation will be discussed.

MATERIALS AND METHODS

Materials

Methylglyoxal (40% aqueous solution, CAS no. 78-98-8) was purchased from Sigma-Aldrich. Glyoxal (40% aqueous solution, CAS no. 107-22-2) was purchased from Nacalai Tesque. KF^{exo-} was purchased from New England Biolabs. 2′-Deoxyguanosine (dG) was purchased from Wako Pure Chemical Industries. 2′-Deoxyguanosine 5′-monophosphate (dGMP) was purchased from Sigma-Aldrich. *N*α-acetyl-L-asparagine (AcAsn), *N*-acetyl-L-aspartic acid (AcAsp), *N*-acetyl-L-cysteine (AcCys) and *N*-acetyl-L-tryptophan (AcTrp) were purchased from Wako Pure Chemical Industries. *N*-Acetyl-L-alanine

*To whom correspondence should be addressed. Tel: +81 11 706 3733; Fax: +81 11 706 4879; Email: hirokam@pharm.hokudai.ac.jp
Present address:
Naoko Murata-Kamiya, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan and CREST, Japan Science and Technology, Japan

Table 1. Sequence of oligonucleotides

Oligonucleotide	Sequence
P-1	5′-ATGACGGAATAT-3′
P-2	5′-ATGACGGAATATTAGC-3′
T-1	3′-TACTGCCTTATAATCG-5′
T-2A	3′-TACTGCCTTATAAAAA-5′
T-2G	3′-TACTGCCTTATAGGGG-5′
T-2C	3′-TACTGCCTTATACCCC-5′
T-2T	3′-TACTGCCTTATATTTT-5′

(AcAla), *N*α-acetyl-L-arginine (AcArg), *N*α-acetyl-L-histidine (AcHis), *N*α-acetyl-L-lysine (AcLys), *N*α-acetyl-L-tyrosine (AcTyr) and *N*-acetylglycine (AcGly) were purchased from Sigma-Aldrich.

Crosslink assay

The oligonucleotides used in the present study are listed in Table 1. A 'template' DNA (120 pmol) annealed with a 'primer' (100 pmol) was mixed with KF^{exo-} (5.52 pmol) in a buffered solution containing 100 mM sodium phosphate (pH 7.4) and 1 mM EDTA and the mixture was incubated on ice for 10 min. The crosslinking reaction was started by addition of methylglyoxal or glyoxal to the DNA/KF^{exo-} mixture and was incubated at 37°C. The reaction was stopped by addition of an equal volume of 2× SDS loading solution (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 0.04% bromophenol blue). These mixtures were then fractionated by 0.1% SDS–12% PAGE. Proteins were visualized by staining with a Silver Stain II Kit (Wako Pure Chemical Industries). The gel image was analyzed with NIH Image v.1.61 software. Under our conditions the band intensities were proportional to the amounts of KF^{exo-} loaded on the gel (data not shown).

$NaBH_4$ reduction of the reaction intermediates

The T-1/P-1 'primed template' and KF^{exo-} were treated with 10 mM methylglyoxal at 37°C for 60, 90 and 120 min as described above and were additionally incubated at 37°C for 60 min in the presence of 50 mM $NaBH_4$.

Reactions of amino acids with 2′-deoxyguanosine in the presence of methylglyoxal

dG (5 mM) and each of the following *N*α-acetyl-L-amino acids, AcGly, AcAla, AcLys, AcArg, AcHis, AcCys, AcTrp, AcTyr, AcAsn and AcAsp (5 mM), were mixed in a buffered solution containing 100 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Methylglyoxal (20 mM) was then added and the mixture was incubated at 37°C for 1–10 days. The reaction mixture was subjected to anion exchange HPLC using a TSK-GEL DEAE-2SW (4.6 × 250 mm) column. A linear gradient of 1 M $HCOONH_4$ (0–40%) in 20% CH_3CN was used from 0 to 40 min. The flow rate was 0.8 ml/min and the detection wavelength was 254 nm.

Reaction of dG derivatives and Nα-acetyl-L-lysine in the presence of methylglyoxal

dG or dGMP (5 mM) and AcLys (5 mM) were mixed in a buffered solution containing 100 mM sodium phosphate (pH 7.0, 7.4, 8.0 or 9.0). Methylglyoxal (20 mM) was then added and the mixture was incubated at 37°C for 24 h. Analysis by HPLC was conducted as described above.

