Chemical cleavage of plasmid DNA by glutathione in the presence of Cu(II) ions

The Cu(II)–thiol system for DNA strand scission

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In the presence of Cu(II) ions, supercoiled DNA is cleaved in neutral solution by low concentrations of thiols. Supercoiled plasmid DNA is cleaved first to open circular DNA, which in turn produces linear DNA and eventually fragments. Cleavage is strongly temperature-dependent and is maximal at 0.10–0.25 M-NaCl concentration. In the presence of excess of either component of the Cu(II)–thiol pair, the extent of cleavage depended on the concentration of the limiting partner, and was easily detectable down to micromolar concentrations of limiting GSH. Scavengers of oxygen-derived species (such as hydrogen peroxide, superoxide radical ion and hydroxyl radical) indicated that the hydroxyl radical may be involved in the cleavage mechanism. DNA cleavage leads to some production of 2-thiobarbituric acid-reactive species and some of the cleavage sites, at least, had 5'-hydroxy and/or 3'-hydroxy groups. There was extensive base damage before cleavage. Studies with S1 nuclease indicated no gross sequence preference for Cu(II)–GSH cleavage of pSP64 plasmid DNA. The Cu(II)–thiol system did not appear to target special structural features in the DNA such as Z-DNA inserts, cruciform structures or left-handed (but non-Z) DNA. Cleavage might arise from a reagent generated either by the Cu(II)–thiol combination in free solution or by attack involving Cu(II) ions pre-bound to DNA. The attack of GSH plus Cu(II) ions on DNA may be a potential toxic lesion under physiological conditions unless special protective measures operate efficiently in the cell.

INTRODUCTION

Sequence-specific cleavage of DNA has many applications in molecular biology, but is limited by the specificities and accessibility of natural restriction endonucleases. One approach to overcoming this has been to modify a DNA-recognizing molecule chemically with a reagent capable of chemical cleavage of DNA. The most commonly used reagent for such chemical cleavage is probably the Fe(II)–EDTA system, which has been attached to oligonucleotides (Boutorin *et al.*, 1984; Dreyer & Dervan, 1985), to intercalators (Hertzberg & Dervan, 1984) and to a combination of these binding species (Boidet-Forget *et al.*, 1988). Minor-groove-directed drugs (Taylor *et al.*, 1984) and anti-sense oligodeoxyribonucleoside methanephosphonates (Lin *et al.*, 1989) have joined the catalogue. The other commonly used chemical cleavage systems include Cu(I)–bis-(1,10-phenanthroline) (Spassky & Sigman, 1985; Chu & Orgel, 1985; François *et al.*, 1989; Veal & Rill, 1989), metal–porphyrin complexes (Lown & Joshua, 1982; Lown *et al.*, 1986; Bernadou *et al.*, 1989; Groves & Farrell, 1989) and rhodium complexes (Kirshenbaum *et al.*, 1988). Photochemical cleaving systems for DNA have also been described (Doan *et al.*, 1987; Jeppesen *et al.*, 1988; Saito *et al.*, 1989; Jeppesen & Nielsen, 1989; Benimetskaya *et al.*, 1989). The metal-complex systems above all require activation to induce strand scission, frequently achieved by means of a reducing agent but also by oxidizing agents in some situations (Sagripanti & Kraemer, 1989; Yamamoto *et al.*, 1989). Sequence-directed cleavage of single-stranded DNA has been achieved by means of complementary oligonucleotides bearing terminal alkylating agents (Meyer *et al.*, 1989).

One use for DNA- and RNA-sequence-specific reagents capable of controlled chemical cleavage is as molecular-biological tools either in the sense of standard artificial restriction

endonucleases or for larger-scale genome mapping. In addition, the genetic basis of several major diseases has been recognized lately, and it seems a realistic goal to target aberrant DNA base sequences for occlusion by direct binding (either at DNA or, more likely, at mRNA level) or by chemical excision.

With this in mind, it is important to develop not only novel DNA-sequence-reading systems but also chemical cleavage systems that are easily amenable to chemical synthetic manipulation and have suitable biocompatibility (e.g. stability, cellular penetration, recycling). Thiols have long been known to autoxidize, especially in the presence of certain transition-metal ions, frequently to the dismay of the synthetic chemist. Thus we considered that metal ion–thiol systems might be suitable reagents for DNA cleavage. The present paper gives details of the cleavage of plasmid DNA by an efficient combination, the Cu(II)–thiol system, some aspects of which we have already reported (Reed & Douglas, 1989).

