# Site-Specific DNA Damage Induced by NADH in the Presence of Copper(II): Role of Active Oxygen Species†

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ABSTRACT: Oxidative DNA damage by NAD(P)H in the presence of metal ions has been characterized by using $^{32}$P 5′ end-labeled DNA fragments obtained from human p53 tumor suppressor gene and c-Ha-*ras*-1 protooncogene. NADH, as well as other endogenous reductants, induced DNA damage in the presence of Cu(II). The order of inducing effect on Cu(II)-dependent DNA damage was ascorbate > reduced glutathione (GSH) > NADH > NADPH. Although NADH caused no or little DNA damage in the presence of Fe(III)-EDTA, the addition of $H_2O_2$ induced the DNA damage. The Cu(II)-mediated DNA damage induced by NADH was inhibited by catalase and bathocuproine, a Cu(I)-specific chelator; but not by scavengers of hydroxyl free radical (•OH), suggesting the involvement of active species derived from hydrogen peroxide ($H_2O_2$) and Cu(I) rather than •OH. The predominant cleavage sites were thymine residues located 5′ and/or 3′ to guanine. The cleavage pattern was similar to that induced by Cu(II) plus GSH, Cu(II) plus ascorbate, or Cu(I) plus $H_2O_2$. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine by NADH increased with its concentration in the presence of Cu(II). UV−visible spectroscopy indicated the facilitation of reduction of Cu(II) by NADH under some conditions. ESR spin-trapping experiments and mass spectrometry showed that the carbon-centered radical was formed during the reaction of NADH with Cu(II). These results suggest that optimal molar ratios of DNA/metal ion yield copper with a high redox potential which catalyzes NADH autoxidation to NAD• being further oxidized to $NAD^+$ with generation of superoxide radical and that $H_2O_2$ reacts with Cu(I) to form active oxygen species such as copper(I)−peroxide complex causing DNA damage.

The biological significance of oxygen free radicals has recently attracted much interest (Halliwell & Gutteridge, 1990), especially in connection with carcinogenesis (Tchou & Grollman, 1993; Floyd, 1990; Feig *et al*., 1994). Excess generation of these radicals in mammalian cells results in damage to DNA (Dizdaroglu, 1992). Fe(II) reacts with hydrogen peroxide ($H_2O_2$) to generate hydroxyl free radical (•OH). On the other hand, recent studies on the reaction of $H_2O_2$ with Cu(I) have suggested that Cu(III) or copper(I)−peroxide [$Cu^{I}OOH$] is formed as a reactive intermediate (Masarwa *et al*., 1988). We have suggested that the main active species causing DNA damage induced by Cu(II) plus $H_2O_2$ are copper−oxygen complexes with reactivity similar to that of •OH and/or singlet oxygen (Yamamoto & Kawanishi, 1989).

Ascorbate and reduced glutathione (GSH) play important roles in the metal-dependent production of oxygen free radicals. It has been demonstrated that, in the presence of Cu(II), ascorbate very efficiently generates active oxygen species which have DNA scission activities (Chiou, 1983; Kazakov *et al*., 1988). GSH, which is present in cell nuclei at relatively high concentrations (Bellomo *et al*., 1992), was found to cause DNA damage in the presence of Cu(II) (John & Douglas, 1993; Milne *et al*., 1993; Prütz, 1994). Thus, it is generally accepted that GSH and ascorbate but not NADH are able to induce oxidative stress (Steinkühler *et al*., 1991). On the other hand, the biological importance of NADH and NADPH as nuclear reductants has been pointed out (Kukielka & Cederbaum, 1994). NADH participates in both electron transport chains and enzymatic reactions only as a two-electron donor, whereas it can function as a one-electron reductant. It was reported that very powerful one-electron oxidants would be able to oxidize NADH by a mechanistic pathway involving one-electron transfer (Carlson *et al*., 1984). Several studies indicate that NADH may react nonenzymatically with some xenobiotics and mediate their reduction (Test, 1995). Thus, a possibility that metal ion plays an important role in nonenzymatic activation of NADH should be considered. Therefore, it is very interesting to clarify whether NADH, as well as ascorbate and GSH, can cause metal-dependent DNA damage.

In this study, the mechanism of DNA damage by NADH in the presence of metal ions has been investigated by DNA-sequencing technique by using $^{32}$P 5′ end-labeled DNA fragments obtained from human p53 tumor suppressor gene and c-Ha-*ras*-1 protooncogene. We have also investigated 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG)[1] formation, a marker of oxidative DNA damage, in calf thymus DNA by an electrochemical detector coupled to a high-pressure liquid chromatograph (HPLC-ECD) and studied the partici-

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[1] Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine (and also known as 8-hydroxy-2′-deoxyguanosine); $O_2^-$, superoxide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; HPLC-ECD, electrochemical detector coupled to a high-pressure liquid chromatograph; ESR, electron spin resonance; SOD, superoxide dismutase; POBN, α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone.

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pation of free radicals in the reaction using the electron spin resonance (ESR) spin-trapping technique. This is the first report providing evidence to support that NAD(P)H can be one of the endogenous reductants inducing Cu(II)-dependent DNA damage.

