Specific Cleavages of DNA by Ascorbate in the Presence of Copper Ion or Copper Chelates

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The DNA-cleavage specificity of ascorbate in the presence of copper ion is analyzed with end-labeled pBR322 DNA fragments. The nonenzymatic reaction of Cu(II)/ascorbate and DNA shows certain degrees of cleavage preference toward purine-containing short segments in the labeled DNA under mild conditions (at 0°C and 10 min). The segments of pyrimidine clusters are least susceptible to cleavage. The DNA scission cannot be detected in the absence of metal ions, and is greatly diminished in the presence of EDTA and metal-chelating peptide. It is more specific than the nuclease-like scission activity induced by cuprous-phenanthroline complex. This scission activity in relation to the antiviral and antitumor activities of vitamin C reported in the literature deserves a crucial consideration.

The controversy surrounding the claim of the antitumor effect of vitamin C has attracted a lot of public attentions (1-3). Despite reports on the antiviral (4-6) and antitumor (7-9) activities of ascorbic acid there is as yet no persuasive explanations of these biological effects at the cellular and molecular levels. Previous studies (9, 10) indicated a preferential cytotoxicity of ascorbate and copper ion against malignant melanoma cells with little effect on the proliferation of normal cells. It is also reported that several biological reductones including ascorbic acid inhibit the growth of sarcoma-180 implanted in mice and cause the breakage of DNA molecules (11-13). However, there is so far no direct evidence of the specific cleavages of DNA by ascorbate and copper ion to account for these observations. We have been interested

in the physiological effect of ascorbate and its possible clinical applications in the prevention of viral diseases and inhibition of tumor growth. The impressive effectiveness of vitamin C, in combination with copper ion and copper chelates in *in vitro* DNA-cleaving and protein-scission activities (14, 15) has prompted us to study the DNA-scission specificity.

DNA fragments of defined sequence were obtained from pBR322 plasmid. pBR322 DNA were prepared as described (16) and digested with restriction endonucleases *Cla*I, *Bam*HI, and *Pst*I to produce three fragments with the molecular size of 352, 777, and 3,233 base pairs, respectively. The fragment mixture was added the [α -³²P]dCTP (10 μ Ci/ μ l) and Klenow fragment of DNA polymerase I (5 U/ μ l) to label the recessed 3' termini created

by cleavage of DNA at *Cla*I site (16). The resulting end-labeled fragments of 352 and 777 base pairs were purified on an 8% polyacrylamide gel and the fragments were extracted from the gel slices of the separated bands and precipitated three times with ethanol after centrifugation.

The reaction mixtures for the scission experiments contain the 3'-labeled DNA fragment (800–1,000 cpm/ng) of 777 base pairs in 2 mM Tris-HCl buffer (pH 7.5). The final concentrations of ascorbate, copper ion, copper peptide or copper-1,10-phenanthroline are indicated in the legends of Figs. 1 and 2. The reactions were started by the addition of a freshly prepared Na ascorbate at 0°C or 25°C, and terminated by the addition of 25 mM EDTA, pH 8.0 after 2 or 10 min. The stopped reaction mixtures were dried under vacuum and dissolved in the loading buffer for electrophoresis in 20% polyacrylamide sequencing gels according to the procedure of Maxam and Gilbert (17). Autoradiography was done on a Fuji X-ray film at -70°C. The cleaved oligonucleotides induced by ascorbate and copper ion were compared directly with the four standard chemical reactions run concurrently on the same sequencing gel. The relative reactivities under various conditions were quantitated with a laser scanning densitometer (Biomed Instruments, Inc., California, U.S.A.) on the developed autoradiograms.

