Sequence-dependent reactivity of linear DNA to chemical cleavage by Cu(II):thiol combinations including cysteine or glutathione

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The chemical cleavage of linear DNA by Cu(II):thiol combinations was investigated using end-labelled double-stranded restriction fragments. Single-strand cleavage of the target DNA occurred with apparent sequence preference, but there appeared to be no apparent consensus sequence for such preferentially cleaved sites based on studies of three different restriction fragments. For any given restriction fragment, the observed Cu(II):thiol DNA-cleavage sequence preference was found to be independent of the nature of the thiol used. Cleavage of 3'-end-labelled DNA generated fragments bearing 5'-phosphoryl termini, and cleavage of 5'-end-labelled DNA gave rise to fragments bearing 3'-phosphoryl and 3'-'phosphatase-inert' termini in equal proportions. No appreciable amounts of fragments bearing 3'- or 5'-hydroxy termini were detected. This pattern of cleavage products is similar to that observed using the hydroxyl-radical-generating systems, ⁶⁰Co γ -irradiation or

methidium propyl EDTA:Fe(II), but is dissimilar to that found with the Cu(II):phenanthroline or bleomycin systems. Cu(II):thiol cleavage of DNA fragments containing inserts of repeated dinucleotide sequence showed alternating patterns of relative cleavage intensities within the insert-sequence regions which were not seen in the sequences flanking the insert sequence. The assembled experimental data indicate that a reaction product of the Cu(II):thiol interaction, probably the hydroxyl radical, causes DNA cleavage. The Cu(II):thiol interaction may occur free in solution or via a copper species which is bound non-specifically to DNA. The observed low sequence-dependence of the cleavage reaction probably reflects the nature of the DNA structure itself, the Cu(II):thiol system serving as a structural probe of sequence-dependent structural variations in the DNA structure.

INTRODUCTION

With the surge of molecular biological techniques, an apparently realistic goal for therapies of certain diseases may be gene-replacement therapy, substituting a healthy gene for a miscreant one. For example, adenosine deaminase deficiency may be treatable by insertion of the gene into haematopoietic stem cells, which would then differentiate and express the gene throughout all the multiple lineages of stem-cell-derived blood cells (Wilson et al., 1990).

To achieve replacement-gene insertion into the genome will require the development of techniques capable of site-selective excision of faulty genes for insertion/ligation of the correct DNA sequence. Various strategies are being considered, including viral carriers, but one possibility is to develop chemical methods. Such site-specific DNA-cleavage reagents would have to combine both accurate sequence-reading ability with precise chemical cutting, but would be of lower molecular mass than (restriction) enzymes and may be tunable for cellular penetration or the avoidance of immune response.

The clinical success of bleomycin against some cancers led to an understanding of its mechanism of action as binding to DNA followed by strand cleavage via an activated oxygen free radical (Stubbe and Kozarich, 1987). Bleomycin has served as a model and stimulus to workers in this area, and the bulk of chemical systems currently used for DNA cleavage are based on free radicals, e.g. the Fe(II):EDTA system for hydroxyl radical generation (Tullius, 1989; Dervan et al., 1982) and the Cu(I):phenanthroline system (Sigman et al., 1979; Downey et

al., 1980). These, the most commonly used cleavage reagents, have been directed to DNA by attachment to intercalators (Hertzberg and Dervan, 1984), minor groove-directed ligands (Schultz et al., 1982) and major groove-binding proteins (Sluka et al., 1987; Ebright et al., 1990) as well as oligonucleotides (Chu and Orgel, 1985; Dreyer and Dervan 1985; Chen and Sigman, 1986). Recently, oligonucleotide binding to DNA via triple-helix formation has been exploited (Moser and Dervan, 1987; François et al., 1988; Strobel and Dervan, 1989).

Other methods of DNA cleavage have been developed including ruthenium and rhodium complexes (Mei and Barton, 1986; Kirschenbaum et al., 1988), the UO₂²⁺ ion (Nielsen et al., 1988) and porphyrins (Fiel et al., 1982; Fouquet et al., 1987). Quite recently our laboratory reported that low concentrations of thiols plus Cu(II) ions cleave DNA efficiently (Reed and Douglas, 1989, 1991; John and Douglas, 1989). We now report a detailed study of the nature of the cleavage of double-stranded DNA (ds DNA) by the Cu(II):thiol system using linear ds DNA fragments of defined sequence.

