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Mechanism of Site-Specific DNA Damage Induced by Methylhydrazines in the Presence of Copper(II) or Manganese(III)†

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ABSTRACT: DNA damage induced by methylhydrazines (monomethylhydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine) in the presence of metal ions was investigated by a DNA sequencing technique. 1,2-Dimethylhydrazine plus Mn(III) caused DNA cleavage at every nucleotide without marked site specificity. ESR–spin-trapping experiments showed that the hydroxyl free radical (•OH) is generated during the Mn(III)-catalyzed autoxidation of 1,2-dimethylhydrazine. DNA damage and •OH generation were inhibited by •OH scavengers and superoxide dismutase, but not by catalase. The results suggest that 1,2-dimethylhydrazine plus Mn(III) generates •OH, not via H_2O_2, and that •OH causes DNA damage. In the presence of Cu(II), DNA cleavage was caused by the three methylhydrazines frequently at thymine residues, especially of the GTC sequence. The order of Cu(II)-mediated DNA damage (1,2-dimethylhydrazine > monomethylhydrazine ~ 1,1-dimethylhydrazine) was not correlated with the order of methyl free radical ($\bullet CH_3$) generation during Cu(II)-catalyzed autoxidation (monomethylhydrazine > 1,1-dimethylhydrazine ≫ 1,2-dimethylhydrazine). Catalase and bathocuproine, a Cu(I)-specific chelating agent, inhibited DNA damage while catalase did not inhibit the $\bullet CH_3$ generation. The order of DNA damage was correlated with the order of ratio of H_2O_2 production to O_2 consumption observed during Cu(II)-catalyzed autoxidation of methylhydrazines. These results suggest that the Cu(I)–peroxide complex rather than the $\bullet CH_3$ plays a more important role in methylhydrazine plus Cu(II)-induced DNA damage.

A number of hydrazine derivatives are found in nature and used in industry, agriculture, and medicine. Most of the hydrazine derivatives have been shown to be carcinogenic and/or mutagenic (Kimball, 1977; Toth, 1980; Parodi et al., 1981). Methylhydrazines such as 1,2-dimethylhydrazine, 1,1-dimethylhydrazine, and monomethylhydrazine are carcinogenic in the large bowel of rodents (Toth, 1977, 1980). It was reported that 1,2-dimethylhydrazine was 100 times more carcinogenic, producing tumors selectively in the colon, than 1,1-dimethylhydrazine (Druckrey et al., 1967; Balő, 1979). Although 1,2-dimethylhydrazine has been widely used for the study of colon cancer, its carcinogenic mechanism remains to be clarified. It has been proposed that 1,2-dimethylhydrazine is enzymatically metabolized to the proximate carcinogen (methylazoxy)methanol, which is further transformed to an ultimate carcinogen, methyldiazonium, that methylates DNA (Fiala, 1977). However, there are several papers against this hypothesis. Rogers and Pegg (1977) reported that since DNA was methylated to a much lesser extent in the colon than in the liver of rats treated with 1,2-dimethylhydrazine, factors other than the production of methylguanine must be important in the initiation of colon cancer. Recently, the participation of the methyl free radical ($\bullet CH_3$)[1] in 1,2-dimethylhydrazine-induced carcinogenesis has been suggested (Kang et al., 1988; Albano et al., 1989). Relevantly, Augusto et al. (1984) reported that DNA strand scission was caused by the carbon radical derived from 2-phenylethylhydrazine metabolism. In addition, metal was shown to enhance 1,2-dimethylhydrazine-induced sister-chromatid exchanges and unscheduled DNA synthesis in cultured cells (MacRae & Stich, 1979; Whiting & Wei, 1979), and nonenzymatic biotransformation of monomethylhydrazine and 1,1-dimethylhydrazine was reported (Godoy et al., 1983). Therefore, regarding the mechanism of carcinogenicity and mutagenicity of methylhydrazines, it can be speculated that methylhydrazines are activated nonenzymatically by endogenous substances, such as metal ions, to produce active species causing DNA damage.

In this study, the mechanism of DNA damage by methylhydrazines (monomethylhydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine) in the presence of metal ions was investigated by both the DNA sequencing technique and the ESR–spin-trapping method.

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[1] Abbreviations: $\bullet CH_3$, methyl free radical; DTPA, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; •OH, hydroxyl free radical; DMPO–OH, •OH adduct of 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO–CH_3, $\bullet CH_3$ adduct of DMPO; POBN, α-(1-oxo-4-pyridyl)-*N*-*tert*-butyl nitrone; POBN–CH_3, $\bullet CH_3$ adduct of POBN; O_2^-, superoxide radical.

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Materials and Methods

Materials. [γ-^{32}P]ATP (6000 Ci/mmol) was supplied by Du Pont–New England Nuclear. Restriction enzymes (*Ava*I, *Xba*I, *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs. $CuCl_2$, other metal chlorides, monomethylhydrazine sulfate, 1,2-dimethylhydrazine dihydrochloride, ethanol, D-mannitol, and dimethyl sulfoxide were from Nacalai Tesque, Inc., Kyoto, Japan. 1,1-Dimethylhydrazine was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Bathocuproinedisulfonic acid, disodium salt, and DTPA were from Dojin Chemicals Co., Kumamoto, Japan. SOD (3150 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were from Sigma Chemical Co. POBN was purchased from Aldrich Chemical Co. DMPO was purchased from Labotec, Co. Ltd., Tokyo, Japan. Mn(III)–pyrophosphate was prepared according to Archibald and Fridovich (1982), and the concentration was estimated by using a value for the molar extinction coefficient at 259.5 nm of 6.2×10^3 M^{-1} cm^{-1}.

