Hydroxyl Free Radical Is Not the Main Active Species in Site-specific DNA Damage Induced by Copper(II) Ion and Hydrogen Peroxide*

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Site-specific DNA damage by Cu(II) plus H₂O₂ was investigated by a DNA-sequencing technique. Cu(II) plus H₂O₂ induced strong DNA cleavage even without piperidine treatment. Piperidine-labile sites were induced frequently at thymine and guanine residues and rarely at adenine residue. A Cu(I)-specific chelating agent, bathocuproine, inhibited the DNA damage. Neither ethanol nor mannitol inhibited it. Of alcohols, tert-butyl alcohol, having relatively low reactivity to hydroxyl free radical, inhibited the DNA damage most strongly. Sodium azide and 1,4-diazobicyclo[2.2.2]octane completely inhibited cleavages at residues of the bases other than guanine. Tris inhibited the DNA damage. The enhancing effect of D₂O on DNA damage was not observed. ESR studies using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) showed that the hydroxyl radical adduct of DMPO was formed during the reaction of Cu(II) with H₂O₂, and that the addition of sodium formate produced the CO₂ radical adduct of DMPO more efficiently than expected. ESR studies showed that the nitroxide radical was formed from 2,2,6,6-tetramethyl-4-piperidone in the presence of Cu(II) plus H₂O₂, indicating the formation of singlet oxygen or its equivalent. The effects of scavengers on DNA damage have considerable correlation with the effects of scavengers on the nitroxide radical production and DMPO-OH formation. The results suggest that the main active species causing DNA damage are more likely copper-peroxide complexes, with similar reactivity to singlet oxygen and/or hydroxyl radical rather than hydroxyl free radical.

Recently, the biochemistry of oxygen activation and the biological significance of the reactive oxygen species have drawn much interest (1, 2). Hydroxyl free radicals are generated from H₂O₂ by means of Fenton reaction with reduced iron, but whether copper acts like iron remains to be clarified (3). The observation that DNA cleavage is efficiently induced by the treatment with Cu(II) ion, H₂O₂, and various reducing agents has stimulated interest in the mechanism of the reaction of Cu(I) with H₂O₂ (5, 6). It has been suggested that the Cu(I) complex binds with DNA, and subsequent oxidation by H₂O₂ causes the damage due to the hydroxyl free radicals at the binding site (4–7). It was reported that hydroxyl free radical was involved in the reaction of supercoiled plasmid DNA with Cu(II) plus H₂O₂ (8). However, recent studies on the reaction of H₂O₂ with the Cu(I) ion or Cu(I) complex have indicated that under some conditions hydroxyl free radicals are not formed (9, 10).

In this study, we examined site specificity of Cu(II)-induced DNA cleavage in the presence of H₂O₂ by using ²P 5´ end-labeled DNA fragments of defined sequence and investigated the participation of activated oxygen species in the reaction mechanism using an ESR spin-trapping technique.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes (BstEII, Aval, XbaI, PstI) and T₄ polynucleotide kinase were purchased from New England BioLabs. [²P]ATP (6000 Ci/mmol) was supplied by Du Pont-New England Nuclear. DTPA, CuCl₂, sodium formate, sodium azide, Tris, d-mannitol, and alcohols were purchased from Nakarai Chemicals Co., Kyoto, Japan. A solution of 30% H₂O₂ was obtained from Sankoutu Chemical Industries Co., Miyagi, Japan. DMPO, 4-POBN, 2,2,6,6-tetramethyl-4-piperidone hydrochloride, 4-0xo-2,2,6,6-tetramethyl-1-piperidinyloxy, and Dabco were purchased from Aldrich. Bathocuproine disulfonic acid, 2Na salt was obtained from Wako Chemicals Co., Osaka, Japan. D₂O (99.95%) was obtained from Commissariat à l’énergie atomique in France.