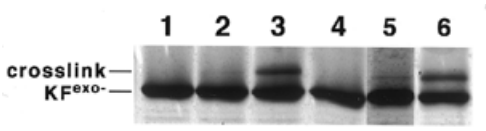

Figure 1. Formation of a DNA–KF^{exo-} crosslink induced by methylglyoxal. The mixture of DNA (T-1/P-1) and KF^{exo-} was incubated with H_2O (lane 1), 10 mM glyoxal (lane 2) or 10 mM methylglyoxal (lane 3) at 37°C for 60 min, as described in Materials and Methods. Lane 4 was identical to lane 3, except that the DNA was absent. Lanes 5 and 6 were identical to lanes 2 and 3, respectively, except that the incubation was for 120 min.

RESULTS

Methylglyoxal crosslinks DNA and DNA polymerase

The DNA polymerase KF^{exo-} and the 'primed template' (P-1 plus T-1, Table 1) were incubated on ice and then treated with methylglyoxal or glyoxal. The reaction mixture was analyzed by SDS–PAGE. When the DNA and KF^{exo-} were incubated with 10 mM methylglyoxal at 37°C for 60 min, a novel band with lower electrophoretic mobility was detected (Fig. 1, lane 3). This band was not detected in the absence of methylglyoxal or DNA (Fig. 1, lanes 1 and 4), suggesting that this band corresponds to a DNA–KF^{exo-} crosslink induced by methylglyoxal. However, when 10 mM glyoxal, an analogous compound, was used this band with lower electrophoretic mobility was not detected after a 60 min incubation (Fig. 1, lane 2). The band corresponding to the DNA–KF^{exo-} crosslink was detectable after incubation with 10 mM glyoxal at 37°C for 120 min (Fig. 1, lane 5). The yield of the crosslink induced by glyoxal (1.5%) was much lower than that induced by methylglyoxal (14.8%, Fig. 1, lanes 5 and 6). Thus, methylglyoxal was a more potent DNA–KF^{exo-} crosslinking reagent than glyoxal. Formation of the DNA–KF^{exo-} crosslink was observed on incubation with 1 mM methylglyoxal at 37°C for 60 min, but not in a similar incubation with 100 µM methylglyoxal (data not shown).

Next, we examined the influence of DNA structure upon the methylglyoxal-induced DNA–DNA polymerase crosslink. When KF^{exo-} and the annealed oligonucleotides T-1 and P-2 (blunt-ended double-stranded DNA) were treated with methylglyoxal the DNA–KF^{exo-} crosslink was not detected (data not shown). Moreover, methylglyoxal did not crosslink single-stranded oligonucleotides (P-1, P-2 and T-1) and KF^{exo-} (data not shown). It is reasonable that a crosslink was formed when the DNA polymerase was incubated with the substrate DNA in the presence of methylglyoxal. Thus, the crosslink observed was the result of covalent bond formation between the specific binding complex of KF^{exo-} and the primed template DNA, thus excluding the possibility that methylglyoxal induced random crosslinking. Moreover, these results suggest that the single-stranded portion of T-1/P-1 (4 nt from the 5′-end of T-1) was involved in crosslink formation.

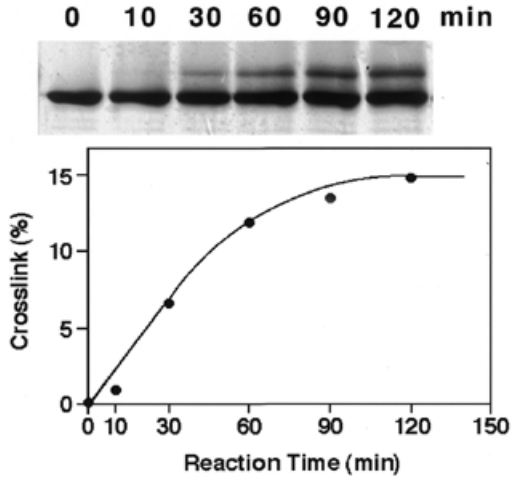

Figure 2. Time course of DNA–KF^{exo-} crosslink formation by methylglyoxal. T-1/P-1 and KF^{exo-} were incubated with 10 mM methylglyoxal, as described in Materials and Methods, for the indicated time periods.