MATERIALS AND METHODS

AnalaR-grade sodium phosphate and copper(II) sulphate pentahydrate were from BDH. Dithiothreitol and glutathione (free acid) were from Sigma. Cysteine (L- and D-forms) and 3-mercaptopropionic acid (3-MPA) were from Aldrich. Thiol solutions were prepared freshly and assayed immediately before use using Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] to determine free thiol concentrations (Riddles et al., 1983). All

Abbreviations used: dsDNA, double-stranded DNA; 3-MPA, 3-mercaptopropionic acid.

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(**a**) *tyrT* 163 bp fragment; (**b**) pBR322 167 bp fragment; (**c**) pBR322 517 bp fragment; (**d**) pRW1011 70 bp fragment; (**e**) pRW1015 70 bp fragment. Strands were labelled as follows (labelling sites not shown). 3'-End-labelling: (**a**): positions -2, -3 lower strand; (**b**): positions 0 and -1, lower strand; (**c**): positions 0 and +1, upper strand; (**d**) and (**e**), positions 0 and -1, lower strand. 5'-End-labelling: (**c**) position -2, lower strand (see the text for details).

Calf intestinal alkaline phosphatase, DNAase I (bovine pancreatic), T4 polynucleotide kinase and the restriction endonucleases *Hind*III and *Rsa*I were obtained from Boehringer-Mannheim. DNA polymerase I large fragment (Klenow fragment, labelling grade) and the restriction endonucleases *Ava*I and *Eco*RI were from Gibco BRL. Calf thymus DNA (type I, highly polymerized) was from Sigma. 5'-[α - 32 P]dATP and 5'-[γ - 32 P]ATP, both of specific radioactivity ≥ 6000 Ci/mmol, were from Amersham. Plasmids pBR322 (Maniatis et al., 1982), pKM Δ 98 (Drew and Travers, 1985) and pRW1011 and pRW1015 (McLean and Wells, 1988) were prepared in predominantly supercoiled form by standard procedures (Maniatis et al., 1982). 3'-End- 32 P-labelled fragments were generated by digestion of supercoiled plasmid DNA with *Eco*RI, 3'-end-labelling at the *Eco*RI sites with 5'-[α - 32 P]dATP using the Klenow fragment of DNA polymerase I (Maniatis et al., 1982) followed by a second restriction digestion (*Rsa*I for pBR322, *Ava*I for pKM Δ 98 or

Cu(II):thiol-mediated scission of DNA was carried out essentially as previously described (John and Douglas, 1989). Briefly, a portion (5 μ l) of thiol solution (0.5 mM free SH) was added to 20 μ l of sodium phosphate buffer (12.5 mM, pH 8.2) containing sonicated calf thymus DNA (50 μ M bp), end-labelled DNA fragment (approx. 100 c.p.s., < 10 pmol bp) and CuSO₄ (0.25 mM). Reactions, generally run for 15 min at 37 °C, were terminated by addition of EDTA to 0.8 mM and/or precipitation of the DNA with ethanol. Determination of the nature of the DNA termini generated by Cu(II):thiol cleavage of DNA was carried out by the procedures of Hertzberg and Dervan (1984). Briefly, calf intestinal alkaline phosphatase was used to remove 5'-phosphoryl groups and T4 polynucleotide kinase to remove 3'-phosphoryl groups present in 3'- or 5'-end-labelled DNA samples respectively that had been treated with Cu(II):thiol under standard conditions. DNAase I digestion of DNA was carried out under conditions described by Ford et al. (1987). Both 'phosphatase' and DNAase I reactions were terminated by DNA precipitation with ethanol.