Subcloning of Aval Fragments of c-Ha-ras-1 Protooncogene—Plasmid pbcNI, which carries a 6.6-kilobase BamHI chromosomal DNA segment containing human c-Ha-ras-1 protooncogene, was purchased from the American Type Culture Collection (11, 12). The plasmid was digested with BstEII and Aval, and the resulting DNA fragments were fractionated by electrophoresis on 3% agarose gels. A 602-base pair Aval fragment (Aval 1645–Aval 2246) and a 435-base pair Aval fragment (Aval 2247–Aval 2681) were ligated into Smal-cleaved pUC18 plasmid and then transferred to Escherichia coli MC1061.

Preparation of ²P 5´ end-labeled DNA Fragments—The plasmid pUC18 was digested with Aval, and the resulting DNA fragments were fractionated by electrophoresis on 3% agarose gels. An Aval fragment (Aval 1645–Aval 2246) and an Aval fragment (Aval 2247–Aval 2681) were labeled at the 5´ termini with [²P]ATP and T₄ polynucleotide kinase (13–15). The ²P 5´ end-labeled 602-base pair Aval fragment was again digested with XbaI to obtain a singly labeled 261-base pair fragment (Aval 1645–XbaI 1905) and a singly labeled 341-base pair fragment (XbaI 1906–Aval 2246). The ²P 5´ end-labeled 435-base pair Aval fragment was further digested with PstI to obtain a singly labeled 98-base pair fragment (Aval 2247–PstI 2344) and a singly labeled 337-base pair fragment (PstI 2345–Aval 2681). The asterisk indicates ³²P-labeling, and nucleotide numbering starts with the BamHI site (12).

Analysis of DNA Damage Induced by Cu(II) plus H₂O₂—The standard reaction mixture in a microtub (1.5-ml Eppendorf) contained 0.2 mM H₂O₂, 20 mM CuCl₂, and [²P]DNA fragment in 200 μl of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA. After incubation at 37 °C for indicated durations, the DNA fragments were electrophoresed using a 12 × 16-cm slab gel, and the autoradiograms

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‡ The abbreviations used are: DTPA, diethylenetriaminepentaaetetic acid; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-OH, hydroxyl radical adduct of 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-CO₂; CO₂ radical adduct of 5,5-dimethyl-1-pyrroline-N-oxide; 4-POBN, α-(4-pyridyl 1-oxide)-N-tert-butylnitroso; Dabco, 1,4-diazobicyclo[2.2.2]octane.

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were obtained by overnight exposure of x-ray film to the gels at −85 °C described previously (13-15).

The preferred cleavage sites by Cu(II) plus H_2O_2 were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure (16) using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltraScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

ESR Spectra Measurements—ESR spectra were measured at room temperature using a JES-FE-3XG spectrometer with 100-kHz field modulation according to the method described previously (15, 17). Spectra were recorded with a modulation amplitude of 1.0 G. The magnetic fields were calculated by the splitting of Mn^{2+} in MgO (\Delta H_m = 86.9 G).

The relative yield of singlet oxygen or its equivalent was measured by using 2,2,6,6-tetramethyl-4-piperidone (15, 17). Typical sample (100 μL) contained 20 mM sodium phosphate buffer (pH 7.9), 20 μM CuCl_2, 8 mM H_2O_2, and 100 mM 2,2,6,6-tetramethyl-4-piperidone. The relative yield of radicals was measured by using DMPO and 4-POBN (15). Typical sample (100 μL) contained 20 mM sodium phosphate buffer (pH 7.9), 10 μM CuCl_2, 8 mM H_2O_2, and 146 mM DMPO. Where indicated, scavenger was added to the sample solution.

RESULTS

Cleavages of 32P-labeled DNA Fragments Induced by Cu(II) plus H_2O_2—The extent of DNA damage was estimated by gel electrophoretic analysis. Fig. 1 shows the autoradiogram of double-stranded DNA fragments treated with Cu(II) plus H_2O_2. Oligonucleotides were clearly detected on the autoradiogram as a result of Cu(II) plus hydrogen peroxide-induced DNA cleavage. Cu(II) or H_2O_2 alone caused no DNA damage. The cleavage increased with time (Fig. 1) and with the concentration of H_2O_2 (data not shown). The cleavage without piperidine treatment suggests the breakages of deoxyribose phosphate backbone by active species (Fig. 1B). The increased amount of oligonucleotides with piperidine treatment (Fig. 1A) suggests that the base alterations and/or liberations were induced by Cu(II) plus H_2O_2.