## EXPERIMENTAL PROCEDURES

*Materials*. [$\gamma$-$^{32}$P]ATP (222 TBq/mmol) was from New England Nuclear. Restriction enzymes (*Hpa*II, *Ava*I, *Xba*I, and *Pst*I) and $T_4$ polynucleotide kinase were purchased from New England Biolabs. Alkaline phosphatase from calf intestine was purchased from Boehringer Manheim GmbH. NADH and NADPH were from Kohjin, Co. Ltd., Tokyo, Japan. L-Ascorbic acid was from Wako Chemical Industries, Ltd., Osaka, Japan. $CuCl_2$, ethanol, D-mannitol, and sodium formate were from Nacalai Tesque, Inc., Kyoto, Japan. DTPA and bathocuproinedisulfonic acid were from Dojin Chemicals Co., Kumamoto, Japan. GSH, calf thymus DNA, SOD (3000 units/mg from bovine erythrocytes), and catalase (45 000 units/mg from bovine liver) were from Sigma Chemical Co. POBN and dimethyl sulfate were purchased from Aldrich Chemical Co. Nuclease $P_1$ (400 units/mg) was from Yamasa Shoyu Co., Chiba, Japan.

*Preparation of $^{32}$P 5′ End-Labeled DNA Fragments*. DNA fragments were obtained from human p53 tumor suppressor gene (Chumakov, 1990) and c-Ha-*ras*-1 protooncogene (Capon *et al*., 1983). Exon 8 of human p53 tumor suppressor gene, a 200-base pair fragment (PU8 14404−PD8 14603), was obtained by PCR amplification performed on human genomic DNA by using a 5′ amplimer (PU8) and a 3′ amplimer (PD8) (Clontech Lab). A $^{32}$P 5′ end-labeled 200-base pair fragment (PU8* 14404−PD8* 14603) was obtained by phosphorylation with [$\gamma$-$^{32}$P]ATP and $T_4$ polynucleotide kinase. The $^{32}$P-labeled 200-base pair fragment (PU8*−PD8*) was further digested with *Hpa*II to obtain a singly labeled 109-base pair fragment (PU8* 14404−*Hpa*II 14512) and a 91-base pair fragment (*Hpa*II 14513−PD8* 14603).

Plasmid pbcNI which carries a 6.6-kilobase *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 protooncogene (Capon *et al*., 1983) was purchased from American Type Culture Collection. 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 from the c-Ha-*ras*-1 according to the method described previously (Yamamoto *et al*., 1989). The asterisk indicates $^{32}$P labeling.

*Analyses of DNA Damage Induced by Reductants in the Presence of Cu(II)*. The standard reaction mixture in a microtube (1.5 mL Eppendorf) contained [$^{32}$P]DNA fragment, 20 $\mu$M per base of sonicated calf thymus DNA, 20 $\mu$M $CuCl_2$, and reductant (NADH, GSH, or ascorbate) in 200 $\mu$L of 10 mM sodium phosphate buffer (pH 7.9) containing 5 $\mu$M DTPA. For the experiment with denatured DNA, 5′ end-labeled DNA fragment was treated at 90 °C for 10 min and quickly chilled before the addition of $CuCl_2$ and endogenous reductant. After incubation at 37 °C for indicated durations, the DNA fragments were heated at 90 °C in 1 M piperidine where indicated and treated as previously described (Yamamoto *et al*., 1989). The DNA fragments were subjected to electrophoresis in 8% denatured polyacrylamide gel.

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam−Gilbert procedure (Maxam & Gilbert, 1980) using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

*Analysis of 8-oxodG Formation in Calf Thymus DNA by NADH plus Cu(II)*. The amount of 8-oxodG was measured by a modified method of Kasai *et al*. (1987). Calf thymus DNA (50 $\mu$M per base) was incubated with various concentrations of NADH and 50 $\mu$M $CuCl_2$ at 37 °C for 60 min. After ethanol precipitation, DNA was digested to the nucleoside by incubation with nuclease $P_1$ and alkaline phosphatase and analyzed by HPLC-ECD as previously described (Ito *et al*., 1993).

*UV−Visible Spectra Measurement*. UV−visible spectra were measured at 37 °C with a UV−Vis-NIR recording spectrophotometer (Shimadzu UV-365). Sodium phosphate buffer (10 mM) containing 0.6 mM $CuCl_2$ in the presence or absence of 1.2 mM bathocuproine was kept at 37 °C, and the spectral tracing was started by the addition of 0.6 mM NADH.

*ESR Spectra Measurement*. 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 & Yamamoto, 1991). Spectra were recorded with a microwave power of 16 mW and a modulation amplitude of 1.0 G. The magnetic fields were calculated by the splitting of Mn(II) in MgO ($\Delta H_{3-4}$ = 86.9 G). POBN was used as a radical-trapping reagent.

*Mass Spectrometry*. Laser desorption mass spectrometry was performed on a Kompact MALDI III equipped with a nitrogen laser (337 nm, 3 ns pulse) to determine the molecular weight of the spin adduct of POBN. Reaction mixture, containing 10 mM NADH, 200 mM POBN, and 100 $\mu$M Cu(II), was incubated for 1 h at 37 °C, which was air-dried on a stainless-steel probe tip. No matrix solution was added to the sample.