Oligonucleotide	T-1	T-2A	T-2G	T-2C	T-2T
	P-1	P-1	P-1	P-1	P-1
MG (mM)	0 10	0 10	0 10	0 10	0 10

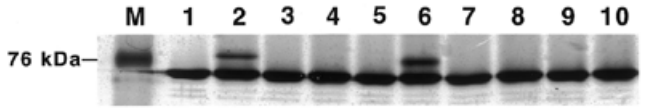

Figure 3. Influence of DNA sequence upon the methylglyoxal-induced DNA–KF^{exo-} crosslink. The 'primed template' and KF^{exo-} were incubated with the indicated concentrations of methylglyoxal (MG) at 37°C for 60 min.

Next, we examined the time course of DNA–KF^{exo-} crosslink formation induced by methylglyoxal. DNA and KF^{exo-} were incubated with 10 mM methylglyoxal at 37°C for 10, 30, 60, 90 and 120 min and the crosslink ratios were measured. As shown in Figure 2, the yield of the DNA–KF^{exo-} crosslink increased in a time-dependent manner.

Guanine is involved in crosslink formation by methylglyoxal

Methylglyoxal reacts with A and C, in addition to G, in polynucleotides (25). To investigate which base is involved in the crosslink, four 'templates' (T-2A, T-2G, T-2C and T-2T, Table 1) were used in crosslink experiments. These oligonucleotides contain a run of the same base 4 nt from the 5′-end of the 'template' T-2 (Table 1). When annealed DNA and KF^{exo-} were incubated with 10 mM methylglyoxal at 37°C for 60 min, formation of a DNA–KF^{exo-} crosslink was observed in the cases of T-2G/P-1 and T-1/P-1 (Fig. 3, lanes 2 and 6). On the other hand, the crosslink was not detected in the cases of the T-2A/P-1, T-2C/P-1 and T-2T/P-1 oligonucleotides (Fig. 3, lanes 4, 8 and 10). These results suggest that methylglyoxal reacts with G residues in the DNA and amino acid residues in the polymerase to form the DNA–KF^{exo-} crosslink. The yields of crosslink induced by methylglyoxal in both T-2G/P-1 and T-1/P-1 were similar: 10.2% and 11.7%, respectively. The T-2G/P-1

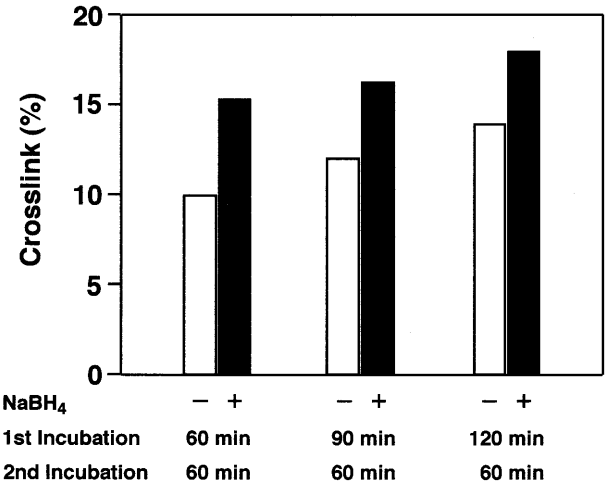

Figure 4. Influence of $NaBH_4$ reduction of the methylglyoxal-induced DNA–KF^{exo-} crosslink. T-1/P-1 and KF^{exo-} were incubated with 10 mM methylglyoxal at 37°C for the indicated period (1st incubation) and were additionally incubated for 60 min in the presence or absence of $NaBH_4$ (2nd incubation).

crosslink band appeared broader than that of T-1/P-1. This result suggests that the crosslink of T-2G/P-1 contains multiple species in which different G bases participate in covalent binding.

$NaBH_4$ reduction of the reaction intermediates

Methylglyoxal is known to form reversible imine intermediates (Schiff base) in reactions with proteins under physiological conditions (16). Reduction of the imine bond by $NaBH_4$ leads to the formation of irreversibly bound complexes between the protein and methylglyoxal. Similar Schiff base intermediates were expected to form between G and methylglyoxal during formation of the crosslink, as shown above. Thus, the influence of $NaBH_4$ reduction on crosslink formation by methylglyoxal was examined using KF^{exo-} and the T-1/P-1 oligonucleotide.

DNA (T-1/P-1) and KF^{exo-} were incubated with 10 mM methylglyoxal at 37°C for 60, 90 and 120 min and then were additionally incubated at 37°C for 60 min in the presence or absence of $NaBH_4$. The reaction mixtures were then analyzed by SDS–PAGE. As shown in Figure 4, for each incubation time the yield of DNA–KF^{exo-} crosslink was higher in the presence than in the absence of $NaBH_4$. These results suggest that the Schiff base was involved in the crosslinking of DNA and KF^{exo-} induced by methylglyoxal. Although formation of the Schiff base is a reversible reaction, the equilibrium may be biased by formation of the DNA–KF^{exo-} crosslink.