Effects of Concentration of Bathocuproine on DNA Damage Induced by Cu(II) plus H_2O_2—Fig. 2 shows the effects of the bathocuproine concentration on DNA damage induced by Cu(II) plus H_2O_2. When 40 μM bathocuproine was added to the reaction solution containing 20 μM Cu(II), DNA damage was completely inhibited. Since bathocuproine is a specific chelating agent for Cu(II), Cu(II) is considered to participate in DNA damage.

When DTPA in a concentration of at least 20 μM was added to the reaction solution containing 20 μM Cu(II), DNA cleavage was inhibited (data not shown). The results suggest that Cu(II) binds to DNA and reacts with H_2O_2, resulting in DNA damage.

Site Specificity of DNA Damage Induced by the Treatment with Cu(II) plus H_2O_2—To estimate the site specificity of DNA damage by Cu(II) plus H_2O_2, 5'P 5'-end-labeled DNA fragments treated with Cu(II) plus H_2O_2 followed by the piperidine treatment were electrophoresed, and the autoradiogram was obtained as shown in Fig. 3. The autoradiogram was scanned with a laser densitometer (Fig. 4). The DNA cleavage sites were determined by using the Maxam-Gilbert procedure (16). Cu(II) plus H_2O_2 induced piperidine-labile sites frequently at thymine and guanine residues especially located at 5' to guanine residues and rarely at adenine residue. The cleavages at the positions of cytosine were variable according to the sequence.

Effects of Scavengers on DNA Damage Induced by Cu(II) plus H_2O_2—Fig. 5 shows the effects of various scavengers of singlet oxygen and hydroxyl radical on DNA damage induced by Cu(II) plus H_2O_2. Addition of 0.05 M sodium azide (Fig. 5A, lane 2) and 0.2 M Dabco (Fig. 5A, lane 3) inhibited DNA damage. None of the 0.8 M ethanol (Fig. 5A, lane 4), 0.2 M mannitol (Fig. 5A, lane 5), or 0.2 M sodium formate (Fig. 5A, lane 6) inhibited it. Tris inhibited DNA damage (Fig. 5B). Table I summarizes the inhibitory effects of various scavengers on DNA damage induced by Cu(II) plus H_2O_2 and rate constants in reactions of various scavengers with hydroxyl free radical. tert-Butyl alcohol, which has been reported to have a relatively small rate constant in reaction with hydroxyl free radical (18), inhibited the DNA damage most strongly. The results indicate that hydroxyl free radical is not the main active species in DNA damage induced by Cu(II) plus H_2O_2.

The effects of singlet oxygen scavengers on the site-specific DNA damage.
DNA Damage by Cu(II) Plus $H_2O_2$

Fig. 3. Site specificity of DNA cleavage induced by Cu(II) plus $H_2O_2$. The $^3P$ 5' end-labeled 98-base pair fragment (Avul$^*$
2247—PstI 2344) in 200 $\mu l$ of 10 mM sodium phosphate buffer at pH 7.9 containing 5 $\mu M$ DTPA was incubated with 0.4 mM $H_2O_2$ plus 20 $\mu M$ CuCl$_2$ at 37°C for 10 min. After the piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide, 8 $M$ urea gel, and the autoradiogram was obtained by exposing x-ray film to the gel. The large G, G + A, and T + C represent the patterns obtained for the same fragment after cleavage by the chemical methods of Maxam and Gilbert (16). The nucleotide number of human c-Ha-ras-1 protooncogene starts with the BamHI site (12).

