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ZINC(II) PROTECTS AGAINST METAL-MEDIATED FREE RADICAL INDUCED DAMAGE: STUDIES ON SINGLE AND DOUBLE-STRAND DNA BREAKAGE

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M13 DNA was used as a source for single and double-stranded DNA. Free radical-induced damage to single and double stranded DNA was caused by ascorbate/iron and ascorbate/copper oxidative systems. The degree of breakage was estimated by running samples on an agarose gel and staining with ethidium bromide, followed by photographic analysis. DNA breakage was dependent on time and concentration of iron or copper ions. Zinc ions protected against damage caused by iron/ascorbate both to single-stranded and double-stranded DNA. In contrast, in the copper/ascorbate system zinc ions protected only against the double-stranded DNA (replicative form of M13) breakage, and not against copper-mediated single-stranded DNA breakages. It seemed to amplify the efficiency of breakage. The protection provided to the replicative form in the copper/ascorbate system is much less effective than the protection to DNA in the iron/ascorbate system. These results support the notion that redox-inactive metal ions, that compete for iron or copper binding sites, could provide protection against transition metal-mediated and free radical-induced damage.

KEY WORDS: DNA, zinc(II), free radicals, Fenton reaction.

ABBREVIATIONS: TBE-10 mM Tris (pH = 8.3), 10 mM Boric acid, 2.5 mM EDTA; TE-10 mM Tris (pH = 7.5), 0.1 mM EDTA; SS DNA-Single Stranded DNA; DS DNA-Double Stranded DNA; RF-Replicative form.

INTRODUCTION

Oxygen free radicals are considered etiological factors involved in several pathological conditions some of which include aging, ischemia/reperfusion, carcinogenesis and other diseases. Superoxide or hydroxyl free radicals react with DNA and cause its damage.[1,2] Hydroxyl radicals are highly effective at causing DNA scission via addition to a DNA base that forms with oxygen a base peroxyradical intermediate.[3] Thymine and guanine in DNA are oxidized to thymine 5,6 glycol and 8-hydroxyguanine, respectively.[4] Lipid peroxy radicals probably cause similar changes.[5] Transition metal ions, particularly iron and copper, are involved in generating oxygen radicals and in their damaging reactions.[6] It has been shown that traces of redox-active iron or copper can catalyze the transformation of superoxide radical anion ($\cdot O_2^-$) to the highly reactive hydroxyl radical ($OH\cdot$) via the metal catalyzed Haber-Weiss reaction.[7,8]

$$\cdot O_2^- + \text{Fe(III)/Cu(II)} \longrightarrow O_2 + \text{Fe(II)/Cu(I)} \xrightarrow{H_2O_2} \text{Fe(III)/Cu(II)} + OH^- + OH\cdot$$

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In biological systems the concentration of free transition metal ions appear to be very low and the metal ions are mostly bound to ligands such as proteins or DNA.[9] DNA/iron and copper complexes are involved in DNA damaging reactions. Intervention and prevention of biological damage caused by a mechanism that depends on the presence of both superoxide radicals and transition metals can be achieved by various approaches.

A new approach is currently being developed in our laboratory. This is based upon the idea that displacement of redox-active metal from its binding site will also shift the site of hydroxyl radical production. As hydroxyl radicals react with biological targets at the site of their formation, this displacement could provide protection (or enhancement) against "site specific" Fenton mechanism. Based upon the similarity between the coordination chemistry of iron or copper, on the one hand, and zinc, on the other, and considering that zinc ions are redox-inactive,[10] it is expected that zinc could displace copper(II) and iron(III) from the biological binding sites. Once it replaces copper or iron it will divert the site of formation and reaction of free radicals and could migrate the deleterious effects induced by them. We have used M13 bacteriophage as a source for single and double stranded DNA and ascorbate/iron or ascorbate/copper as hydroxyl radical generating systems. We have shown the effect of protection against DNA degradation in the presence of zinc ions.

MATERIALS AND METHODS

Strains and Maintenance

JM103 is an *Escherichia coli* K-12 derivative. For short term storage (2–4 weeks at 4°C) bacteria were plated on M-9 minimal plates[11] supplemented with 0.2% glucose as a carbon source. M-13 bacteriophage served as a source of DNA and was maintained either as a free virus or as its replicative form inside the infected cells.