## RESULTS

*Cleavages of $^{32}$P-Labeled DNA Fragments Induced by NADH in the Presence of Cu(II)*. The extent of DNA damage induced by NADH in the presence of metal was estimated by gel electrophoretic analysis. NADH induced DNA damage in the presence of Cu(II) (Figure 1). Even without piperidine treatment (Figure 1A), oligonucleotides were formed by NADH in the presence of Cu(II), suggesting breakage of the deoxyribose phosphate backbone. The amount of oligonucleotides increased with piperidine treatment (Figure 1B). Since altered base is readily removed from its sugar by the piperidine treatment, it is considered that the base alteration and/or liberation were induced by NADH in the presence of Cu(II).

*Comparison of Metal-Dependent DNA Damage by NADH, GSH, and Ascorbate*. Cu(II)-dependent DNA damage induced by NADH was compared with that induced by GSH or ascorbate. The extent of DNA damage was calculated from origin in the band from the treated DNA fragment as compared with that from the untreated DNA fragment. The

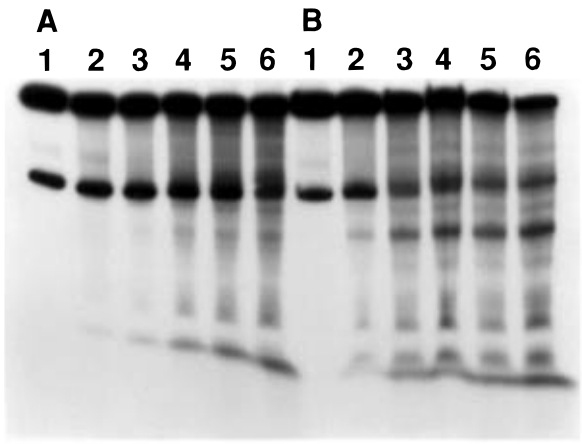


FIGURE 1: Time course of DNA cleavage induced by NADH in the presence of Cu(II). The reaction mixture contained the $^{32}$P 5′ end-labeled 261-base pair fragment (*Ava*I* 1645−*Xba*I 1905), 20 $\mu$M per base of sonicated calf thymus DNA, 2 mM NADH, 20 $\mu$M $CuCl_2$, and 5 $\mu$M DTPA in 200 $\mu$L of 10 mM sodium phosphate buffer at pH 7.9. After the incubation at 37 °C for the indicated durations, followed by piperidine treatment (B) or without piperidine treatment (A), 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 2, 15 min; lane 3, 30 min; lane 4, 45 min; lane 5, 60 min; lane 6, 90 min; lane 1, incubation time was 90 min without NADH.

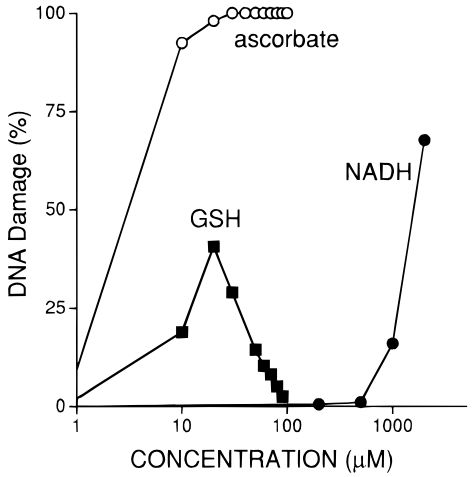


FIGURE 2: Comparison of copper(II)-dependent DNA damage by NADH with that by GSH and ascorbate. The $^{32}$P 5′ end-labeled 261-base pair fragment (*Ava*I* 1645−*Xba*I 1905) and 20 $\mu$M per base of sonicated calf thymus DNA were incubated with various concentrations of NADH, GSH, or ascorbate in the presence of 20 $\mu$M $CuCl_2$ in 200 $\mu$L of 10 mM sodium phosphate buffer (pH 7.9) containing 5 $\mu$M DTPA. After the incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the Figure 1 legend. The densities of individual bands were determined by densitometry (ATTO Densitograph AE-6910) on the resulting autoradiograms.

order of inducing effect on Cu(II)-dependent DNA damage was estimated to be ascorbate > GSH > NADH (Figure 2). Cu(II)-dependent DNA damage was apparently induced by more than 1 mM NADH. Although NADPH also caused Cu(II)-dependent DNA damage, the activity was weaker than that of NADH (data not shown). Neither NADH nor NADPH caused DNA damage in the presence of Fe(III) or Fe(III)−EDTA complex. Metal-dependent DNA damage induced by ascorbate was very strong. Ascorbate induced DNA damage in the presence of Fe(III) or Fe(III)−EDTA complex, but it showed activity weaker than that induced in