Before electrophoresis, all samples were resuspended in 90 % formamide loading buffer, heated at 90 °C for 5 min and snap-cooled in ice/water before being loaded on to denaturing polyacrylamide gels (8 % or 20 % polyacrylamide, 1:20 cross-linked, 7 M urea and 90 mM Tris/borate/2 mM EDTA buffer, pH 8.3). After electrophoresis (2 h, 1500 V), gels were fixed in 10 % acetic acid, washed in distilled water and dried down on to glass plates. Dried gels were exposed to preflashed (Laskey, 1980) Fuji RX100 X-ray film at -80 °C with an intensifying screen. Digestion pattern bands were assigned to particular nucleotides of the DNA fragment by comparison with a control lane of G-track DNA sample (showing the positions of guanines) and with reference to the published nucleotide sequences of the fragments.

RESULTS

Cleavage of 3-end-labelled linear dsDNA fragments was examined by denaturing PAGE to detect sites of single-strand breaks on the labelled strand of the DNA duplex.

Time course

Treatment of the *tyrT* 163 bp fragment by the Cu(II):thiol system caused single-strand breaks to occur at all positions within the fragment (Figure 2, lanes 5–9). Not all positions were cleaved with the same frequency, as judged by the variation in band intensities. There was, however, a definite pattern of preferred sites of cleavage, a pattern that remained constant over the time course. Standard conditions for fragment cleavage were chosen to provide limited digestion of the intact DNA (i.e. where, statistically, only one cut per DNA strand occurred.

Figure 2 Time course of Cu(II):GSH cleavage of linear *tyrT* DNA

Incubations were carried out under standard DNA and buffer conditions (see the Materials and methods section) and contained 3'-end-labelled *tyrT* DNA with the following additions: lane 1, none; lanes 2 and 5-9, Cu(II) (200 μ M); lanes 3 and 5-9, GSH (100 μ M). Incubations were terminated after 20 min (lanes 1-3 and 9) or 2, 4, 8 and 12 min (lanes 5-8 respectively), analysed by denaturing PAGE (8% gels) and autoradiographed as in the text. Lane 4, *tyrT* DNA G-track. Nucleotide numbering refers to G-track fragments and is as shown in Figure 1.

approx. 80% of the fragment remained intact) and further reactions were standardized to 10 min duration under the above conditions to provide this.

Cleavage of pBR322 DNA fragments

A range of thiols when tested for cleavage of *tyrT* DNA under such conditions as described above showed apparent sequence selectivity, independent of the nature of the thiol (John and Douglas, 1989). To examine whether this was a true sequence-dependent cleavage, additional linear fragments of pBR322 DNA (167 bp and 517 bp fragments) were cleaved using the Cu(II):thiol system.

Figures 3 and 4 show the patterns of cleavage obtained with four different thiols and Cu(II) for the 167 bp and 517 bp fragments respectively (partial sequences shown in Figure 1). The strong band at position 68/69 was attributed to a band compression resulting in the co-migration of bands 68 and 69. This effect is evident in published work using this DNA fragment (Harshman and Dervan, 1985), but was not commented on, and it was therefore concluded that in this present work this band did not correspond to a preferentially cleaved DNA site.

As was the case for the *tyrT* DNA fragment (John and Douglas, 1989), both the 167 bp and 517 bp fragments were cleaved by Cu(II):thiol with apparent sequence preferences (Figures 3 and 4 respectively). Moreover, these preferences were independent of the nature of the thiol used, i.e. compare the similar cleavage patterns obtained using four different thiols and Cu(II) to cleave the 167 bp fragment (lanes 5-8, Figure 3). However, comparison of preferred cleavage sites on all three DNA fragments (Table 1) shows that all these fragments were cleaved with different apparent sequence preferences and that no

Figure 3 Cleavage of 167 bp fragment of pBR322 by Cu(II):thiols

Incubations (10 min, 37 $^{\circ}$ C) were carried out under standard DNA and buffer conditions (see the Materials and methods section) and contained 3'-end-labelled 167 bp fragment with the following additions: lane 1, none; lanes 2 and 5-8 Cu(II) 200 μ M; lanes 3 and 5-8 GSH, *D*-Cys, *L*-Cys, GSH and 3-MPA respectively at 100 μ M. Incubations were terminated, analysed by denaturing PAGE (8% gels) and autoradiographed as in the text. Lane 4, 167 bp G-track. Nucleotide numbering refers to G-track fragments and is as shown in Figure 1.

consensus sequence was apparent. Thus the sequence preference appeared to be characteristic of the piece of DNA used.