Preparation of DNA Fragments. ^{32}P 5′ end-labeled DNA fragments were prepared from plasmid pbcNI which carries a 6.6-kilobase *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 protooncogene (Capon et al., 1983) according to the method described previously (Yamamoto et al., 1989). Singly labeled 261 base pair fragment (*Ava*I* 1645–*Xba*I 1905), 341 base pair fragment (*Xba*I 1906–*Ava*I* 2246), 98 base pair fragment (*Ava*I* 2247–*Pst*I 2344), and 337 base pair fragment (*Pst*I 2345–*Ava*I* 2681) were obtained. The asterisk indicates ^{32}P labeling, and nucleotide numbering starts with the *Bam*HI site (Capon et al., 1983).

Analysis of DNA Damage. DNA damage was analyzed as previously described (Yamamoto & Kawanishi, 1989). The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the procedure of Maxam and Gilbert (1980) using a DNA sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Measurements of Oxygen Consumption. The O_2 consumption during the autoxidation of methylhydrazines was measured in a thermostated (37 °C) water-jacketed glass vessel, fitted with a Clark electrode (Gilson) according to the method described previously (Kawanishi et al., 1989).

ESR Spectra Measurements. ESR spectra were measured at room temperature using a JES-FE-3XG (JEOL, Tokyo, Japan) spectrometer with 100-kHz field modulation according to the method described previously (Kawanishi et al., 1986; Inoue & Kawanishi, 1987). Spectra were recorded with a microwave power of 16 mW, a modulation amplitude of 1.0 G, and a receiver gain of 1×1000 unless otherwise indicated. The magnetic fields were calculated by the splitting of Mn(II) in MgO (ΔH_{3-4} = 86.9 G). DMPO and POBN were used as radical trapping reagents.

Results

Cleavage of ^{32}P-Labeled DNA Fragments Induced by Methylhydrazines in the Presence of Metal Ions. The extent of DNA damage induced by methylhydrazines in the presence of metal ions [Cu(II), Mn(II), Mn(III), Fe(III), Co(II), and Ni(II)] was estimated by gel electrophoresis analysis. Figure 1 shows the effect of metal ions on methylhydrazine-dependent DNA damage. The upper band and lower band in the control show single-stranded and double-stranded forms of intact DNA fragment, respectively. No oligonucleotide was observed with monomethylhydrazine, 1,2-dimethylhydrazine, or 1,1-dimethylhydrazine alone, showing that methylhydrazines themselves are not DNA-damaging agents (lane 2). Monomethylhydrazine induced DNA damage in the presence of Cu(II) (Figure 1A, lane 3), whereas monomethylhydrazine induced little or no DNA damage in the presence of Mn(II), Mn(III), Fe(III) (Figure 1A, lanes 4–6), Co(II), or Ni(II) (data not shown) under the present conditions. 1,2-Dimethylhydrazine induced DNA damage in the presence of Cu(II) or Mn(III) (Figure 1B, lanes 3 and 5). 1,1-Dimethylhydrazine induced DNA damage in the presence of Cu(II) (Figure 1C, lane 3). Metal ion alone induced little or no DNA damage.

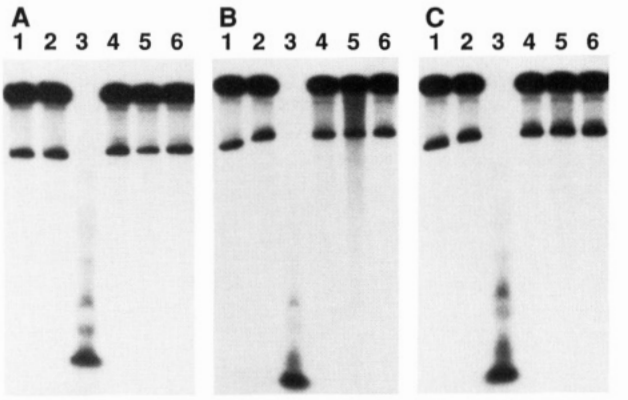

FIGURE 1: Autoradiogram of ^{32}P-labeled DNA fragments incubated with methylhydrazines in the presence of metal ions. The ^{32}P 5′ end-labeled 337 base pair fragment (*Pst*I 2345–*Ava*I* 2681) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 50 μM per base of sonicated calf thymus DNA was incubated with 20 μM metal ion and 0.5 mM monomethylhydrazine (A), 1,2-dimethylhydrazine (B), or 1,1-dimethylhydrazine (C) at 37 °C for 30 min. After being heated at 90 °C in 1 M piperidine for 20 min, the treated DNA fragments were electrophoresed on an 8% polyacrylamide–8 M urea gel (12 × 16 cm), and the autoradiogram was obtained by exposing X-ray film to the gel. Lane 1, no metal, no methyhydrazines; lane 2, no metal; lane 3, $CuCl_2$; lane 4, $MnCl_2$; lane 5, Mn(III)–pyrophosphate; lane 6, $FeCl_3$.

The Cu(II)-mediated DNA cleavage by methylhydrazines increased with time (Figure 2). Even without piperidine treatment, oligonucleotides were formed (Figure 2, lanes 4–6), suggesting the breakage of the deoxyribose phosphate backbone. The increased amount of oligonucleotides with piperidine treatment (Figure 2, lanes 1–3) suggests that base alteration and/or liberation was induced by methylhydrazines in the presence of Cu(II). The order of ability to induce DNA damage in the presence of Cu(II) was 1,2-dimethylhydrazine > monomethylhydrazine ~ 1,1-dimethylhydrazine.