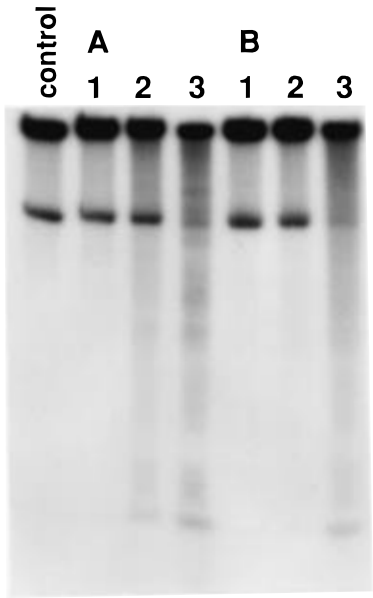


FIGURE 3: Effect of $H_2O_2$ on NADH-dependent DNA damage in the presence of Cu(II) and Fe(III)−EDTA. The $^{32}$P 5′ end-labeled 337-base pair fragment (*Pst*I 2345−*Ava*I* 2681) and 20 $\mu$M per base of sonicated calf thymus DNA were incubated with 20 $\mu$M metal [$CuCl_2$ (A) or Fe(III)−EDTA (B)] in the presence of 200 $\mu$M NADH and/or 20 $\mu$M $H_2O_2$ in 200 $\mu$L of 10 mM sodium phosphate buffer (pH 7.9) containing 5 $\mu$M DTPA. After the incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the Figure 1 legend: lane 1, NADH; lane 2, $H_2O_2$; lane 3, $H_2O_2$ + NADH. Control contains DNA without metal, NADH, and $H_2O_2$.

the presence of Cu(II) (data not shown). Ascorbate showed a reactivity different from those of NADH and GSH which could not induce Fe(III)-dependent DNA damage. GSH was observed to be less effective than ascorbate in promotion of Cu(II)-dependent DNA damage. DNA damage was maximal with a combination of 20 $\mu$M GSH and 20 $\mu$M Cu(II). The decrease of DNA damage at higher GSH concentrations is explained by the scavenging of active species and/or copper by GSH itself (Prütz, 1994).

When denatured single-stranded DNA was incubated with NADH, GSH, or ascorbate in the presence of Cu(II), the DNA damage was observed in each case (data not shown). Damage to denatured single-stranded DNA was stronger than that to native double-stranded DNA.

*Effects of $H_2O_2$ on Metal-Dependent DNA Damage by NADH*. Effects of $H_2O_2$ on metal-dependent DNA damage in the presence of NADH were examined, and the results are shown in Figure 3. Cu(II)-dependent DNA damage was not induced by 200 $\mu$M NADH (Figure 3A, lane 1). Although Cu(II) plus $H_2O_2$ induced DNA damage slightly (Figure 3A, lane 2), NADH enhanced Cu(II) plus $H_2O_2$-induced DNA damage (Figure 3A, lane 3). Neither NADH nor $H_2O_2$ caused DNA damage in the presence of Fe(III)−EDTA complex (Figure 3B, lanes 1 and 2). A combination of NADH and $H_2O_2$ induced Fe(III)−EDTA-dependent DNA damage (Figure 3B, lane 3). Similarly, Imlay and Linn (1988) reported that, when DNA was incubated in an NADH−Fe(III)−EDTA−$H_2O_2$ system, single-strand breaks were ensured.

*Effects of Scavengers and Bathocuproine on NADH plus Cu(II)-Induced DNA Damage*. Figure 4 shows the effects of radical scavengers, SOD, and catalase on NADH plus Cu(II)-induced DNA damage. Catalase and bathocuproine,

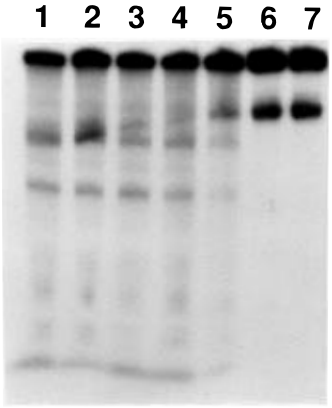


FIGURE 4: Effects of scavengers and bathocuproine on DNA cleavage induced by NADH in the presence of Cu(II). The reaction mixture contained the $^{32}$P 5′ end-labeled 261-base pair fragment (*Ava*I* 1645−*Xba*I 1905), 20 $\mu$M per base of sonicated calf thymus DNA, 2 mM NADH, 20 $\mu$M $CuCl_2$, scavenger, and 5 $\mu$M DTPA in 200 $\mu$L of 10 mM sodium phosphate buffer at pH 7.9. Scavenger or bathocuproine was added where indicated. After the incubation at 37 °C for 60 min, followed by 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, 0.2 M mannitol; lane 4, 0.2 M sodium formate; lane 5, 30 units of SOD; lane 6, 30 units of catalase; lane 7, 100 $\mu$M bathocuproine.

a Cu(I)-specific chelator, inhibited DNA damage completely (lanes 6 and 7), whereas •OH scavengers (ethanol, mannitol, and sodium formate) did not (lanes 2−4). These results suggest that Cu(I) and $H_2O_2$ but not •OH play important roles in the DNA damage. SOD did not significantly inhibit DNA damage by NADH plus Cu(II) (lane 5). Addition of DTPA of a molar concentration greater than that of Cu(II) inhibited the DNA damage (data not shown).