Figure 1 shows the cleavage patterns of the labeled DNA induced by copper/ascorbate (lane 4) and its comparison with that induced by copper-phenanthroline complex (lane 6). It clearly indicates that under the same conditions Cu(II)-ascorbate has a much milder DNA-scission than that of cuprous-phenanthroline. It is noteworthy that DNA in the presence of ascorbate alone is not cleaved to a significant amount (lane 2 of Fig. 1 and lane 8 of Fig. 2). This is in contrast to the previous report which indicated the strand breakage of DNA with ascorbic acid in the presence of oxygen (18). We believe that the DNA cleavage of ascorbate observed in the absence of copper ion is probably caused by the trace metal contaminants present in the solutions; removal of these trace metal ions by 1–2 μ M of EDTA or 2,2'-biquinoline (cuproine) and use of deionized, double-distilled water effectively eliminate the minor cleavage patterns sometimes occurring in the ascorbate-treated DNA. It also occurs in the phenanthroline-con-

taining sample treated with ascorbate (lane 7 of Fig. 1) in the absence of copper ion; the obvious DNA scission could be ascribed to the presence of trace copper ions in the solution and the high cleavage activity of Cu(I)-phenanthroline complex thus formed. The scission reaction is temperature dependent; the cleavage can be controlled by lowering the reaction temperature to 0°C (Fig. 1, lanes A and B). However the specific scission patterns are essentially the same at the two different temperatures albeit with the much lower reaction rate at the lower temperature, which is more appropriate for the study of this fast DNA-scission reaction.

We have investigated the cleavage specificity by comparing the patterns with those of the base-specific chemical cleavages of DNA-sequencing gel (Fig. 2). The relative intensity of each cleaved fragment should reflect the reactivities of the scission reaction toward the different sequence segments or different bases in the labeled DNA. Although the scission activity is not highly specific as those exhibited by the restriction enzymes, Jessee *et al.* (19) have shown an artificial micrococcal nuclease-like activity associated with the cleavage reaction induced by 1,10-phenanthroline-cuprous complex (Fig. 2, lane 7). The scission reaction of ascorbate/copper is milder and more specific than that of phenanthroline/copper which shows the cleaved products mainly consisted of short segments of oligonucleotides. The quantitative densitometric scans of each individual lane reveal the relative susceptibilities of the different sequence segments (Fig. 3). DNA was cleaved more extensively at the 3' sides of deoxyguanosines and both 3' and 5' sides of deoxyadenosines than those of other bases, with the pyrimidine clusters the least susceptible to cleavage (segments of #21–25 (TTCTC), #31–35 (TCTTC), and #39–43 (CCCTT) numbered starting from the labeled 3' end at the *Cla*I site). The DNA-cleavage reaction is greatly inhibited by the addition of the copper-chelating tripeptide, diglycyl-L-histidine (Fig. 1 and Refs. 14, 15). The reaction rate is also markedly decreased by the decrease in the concentrations of ascorbate and copper ions (Fig. 2), which suggests that the reaction is dependent on the oxidation products of ascorbate in the presence of copper and oxygen, including hydrogen peroxide and some unidentified, activated oxygen radicals. The oligo-

Fig. 1. DNA scissions of ascorbate in the presence of copper ion, copper tripeptide and copper-1,10-phenanthroline. The 3'-end labeled DNA fragment of 777 base pairs was incubated with CuCl_2 , Cu-diglycyl-L-histidine, and Cu-1,10-phenanthroline (final concentrations in the reaction mixtures were $25 \mu\text{M}$ in 2 mM Tris-HCl buffer, pH 7.5) at 0°C , followed by the addition of freshly-prepared Na ascorbate (1 mM after mixing) to start the reactions. The reactions were terminated by the addition of 25 mM EDTA (5 mM after mixing) after 10 min at 0°C or 2 min at 25°C (lane A shown on the right). Lane 1, DNA without treatment; lane 2, DNA plus ascorbate (the same pattern as that of DNA plus copper ion alone); lane 3, DNA plus Cu(II)-phenanthroline in the absence of ascorbate; lane 4, DNA plus Cu(II) in the presence of ascorbate; lane 5, DNA plus Cu-tripeptide and ascorbate; lane 6, DNA plus Cu-phenanthroline and ascorbate; lane 7, DNA plus 1,10-phenanthroline ($100 \mu\text{M}$ after mixing) and ascorbate without the addition of copper ion; lane 8, DNA in the presence of 1,10-phenanthroline ($100 \mu\text{M}$) alone. Lanes A and B, DNA plus copper ($50 \mu\text{M}$) and ascorbate (1 mM) incubated for 2 min at 25°C (A) and 10 min at 0°C (B), respectively. Electrophoresis was run at $1,500 \text{ V}$ for 2 h in the left panel and 3 h in the right panel (lanes A and B). The films were exposed for 96 h at -70°C .