Effects of Scavengers on DNA Damage Induced by 1,2-Dimethylhydrazine plus Mn(III) or Methylhydrazines plus Cu(II). Figure 3A shows the effects of •OH scavengers, SOD, and catalase on DNA damage by 1,2-dimethylhydrazine plus Mn(III). The DNA damage was inhibited by ethanol (lane 2), mannitol (lane 3), dimethyl sulfoxide (lane 4), or SOD (lane 5), whereas it was not inhibited by catalase (lane 6). Heat-denatured SOD did not inhibit the DNA damage. On the other hand, DNA damage by 1,2-dimethylhydrazine plus Cu(II) was inhibited by catalase, whereas it was not inhibited by •OH scavengers or SOD (Figure 3B). Heat-denatured catalase did not inhibit the DNA damage. In the case of monomethylhydrazine or 1,1-dimethylhydrazine, similar scavenger effects on Cu(II)-mediated DNA damage were observed (data not shown). Catalase activity, determined by measuring O_2 produced from H_2O_2, was not inhibited by the methylhydrazines under the conditions used (data not shown), although it was reported that some hydrazines inhibited

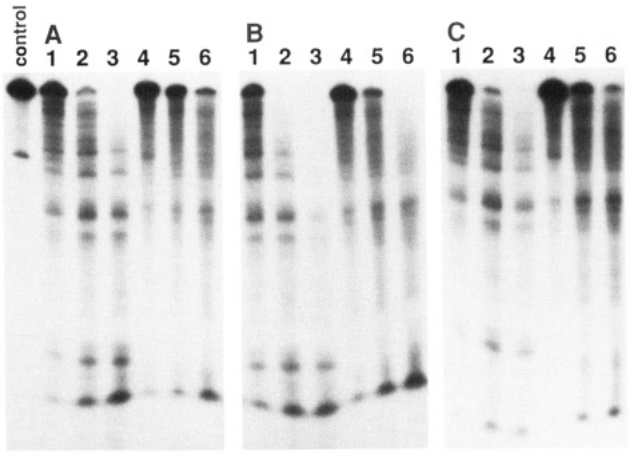

FIGURE 2: Time course of DNA cleavage induced by methylhydrazines in the presence of Cu(II). The ^{32}P 5′ end-labeled 337 base pair fragment (*Pst*I 2345–*Ava*I* 2681) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 50 μM per base of sonicated calf thymus DNA was incubated with 20 μM $CuCl_2$ and 0.5 mM monomethylhydrazine (A), 1,2-dimethylhydrazine (B), or 1,1-dimethylhydrazine (C) at 37 °C for the indicated durations. After the piperidine treatment (lanes 1–3), or without piperidine treatment (lanes 4–6), the treated DNA fragments were analyzed by the method described in the Figure 1 legend. Lane 1, 10 min; lane 2, 20 min; lane 3, 30 min; lane 4, 10 min; lane 50, 20 min; lane 6, 30 min; control lane, 30 min without methylhydrazines.

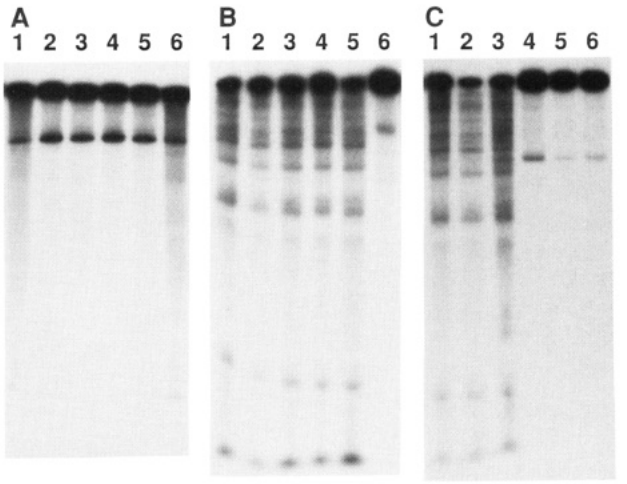

FIGURE 3: Effects of •OH scavengers, SOD, catalase, and bathocuproine on DNA cleavage induced by methylhydrazines in the presence of Cu(II) or Mn(III). (A) The ^{32}P 5′ end-labeled 337 base pair fragment (*Pst*I 2345–*Ava*I* 2681) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 5 μM per base of sonicated calf thymus DNA was incubated with 20 μM Mn(III)–pyrophosphate, scavenger, and 0.5 mM 1,2-dimethylhydrazine at 37 °C for 30 min. After the piperidine treatment, the DNA fragments were analyzed by the method described in the Figure 1 legend. Lane 1, no scavenger; lane 2, 0.8 M ethanol; lane 3, 0.1 M mannitol; lane 4, 0.8 M dimethyl sulfoxide; lane 5, 30 units of SOD; lane 6, 30 units of catalase. (B) The ^{32}P 5′ end-labeled fragment in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 50 μM per base of sonicated calf thymus DNA was incubated with 20 μM $CuCl_2$, scavenger, and 0.5 mM 1,2-dimethylhydrazine at 37 °C for 10 min. After the piperidine treatment, the DNA fragments were analyzed. Scavenger was added as described in (A). (C) The ^{32}P 5′ end-labeled fragment in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 50 μM per base of sonicated calf thymus DNA was incubated with 20 μM $CuCl_2$, and 0.5 mM each of methylhydrazines at 37 °C for 10 min. Where indicated, 50 μM bathocuproine was added. After the piperidine treatment, the DNA fragments were analyzed. Lane 1, $CuCl_2$ + monomethylhydrazine; lane 2, $CuCl_2$ + 1,2-dimethylhydrazine; lane 3, $CuCl_2$ + 1,1-dimethylhydrazine; lane 4, $CuCl_2$ + monomethylhydrazine + bathocuproine; lane 5, $CuCl_2$ + 1,2-dimethylhydrazine + bathocuproine; lane 6, $CuCl_2$ + 1,1-dimethylhydrazine + bathocuproine.

catalase (Ortiz de Montellano & Kerr, 1983).