*Site Specificity of DNA Cleavage Induced by NADH in the Presence of Cu(II).* NADH plus Cu(II) induced piperidine-labile sites frequently at thymine residues, especially located 5′ and/or 3′ to guanine (Figures 5A,B and 6A). The site specificity was similar to that of DNA cleavage induced by $H_2O_2$ in the presence of Cu(I) (data not shown). When denatured DNA was used, the site specificity of DNA cleavage was changed (Figure 5). Cleavage at guanine residues was increased, and cleavage at 5′-GG-3′ in the 12th codon of c-Ha-*ras*-1 protooncogene was noteworthy (Figure 5B). The site specificity of DNA cleavage was compared with that in the case of GSH or ascorbate (Figure 6). GSH and ascorbate gave a DNA cleavage pattern similar to that of NADH.

*Formation of 8-oxodG in Calf Thymus DNA by NADH plus Cu(II).* A recent study has shown that piperidine treatment of 8-oxodG-containing DNA results in breakage of the deoxyribose phosphate backbone (Tchou *et al*., 1991). NADH plus Cu(II) increased 8-oxodG content in calf thymus DNA (Figure 7). The amount of 8-oxodG increased with increasing NADH concentration. The formation of 8-oxodG was increased about 2.5-fold with DNA denaturation. This is in agreement with the above-mentioned observation that, when denatured [$^{32}$P]DNA fragments were used, cleavage at guanine residues was increased. Relevantly, Fuciarelli *et al*. (1990) have shown that the yields of 8-oxodG and some other oxidized base were higher in denatured DNA than in native DNA.

The addition of $H_2O_2$ enhanced NADH plus Cu(II)-induced oxidative DNA damage, including formation of 8-oxodG (data not shown). Without Cu(II), NADH did not induce the increase of 8-oxodG formation.

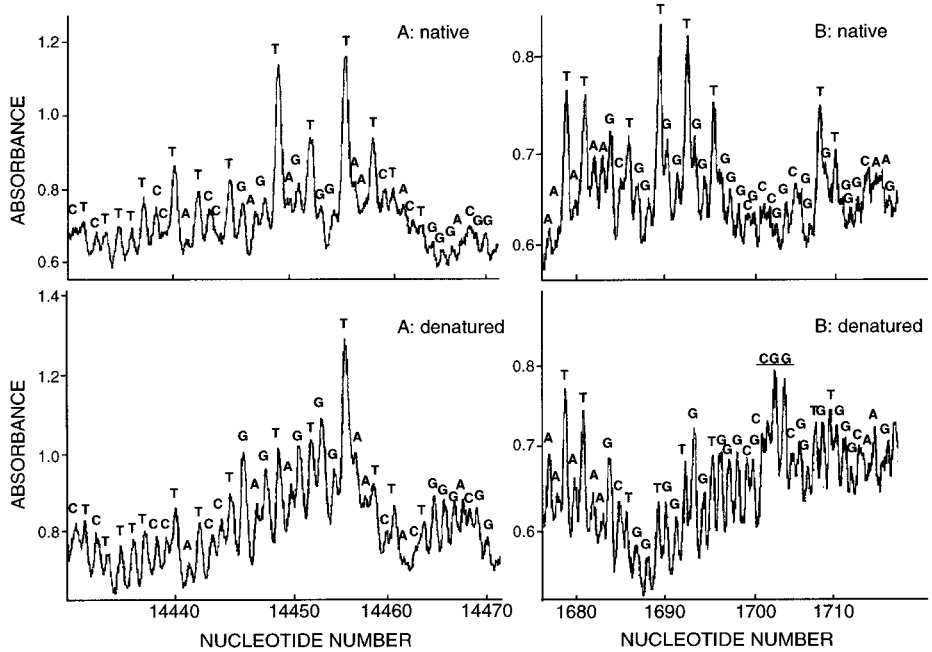


FIGURE 5: Site specificity of DNA cleavage induced by NADH plus Cu(II). The $^{32}$P 5′ end-labeled 109-base pair fragment (PU8* 14404−*Hpa*II 14512) from p53 (A) or 261-base pair fragment (*Ava*I* 1645−*Xba*I 1905) from c-Ha-*ras*-1 (B) in 200 $\mu$L of 10 mM sodium phosphate buffer at pH 7.9 containing 5 $\mu$M DTPA and 10 $\mu$M per base of sonicated calf thymus DNA was incubated with 2 mM NADH plus 20 $\mu$M $CuCl_2$ at 37 °C for 60 min. For the experiment with denatured DNA, the 5′ end-labeled DNA fragment was treated at 90 °C for 10 min and quickly chilled before the addition of NADH plus $CuCl_2$. 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 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. The horizontal axis shows the nucleotide number of human p53 tumor suppressor gene and c-Ha-*ras*-1 protooncogene. Underscoring indicates the 12th codon of human c-Ha-*ras*-1 protooncogene.