Nature of DNA termini generated by Cu(II):thiol cleavage

5'-End determination

Careful inspection of Figures 2-4 reveals that Cu(II):thiol degradation of 3'-end-labelled DNA produces DNA fragments of similar electrophoretic mobility to G-track DNA fragments (which bear terminal 5'-phosphate groups; Maxam and Gilbert, 1980). Proof of the nature of the 5'-end of Cu(II):thiol-digested DNA is shown in Figure 5(a) where samples of G-track and Cu(II):thiol-treated 3'-end-labelled 517 bp fragment are shown before and after treatment with calf intestinal alkaline phosphatase (to remove the 5'-phosphate groups; Hertzberg and Dervan, 1984). The decrease in electrophoretic mobility and co-migration of both G-track and Cu(II):thiol-treated DNA fragments on treatment with this enzyme proves that Cu(II):thiol-treated DNA samples possessed terminal 5'-phosphate groups which were subsequently converted into 5'-hydroxy groups, thereby decreasing the mobility of the DNA on electrophoresis. Note that no significant amounts of fragments bearing 5'-hydroxy termini are seen before treatment with calf intestinal alkaline phosphatase (compare lanes 3 and 4, Figure 5a).

3'-End determination

Using 5'-end-labelled 517 bp fragment DNA and T4 polynucleotide kinase as a 3'-phosphatase (Hertzberg and Dervan, 1984), the apparently single electrophoretic bands formed by Cu(II):thiol degradation of 5'-end labelled DNA (lane 2, Figure 5b) were found to be made up of two separate

Figure 4 Cleavage of 517 bp fragment of pBR322 by Cu(II):thiols

Incubations (10 min, 37 °C) were carried out under standard DNA and buffer conditions (see the Materials and methods section) and contained 3'-end-labelled 517 bp fragment with the following additions: lane 1, none; lanes 2 and 5–8, Cu(II) (200 μ M); lanes 3 and 5–8, GSH, D-Cys, L-Cys, GSH and 3-MPA respectively (all 100 μ M). Incubations were terminated, analysed by denaturing PAGE (8% gels) autoradiographed as in the text. Lane 4, 517 bp G-track. Nucleotide numbering refers to G-track fragments and is as shown in Figure 1.

Table 1 Preferred cleavage sites for the Cu(II):thiol system

DNA fragment	Cleavage site*	Sequence (5'–3')†
<i>tyrT</i> *163bp)	45–47	AAAAATGAAC
	64–67	TGTAAGTGT
	80–83	TCAAATGACG
	97–99	AAGCGGGGCG
	107–109	CCTTATCGGG
167bp	53–58	AATTTAACTG
	79–84	CATACACGGT
	91–97	ATTGTTAGAT
	108–111	GGATGACGAT
	123–124	GGTGACGGTG
517bp	83–87	AAAGTGCCAC
	111–113	AATAGGGGTT
	123–124	GAAAAATAAA
	129–134	GTATTTAGAA
	171–174	TTATCAGGGG

* Cleavage sites on the 3'-end-labelled strand; data taken from Figures 2–4.

† Preferred cleavage sites (underlined) and flanking sequences are shown.

species (lane 3, Figure 5b). On dephosphorylation by T4 polynucleotide kinase, the electrophoretic mobility of one of the two bands (the 'phosphatase-inert' band) was unaffected, whereas the other band became less mobile and co-migrated with fragments generated by DNAase I digestion (3'-hydroxy ends; lane 4, Figure 5b). The 'phosphatase-inert' band migrated just in front of the G-track marker fragments, indicating a similarly charged terminal group. Thus Cu(II):thiol digestion of 5'-end-labelled DNA produced 3'-phosphate termini and '3'-phosphate-

Figure 5 Determination of the nature of the DNA termini generated by Cu(II):thiol cleavage