Fig. 4. Site specificity of DNA cleavage induced by Cu(II) plus $H_2O_2$. A, the $^3P$ 5' end-labeled 261-base pair fragment (Avul$^*$
1645—XbaI 1906) in 200 $\mu l$ of 10 mM sodium phosphate buffer at pH 7.9 containing 5 $\mu M$ DTPA was incubated with 0.2 mM $H_2O_2$ plus 10 $\mu M$ CuCl$_2$ at 37°C for 6 min. B, the $^3P$ 5' end-labeled 342-base pair fragment (XbaI 1906—Avul$^*$ 2246) was used. After the piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide, 8 $M$ urea gel, and the autoradiogram was obtained by exposing x-ray film to the gel. The relative amounts of oligonucleotides produced were measured by a laser densitometer (LKB 2222 UltroScan XL). The piperidine-labile sites of the treated DNA were determined by direct comparison with the same DNA fragment after undergoing DNA sequence reaction according to the Maxam-Gilbert procedure (16). The horizontal axis, the nucleotide number of human c-Ha-ras-1 protooncogene starting with the BamHI site (12). Underlining, the 12th codon of human c-Ha-ras-1 protooncogene.

Fig. 5. Production of the Nitroxide Radical during the Reaction of Cu(II) with $H_2O_2$. The reaction mixture contained 2,2,6,6-tetramethyl-4-piperidone—Fig. 5a shows that nitroxide radical was produced from 2,2,6,6-tetramethyl-4-piperidine during the reaction of Cu(II) with $H_2O_2$. The spectrum of the nitroxide radical was reported by Kalyanaraman et al. (20) and Kremers and Singh (21). The formation of DMPO-OH was inhibited completely by Dabco (Fig. 7c), partially by ethanol (Fig. 7d), and little by mannitol (Fig. 7e). Fig. 7f shows that in spite of little inhibitory effect, the addition of sodium formate resulted in the appearance of CO$_3^-$ radical adduct of DMPO with $a_N = 15.6$ G and $a_H = 18.7$ G (22).

Tris inhibited the DMPO-OH formation considerably (Fig. 7g). When 4-POBN was used instead of DMPO, spectrum of the spin adduct showed hyperfine splitting from one nitrogen and one hydrogen: $a_N = 15.4$ G, $a_H = 2.8$ G (Fig. 7h). Since this signal was dependent upon the presence of Tris, it may be assigned to the Tris-derived carbon-centered radical adduct of 4-POBN by reference to the reported constants (18, 23, 24).

There was a considerable correlation between the effects of scavengers on DMPO-OH formation and those on DNA damage.

Production of the Nitroxide Radical during the Reaction of Cu(II) with $H_2O_2$ in the Presence of 2,2,6,6-Tetramethyl-4-piperidine—Fig. 8a shows that nitroxide radical was produced from 2,2,6,6-tetramethyl-4-piperidine during the reaction of Cu(II) with $H_2O_2$. The spectrum of the nitroxide radical was
Fig. 5. Effects of singlet oxygen scavengers and hydroxyl radical scavengers on DNA cleavage induced by Cu(II) plus H₂O₂. A, the reaction mixture contained the ³²P 5’ end-labeled 337-base pair fragment (PstI 2345–AvaI* 2681), 20 μM CuCl₂, 0.4 mM H₂O₂, 5 μM DTPA in 200 μl of 10 mM sodium phosphate buffer at pH 7.9. Singlet oxygen scavenger or hydroxyl radical scavenger was added where indicated. Lane 1, no scavenger; lane 2, 0.05 mM sodium azide; lane 3, 0.2 M Dabco; lane 4, 0.8 M ethanol; lane 5, 0.2 M mannitol; lane 6, 0.2 M sodium formate. After incubation at 37 °C for 12 min followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the Fig. 1 legend. B, the reaction mixture contained the ³²P 5’ end-labeled 341-base pair fragment (XbaI 1906–AvaI* 2246), 20 μM CuCl₂, 0.4 mM H₂O₂, and 5 μM DTPA in 200 μl of 10 mM sodium phosphate buffer at pH 7.9. Tris was added where indicated in the following mM concentrations: lane 1, 0; lane 2, 15; lane 3, 50. After incubation at 37 °C for 10 min followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the Fig. 1 legend.