The addition of bathocuproine, a Cu(I)-specific chelating agent, completely inhibited DNA damage by methylhydrazines plus Cu(II) (Figure 3C). The addition of DTPA of a concentration greater than that of Cu(II) also inhibited the DNA damage completely (data not shown).

Site Specificity of DNA Cleavage Induced by 1,2-Dimethylhydrazine plus Mn(III) or Methylhydrazines plus Cu(II). The ^{32}P 5′ end-labeled DNA fragments incubated with methylhydrazines in the presence of Cu(II) followed by the piperidine treatment were electrophoresed, and the autoradiogram was obtained as shown in Figure 4. The autoradiograms were scanned with a laser densitometer (Figure 5). The DNA cleavage sites were determined by using the procedure of Maxam and Gilbert (1980). 1,2-Dimethylhydrazine plus Cu(II) induced piperidine-labile sites frequently at thymine residues. The most preferred site is the thymine residue of the GTC sequence, followed by thymine residues between purine residues. Monomethylhydrazine or 1,1-dimethylhydrazine showed a Cu(II)-mediated DNA cleavage pattern similar to that of 1,2-dimethylhydrazine (Figure 4).

The site specificity of DNA cleavage induced by 1,2-dimethylhydrazine plus Mn(III) is shown in Figure 6. The DNA cleavage occurred at positions of every nucleotide without marked site specificity.

Autoxidation of Methylhydrazines in the Presence of Cu(II). Figure 7 shows the effect of Cu(II) on O_2 consumption during the autoxidation of methylhydrazines. When catalase was added, an immediate increase of O_2 concentration was observed, indicating that H_2O_2 was produced during the Cu(II)-catalyzed autoxidation. The order of ratio of H_2O_2 production to O_2 consumption was 1,2-dimethylhydrazine > monomethylhydrazine ~ 1,1-dimethylhydrazine.

Production of •CH_3 during the Autoxidation of Methylhydrazines in the Presence of Cu(II) or Mn(III). The spin-trapping method was used to detect free radicals produced during the autoxidation of methylhydrazines. Figure 8A shows an ESR spectrum of a spin adduct observed when monomethylhydrazine was added to a buffer solution containing POBN and Cu(II). The signals (α_N = 16.0 G, α_H = 2.7 G) can be assigned to the •CH_3 adduct of POBN (Buettner, 1987). Catalase did not inhibit the formation of POBN–CH_3 (data not shown). When 1,2-dimethylhydrazine or 1,1-dimethylhydrazine was added, POBN–CH_3 was also observed (spectra B and C). In the case of Mn(III), only monomethylhydrazine was activated to produce a small amount of POBN–CH_3 among the three methylhydrazines (spectrum D).

When DMPO was used instead of POBN, •CH_3 adduct was also observed. Figure 9A shows an ESR spectrum of a spin adduct observed when monomethylhydrazine was added to a buffer solution containing DMPO and Cu(II). The signals (α_N = 16.3 G, α_H = 23.3 G) can be assigned to DMPO–CH_3 (Buettner, 1987). Catalase did not inhibit the production of DMPO–CH_3 (spectrum B). When 1,2-dimethylhydrazine (spectrum C) or 1,1-dimethylhydrazine (spectrum D) was added instead of monomethylhydrazine, little DMPO–CH_3 was observed. The experiments using DMPO and POBN showed that the order of •CH_3 generation during Cu(II)-catalyzed autoxidation of methylhydrazines is monomethylhydrazine > 1,1-dimethylhydrazine ≫ 1,2-dimethylhydrazine.

Production of •OH during the Autoxidation of 1,2-Dimethylhydrazine in the Presence of Mn(III). Figure 10A shows an ESR spectrum of a spin adduct observed when DMPO was added to a buffer solution containing 1,2-di-