MATERIALS AND METHODS

Plasmids

Unless otherwise specified, all experiments were carried out on plasmid pSP64 DNA. The following plasmids, containing synthetic inserts with specific conformational features, were kindly given by Dr. M. McLean (I.C.I. Diagnostics, Northwich, Cheshire, U.K.) (for further information see references): pRW1001 [insert (CG)₈, Z-forming] (McLean *et al.*, 1986); pRW1002 [insert (CG)₄, possibly Z-forming] (McLean *et al.*, 1986); pRW1011 [insert (TG)₁₂, Z-forming] (McLean & Wells, 1988); pRW1015 [insert (TG)₆(AC)₆, left-handed non-Z] (McLean & Wells, 1988); pRW1155 [insert GGATCC(TG)₂₀-AATT(CA)₂₀ → GGATCC cruciform-forming] (Blaho *et al.*, 1988). In most cases the sequence of the insert was verified by

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excision and 3'-end-labelling of a fragment containing the insert, followed by G-tracking (Maniatis *et al.*, 1982).

Reaction conditions for thiol-dependent DNA cleavage

Plasmid DNA was purified from *Escherichia coli* HW82 (pSP64) or *E. coli* K12 TG1 (all other plasmids) by CsCl-density-gradient centrifugation (Reed & Douglas, 1989). The plasmid DNA was not contaminated with RNA or chromosomal DNA, and was predominantly (> 95%) in the supercoiled form (as judged by agarose-gel electrophoresis). DNA concentrations as base-pairs were based on ϵ_{260} $13\,100\text{ M}^{-1}\cdot\text{cm}^{-1}$ (John & Douglas, 1989). Thiol concentrations were determined (Riddles *et al.*, 1983) immediately before incubation with DNA by titration of stock thiol solutions with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid); Sigma Chemical Co., Poole, Dorset, U.K.]. Aqueous solutions (20 mM) of CuSO_4 were prepared and diluted to the appropriate concentration immediately before use. All thiols and metal salts were of the highest quality available commercially. Water and all solutions used throughout were stored in plastic containers to prevent contamination by adventitious metal ions leached from glass.

Plasmid DNA (final concentration $30\text{ }\mu\text{M}$ -base-pairs unless stated otherwise) was incubated at 37°C with thiol plus Cu(II) ions in 10 mM-sodium phosphate buffer, pH 8.0, without added NaCl (unless stated otherwise). Specific details of incubation period and thiol and Cu(II) ion concentrations are given in the legends to Figures and the Table. Quenching of reactions was achieved either by adding EDTA (final concentration $\geq 12\text{ mM}$) or by addition of Bromophenol Blue and immediate loading and agarose-gel electrophoresis.

Quantification of DNA cleavage

The relative amounts of supercoiled, open circular and linear DNA were analysed by submarine agarose-gel electrophoresis (0.7% agarose; running buffer 90 mM-Tris/HCl/90 mM-boric acid/2 mM-EDTA, pH 8.2) and quantified by ethidium bromide staining and densitometry (LKB microdensitometer). The film used to photograph the gels (Polaroid type 665) was shown to have a linear response in the range of DNA quantities used. Since supercoiled DNA is restricted in its ability to bind ethidium bromide relative to open circular and linear forms, it was necessary to correct values obtained for supercoiled DNA by using the factor 1.28, determined by the method of Haidle *et al.* (1979), i.e. 1.28:1:1 proportions for ethidium bromide binding to supercoiled, open circular and linear DNA respectively.

Some pSP64 preparations were found to be inherently more sensitive to Cu(II)-thiol cleavage than others. The available evidence suggests that this is a function of the DNA rather than the Cu(II)-thiol cleavage chemistry. Appropriate internal controls were included in every experiment to standardize the system, and comparison of results was always made within a single experiment.