Table I
Effects of alcohols on DNA damage induced by Cu(II) plus H₂O₂.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>Rate of reaction with ·OH⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>0.8 %</td>
<td>0 , 6.1 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0</td>
<td>1, 0</td>
<td></td>
</tr>
<tr>
<td>1-Propanol</td>
<td>0.8 %</td>
<td>10 , 16.5 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>2-Propanol</td>
<td>0.8 %</td>
<td>25, 12 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>1-Butanol</td>
<td>0</td>
<td>1 , 22 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>2-Butanol</td>
<td>0.8 %</td>
<td>30 , 14 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>tert-Butyl</td>
<td>0.8 %</td>
<td>60 , 2.8 × 10⁻⁶</td>
<td></td>
</tr>
</tbody>
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*From Ref. 18.

identical with that of 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy. Neither Cu(II) nor H₂O₂ alone produced significant amounts of the nitroxide radical. The nitroxide production was inhibited by 0.05 mM sodium azide (Fig. 8b) and 0.4 M Dabco (Fig. 8c) but not by 0.8 mM ethanol (Fig. 8d), 0.4 M mannitol (Fig. 8e), or 0.4 M sodium formate (Fig. 8f). Tris inhibited nitroxide production (Fig. 8g), whereas superoxide dismutase (15 units) did not (data not shown).

There was a considerable correlation between the effects of scavengers on the nitroxide production and those on DNA damage.

**DISCUSSION**

The present study has shown that the Cu(II) ion binds to DNA and subsequently reacts with H₂O₂ to cause strong DNA damage. The DNA-sequencing experiments on DNA fragments treated with Cu(II) plus H₂O₂ revealed that thymine and guanine residues especially located at 5’ to guanine residue were the piperidine-labile sites, and the extent of cleavages at the positions of cytosine residue(s) was dependent on the sequence. Adenine was resistive. These results suggest that the order of base alterations and/or liberation is thymine > guanine > cytosine > adenine. In previous papers, we proposed that hydroxyl radicals cause cleavages at every nucleotide with a little stronger cleavages at positions of every guanine and thymine (14) and that singlet oxygen causes alterations at positions of every guanine (15, 17). The present result may not be fully explained on the basis of the site specificity of DNA cleavage by hydroxyl radicals (14) or singlet oxygen only (15, 17). The inhibitory effects of various alcohols on DNA cleavage induced by Cu(II) plus H₂O₂ were not correlated with the rate constants in reaction of various alcohols with hydroxyl free radicals. The result suggests that the participation of hydroxyl free radicals in DNA damage is small, if any.

ESR studies using DMPO showed that the hydroxyl radical...
adduct of DMPO was formed during the reaction of Cu(II) with H$_2$O$_2$ and that the addition of sodium formate produced the CO$_2$ radical adduct of DMPO more efficiently than expected for the reaction of hydroxyl free radical. Both the great yield of DMPO-CO$_2$ and little inhibition of DMPO-OH formation by formate suggest that the active species is hydroxyl radical bound in some way to copper rather than hydroxyl free radical. Relevantly, Youngman (25) has regarded metal-oxygen complex as complexed hydroxyl radical. Azide, Dabco, and Tris, which inhibited the DNA damage, showed the inhibitory effects on the nitroxide production and subsequently sequence-specific DNA cleavage and subsequent sequence-specific DNA cleavage. The present ESR data show that Cu(II) added to H$_2$O$_2$ converts azide anion to azide radical, which is known to be a strong one-electron oxidant (30). Then it is presumed that sodium azide and Dabco may react with copper-peroxide complexes to produce active species such as nitrogen-centered radicals, some of which oxidize guanine more readily than other bases. Relevantly, Nakayama et al. (31) reported that the energy level of the highest occupied molecular orbital of guanine is highest among the nucleic acid bases, and accordingly, guanine is oxidized most easily. Similarly, Tris caused the strong inhibition of DNA damage and subsequent sequence-specific DNA damage.