Fig. 2. Effects of the concentrations of ascorbate and copper ion on the DNA-scission and the comparison of cleavage sites with base-specific chemical reactions of Maxam and Gilbert (17). The conditions are the same as those described in Fig. 1 and the reactions were done at 0°C for 10 min. The four chemical cleavages of sequencing methods were as described (17), and the lanes on the left were the samples of first loading whereas those on the right corresponding to the same samples applied to the gel after 5 h electrophoresis (at $1,200 \text{ V}$) of first loading; the electrophoresis was continued for another 3.5 h after second loading. The autoradiography was done at -70°C for 44 h. Lane 1, control DNA without treatment; lane 2, DNA plus ascorbate (1 mM) and copper (0.5 mM); lane 3, DNA plus ascorbate (0.1 mM) and copper (0.5 mM); lane 4, DNA plus ascorbate (0.01 mM) and copper (0.5 mM); lane 5, DNA plus ascorbate (1 mM) and copper (0.05 mM); lane 6, DNA plus ascorbate (1 mM) and copper ($5 \mu\text{M}$); lane 7, DNA plus ascorbate (1 mM) and copper-1,10-phenanthroline ($25 \mu\text{M}$); lane 8, DNA plus ascorbate (1 mM) alone. Lanes G, A+G, C+T, and C correspond to the four base-specific sequencing reactions.

Fig. 3. Cleavage specificity of 3'-end-labeled DNA fragment of pBR322 plasmid induced by ascorbate/copper. Arrows locate the most susceptible sites of scission from the densitometric scans of the autoradiograms in the lane B of Fig. 1 and the lane 5 of Fig. 2. The numbers indicated below the arrows denote the relative cleavage intensities from the densitometric scans with the most preferred site of scission at nucleotide #19 from the 3' end rated as 100. Other minor cleavage sites with their corresponding cleavage intensities are as follows: #1-2 (32), #2-3 (30), #3-4 (39), #4-5 (45), #5-6 (38), #6-7 (35), #7-8 (25), #8-9 (32), #10-11 (65), #14-15 (68), #15-16 (38), #16-17 (50), and #17-18 (62).

nucleotides produced under this kind of mild conditions have the same electrophoretic mobilities as the markers of the four specific chemical reactions, indicating that the phosphodiester bonds are the main targets for the cleavage by ascorbate/copper and the 5' termini of the breaks are probably phosphorylated as those produced from the four base-specific reactions (17). The detailed characterization of the structures of the cleaved products will be reported elsewhere.

The biological significance of the relatively specific DNA-cleavage by ascorbate/copper is still open to speculation. Attention is drawn to the recent reports of the sequence-specific cleavages of DNA by the antitumor drugs such as bleomycin (20, 21) and mitomycin C (22), which indicated that the dinucleotide sequences GT and GC are two of the most specific sites of cleavage in both cases. In our system, the most preferred sites seem to locate at G-C, G-A, A-C, and A-T of purine-containing segments similar to the G-T and G-C cleavage sites of these two drugs. This is also reflected in the observation by Kasai and Nishimura (23) that ascorbic acid in the presence of oxygen specifically hydroxylated the C-8 position of deoxyguanosines in DNA. It is of great interest to explore the possibility of utilizing this DNA-scission activity as the basis for new free-radical generating antiviral and antitumor drugs.

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