DNA Preparation

Single and double stranded DNA were purified from the two biological forms of M-13. The circular single stranded DNA of the phage was isolated from the mature free phage according to a modification of Shreier and Cortese.[12] 2 ml of medium (8 g bactotryptone, 5 g yeast extract, 5 g NaCl in 1 liter H_2O) containing 10 μl of fresh JM103 plating culture was inoculated with a well isolate M13 plaque. 20–40 culture samples were grown at 37°C, while shaking, for 6 hours, then spinned down at 10,000 g for 5 min to totally separate between cell precipitate and the clear supernatant containing the free matured phage. Supernatants were collected in 30 ml corex tubes. 0.25 volume of 2.5M NaCl, 20% polyethylene glycol (8000) solution was added to each supernatant sample, mixed and incubated on ice for approximately one hour. Phages were precipitated at 10,000 g for 30 min at 4°C. Supernatant was discarded totally and the pellet was resuspended in 0.1 volume of TE. DNA was further purified by extracting once with an equal volume of phenol and twice with an equal volume of phenol:chloroform:isoamyl alcohol mixture (in a volume ratio of 50:48:2 respectively). To the final aqueous phase 0.1 volume of 3M sodium acetate (pH = 5.2) and 2 volumes of cold ethanol were added. samples were mixed gently by inversion. Following 1–2 hours at —20°C, DNA was precipitated by spinning at 12,000 g. The

pellet was washed with 70% ethanol then dried under vacuum. Pellet was resuspended at 0.05 volume of original culture sample in water.

The supercoiled double stranded DNA, was isolated from infected bacteria containing the M13 replicative form according to Felsenstein protocol.[13] After isolation of DNA it was further purified on a pZ523 column, and residual contaminating RNA was removed by RNAse treatment as described by the manufacturer (5 Prime⟶ 3 Prime,Inc.).

Preparation of Copper and Iron Solutions

a. A 0.1 M stock solution of $CuSO_4$ was prepared in H_2O. Immediately prior to the experiment, the stock solution was diluted to 200 μM and samples were taken for different experiments. The final concentration used in most of the experiments was 20 μM. DNA was incubated in 5 mM Tris (pH = 7.4) and 20 μM Cu(II) for 5 minutes at 37°C before starting of the reaction.

b. When Fe(III) was needed, $FeNH_4(SO_4)_2$ was used. 4.98 mg of the salt were dissolved in 20 ml H_2O (which was acidified to pH = 3.2) to make a 500 μM solution. DNA was incubated with 500 μM Fe(III) for 5 min at 37°C before the addition of 5 mM phosphate buffer.

Nicking Reactions

Variations in nicking reactions are described in the legends to the specific figures. In general, 1–2 μl DNA (1 mg/ml) were mixed with 1 μl of 500 μM Fe(III), incubated 5 min at 37°C, and then 59 mM sodium phosphate buffer (pH = 7.4) was added to a final volume of 10 μl. Similarly, 20 μM Cu(II) in 59 mM Tris buffer (pH = 7.4) in a final volume of 10 μl were incubated with DNA at 37°C for 5 minutes. The reaction was initiated by the addition of 2.5 mM ascorbate and was followed by incubation at 37°C for the desired length of time. In the case of Fe(III), the reaction was terminated by the addition of 10 mM Desferal®. When Cu(II) was used the reaction was stopped with 10 mM EDTA (pH = 8.0). Three μl of loading buffer (0.25% Bromo Phenol Blue, 0.25% xylene cyanol, 30% glycerol) were then added to each reaction mixture and samples were loaded on agarose gels.

For the protection by zinc, the sample were prepared as followed: Fe(III) was let to interact with DNA, at pH 3.2 for 5 min, then sodium phosphate buffer (pH 7.4) (5 mM) was added. Only subsequently, Zn(II) was added followed by the addition of ascorbate, in order to initiate the DNA-breakage reaction. Cu(II) was incubated 5 min with the DNA in Tris buffer (5 mM, pH 7.4), followed by the addition of Zn(II) and ascorbate.

Agarose Gel Electrophoresis

DNA sample (0.5-1 μg in 10–15 μl volume) were run on 0.7–1% agarose gel in Tris-borate/EDTA (TBE) buffer. Electrophoresis was performed at 100 V for 6–8 hours. Gels were stained at the end of the run with 0.5 μg/ml Ethidium Bromide in TBE buffer for 30 minutes. Pictures were taken with a Polaroid Land Camera using Polaroid 677 film.