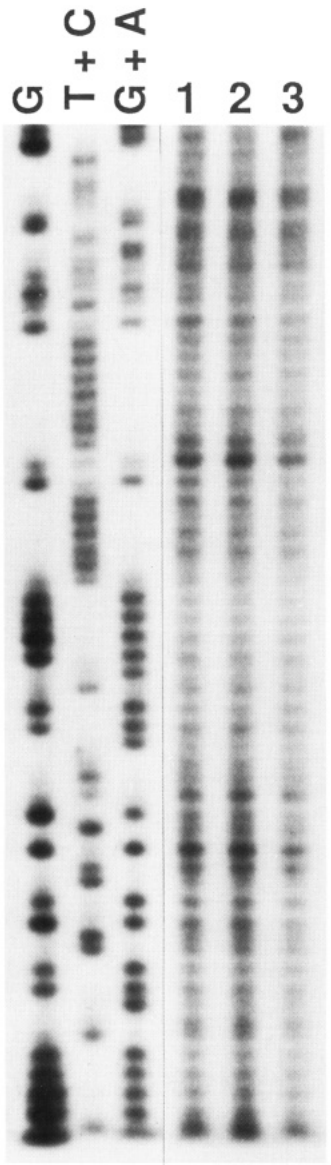

FIGURE 4: Site specificity of DNA cleavage induced by methylhydrazines plus Cu(II). The ^{32}P 5′ end-labeled 98 base pair fragment (*Ava*I* 2247–*Pst*I 2344) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 50 μM per base of sonicated calf thymus DNA was incubated with 20 μM $CuCl_2$ and 0.5 mM monomethylhydrazine (lane 1), 1,2-dimethylhydrazine (lane 2) or 1,1-dimethylhydrazine (lane 3) at 37 °C for 10 min. After the piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide–8 M urea gel using a DNA sequencing system, and the autoradiogram was obtained by exposing X-ray film to the gel. The lanes G, G + A, and T + C represent the patterns obtained for the same fragment after cleavage by the procedure of Maxam and Gilbert (1980).

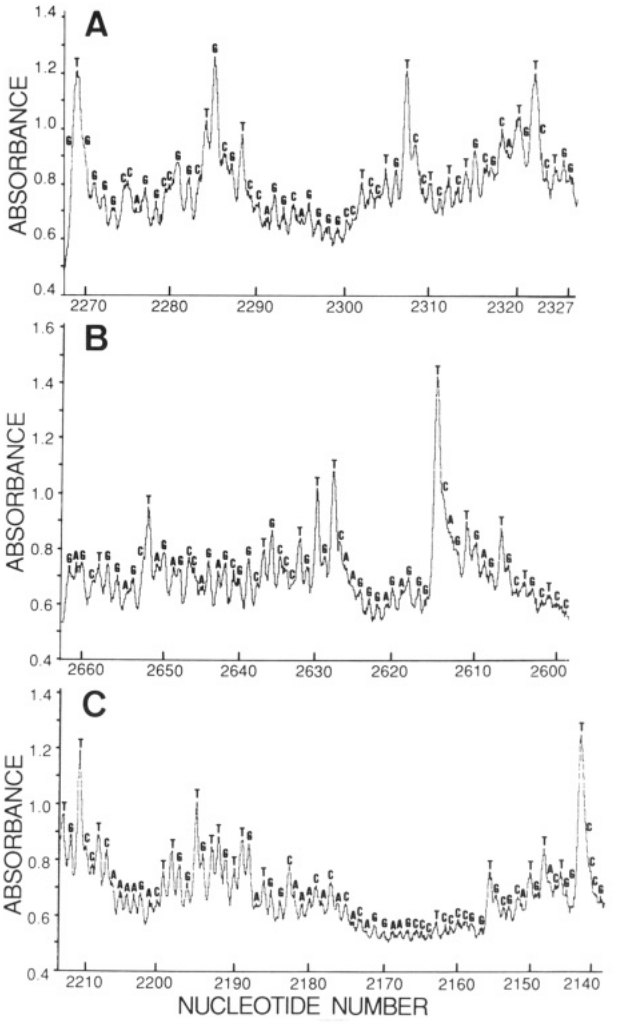

FIGURE 5: Site specificity of DNA cleavage induced by 1,2-dimethylhydrazine plus Cu(II). (A) The ^{32}P 5′ end-labeled 98 base pair fragment (*Ava*I* 2247–*Pst*I 2344) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 50 μM per base of sonicated calf thymus DNA was incubated with 0.5 mM 1,2-dimethylhydrazine plus 20 μM $CuCl_2$ at 37 °C for 10 min. (B) The ^{32}P 5′ end-labeled 337 base pair fragment (*Pst*I 2345–*Ava*I* 2681) was used. (C) The ^{32}P 5′ end-labeled 341 base pair fragment (*Xba*I 1906–*Ava*I* 2246) was used. After piperidine treatment, DNA fragments were electrophoresed, and the autoradiograms were obtained as described in the Figure 4 legend. The relative amounts of oligonucleotides produced were measured by a laser densitometer. *The horizontal axis*, the nucleotide number of human c-Ha-*ras*-1 protooncogene starting with *Bam*HI site (Capon et al., 1983).

methylhydrazine and Mn(III). The spin adduct ($\alpha_N = \alpha_H$ = 14.8 G) is assigned to the •OH adduct of DMPO by referring to the reported constants (Buettner, 1987). Addition of ethanol inhibited the yield of DMPO–OH, resulting in the appearance of new signals due to trapping of the α-hydroxyethyl radical (spectrum B), which would be produced by the reaction of ethanol with •OH (Inoue & Kawanishi, 1987; Kawanishi et al., 1986). SOD inhibited the yield of DMPO–OH by about 90% (spectrum C) whereas catalase did not inhibit it (spectrum D). Heat-denatured SOD inhibited it by about 20% (data not shown), which may be due to a nonspecific protein effect.

Discussion

The present results have shown that although methylhydrazines themselves do not cause DNA damage, they induce DNA damage in combination with Cu(II). 1,2-Dimethylhydrazine plus Mn(III) also induced DNA damage.