Fig. 7. ESR spectra of the radical adduct of DMPO or 4-POBN produced from H$_2$O$_2$ catalyzed by Cu(II) in the presence of various scavengers. Spectrum a, the sample (100 µl) contained 8 mM H$_2$O$_2$ and 20 µM CuCl$_2$ in 20 mM sodium phosphate buffer at pH 7.9; spectrum b, 0.05 M sodium azide was added; spectrum c, 0.2 M Dabco was added; spectrum d, 0.8 M ethanol was added; spectrum e, 0.2 M mannitol was added; spectrum f, 0.2 M sodium formate was added, and the spectrum is assigned to a mixture of DMPO-OH (●) and DMPO·CO$_2$ (○); spectrum g, 0.05 M Tris was added; spectrum h, the sample contained 8 mM H$_2$O$_2$, 20 µM CuCl$_2$, and 0.05 M Tris in 20 mM sodium phosphate buffer at pH 7.9. After the addition of 146 mM DMPO (spectra a-g) or after addition of 100 mM 4-POBN and incubation at 37°C for 10 min, aliquots of the solution were taken in a calibrated capillary, and ESR spectra were measured at room temperature as described under "Experimental Procedures."

Fig. 8. ESR spectra of nitroxide radical formed from 2,2,6,6-tetramethyl-4-piperidone during the reaction of the Cu(II) ion with H$_2$O$_2$ in the presence of various scavengers. Spectrum a, the sample (100 µl) contained 8 mM H$_2$O$_2$ and 20 µM CuCl$_2$ in 20 mM sodium phosphate buffer at pH 7.9; to the remaining spectra, the following additions were made: spectrum b, 0.05 M sodium azide; spectrum c, 0.4 M Dabco; spectrum d, 0.8 M ethanol; spectrum e, 0.4 M mannitol; spectrum f, 0.4 M sodium formate; spectrum g, 0.3 M Tris. After the addition of 100 mM 2,2,6,6-tetramethyl-4-piperidone and incubation at 37°C for 10 min, aliquots of the solution were taken in a calibrated capillary, and ESR spectra were measured at room temperature as described under "Experimental Procedures."

An ESR method of detecting singlet oxygen production has been reported by Lion et al. (27; see also 26). It is based on the reaction of singlet oxygen with sterically hindered 2,2,6,6-tetramethyl-4-piperidone leading to a stable nitroxide free radical. We reported previously that this method is useful for detecting singlet oxygen (15, 17, 28). The present result showed that the nitroxide is produced during the reaction of Cu(II) with H$_2$O$_2$ and that it was markedly decreased by sodium azide, Dabco, and Tris, which are known as singlet oxygen scavengers (29). The correlation between the inhibitory effects of scavengers on the nitroxide production and those on the DNA cleavage lead us to speculate that singlet oxygen is involved in DNA damage. However, no enhancing effect of D$_2$O on the DNA damage indicates that the DNA damage is caused by an equivalent of singlet oxygen, which may be termed as bound singlet oxygen, rather than singlet oxygen. Copper-peroxide complexes may be considered to be such bound singlet oxygen.

Sodium azide and Dabco completely inhibited the cleavages at thymine, cytosine, and adenine residues and resulted in preferring cleavage at polyguanosine sequences. Because singlet oxygen causes specific alteration at guanine residues (15, 17), it seems unlikely that a singlet oxygen scavenger did not inhibit the cleavage at guanine residues. The present ESR data show that Cu(II) added to H$_2$O$_2$ converts azide anion to azide radical, which is known to be a strong one-electron oxidant (30). Then it is presumed that sodium azide and Dabco may react with copper-peroxide complexes to produce active species such as nitrogen-centered radicals, some of which oxidize guanine more readily than other bases. Relevantly, Nakayama et al. (31) reported that the energy level of the highest occupied molecular orbital of guanine is highest among the nucleic acid bases, and accordingly, guanine is oxidized most easily. Similarly, Tris caused the strong inhibition of DNA damage and subsequent sequence-specific DNA damage
cleavages. We speculate that Tris can react with copper-peroxide complexes to produce the carbon-centered radicals that attack the thymine residue of the TGG sequence. This is supported by ESR data that showed that Tris inhibits both DMPO·OH formation and nitrooxide radical production and that carbon-centered radicals are derived from Tris.