Inhibition of DNA cleavage

DNA was incubated (at 37°C for 90 min) with GSH plus Cu(II) ions (final concentrations $5\text{ }\mu\text{M}$ and $100\text{ }\mu\text{M}$ respectively), and the conversion of the supercoiled form into the open circular form was analysed by agarose-gel electrophoresis and densitometry. Inhibition of cleavage by the following scavengers of reactive O_2 -derived species was assessed (final concentrations in parentheses): mannitol (0.1 M), glycerol (10%, v/v), NaN_3 (0.1 M), catalase [5 ng/ml (0.1 unit) or $1\text{ }\mu\text{g/ml}$ (23 units)] and superoxide dismutase [1 mg/ml (3000 units)]. Protection against cleavage was calculated as:

$$\text{Protection} = 1 - (S_c - S_{G+1}) / (S_c - S_G)$$

where S_c is the percentage supercoiled form present in the control (untreated) plasmid DNA, S_G is the percentage supercoiled form present in plasmid DNA treated with GSH plus Cu(II) ions and S_{G+1} is the percentage supercoiled form present in plasmid DNA treated with GSH plus Cu(II) ions plus the appropriate putative inhibitor.

Investigation of the nature of the cleavage products

To determine the nature of the DNA ends formed by Cu(II)-thiol cleavage, pSP64 DNA ($8\text{ }\mu\text{g}$) was digested with GSH plus Cu(II) ions (final concentrations $25\text{ }\mu\text{M}$ and $100\text{ }\mu\text{M}$ respectively) for 1 h at 37°C . This resulted in complete conversion of the supercoiled form into the open circular and linear forms. The sample, divided into four equal portions, was ethanol-precipitated. One sample was labelled by using the Klenow fragment of DNA polymerase I and [α - ^{32}P]dATP according to standard procedures (Maniatis *et al.*, 1982). Two other samples were labelled by using T4 polynucleotide kinase and [γ - ^{32}P]dATP, one by a protocol for 5'-protruding ends (Maniatis *et al.*, 1982) and the other by a protocol for 3'-protruding or blunt ends (Maniatis *et al.*, 1982). The fourth sample was not labelled after incubation. All samples were ethanol-precipitated, resuspended in water and electrophoresed on a 0.7% agarose gel. The gel was then cut into two. The piece containing the control samples was stained in ethidium bromide, and the rest was dried and autoradiographed (Fuji RX X-ray film with one or two calcium tungstate intensifying screens, exposure overnight at -80°C).

Formation of base propenals was assessed as follows. Calf thymus DNA (final concentration 1 mM) was incubated for periods up to 3 h at 37°C with GSH (final concentration 0–5 mM) and Cu(II) ions (final concentration 0–5 mM) in a final volume of $90\text{ }\mu\text{l}$. 2-Thiobarbituric acid ($900\text{ }\mu\text{l}$ of a 0.6% solution, pH 2) was added, and samples were heated at 90°C for 30 min. The absorbance at 532 nm was measured and the concentration of the chromophore was calculated by using ϵ_{532} $158\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$. Bleomycin was standardized optically before use by using ϵ_{292} $14\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Hertzberg & Dervan, 1984). Incubation mixtures contained calf thymus DNA and bleomycin (final concentrations 1 mM and 0.5 mM respectively). The reaction was initiated by the addition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (final concentration 0.5 mM) and incubations were at 37°C for 15 min.

To ascertain if incubation with the Cu(II)-thiol system leads to base damage not resulting in strand cleavage, the following procedure was carried out. Plasmid DNA ($5\text{ }\mu\text{g}$) was incubated with GSH plus Cu(II) ions (final concentrations $20\text{ }\mu\text{M}$ and $100\text{ }\mu\text{M}$ respectively) for 1 h at 37°C . The DNA was separated from the cleavage system by centrifugation through a column (1 ml volume) of Sephadex G-50 (Maniatis *et al.*, 1982), divided into two portions and ethanol-precipitated. Piperidine (100 μl of 1 M) was added to one sample, which was then heated at 90°C for 30 min and dried (Savant Speedvac SVC100H concentrator). Each sample was washed twice with water ($2 \times 10\text{ }\mu\text{l}$), with drying between washes, resuspended in water and analysed by agarose-gel electrophoresis.