Cleavage of the 517 bp fragment, either 5'- or 3'-end-labelled, was carried out using Cu(II) (200 μ M) and GSH (100 μ M) under standard reaction conditions (see the Materials and methods section). Incubations (10 min, 37 °C) were terminated and further treated with calf intestinal alkaline phosphatase or polynucleotide kinase (where appropriate) as in the text. Samples were analysed by denaturing PAGE (20% gels) and autoradiographed as in the text. (a) 5'-end determination using 3'-end-labelled 517 bp fragment: lanes 1 and 2, 517 bp G-tracks without and with phosphatase treatment respectively. Lanes 3 and 4, Cu(II):GSH-treated 517 bp fragment with and without phosphate treatment respectively. (b) 3'-end determination using 5'-end-labelled 517 bp fragment: lane 1, 517 bp G-track. Lanes 2 and 3, Cu(II):GSH-treated 517 bp fragment without and with polynucleotide kinase treatment respectively. Lane 4, 517 bp fragment treated with DNAase I (see the text). Nucleotide numbering refers to G-track fragments and is as shown in Figure 1.

like' 'phosphatase-inert' termini, which probably possess a similar charge to 3'-phosphate-terminated fragments.

Cu(II):thiol cleavage of fragments containing repeating dinucleotide sequences

Plasmids pRW1011 and pRW1015 contain specific inserts of repeating dinucleotide sequences which, when present in supercoiled DNA molecules and under certain conditions, such as high NaCl concentration, can adopt Z-DNA or other non-B-DNA conformations (McLean and Wells, 1988). However, when these insert sequences are present in linear dsDNA fragments under normal solution conditions (i.e. neutral pH, low NaCl concentration), these conformational changes do not occur. Thus Cu(II):thiol cleavage of 3'-end-labelled 70 bp fragments containing these sequences was carried out to examine the effect of alternating sequence on the pattern of DNA cleavage. Three important features are apparent from the cleavage patterns shown in Figure 6: (i) severe compression of bands occurred in the region of positions 37–48 (lanes 4, 5 and 6) of the pRW1015-derived fragment, (ii) for both pRW1011- and pRW1015-derived fragments, a regularly alternating pattern of band intensities (light–dark–light, etc.) was seen in the region of the inserted sequences (positions 19–42, lanes 9, 10 and 11 and positions 19–28, lanes 3–6), and (iii) no other such marked differences in cleavage intensities were observed for sequences outside of the insert region. The most intense bands of the alternating patterns were at positions corresponding to the underlined residues in the following sequences:

Figure 6 Cu(II):thiol cleavage of 70 bp fragments derived from pRW1011 and pRW1015

Incubations were carried out under standard DNA and buffer conditions and contained 3'-end-labelled pRW1015-derived 70 bp fragment (lanes 1–7) or pRW1011-derived 70 bp fragment (lanes 8–11) with the following additions: lanes 1 and 8, none. Lane 2, Cu(II) (200 μ M); lane 3, GSH (100 μ M); lanes 4–6 and 9–11, Cu(II) (200 μ M) plus GSH (100 μ M). Incubations were for 10 min (lanes 4 and 9), 20 min (lanes 5 and 10) or 40 min (lanes 1–3, 6, 8 and 11), all at 37 °C and were terminated, analysed by denaturing PAGE (8% gels) and autoradiographed as in the text. Lane 7, pRW1015-derived 70 bp fragment G-track. The insert sequence region (residues 19–42, inclusive) is denoted by the bracket and nucleotide numbering is as shown in Figure 1.

Cleavages in the region 32–42 of pRW1015 were obscured by the compression of bands. Resolution of such band compressions was achieved by electrophoresis in denaturing gels containing 30% formamide in addition to urea (Shafer et al., 1989), and showed that the alternating band pattern did not extend into the previously unresolved section (results not shown). Cleavage sites on the complementary strands were not determined.