It has been assumed (32–34) that H2O2 reduces Cu(II) to Cu(I) followed by the reaction of Cu(I) with H2O2 and the formation of hydroxyl free radicals as shown in Equations 1 and 2.

\[
\text{Cu}^{(II)} + \text{H}_2\text{O}_2 \rightleftharpoons \text{Cu}^{(I)} + \text{HO}_2 + \text{H}^+ \quad (1)
\]

\[
\text{Cu}^{(I)} + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^- + \text{Cu}^{(II)} \quad (2)
\]

The inhibitory effect of bathocuproine on DNA damage suggests that an interconversion of Cu(II) and Cu(I) has an important role in the DNA damage. In the ESR studies using DMPO, the superoxide radical adduct of DMPO was not detected. The result lowers the possibility of Equation 1.

Relevantly, Barb et al. (35) reported that the catalytic decomposition of H2O2 by Cu(II) was much slower than that by Fe(III). Alternatively, the possible mechanism of Equations 3–7 can be envisioned as accounting for most of the observations.

Equation 3.

\[
\text{DNA} + 2\text{Cu}^{(II)} \rightarrow \text{DNA} + 2\text{Cu}^{(I)}
\]

Equation 4.

\[
\text{Cu}^{(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{(I)} + \text{HO}_2 + \text{H}^+ \quad (4)
\]

Equation 5.

\[
\text{Cu}^{(II)} \rightarrow \text{Cu}^{(I)} + \text{O}^\cdot \quad (5)
\]

Equation 6.

\[
\text{Cu}^{(II)} \rightarrow \text{Cu}^{(I)} + \text{O}_2 + \text{H}^+ \quad (6)
\]

Equation 7.

\[
\text{DNA} \rightarrow \text{DNA} \quad (7)
\]

Cu(II) ion binds to DNA (Equation 3). Addition of DTPA of molar concentration greater than that of Cu(II) ion inhibited the DNA damage, indicating that Cu(I) bound to DNA is necessary for hydrogen peroxide-dependent DNA damage. Two Cu(II) ions bound to DNA react with H2O2 resulting in the formation of copper-peroxide complex, which may participate in DNA damage to some extent (Equation 4). Relevantly, Karlin et al. (36) reported that a hydroperoxo-diocuppero complex can undergo transfer of a single oxygen atom to the substrate. Equation 4 may be supported by the kinetic study of the binuclear Cu(II) complexes, which showed high catalytic activities for the decomposition of H2O2 (37). The formation of dioxynitrogen could occur by the sequential decomposition of the complex (Equation 5). The possibility that the dioxynitrogen contains singlet oxygen cannot be excluded (38).

Two electron transfer in Equation 5 is considered to be more favorable than one-electron transfer in Equation 1 (39, 40). Cu(I) reacts with H2O2 to give a ternary Cu(I)-peroxide complex (Equation 6), which may cause DNA damage (Equation 7). Equations 6 and 7 may be supported by a recent report that the reaction of Cu(I) with H2O2 yields Cu(I)-OOH (10).

Further research is necessary to confirm these mechanisms.

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REFERENCES


DNA (7).

\[
\text{Cu}^{(II)} \rightarrow \text{Cu}^{(I)} \rightarrow \text{Cu}^{(I)} - \text{OH} \rightarrow \text{Cu}^{(I)} - \text{OH}^+ \rightarrow \text{Cu}^{(I)} \rightarrow \text{Cu}^{(I)} \rightarrow \text{Cu}^{(I)} \rightarrow \text{Cu}^{(I)}
\]