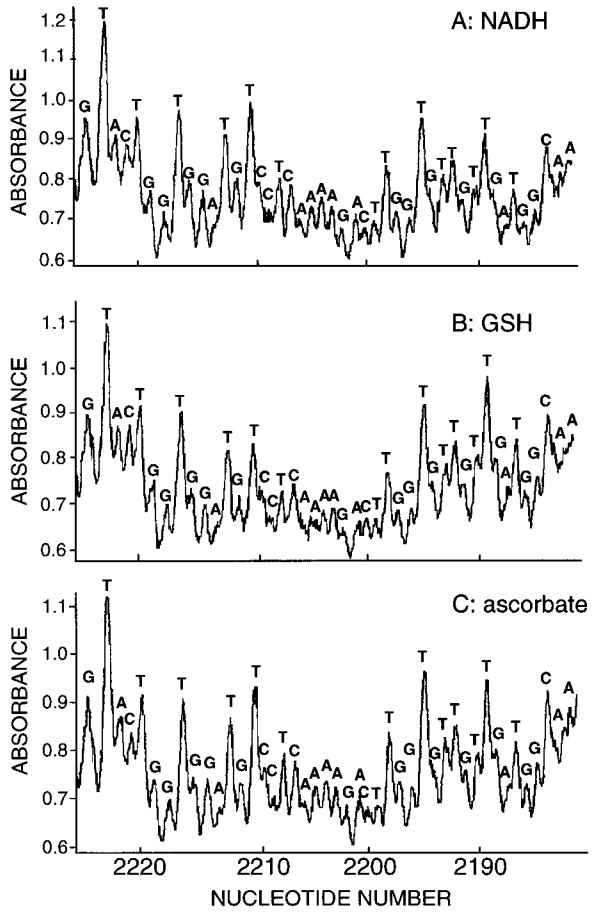


FIGURE 6: Comparison of site specificity of DNA cleavage induced by NADH, GSH, and ascorbate in the presence of Cu(II). The $^{32}$P 5′ end-labeled 341-base pair fragment (*Xba*I 1906−*Ava*I* 2246) and sonicated calf thymus DNA in 200 $\mu$L of 10 mM sodium phosphate buffer at pH 7.9 containing 5 $\mu$M DTPA was incubated with reductant in the presence of 20 $\mu$M $CuCl_2$ at 37 °C for 60 min. After the piperidine treatment, DNA fragments were analyzed as described in the Figure 5 legend: (A) 2 mM NADH + 10 $\mu$M per base of calf thymus DNA, (B) 20 $\mu$M GSH + 5 $\mu$M per base of calf thymus DNA, (C) 10 $\mu$M ascorbate + 20 $\mu$M per base of calf thymus DNA.

*Changes in UV−Visible Spectra during Oxidation of NADH by Cu(II).* Figure 8A shows changes in the UV−visible spectra of NADH plus Cu(II) with time. NADH showed very slow autoxidation in the buffer solution without addition of metal ion. The addition of Cu(II) more than 1 mM apparently induced the oxidation of 1 mM NADH at pH 6.0. The oxidation was increased with decreasing pH (data not shown). The inhibitory effect of catalase on the reaction was not observed. These results show that NADH was directly oxidized by Cu(II) as shown in reaction 1. Relevantly, Park *et al.* (1991) studied oxidation of NADH analogues with Cu(II) to find that the reaction follows first-order kinetics with respect to both NADH analogue and Cu(II).

$$\text{Cu(II)} + \text{NADH} \rightarrow \text{Cu(I)} + \text{NAD}^{\bullet} + \text{H}^{+} \quad (1)$$

$$\text{Cu(I)} + \text{bathocuproine} \rightarrow \text{Cu(I)}-\text{bathocuproine} \quad (2)$$

Addition of bathocuproine, a Cu(I)-specific chelator, dramatically enhanced the rate of oxidation of NADH to form a yellow compound with an absorption maximum at 482 nm (Figure 8B), which is assigned to the complex of Cu(I) with bathocuproine (Blair & Diehl, 1961). This indicates that bathocuproine rapidly reacts with Cu(I) to form a stable complex as shown in reaction 2, resulting in acceleration of reaction 1.

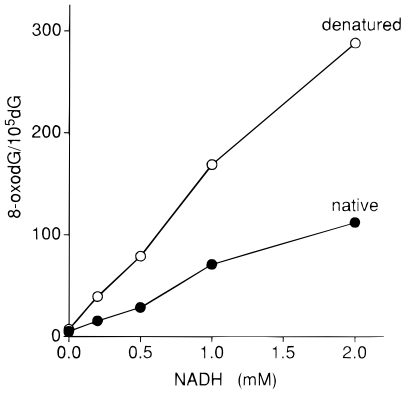


FIGURE 7: Formation of 8-oxodG in DNA incubated with NADH in the presence of Cu(II). Calf thymus DNA (50 $\mu$M per base) was incubated with NADH of the indicated concentrations in the presence of 50 $\mu$M $CuCl_2$ in 200 $\mu$L of 4 mM phosphate buffer (pH 7.8) containing 5 $\mu$M DTPA at 37 °C for 60 min. For the experiment with denatured DNA, calf thymus DNA was treated at 90 °C for 10 min and quickly chilled before the addition of NADH and $CuCl_2$. After ethanol precipitation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described under Experimental Procedures.