DISCUSSION

Previous workers have shown that in the presence of Cu(II), thiols can cause single-strand breaks in supercoiled plasmid DNA (Reed and Douglas, 1989). Of the metal ions tested, only Cu(II) was effective for the thiol-induced DNA-cleavage reaction. The Cu(II):thiol system did not appear to cleave supercoiled DNA preferentially at special structural features of plasmids containing certain insert sequences (Reed and Douglas, 1991), e.g. Z-DNA inserts, cruciform structures or left-handed (but non-Z) DNA regions. Base damage was detected, together with DNA fragments possessing 5'-hydroxy and 3'-hydroxy groups, as well as 2-thiobarbituric acid-reactive species (probably base propenals) as products of the cleavage reaction. The Cu(II):thiol-cleavage reaction was further examined in detail (at single nucleotide resolution) using radioactively labelled linear dsDNA restriction fragments (John and Douglas, 1989). In agreement with Reed and Douglas (1989) it was found that the Cu(II):thiol system caused single-strand cleavages within the linear dsDNA substrate and that the extent of DNA cleavage was independent of the nature of the thiol used. In addition, and surprisingly at first, it was observed that the Cu(II):thiol-induced degradation of the *tyrT* DNA fragment did not occur equally at every nucleotide position. This apparent sequence preference of the

DNA cleavage caused by the Cu(II):thiol system was independent of the nature of the thiol.

The work described in this paper shows that the 167 bp and 517 bp fragments derived from pBR322 are cleaved by Cu(II):thiol with a thiol-structure-independent apparent sequence preference. These results indicate that the ultimate DNA-cleaving species must be a common product of the reaction of Cu(II) and thiol and is therefore not likely to be a copper–thiol complex or a copper-derived thiol species (e.g. a thiyl radical, RS $\cdot$). The inhibition of cleavage by hydroxyl radical scavengers or removal of dissolved oxygen (Reed and Douglas, 1991), the inhibition of cleavage by both Cu(II)- and Cu(I)-specific chelators (unpublished results) and the documented hydroxylation of aromatic molecules by Cu(II):ascorbate (Udenfriend et al., 1954) suggest that the DNA-cleaving species is the hydroxyl radical.

Cleavage by Cu(II):thiol produced 5'-phosphoryl, 3'-phosphoryl and 3'-'phosphatase-inert' termini (the last two in roughly equal proportions). The 3'-'phosphatase-inert' species has not yet been further identified. However, hydroxyl-radical-mediated DNA damage produced by either ^{60}Co γ -irradiation (Kuwabara et al., 1986) or methidium propyl EDTA:Fe(II) treatment (Hertzberg and Dervan, 1984) both lead to production of 5'-phosphoryl termini and equivalent proportions of 3'-phosphoryl and 3'-phosphoglycollate termini ('phosphatase-inert' termini); unequal proportions are produced by Cu(I):phenanthroline cleavage (3'-phosphoglycollate is a minor product; Kuwabara et al., 1986) and bleomycin cleavage (3'-phosphoglycollate is the primary stable product; Stubbe and Kozarich, 1987) where hydroxyl radical is not the ultimate DNA-cleaving species. Thus Cu(II):thiol DNA-cleavage mechanism is similar to that of the hydroxyl-radical-generating systems, as both systems produce similar products in similar proportions. The 3'-'phosphatase-inert' species observed in the present work can therefore be inferred to be a 3'-phosphoglycollate species. Reactions consistent with this pattern of cleavage products are attack of hydroxyl radicals equally at both C-1' and C-4' of deoxyribose.

Previous work by Reed and Douglas (1991) showed the presence of 5'-hydroxy and 3'-hydroxy termini in plasmid DNA treated with Cu(II):thiol, but detection of 3'- and 5'-phosphoryl termini was not carried out. In this present work, however, end-determination experiments showed that significant quantities of 3'- or 5'-hydroxy termini were not produced. Thus DNA fragments bearing 3'- or 5'-hydroxy termini are likely to be very minor products of Cu(II):thiol DNA cleavage, only detected in the plasmid system by its very sensitive assay (Reed and Douglas, 1991).