It is generally considered that •OH causes DNA cleavage uniformly at every nucleotide, regardless of sequence (Henner et al., 1982; Kawanishi et al., 1986; Inoue & Kawanishi, 1987). The present study showed that 1,2-dimethylhydrazine plus Mn(III) causes cleavage at every nucleotide without marked site specificity. It is speculated that the Mn(III)-mediated DNA cleavages are mainly caused by •OH. DNA damage induced by 1,2-dimethylhydrazine in the presence of Mn(III) was inhibited by •OH scavengers and SOD but not by catalase. Consistently, in ESR–spin-trapping experiment using DMPO, •OH production by 1,2-dimethylhydrazine plus Mn(III) was detected. The •OH production was inhibited by •OH scavengers (ethanol, mannitol, and dimethyl sulfoxide) and SOD but not by catalase. These results show that 1,2-dimethylhydrazine plus Mn(III) produces •OH, which causes DNA damage, via $O_2^{\cdot-}$—not via H_2O_2. The formation of •OH

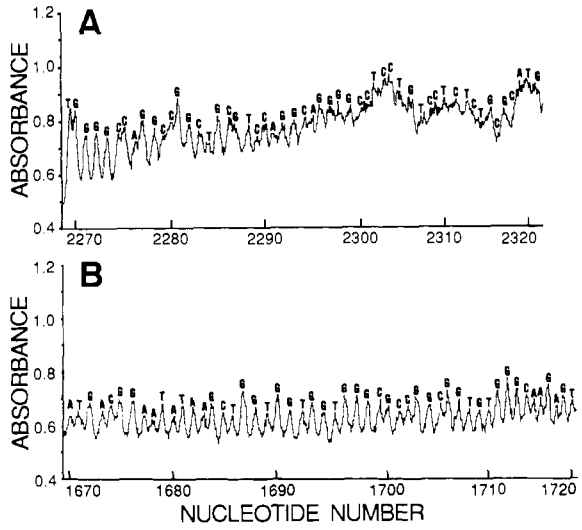

FIGURE 6: Site specificity of DNA cleavage induced by 1,2-dimethylhydrazine plus Mn(III). (A) The ^{32}P 5′ end-labeled 98 base pair fragment (*AvaI** 2247–*PstI* 2344) in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 5 μM per base of sonicated calf thymus DNA was incubated with 0.5 mM 1,2-dimethylhydrazine plus 20 μM Mn(III)–pyrophosphate at 37 °C for 30 min. (B) The ^{32}P 5′ end-labeled 261 base pair fragment (*AvaI** 1645–*XbaI* 1905) was used. After piperidine treatment, DNA fragments were analyzed as described in the Figure 5 legend.

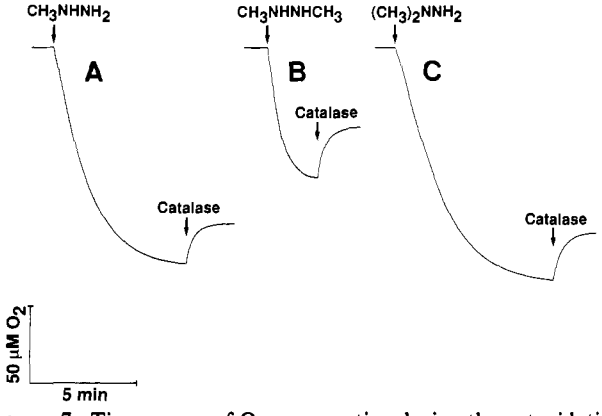

FIGURE 7: Time course of O_2 consumption during the autoxidation of methylhydrazines in the presence of Cu(II). The O_2 consumption was measured in a thermostated (37 °C) water-jacketed glass vessel, fitted with a Clark electrode (Gilson). One hundred micromolar monomethylhydrazine (A), 1,2-dimethylhydrazine (B), or 1,1-dimethylhydrazine (C) was added to 1.8 mL of 20 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA and 20 μM $CuCl_2$. After the O_2 consumption, 54 units of catalase was added.

without H_2O_2 is noteworthy, since it is generally accepted that •OH is generated by the metal-catalyzed decomposition of H_2O_2. Regarding the reaction mechanism, it can be speculated that during the Mn(III)-mediated autoxidation of 1,2-dimethylhydrazine, Mn(II) and O_2^- are generated to form MnO_2^+ and that the complex reacts with 1,2-dimethylhydrazine to generate •OH. Relevantly, Cabelli and Bielski (1984) proposed that Mn(II) reacts with O_2^- to form MnO_2^+. Further research is necessary to clarify the mechanism.

Methylhydrazines plus Cu(II) caused cleavage frequently at thymine residues especially of the GTC sequence. The site specificity cannot be explained by •OH. ESR–spin-trapping experiments showed that the $\bullet CH_3$ adduct is generated during the Cu(II)-catalyzed autoxidation of methylhydrazines. The $\bullet CH_3$ is a reactive species which has a potential to induce modification of biomolecules (Freeman & Crapo, 1982). Regarding DNA base alteration, $\bullet CH_3$ is nucleophilic and would not be expected to methylate guanine in the N^7-position

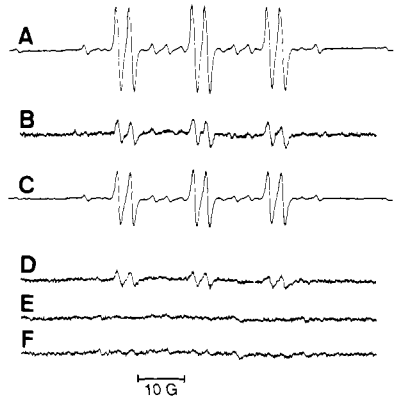

FIGURE 8: ESR spectra of the $\bullet CH_3$ adducts of POBN produced by methylhydrazines in the presence of Cu(II) or Mn(III). The samples contained 10 mM POBN and 5 μM DTPA in 100 μL of 20 mM sodium phosphate buffer at pH 7.9. Spectrum A, 20 μM $CuCl_2$ and 0.5 mM monomethylhydrazine were added; spectrum B, $CuCl_2$ and 0.5 mM 1,2-dimethylhydrazine were added; spectrum C, $CuCl_2$ and 0.5 mM 1,1-dimethylhydrazine were added: spectrum D, 20 μM Mn(III)–pyrophosphate and monomethylhydrazine were added; spectrum E, Mn(III)–pyrophosphate and 1,2-dimethylhydrazine were added; spectrum F, Mn(III)–pyrophosphate and 1,1-dimethylhydrazine were added. After incubation at 37 °C for 10 min, ESR spectra were measured at room temperature as described under Materials and Methods with a receiver gain of 100 (A, C) or 1000 (B, D, E, F).