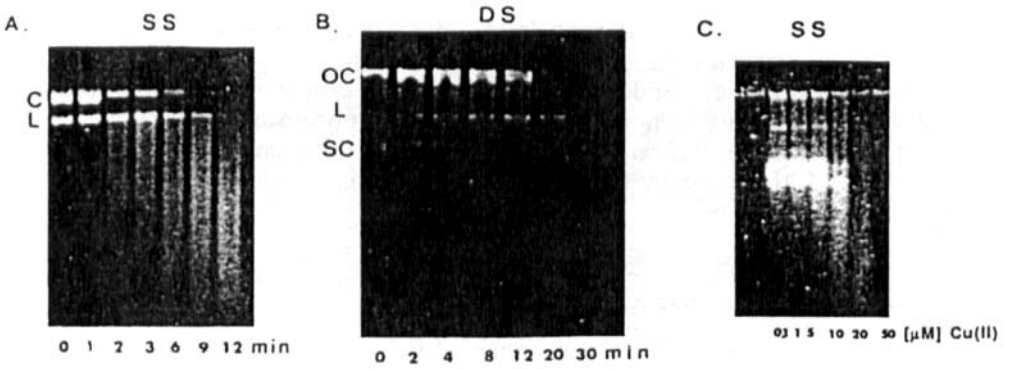

FIGURE 1 Copper(II)/ascorbate-mediated DNA breakage. Reaction mixtures of 10 μl were prepared as described in Methods. Single-stranded (panels A & C) and double-stranded (panel B) DNA (1–2 μg) were incubated with 20 μM Cu(II) (panels A & B) or with the indicated concentrations (panel C) for 5 min at 37°C in 5 mM Tris (pH 7.4). The reaction was initiated by the addition of 2.5 mM ascorbate and was terminated with the addition of 10 mM EDTA (pH = 8.0) at the indicated times (panels A & B) or after 30 min (panel C). Loading buffer (3 μl) was added and the gel was run at conditions described in the Methods. L = linear form M13; C = circular form M13; SC = supercoiled replicative form of M13; OC = open circular form of M13

RESULTS

Two biological forms of the bacteriophage M13 have been used to study the mechanism of DNA damage in a metal ions/ascorbate system. These forms are the single-stranded circular form of the mature phage and the supercoiled double-stranded form of the replicative form of the phage. Figure 1 illustrates the damage caused to both single and double-stranded DNA in the Cu(II)-ascorbate system. There is an absolute requirement for both ascorbate and iron ions for the breakage ot occur. In panel A (Figure 1) the conversion of the circular DNA form to the linear form, and subse-

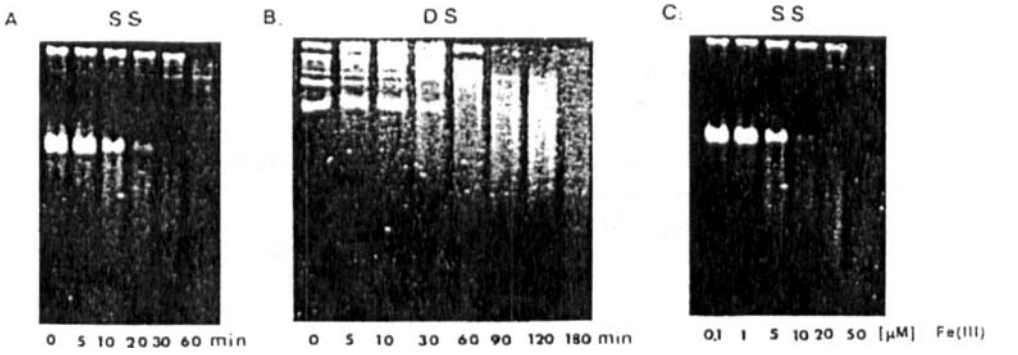

FIGURE 2 Iron(III)/ascorbate-mediated DNA breakage. Reaction mixtures of 10 μl were prepared as described in the Methods. Single-stranded (panels A & C) and double-stranded (panel B) DNA (1–2 μg) were incubated with 50 μM Fe(III) (panels A & B) or with the indicated concentrations (panel C) for 5 min at 37°C, followed by the addition of 5 mM sodium phosphate. The reaction was initiated by the addition of 2.5 mM ascorbate and was terminated by 10 mM Desferal at the indicated times (panels A & B), or after 30 min (panel C). Loading buffer (3 μl) was added and the gel was run at conditions described in the Methods.