Lysine and cysteine react with 2′-deoxyguanosine in the presence of methylglyoxal

To investigate which amino acid residues are involved in the DNA–KF^{exo-} crosslink induced by methylglyoxal, dG and each of the *N*α-acetyl-L-amino acids, AcGly, AcAla, AcLys, AcArg, AcHis, AcCys, AcTrp, AcTyr, AcAsn and AcAsp, were mixed with methylglyoxal in a buffered solution and incubated at 37°C for 1–10 days. The reaction mixture was then analyzed by anion exchange HPLC. In this study we used some of the *N*α-acetyl-L-amino acids to exclude a side reaction with the α-amino group (16). When dG and AcLys were treated with methylglyoxal, a peak corresponding to a dG–AcLys

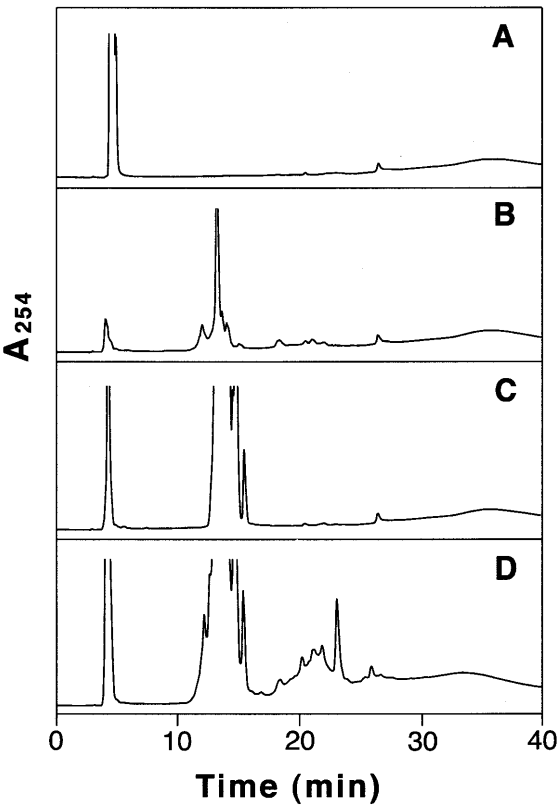

Figure 5. Methylglyoxal crosslinks dG and AcLys. (**A**) dG and AcLys were incubated in the absence of methylglyoxal. (**B**) AcLys, (**C**) dG and (**D**) dG and AcLys were incubated with 20 mM methylglyoxal at 37°C for 10 days. The reaction mixture was analyzed by anion exchange HPLC.

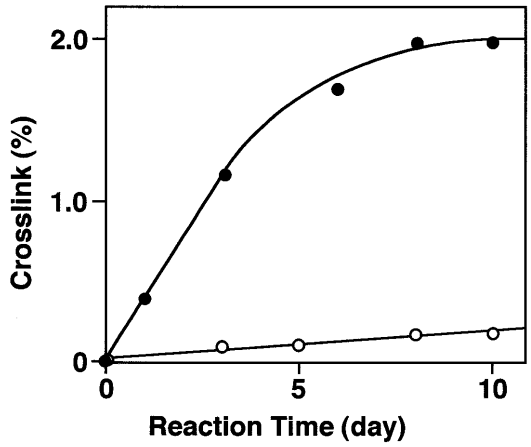

Figure 6. Time course of dG–AcLys (closed circles) and dG–AcCys (open circles) crosslink formation by methylglyoxal. dG and AcLys/AcCys were incubated with 20 mM methylglyoxal as described in Materials and Methods for the indicated time periods.

crosslink was detected (~23 min, Fig. 5D). This peak was not detected in the absence of either methylglyoxal, dG or AcLys (Fig. 5A–C). Yield of the dG–AcLys crosslink increased in a time-dependent manner during a 10 day incubation with methylglyoxal (Fig. 6). By the end of the 10 day incubation, yield of the dG–AcLys crosslink reached 2% (yield of the dG–AcLys crosslink derived from the peak area for the dG–AcLys crosslink versus the total peak area derived from dG is shown as a percentage). When AcCys was incubated with dG and methylglyoxal, a peak corresponding to a dG–AcCys crosslink was detected. As for AcLys, yield of the dG–AcCys crosslink increased in a time-dependent manner during a 10 day incubation with methylglyoxal (Fig. 6). However, yield of the dG–AcCys crosslink was much lower than that of the dG–AcLys crosslink. By the end of the 10 day incubation yield of the dG–AcLys crosslink reached 0.2%. No crosslink was observed in the cases of the other amino acid derivatives tested (data not shown). These results suggest that the Lys and Cys residues of KF^{exo-} are involved in crosslink formation with DNA.