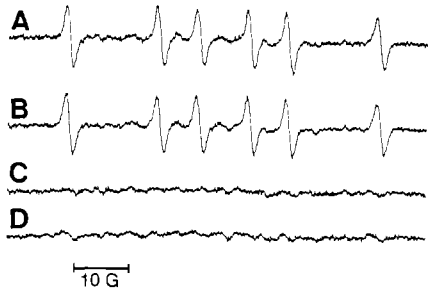

FIGURE 9: ESR spectra of the methyl radical adducts of DMPO produced by methylhydrazines in the presence of Cu(II). The samples contained 20 μM $CuCl_2$, 146 mM DMPO, and 5 μM DTPA in 100 μL of 20 mM sodium phosphate buffer at pH 7.9. Spectrum A, 0.5 mM monomethylhydrazine was added; spectrum B, monomethylhydrazine and 15 units of catalase were added; spectrum C, 0.5 mM 1,2-dimethylhydrazine was added; spectrum D, 0.5 mM 1,1-dimethylhydrazine was added. After incubation at 37 °C for 10 min, ESR spectra were measured at room temperature as described under Materials and Methods.

or the O^6-position (Kang et al., 1988). The $\bullet CH_3$ may be expected to abstract hydrogen atoms of deoxyribose, resulting in DNA backbone breakage. However, $\bullet CH_3$ is not considered to play an important role in Cu(II)-mediated methylhydrazine-dependent DNA damage on the basis of the following three results. First, the order of $\bullet CH_3$ generation during Cu(II)-catalyzed autoxidation of methylhydrazines was not correlated with the order of DNA damage. Second, catalase, which inhibited the Cu(II)-mediated DNA damage, did not inhibit $\bullet CH_3$ formation. Third, monomethylhydrazine plus Mn(III) produced $\bullet CH_3$ but did not cause DNA damage. Little importance of $\bullet CH_3$ in the Cu(II)-mediated DNA damage can be explained by assuming that reactive $\bullet CH_3$ reacts with O_2 to produce relatively inert formaldehyde (Klein et al., 1980) or undergoes dimerization to ethane (Kang et al., 1988). On the other hand, the inhibitory effects of bathocuproine and catalase on Cu(II)-mediated DNA damage indicate that Cu(I) and H_2O_2 have an important role in the production of active species causing DNA damage. The order of ability to induce Cu(II)-mediated DNA damage (1,2-dimethylhydrazine > monomethylhydrazine ~ 1,1-dimethyl-

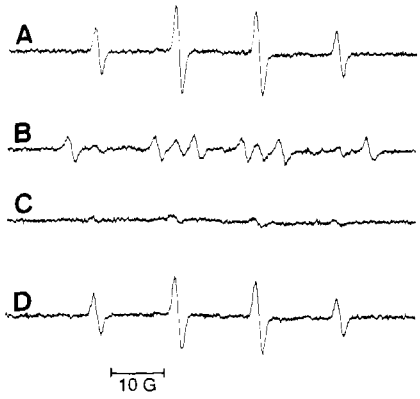

FIGURE 10: ESR spectra of the radical adducts of DMPO produced by 1,2-dimethylhydrazine plus Mn(III) in the presence of various scavengers. The samples contained 20 μM Mn(III)–pyrophosphate, 0.5 mM 1,2-dimethylhydrazine, 146 mM DMPO, and 5 μM DTPA in 100 μL of 20 mM sodium phosphate buffer at pH 7.9. Spectrum A, no scavenger was added; spectrum B, 0.8 M ethanol was added; spectrum C, 15 units of SOD was added; spectrum D, 15 units of catalase was added. After incubation at 37 °C for 10 min, ESR spectra were measured at room temperature as described under Materials and Methods.

hydrazine) was correlated with the ratio of H_2O_2 production to O_2 consumption observed during Cu(II)-catalyzed autoxidation of methylhydrazines. Our previous paper suggested that Cu(I) bound to DNA reacts with H_2O_2 to give ternary Cu(I)–peroxide complex, which causes DNA damage (Yamamoto & Kawanishi, 1989). Relevantly, Prütz et al. (1990) reported the formation of DNA–Cu(I) and the deleterious reaction with H_2O_2. Therefore, it is considered that such a ternary Cu(I)–peroxide complex participates in methylhydrazine plus Cu(II)-induced DNA damage.