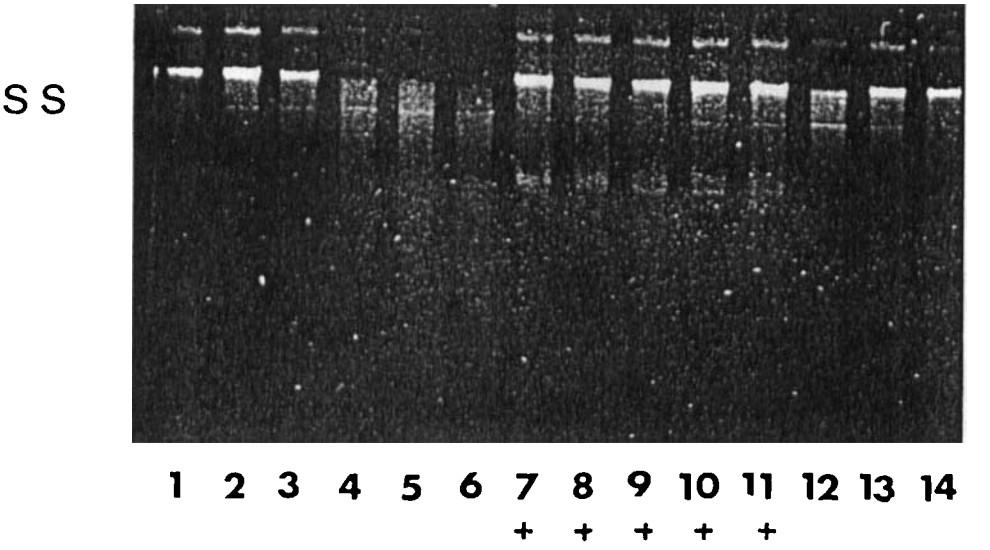

FIGURE 3 Zinc(II) protects against single-strand DNA breakage in Fe(III)/ascorbate system. Reaction mixtures of 10 μl were prepared as described in the Methods for the Fe(III)/ascorbate system. Single-stranded DNA (1 μg/ml) was incubated 30 min at 37°C with the following concentrations of Fe(III): 1 μM (lanes 2,7); 5 μM (lanes 3,8); 10 μM (lanes 4,9); 20 μM (lanes 5,10); and 50 μM (lanes 6,11–14), and with Zn(II) at concentrations of 50 μM (lanes 7–11), and 1, 5, 10 μM in lanes 12–14, respectively. Lane 1 is the control for zero time.