The Cu(II):RSH cleavage of supercoiled plasmids containing specific inserts which produced Z-DNA, cruciform or left-handed non-Z-DNA structures when under supercoil stress, occurred throughout the whole plasmid DNA sequence and not specifically (or preferentially) at the sites of the non-B-DNA insert sequences (Reed and Douglas, 1991). Thus Cu(II):RSH showed no ability to act as a probe for these non-B-DNA structures. Using linear restriction fragments with Cu(II):thiol showed that, within the insert sequences [(TG) $_{12}$ or (TG) $_{6}$ (AC) $_{6}$], there was a preference for cleavage at cytosines in the (AC) $_n$ sequence (only 3'-end-labelled, lower strand examined). On considering cleavage intensities at all nucleotide positions of the DNA fragments, it was clear that not all cytosines were preferentially cleaved, but that only within the insert sequences was cleavage relatively enhanced at cytosines compared with intervening adenines. In the absence of the special conditions required for restriction fragments to form Z-DNA (e.g. 5 M NaCl; Klysik et al., 1981), the cleavage patterns observed for the insert-sequence regions

may be produced in response to some other factor(s), e.g. local helical parameters affecting binding of Cu(II) to DNA at such 3'-(purine·pyrimidine)_n sequences [(CA)_n tracts are known to form unusual non-B-DNA structures, e.g. Timsit et al (1991)]. The lack of a clearly apparent consensus sequence for the Cu(II):thiol-preferred cleavage sites on DNA and the preferential reaction of Cu(II):thiol with alternate bases of the insert regions of the 70 bp fragments may reflect that Cu(II):thiol cleavage preferences are dependent on sequence-determined factors (such as local variations in DNA structure), but not on the DNA sequence itself.

The apparent sequence preference of Cu(II):thiol DNA cleavage is difficult to explain in terms of copper ion binding to specific DNA base sites, e.g. N-7 of guanines (Pezzano and Podo, 1980). Previous work using the analogous Cu(II):H₂O₂ system (Sagripanti and Kraemer, 1989), showing that base-labile sites were generated specifically at poly:(G) sequences, was inferred to be due to the specific Cu(II) binding to G sites and its subsequent reaction. However, when the *tyrT* fragment, which had been previously treated with either Cu(II):thiol or Cu(II):H₂O₂, was treated with piperidine (1 M), no such guanosine-specific base-labile sites were observed (results not shown).

Guanosine positions were not preferentially cleaved and many thymidine, cytosine and guanosine residues were detected as base-labile sites. Thus in our hands there was no evidence of specific binding of copper to guanosine residues.

The 'sequence-dependent' patterns of cleavage observed above are unexpected, since the Cu(II):thiol system is capable of generating hydroxyl radical (Chiou, 1983) and yet does not give the 'sequence-neutral' patterns generated by the Fe(II):EDTA system (Tullius, 1989) which cleaves DNA via hydroxyl radicals. It has been reported that pBR322-derived linear duplex DNA fragments may be cleaved by Cu(II) and amino sugars (Watanabe et al., 1990).

In conclusion, the Cu(II):thiol system cleaves DNA with an apparent sequence preference which is independent of the nature of the thiol used. This demonstrates that the cleavage preference is not due to reaction of either putative Cu(II)-thiol complexes themselves or of oxidized forms of thiol (e.g. thiyl radical) with the DNA, but is due to a common reaction product that bears no structural resemblance to the thiol used. Cu(II):thiol treatment of DNA causes both strand breaks and base damage [the latter occurs before detectable strand cleavage (John, 1991)] and cleavage products include oligonucleotides bearing 5'-phosphoryl, 3'-phosphoryl and 3'-phosphoglycollate-like termini. The similarity of the products of Cu(II):thiol DNA cleavage and those of hydroxyl-radical-cleaved DNA, together with the inhibition by hydroxyl radical scavengers (Reed and Douglas, 1991), strongly suggests hydroxyl radical as the major DNA-cleaving species.

From the low sequence-dependence of Cu(II):thiol cleavage of DNA, it is likely that the majority of DNA-damaging radical production occurs either via Cu(II) bound to DNA non-specifically and/or via Cu(II), free in solution, and from which the cleaving radical may diffuse to react with DNA.

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