Sequence or conformational preference of Cu(II)-thiol cleavage of DNA

Plasmid DNA (100–150 μg) was incubated for 3 h at 37°C with cysteine plus Cu(II) ions (final concentrations 1 mM and 2 mM respectively). The DNA was separated from excess Cu(II)-thiol reagent by centrifugation through Sephadex G-50 (1 ml column volume) (Maniatis *et al.*, 1982), divided into three portions and ethanol-precipitated. The following digestion procedures were carried out: (1) restriction endonuclease with a unique site, namely *Eco*RI for pSP64, *Rsa*I for pRW1001,

pRW1002, pRW1011 and pRW1015, and *Pst*I for pRW1155; (2) restriction endonuclease with multiple sites (number of sites in parentheses), namely *Rsa*I for pSP64 (three), *Pst*I for pRW1001, pRW1002, pRW1011 and pRW1015 (all two), and *Bgl*I for pRW1155 (three); (3) as for (1) followed by *S*I nuclease; (4) as for (2) followed by *S*I nuclease. Control uncleaved plasmid DNA was digested identically. Samples were analysed by agarose-gel electrophoresis.

RESULTS

We have reported previously that, in the presence of Cu(II) ions, low concentrations of thiols cleave DNA, converting the supercoiled form of plasmid DNA into the open circular and linear forms (Reed & Douglas, 1989). We now report the system more fully. Chelating agents for Cu(II) ions, for example sodium citrate and EDTA, totally inhibited DNA cleavage. Addition of EDTA (final concentration 12 mM) after incubation of DNA with thiol plus Cu(II) ions quenched the cleavage, and such samples could be stored at 4 °C for at least 24 h without further DNA strand breakage occurring.

Fig. 1. Temperature-dependence of DNA cleavage by GSH in the presence of Cu(II) ions

Samples contained pSP64 DNA (30 μM) and (a) GSH (20 μM), (b) Cu(II) ions (100 μM) or (c) GSH (20 μM) plus Cu(II) ions (100 μM) and were incubated for 3 h at the temperature indicated. Open circular (oc), supercoiled (sc) and linear (lin) DNA band positions are indicated. DNA in the absence of Cu(II) ions or GSH gave electrophoretic patterns essentially identical with those in (a) or (b) at 0 °C; no evidence of changes in control band intensity with temperature was detected under these conditions for control DNA alone.

Fig. 2. Cu(II)–thiol-dependent conversion of supercoiled into open circular DNA as a function of time

Samples contained pSP64 DNA (30 μM), GSH (5 μM) and Cu(II) ions (100 μM) and were incubated at 37 °C for the times shown. Results are the means for three separate experiments. The error bars are shown for the Cu(II) ion system (■) and the Cu(II) ion plus GSH system (●); the spread of data for the GSH alone system (▲) is comparable with that for Cu(II) ions alone. Clearly within experimental error the Cu(II) ions alone and GSH alone data are indistinguishable.

Temperature-dependence of Cu(II)–thiol cleavage of DNA

Cu(II)–thiol cleavage of DNA is extremely temperature-dependent (Fig. 1). Significant cleavage occurred on incubation at 0 °C with GSH plus Cu(II) ions (final concentrations 20 μM and 100 μM respectively; 2 h incubation): the amount of strand breakage increased as the temperature was raised to 15 °C, 25 °C, 37 °C or 50 °C, until at 65 °C the DNA was completely digested to low-molecular-mass species that appeared as a smear on the gel. Controls [Cu(II) ions only or GSH only] showed similar temperature-dependencies. On the basis of such results, later experiments were carried out at 37 °C, which gave cleavage rates sufficiently low to be reproducible and yet rapid enough for experiments to be concluded within 5–6 h.

Time-dependence of Cu(II)–thiol cleavage of DNA

A time course of the conversion of supercoiled into open circular DNA during incubation at 37 °C with GSH plus Cu(II) ions (final concentrations 5 μM and 100 μM respectively) is shown in Fig. 2. Incubation of DNA with either Cu(II) ions or GSH alone for up to 3 h resulted in little or no conversion of DNA from the supercoiled form into the open circular form. In contrast, when DNA was incubated with both metal ion and thiol there was a steady increase in the percentage of open circular DNA in the sample, and by 3 h almost all the DNA was in this form. Under these conditions, formation of linear DNA was apparent only at the 3 h time point and only to a very limited extent (1%). The slightly greater percentage of open circular form formed at 'zero' incubation time is probably real and caused by a small degree of reaction before completion of quenching.