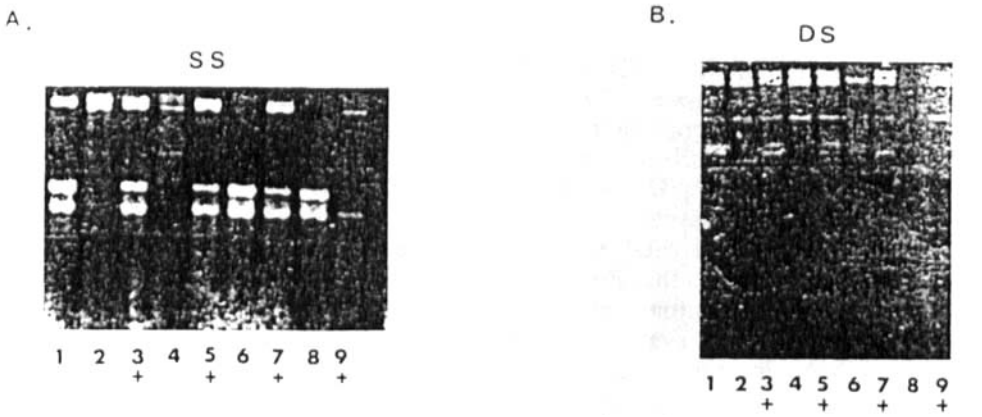

FIGURE 4 The effect of Zinc(II) on metal-mediated ascorbate induced DNA breaks: Panel A- Double-strand breaks in Fe(III)/ascorbate and Cu(II)/ascorbate systems. Panel B- Single-strand breaks in Fe(II)/ascorbate and Cu(II)/ascorbate systems. Reactions mixtures of 10 μl were prepared as described in the Methods. Double-stranded DNA (1 μg/ml) Panel A and single-stranded DNA (1 μg/ml) Panel B were incubated with either 50 μM (Fe(III) (lane 1–5) or 20 mM Cu(II) (lanes 6–9). 500 μM Zinc (II) were added to samples in lanes marked with (+). The reaction was initiated by the addition of 2.5 mM of ascorbate. Lane 1, in each panel, is the control without ascorbate. Samples in lanes 2,3 were incubated for 15 min and in lanes 4,5 for 30 min at 37°C before stopping the reaction by adding 10 mM Desferal. Samples in lanes 6,7 were incubated for 5 minutes and in lanes 8,9 for 10 minutes at 37°C, before the termination of the reactions by the addition of 10 mM EDTA.

quently to smaller fragments, versus time, is observed. Panel B (Figure 1) illustrates the conversion of the supercoiled double-stranded form to the open circular double-stranded form, and later on to the linear double-stranded form and to smaller double-stranded fragments. Panel C demonstrates the nicking efficiency when the metal ions concentrations is increased.

Figure 2 demonstrates, the time course of damage to single-stranded DNA (panel A) and to double-stranded DNA (panel B) in an iron/ascorbate system. As demonstrated above, both ascorbate and iron are necessary for the breakage to occur. Note that the copper/ascorbate system is much more efficient in degrading DNA as compared to the iron/ascorbate system, as is reflected by the differences in the time scales and the metal ion concentrations.

Figure 3 exhibits the protection against single stranded DNA degradation acquired by the addition of zinc ions. The level of protection was dependent upon the relative concentrations of iron and zinc ions. For [Zn(II)]/[Fe(III)] = 5, 100% protection was recorded and no degradation occurred. Zinc ions in combination with ascorbate did not cause any DNA damage. These protective effects were obtained for both single-stranded and double-stranded DNA.

Comparison between the protection of zinc ions in the iron/ascorbate system versus the copper/ascorbate system is presented in Figure 4. In both systems, double *and* single-stranded DNA were used. Clearly, zinc ions protect against single and double-stranded DNA degradation in the iron/ascorbate system. In the copper/ascorbate system, there is inhibition of degradation of double-stranded DNA. However, when single-stranded DNA is used as the substrate, no protection against breakage was observed. In fact, an amplification of this damage was seen.

DISCUSSION

We have demonstrated that Zn(II) ions strongly inhibit DNA degradation caused by a free radical generating system. This is in accord with the previously shown protection by zinc against bacterial killing caused by paraquat and copper,[14] and against arrhythmias induced by ischemia and reperfusion to the isolated rat heart.[15] Others have demonstrated that Zn(II) ions effectively inhibit lipid peroxidation in a membrane system exposed to xanthine, xanthine oxidase and iron.[16] The idea that Zn(II) may inhibit redox metal catalyzed deleterious reactions has already been proposed.[17-19] It was suggested that Zn(II) may compete with iron for crucial phospholipid binding sites on membrane surfaces[17] or that zinc may play a role in a mechanism involving metallothionein synthesis, exclusion of extracellular iron and changes in activities of cytosolic or microsomal enzymes.[19]

In our *in vitro* system, the protective mode of zinc could be due to direct competition and subsequent displacement of iron(III) or copper(II) from their binding sites. As zinc is a redox-inactive metal, it could not serve as a center for site specific production of free radicals like copper and iron. On the other hand, zinc(II) protection could be due to its binding to cellular sites that modulates the affinity of those sites for copper or iron. A property shared by zinc, copper and iron is the ability of each to form complexes of various geometries.[20] The differences in the efficiency of protection by zinc ions in the iron-mediated or copper-mediated DNA degradation processes could be due to different specific binding sites for copper and iron ions on the DNA molecule. It could also be affected by the differences in the chelating capacity for iron or copper of the buffers used in the reactions, and by the different structure that single or double stranded DNA assume in those solutions.

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