Table 2. Influence of pH on crosslink formation by methylglyoxal

pH	Yield	
	dG–AcLys	dGMP–AcLys
7.0	0.19% (1.0)	0.63% (1.0)
7.4	0.43% (2.3)	0.87% (1.4)
8.0	0.81% (4.3)	1.20% (1.9)
9.0	1.08% (5.7)	1.54% (2.4)

Influence of pH on crosslink formation by methylglyoxal

We speculated that the ε-amino group of a Lys residue attacks the carbonyl group of methylglyoxal and thus crosslink formation would be favored under alkaline conditions. Therefore, we examined the influence of pH on crosslink formation by methylglyoxal. We examined crosslink formation of dGMP–AcLys as well as dG–AcLys. 2′-Deoxyguanosine derivatives (dG or dGMP) and AcLys were mixed with methylglyoxal in buffered solutions with different pH values (pH 7.0, 7.4, 8.0 and 9.0) and incubated at 37°C for 24 h. As shown in Table 2, yields of the crosslinks were pH dependent and were higher under more alkaline conditions. At pH 9.0 the dG–AcLys and dGMP–AcLys crosslinks were formed 5.7- and 2.4-fold more efficiently, respectively, than at pH 7.0. Thus, these crosslinks appear to be initiated by nucleophilic attack of an amino group. Accordingly, the observed DNA–KF^{exo-} crosslink probably proceeded via a similar mechanism.

DISCUSSION

In the present study we have investigated the formation of crosslinks induced by methylglyoxal between DNA and a DNA polymerase. We found that methylglyoxal induced a DNA–KF^{exo-} crosslink (Fig. 1). This result suggests that DNA replication would be severely inhibited by a methylglyoxal-induced DNA–DNA polymerase crosslink. Thus, accumulation of this compound in cells may be very cytotoxic and could induce apoptosis. These putative effects may cause neurodegeneration, aging and many age-related human diseases, such as diabetes.

An analogous compound, glyoxal, is also present as an endogeneous mutagen in cells and an environmental mutagen in foods (7,9,10,26). Both glyoxal and methylglyoxal react with a G base to form similar tricyclic compounds (27,28). Thus, we also examined the crosslinking ability of glyoxal. Glyoxal could indeed crosslink DNA and DNA polymerase

(Fig. 1). However, the crosslinking ability of glyoxal was 10-fold less than that of methylglyoxal. Thus, methylglyoxal appears to be a more important toxic aldehyde compound when we consider crosslinking ability, which may be involved in the biological processes described above.

We previously reported that both glyoxal and methylglyoxal induce mutations mainly at G:C base pairs in *E.coli* (12,29,30) and in mammalian cells (13,31). However, the mutations induced by the two compounds show some differences. The ratio of frameshift mutations induced by methylglyoxal was higher than that induced by glyoxal. In the present study we have shown that methylglyoxal is a much more potent crosslinking reagent than glyoxal. Thus, methylglyoxal is expected to produce more DNA–DNA polymerase crosslinks *in vivo*. When this crosslink is formed, polymerase bound covalently to DNA will lose its polymerase activity, while another molecule of DNA polymerase may continue the paused DNA synthesis. Since the DNA polymerase is attached to a guanine base, this modified guanine may behave as a 'bulky DNA lesion' during the putative continued DNA synthesis. Many bulky DNA lesions are known to elicit frameshift mutations (32–34). We hypothesize that formation of DNA–DNA polymerase crosslinks is one of the pathways inducing frameshift mutations and that this may be a reason for the greater number of frameshift mutations induced by methylglyoxal than by glyoxal.