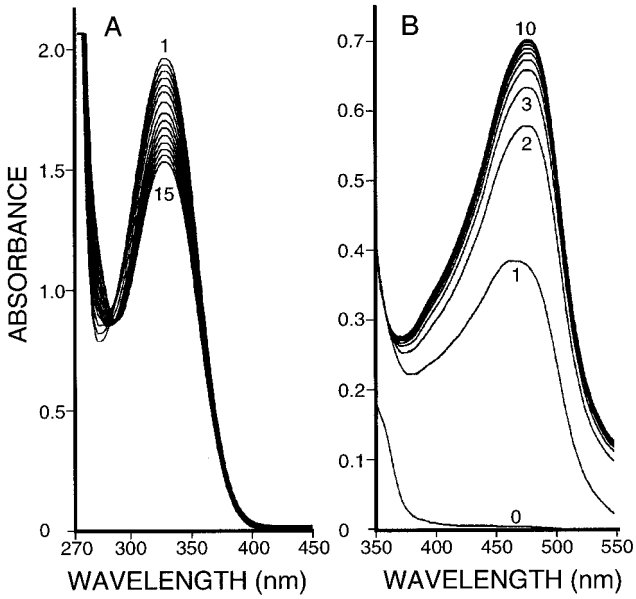


FIGURE 8: Changes in UV−visible spectra with time for reaction of NADH with Cu(II). (A) Sodium phosphate buffer (10 mM) at pH 6.0 containing 1 mM NADH was kept at 37 °C, and the spectral tracing was started by the addition of 1 mM $CuCl_2$. Spectral tracing was repeated every 10 min. (B) Sodium phosphate buffer (10 mM) at pH 7.8 containing 1.2 mM bathocuproine and 0.6 mM $CuCl_2$ was kept at 37 °C, and the spectral tracing was started by the addition of 0.6 mM NADH. Spectral tracing was repeated every 2 min. Spectrum 0 was due to bathocuproine and $CuCl_2$.

*Production of Carbon-Centered Radicals during the Reaction of NADH with Cu(II).* The ESR spin-trapping method was used to detect free radicals produced during the reaction of NADH with Cu(II). Figure 9C shows an ESR spectrum of a spin adduct observed when NADH was added to a buffer solution containing POBN and Cu(II). The signals ($a_N$ = 15.7 G, $a_H$ = 2.7 G) can be assigned to the carbon-centered adduct of POBN (Buettner, 1987). These results suggest

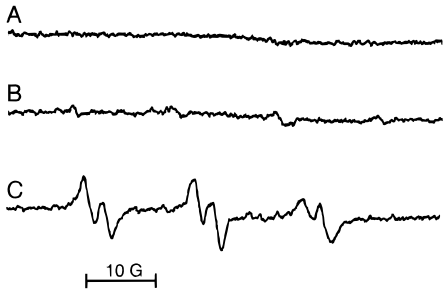


FIGURE 9: ESR spectrum of the carbon-centered radical adduct of POBN produced by NADH in the presence of Cu(II). The samples contained 200 mM POBN and 5 $\mu$M DTPA in 50 $\mu$L of 10 mM sodium phosphate buffer at pH 7.8: (A) 1 mM $CuCl_2$ was added, (B) 10 mM NADH was added, (C) 1 mM $CuCl_2$ and 10 mM NADH were added. After incubation at 37 °C for 60 min, ESR spectra were measured at room temperature as described under Experimental Procedures.

that Cu(II) reduces NADH to generate carbon-centered radical, which may correspond to NAD$^\bullet$ or the radical derived from NAD$^\bullet$.

The adduct obtained from the reaction mixture of POBN and NADH in the presence of Cu(II) showed a mass spectrum with a molecular ion at *m/e* 318 (M + 1). The molecular ion is that expected for the POBN-trapped carbon-centered radical of perhaps the C-4 position of nicotinamide, if a bond between the nicotinamide group and the ribose group in NADH breaks and a hydrogen joins in the ionization process. A combination of ESR spin-trapping experiments with mass spectrometry suggested that the NAD$^\bullet$ was formed during the reaction of NADH with Cu(II).

## DISCUSSION

This study was undertaken to evaluate the ability of NADH in comparison with that of ascorbate or GSH to promote metal-mediated DNA damage. The present results have shown that, although NADH itself does not cause DNA damage, NADH as well as other endogenous reductants can induce DNA damage in the presence of Cu(II). The order of inducing effect on Cu(II)-dependent DNA damage was ascorbate > GSH > NADH > NADPH. Although NADH induced no or little DNA damage in the presence of Fe(III)−EDTA complex, a combination of NADH and $H_2O_2$ induced Fe(III)−EDTA-dependent DNA damage.

It is generally considered that $^\bullet$OH causes DNA cleavage at every nucleotide with no marked site specificity (Kawanishi *et al*., 1986; Inoue & Kawanishi, 1987; Celander & Cech, 1990). NADH plus Cu(II) induced piperidine-labile sites frequently at thymine residues, especially located 5′ and/or 3′ to guanine. The site specificity cannot be explained by $^\bullet$OH. DNA damage by bound Cu(II) in the presence of NADH has been shown to occur primarily at the binding site, indicating a site-specific mechanism of DNA damage. In order to examine what kind of active species cause DNA damage, experiments using various scavengers were performed. Bathocuproine and catalase completely inhibited DNA damage by NADH plus Cu(II), whereas $^\bullet$OH scavengers and SOD did not. These results suggest that active species derived from $H_2O_2$ and Cu(I) rather than $^\bullet$OH play an important role in the DNA damage. However, there remains a possibility that Cu(II) binds to DNA in a site-specific manner and then reacts with $H_2O_2$ to form $^\bullet$OH, which immediately attacks an adjacent constituent of DNA before it can be scavenged by $^\bullet$OH scavengers (Dizdaroglu *et al*., 1991).