Regarding the molecular mechanism of 1,2-dimethylhydrazine-induced cancer, the role of methylation of the DNA base, especially O^6-methylguanine formation through enzymatic activation, has been emphasized (Hawks & Magee, 1974; Bull et al., 1981; Bedell et al., 1982). However, it has been shown that 1,2-dimethylhydrazine is poor substrate for the purified microsomal FAD-containing monooxygenase (Prough et al., 1981) or cytochrome P-450 (Moloney et al., 1984). Furthermore, it has been demonstrated that the extent of DNA strand breakage in the colon correlates with the sensitivity to 1,2-dimethylhydrazine carcinogenesis in different mouse strains (Bolognesi & Boffa, 1986). It is generally accepted that reactions of free radicals with DNA may not lead to covalent carcinogen–DNA adducts but to other types of damage such as strand scission (Ts'o et al., 1977). Therefore, it can be speculated that methylhydrazines induce DNA damage through free radical or related active species produced by a nonenzymatic activation mechanism. It is noteworthy that during the Cu(II)-catalyzed oxidation, methylhydrazine generates $\cdot CH_3$ and H_2O_2 and that Cu(I) and H_2O_2 participate in site-specific cleavage of isolated DNA. Since it was reported that copper exists in nuclei and plays a key role in determining the DNA quaternary structure (Bryan et al., 1981; Lewis & Laemmli, 1982; George et al., 1987; Agarwal et al., 1989), the possibility of Cu(II)-mediated methylhydrazine-induced DNA damage in vivo can be considered. Regarding the molecular mechanism of 1,2-dimethylhydrazine-induced carcinogenesis, it was reported that manganese strongly enhanced sister-chromatid exchanges by 1,2-dimethylhydrazine (MacRae & Stich, 1979). Sun et al. (1988) suggested the involvement of O_2^- in 1,2-dimethylhydrazine-induced carcinogenesis. Relevantly, it is of interest to find that 1,2-dimethylhydrazine plus Mn(II) generates •OH via O_2^- and that the •OH causes DNA damage. A considerable amount of manganese exists in nuclei (Sakurai et al., 1985). Therefore, it can be speculated that manganese-mediated 1,2-dimethylhydrazine-induced DNA damage may be also relevant for the expression of the carcinogenic property of 1,2-dimethylhydrazine.

There are several reports concerning decreased incidence of 1,2-dimethylhydrazine-induced colon cancer in animals fed antioxidants such as vitamin E (Cook & McNamara, 1980), vitamin A (Newberne & Suphakarn, 1977), ascorbic acid (Reddy & Hirota, 1979), selenium (Jacobs et al., 1981), and disulfiram (Wattenberg, 1975). These papers may support the involvement of an active oxidant such as Cu(I)–peroxide complex and •OH in carcinogenesis. Collectively, the present study suggests that DNA damage by methylhydrazines in vivo is metal-mediated at least to some extent.

Registry No. Cu, 7440-50-8; Mn, 7439-96-5; •OH, 3352-57-6; $\cdot CH_3$, 2229-07-4; H_2O_2, 7722-84-1; monomethylhydrazine, 60-34-4; 1,1-dimethylhydrazine, 57-14-7; 1,2-dimethylhydrazine, 540-73-8.

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Interactions of Photoactive DNAs with Terminal Deoxynucleotidyl Transferase: Identification of Peptides in the DNA Binding Domain†

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ABSTRACT: Terminal deoxynucleotidyl transferase (terminal transferase) was specifically modified in the DNA binding site by a photoactive DNA substrate (hetero-40-mer duplex containing eight 5-azido-dUMP residues at one 3′ end). Under optimal photolabeling conditions, 27–40% of the DNA was covalently cross-linked to terminal transferase. The specificity of the DNA and protein interaction was demonstrated by protection of photolabeling at the DNA binding domain with natural DNA substrates. In order to recover high yields of modified peptides from limited amounts of starting material, protein modified with ^{32}P-labeled photoactive DNA and digested with trypsin was extracted 4 times with phenol followed by gel filtration chromatography. All peptides not cross-linked to DNA were extracted into the phenol phase while the photolyzed DNA and the covalently cross-linked peptides remained in the aqueous phase. The ^{32}P-containing peptide–DNA fraction was subjected to amino acid sequence analysis. Two sequences, Asp^{221}–Lys^{231} (peptide B8) and Cys^{234}–Lys^{249} (peptide B10), present in similar yield, were identified. Structure predictions placed the two peptides in an α-helical array of 39 Å which would accommodate a DNA helix span of 11 nucleotides. These peptides share sequence similarity with a region in DNA polymerase β that has been implicated in the binding of DNA template.

The events involved in the rearrangements of immunoglobulin and T-cell receptor genes appear to be mediated in part by terminal deoxynucleotidyl transferase (terminal transferase)[1] (Alt & Baltimore, 1982; Alt et al., 1986). This enzyme is non-template-directed and catalyzes the addition of deoxynucleoside triphosphates onto a 3′-OH group of a DNA initiator in a distributive manner. A 58-kDa polypeptide chain represents the primary translated sequence of this protein, and polymerase activity is the sole catalytic function identified with the protein. Terminal transferase can be cleaved by limited proteolysis to two small polypeptides, α (11 kDa) and β (33 kDa), both of which are required for catalytic activity (Deibel & Coleman, 1980a; Bollum & Chang, 1981; Chang et al., 1982).

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[1] Abbreviations: TdT, terminal deoxynucleotidyl transferase; azido-dU-DNA, random-sequence 40 base pair DNA that contained 5-azidouracil in place of thymidine; DS DNA, nonradioactive DNA(a) shown in Figure 1 in which 5-azidouracil is replaced by thymidine; TS DNA, template strand of DS DNA; CS DNA, complementary strand to TS DNA; 8-azido-dATP, 8-azido-2′-deoxyadenosine 5′-triphosphate; 5-azido-dUTP, 5-azido-2′-deoxyuridine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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