The specific complex of KF^{exo-} and primed template DNA was necessary for formation of the DNA–KF^{exo-} crosslink, because single-stranded and completely double-stranded DNAs failed to form a crosslink (data not shown). Moreover, we found that G residues in the DNA were involved in the crosslinking reaction by methylglyoxal (Fig. 3). Thus, methylglyoxal could crosslink the G residues of a single-stranded portion of a template DNA bound with a DNA polymerase and amino acid residues near the binding site. Taken together with the observation that only Lys and Cys derivatives produced crosslinks with dG in the presence of methylglyoxal (Figs 5 and 6), we concluded that the Lys (and possibly Cys) residues of the DNA polymerase and G bases of the DNA were involved in crosslinking.

The structure of KF^{exo-} bound to duplex DNA has been reported (35). Although we could not identify the single-stranded region of the template based upon this binary complex, Lys residues at 467, 469, 511, 518, 520, 810 and 829 may locate near G residues in the template. Because KF^{exo-} contains only one Cys residue near the C-terminus, the Lys residues described above may be the amino acids involved in the crosslinking reactions observed in this study.

It was reported that methylglyoxal reacts with the polynucleotides poly(G), poly(A) and poly(C) with a reactivity ratio of 100:7:3 (25). In the present study, however, DNA–KF^{exo-} crosslinks were not detected in the cases of DNA containing a run of A, C or T in the single-stranded portion of the template DNA (Fig. 3).

It was reported that methylglyoxal reacts rapidly with Arg residues and also reacts with Lys residues in proteins (18,19). Methylglyoxal reacts with AcArg and AcLys to form glycosylamine derivatives and with AcCys to form a hemithioacetal (17). We found that methylglyoxal reacts with AcLys and AcCys to form a dG–amino acid crosslink. In particular, AcLys was the major amino acid derivative that formed a methylglyoxal-induced crosslink. Yield of the dG–AcLys crosslink was 10-fold higher than that of the dG–AcCys crosslink (Fig. 6). Unexpectedly, in the case of AcArg no peak corresponding to the dG–AcArg crosslink was detected, while peaks corresponding to methylglyoxal-adducted dG and methylglyoxal-adducted AcArg were detected (data not shown). It was reported that the reaction of methylglyoxal with AcArg proceeds via a conversion to form an irreversible imidazolone derivative (17). We speculate that methylglyoxal reacts with the guanidino group of AcArg to form a stable cyclic methylglyoxal–AcArg adduct, rather than a crosslink.

Yields of the dG–AcLys and dGMP–AcLys crosslinks increased in a pH-dependent manner in the pH range 7.0–9.0 (Table 2). These results suggest that the crosslinks are initiated by nucleophilic attack by an amino group. Accordingly, the observed formation of DNA–KF^{exo-} crosslinks probably proceeded by a similar mechanism. Moreover, we showed that crosslink formation between DNA and KF^{exo-} was enhanced by additional incubation with $NaBH_4$ (Fig. 4). This result suggests that the crosslink contained a Schiff base and supports the reaction mechanism described above.

The formation of DNA–histone crosslinks induced by aldehydes *in vitro* has been studied previously, using a filter-binding assay. Formaldehyde, glutaraldehyde, acrolein, acetaldehyde, propionaldehyde, butyraldehyde, crotonaldehyde and *trans*-2-pentenal induce crosslinks (36). Moreover, it was reported that acetaldehyde induced crosslinks between $poly(dG)_6$ and $poly(Lys)_5$ (37). These data are consistent with our present finding that methylglyoxal induces crosslinks between G and Lys.

Methylglyoxal crosslinks G residues in template DNAs and amino acid residues of DNA polymerase. Since transcription complexes involving RNA polymerase bind with the single-stranded portion of DNA, similar reactions may be induced. In addition, single-stranded-binding proteins, such as mammalian RP-A (replication protein A), which play an important role in many biological processes, may be crosslinked with DNA by methylglyoxal. Thus, endogenous formation of methylglyoxal may inhibit many cellular events in addition to DNA replication.

Based on this finding, methylglyoxal may be used in crosslinking experiments to identify (Lys) residues located in or near the template DNA-binding site of a DNA polymerase. Namely, sequencing of the peptides isolated after crosslinking and subsequent proteinase treatment may show which amino acid is crosslinked with a template G residue. Given that a G base could be incorporated at any desired site from the primer–template junction, more information may be available when primed template oligonucleotides with different sequence contexts are used.

In conclusion, methylglyoxal reacts with G residues in DNA and with Lys and Cys residues in DNA polymerase to form a DNA–DNA polymerase crosslink. Thus, DNA replication would be severely inhibited by methylglyoxal. Further studies will reveal the relationship between this crosslink formation and many age-associated diseases.

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