The mechanism of reactions 3−8 can be envisioned as accounting for most of the observations.

$$\text{DNA} + \text{Cu(II)} \rightleftharpoons \text{DNA–Cu(II)} \quad (3)$$

$$\text{DNA–Cu(II)} + \text{NADH} \longrightarrow \text{DNA–Cu(I)} + \text{NAD}^\bullet + \text{H}^+ \quad (4)$$

$$\text{NAD}^\bullet + \text{O}_2 \longrightarrow \text{NAD}^+ + \text{O}_2^- \quad (5)$$

$$2\text{O}_2^- + 2\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (6)$$

$$\text{DNA–Cu(I)} + \text{H}_2\text{O}_2 \xrightarrow{\text{H}^+} \text{DNA–Cu(I)OOH} \quad (7)$$

$$\text{DNA–Cu(I)OOH} + \text{H}^+ \xrightarrow{\text{H}^+} \text{DNA}^\bullet\text{OH} + \text{Cu(II)} + \text{H}_2\text{O} \quad (8)$$

$$\downarrow$$

DNA damage

Cu(II) ion binds to DNA (reaction 3). Addition of DTPA of a molar concentration greater than that of Cu(II) inhibited the DNA damage, indicating that Cu(II) bound to DNA is necessary for induction of NADH-dependent DNA damage. NADH derives one-electron reduction of DNA-bound Cu(II) to give Cu(I) with concomitant formation of NAD$^\bullet$ (reaction 4). The direct oxidation of NADH by Cu(II) is supported by the present UV−visible spectroscopic results. Evidence for NAD$^\bullet$ formation is provided by the ESR spin-trapping experiments and mass spectroscopy. Reaction 4 can be expected to predominately equilibrate to the right by assuming that Cu(II)-induced redox can be dramatically promoted by the binding to DNA. Relevantly, it has been reported that NADH reduces Cu(II) to Cu(I) and the latter interacts with bathocuproine or neocuproine, a Cu(I)-specific chelator, to yield a stable complex (Blair & Diehl, 1961; Morgenstern *et al*., 1965). The redox potential for copper−neocuproine complex is the most positive known for synthetic complexes (Petterson & Holm, 1975), in which the ligand structure should destabilize planar Cu(II) and stabilize tetrahedral Cu(I). Similarly, DNA may form a more stable complex with Cu(I) than with Cu(II), and the very high affinity of DNA for Cu(I) is equivalent to an increase of the one-electron redox potential of Cu(II), as Prütz *et al*. (1993) pointed out. As the following reaction (reaction 5), NAD$^\bullet$ reacts with $O_2$ to form $NAD^+$ and superoxide ($O_2^-$) at an almost diffusion-controlled rate ($2 \times 10^9$ $M^{-1}$ $s^{-1}$) (Land & Swallow, 1971). Then, $O_2^-$ produces $H_2O_2$ by copper-dependent dismutation (reaction 6). Finally, Cu(I) reacts with $H_2O_2$ to give ternary copper(I)−peroxide complex (reaction 7), which causes DNA damage (reaction 8). Reactions 7 and 8 may be supported by recent findings that the reaction of Cu(I) with $H_2O_2$ yields $Cu^{I}OOH$ (Yamamoto & Kawanishi, 1989; Oikawa *et al*., 1995).

In summary, optimal molar ratios of DNA/metal ion at physiological pH yield copper with a high redox potential which oxidizes NADH with generation of $O_2^-$, and that $H_2O_2$ reacts with Cu(I) to form active oxygen species such as copper(I)−peroxide complex causing oxidative DNA damage, including 8-oxodG. This finding is noteworthy in connection with reports that the binding of copper to DNA might serve physiological functions (Lewis & Laemmli, 1982; Dijkwel & Wenink, 1986) and that NADH might function as a nuclear reductant (Kukielka & Cederbaum, 1994). Several papers have pointed out the possibility that chemicals are nonenzymatically reduced by NAD(P)H *in*

*vivo* (Test, 1995). We have also studied DNA damage by quinone plus NADH. Quinone did not induce DNA damage in the presence of Cu(II), but addition of NADH induced the DNA damage even in the absence of enzyme (Kawanishi *et al*., 1995; Naito *et al*., 1994). The concentration of NAD(P)H in certain tissue was estimated to be as high as 100−200 $\mu$M (Malaisse *et al*., 1979). The present study shows that NADH at physiological concentrations induces Cu(II)-dependent formation of 8-oxodG. Thus, we have shown that NADH can be a source of endogenous one-electron reductant to metal, resulting in oxidative DNA damage.

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