The effects of varying the concentration of GSH at a fixed concentration of Cu(II) ions or of varying the concentration of Cu(II) ions at a fixed concentration of GSH are shown in Fig. 3 (in this Figure the GSH and Cu(II) ion controls have been omitted, as no significant strand breakage was observed in these samples). In the presence of 100 μM-Cu(II) ion little or no cleavage was seen with 1 μM-GSH, but with 10 μM-GSH nearly all of the DNA was converted into the open circular and linear forms (Fig. 3a). The extent of cleavage increased as the GSH

Fig. 3. Agarose-gel-electrophoretic demonstration of the dependence of plasmid DNA cleavage on (a) GSH and (b) Cu(II) ion concentrations

pSP64 DNA (30 μM) was incubated for 1 h at 37 $^{\circ}\text{C}$ with the following: (a) Cu(II) ions (100 μM) plus GSH at 0 μM (lane 1), 1 μM (lane 2), 10 μM (lane 3), 25 μM (lane 4), 50 μM (lane 5) 75 μM (lane 6) or 100 μM (lane 7); (b) GSH (100 μM) plus Cu(II) ions at 0 μM (lane 1), 1 μM (lane 2), 10 μM (lane 3), 25 μM (lane 4), 50 μM (lane 5), 75 μM (lane 6) or 100 μM (lane 7).

concentration was increased (25, 50 and 75 μM) and then showed signs of reaching a plateau, there being little difference between the 75 μM - and 100 μM -GSH samples (Fig. 3a). In order to achieve conditions suitable for band quantification by densitometry (intensities of bands of open circular and supercoiled forms comparable) a GSH concentration of 5 μM was chosen, this being based on the above results in the 1–10 μM -GSH region. Such conditions were used for many subsequent studies.

A different trend was observed when the GSH concentration was set at 100 μM and that of Cu(II) ions was increased (Fig. 3b). Up to 25 μM -Cu(II) ion there was little change in the ratio of supercoiled to open circular DNA. At 50 μM -Cu(II) ion all of the DNA was in the open circular and linear forms, and the extent of cleavage continued to increase significantly with 75 μM - and 100 μM -Cu(II) ion (Fig. 3b). This initial cleavage-inactive region of Cu(II) ion concentration was found not to be related to the concentration of DNA present. The experiment was repeated with 15 μM -, 60 μM - or 120 μM -DNA and a similar inactivity region was observed (results not shown). The only differences lay in the extent of cleavage with 75 μM - or 100 μM -Cu(II) ion, which increased as the concentration of DNA decreased. However, the non-cleaving region was related to the GSH concentration; at 50 μM -GSH zero or very little non-cleavage region was observed, whereas at 500 μM -GSH the region extended to 75 μM -Cu(II) ion (results not shown). The concentration-dependence studies showed that at very high concentrations of GSH there was a concentration region with respect to added Cu(II) ions in which little cleavage was detectable, this being described above as a threshold. Given the 'resolution' of the agarose-gel-electrophoretic data, this threshold could be the result of a rate law for cleavage that was second-order in the concentration of

Cu(II) ions. Such a rate law for DNA cleavage would result in a plot of cleavage versus Cu(II) ion concentration that was convex-upward, and, with the use of agarose-gel electrophoresis, the low Cu(II) ion concentration would probably appear as a 'threshold'. A rate law involving $[\text{Cu(II) ion}]^2$ has been reported for the Cu(II)-ion-catalysed oxidation of cysteine (Hanaki & Kamide, 1983; Ehrenberg *et al.*, 1989).

Salt effects on Cu(II)-thiol cleavage of DNA

NaCl was included with samples incubated for 90 min at 37 $^{\circ}\text{C}$ with GSH and Cu(II) ions (final concentrations 5 μM and 100 μM respectively). Low concentrations of NaCl stimulated strand breakage, with maximum DNA cleavage at NaCl concentrations of 0.1–0.25 M. At higher concentrations of NaCl cleavage was increasingly inhibited, until by 1 M-NaCl the percentage of open circular DNA formed was similar to that in the absence of NaCl.

Nature of the chemistry of Cu(II)-thiol-dependent cleavage of DNA

To understand better the chemistry of Cu(II)-thiol-dependent cleavage of DNA, two experimental approaches were adopted, namely the use of scavengers of reactive oxygen species and a study of the nature of the products of cleavage.

Scavengers. Incubation of DNA with Cu(II) ions plus thiol under conditions of minimal O_2 (in sealed vials, all reagents flushed with N_2) significantly diminished the extent of DNA cleavage (results not shown). Various scavengers of reactive oxygen species were included (90 min incubations at 37 $^{\circ}\text{C}$, mixtures containing GSH plus Cu(II) ions at final concentrations 5 μM and 100 μM respectively) (Table 1). The hydroxyl radical ($\text{HO}^{\bullet}$) scavengers mannitol and glycerol gave conflicting results; glycerol protected supercoiled DNA by 85% whereas mannitol afforded no protection. NaN_3 completely inhibited cleavage. Catalase protected at extremely low concentrations; even 5 ng/ml (0.1 unit) afforded 46% protection, and at 1 $\mu\text{g}/\text{ml}$ (23 units) it completely inhibited strand breakage. Boiling the catalase for 5 min abolished its ability to inhibit strand breakage. The inhibition of DNA cleavage by catalase was shown not to be due to a catalase-dependent effect on the GSH in the system. Catalase did not cause any enhanced autoxidation of GSH, as judged by titration of free thiol concentration with Ellman's reagent (results not shown). Superoxide dismutase provided limited protection against DNA cleavage [74% protection at 1 mg/ml (3000 units)], but boiling the enzyme for 5 min did not abolish this inhibitory ability.

Nature of the products of cleavage. Plasmid DNA that has been extensively cleaved by the Cu(II)-GSH system yields products that can be labelled by both the Klenow fragment of DNA polymerase I and T4 polynucleotide kinase (Fig. 4). Thus at least some of the strand breaks have produced fragments bearing a 5'-hydroxy and/or a 3'-hydroxy group. Piperidine treatment of plasmid DNA incubated for 1 h at 37 $^{\circ}\text{C}$ with GSH plus Cu(II) ions (final concentrations 20 μM and 100 μM respectively) resulted in extensive fragmentation of the DNA to low-molecular-mass species. An identical sample, not treated with piperidine, was predominantly in the open circular form. This suggests that extensive base damage occurs on exposure of DNA to GSH plus Cu(II) ions and that this damage does not necessarily lead to strand breakage.

Cleavage of DNA by the Cu(II)-thiol system results in the formation of 2-thiobarbituric acid-reactive species, although only to a very limited extent. Incubation of calf thymus DNA with GSH plus Cu(II) ions (final concentrations 1 mM, 0.5 mM

Fig. 4. Labelling of plasmid DNA cleaved with GSH plus Cu(II) ions

pSP64 DNA (8 μ g) was incubated for 1 h at 37 °C with GSH (25 μ M) plus Cu(II) ions (100 μ M). The sample was divided into four portions, each of which was ethanol-precipitated and treated as follows: lane 2, no further treatment; lane 3, labelled with [γ - 32 P]dATP and T4 polynucleotide kinase by using the protocol for 5'-protruding ends; lane 4, labelled with [γ - 32 P]dATP and T4 polynucleotide kinase by using the protocol for 3'-protruding or blunt ends; lane 5, labelled with [α - 32 P]dATP and the Klenow fragment of DNA polymerase I. Lane 1 is control pSP64 DNA. Lanes 1 and 2 were stained with ethidium bromide after gel electrophoresis, and lanes 3–5 were dried and autoradiographed. Open circular (oc), supercoiled (sc) and linear (lin) DNA band positions are indicated.

and 5 mM respectively) for 3 h at 37 °C resulted in the formation of 0.36 μ M 2-thiobarbituric acid-reactive species. In contrast, incubation of calf thymus DNA with bleomycin, which is known to form propenals (Hertzberg & Dervan, 1984) (final concentrations 1 mM and 0.5 mM respectively), for 15 min at 37 °C resulted in the formation of 3.92 μ M 2-thiobarbituric acid-reactive species. Production of 2-thiobarbituric acid-reactive species by the Cu(II)–thiol system depended on the concentrations of GSH and Cu(II) ions and on the time and temperature of incubation.

Table 1. Inhibition of Cu(II)–thiol-dependent DNA cleavage by scavengers of reactive oxygen species

pSP64 DNA (30 μ M) was incubated with 5 μ M-GSH plus 100 μ M-Cu(II) ion for 1.5 h at 37 °C in the presence of the indicated scavengers of reactive oxygen species. Protection against cleavage was calculated as:

$$\text{Protection} = 1 - (S_c - S_{G+I}) / (S_c - S_G)$$

where S_c is the percentage supercoiled form in control (untreated) plasmid DNA, S_G is the percentage supercoiled form in plasmid DNA incubated with GSH plus Cu(II) ions and S_{G+I} is the percentage supercoiled form in plasmid DNA incubated with GSH plus Cu(II) ions plus inhibitor. Results are means $\pm$ S.E.M. for three separate experiments. The value of S_c was 96 ± 3 and that of S_G was 24 ± 6 .

Inhibitor	Concentration	S_{G+I}	Protection
Mannitol	0.1 M	25 ± 10	0
Glycerol	10 % (v/v)	85 ± 3	85
NaN ₃	0.1 M	94 ± 7	100
Catalase	5 ng/ml	57 ± 9	46
	1 μ g/ml	91 ± 7	100
	1 μ g/ml (boiled)	16 ± 13	0
Superoxide dismutase	1 mg/ml	77 ± 3	74
	1 mg/ml (boiled)	80 ± 4	78

Sequence or conformational preference of Cu(II)–thiol cleavage of DNA

Cleavage of a linear 3'-end-labelled 160 bp fragment of pKMΔ98 plasmid DNA by cysteine plus Cu(II) ions had some apparent sequence preference (John & Douglas, 1989). To reveal any such sequence or conformational preference in the cleavage of supercoiled plasmid DNA, we used the following protocol (from Kirshenbaum *et al.*, 1988). Plasmid DNA was fairly extensively

Fig. 5. Test of sequence or conformational preference of cleavage of pSP64 DNA by cysteine plus Cu(II) ions

Experimental details are given in the text. Lanes 1, 8 and 15, DNA digested with *EcoRI* and *Hind III*; lane 2, pSP64 DNA; lanes 3–7, pSP64 digested with *RsaI* (lane 3), *EcoRI* (lane 4), S1 nuclease (lane 5), *RsaI* followed by S1 nuclease (lane 6) or *EcoRI* followed by S1 nuclease (lane 7); lane 9, Cu(II)–cysteine-treated pSP64; lanes 10–14, Cu(II)–cysteine-treated pSP64 digested with *RsaI* (lane 10), *EcoRI* (lane 11), S1 nuclease (lane 12), *RsaI* followed by S1 nuclease (lane 13) or *EcoRI* followed by S1 nuclease (lane 14). The extra bands in lane 5 represent open circular and linear plasmid DNA; those in lanes 10 and 11 are the products of incomplete digestion with *RsaI* and *EcoRI* respectively. High-molecular-mass bands (e.g. those visible in lanes 5, 9 and 11) probably indicate minor contaminants in the plasmid preparation used and are only detectable because these lanes were heavily overloaded for the purposes of this experiment.

cleaved by cysteine plus Cu(II) ions, so that all three forms (supercoiled, open circular and linear) were present. Samples were then digested with restriction endonucleases specific for either single or multiple sites in the plasmid DNA, and finally incubated with S1 nuclease to cut opposite any Cu(II)-thiol-generated single-strand nicks. In the event of significant sequence or conformational preference new bands, in addition to those resulting from the restriction digest, should be apparent after treatment with S1 nuclease. In the absence of specificity a smear of low-molecular-mass DNA fragments would be expected (Kirshenbaum *et al.*, 1988).

With the use of this protocol pSP64, pRW1001, pRW1002, pRW1011, pRW1015 and pRW1155 were used as the DNA substrates for the Cu(II)-cysteine cleavage. Fig. 5 shows the results for pSP64. In lanes 12–14 [Cu(II)-cysteine digest followed (lane 12) by S1-nuclease digest and (lanes 13 and 14) by restriction-endonuclease digest and then S1-nuclease digest] no new discrete bands are apparent, merely a smear of DNA fragments. Similar results were obtained with all of the other plasmids.

Cu(II)-thiol cleavage of single-stranded DNA

Incubation of single-stranded M13 DNA (30 μ M) with 1 μ M-GSH plus 100 μ M-Cu(II) ions for 1 h at 37 °C resulted in DNA fragmentation. Direct comparison with cleavage of supercoiled plasmid DNA is not possible since with circular single-stranded DNA two nicks lead to fragments, whereas with supercoiled DNA, although a single nick can be detected through generation of the open circular form, tens of nicks may have to occur before fragmentation of the plasmid is seen.

DISCUSSION

The combination of thiols with Cu(II) ions provides a good cleaving agent for plasmid DNA. The reaction is quite general for thiols, but cleavage efficiency does vary with thiol structure (Reed & Douglas, 1989). Of a range of metal ions tested the Cu(II) ion is the most reactive under the mild neutral-pH conditions preferable for such a reagent (Reed & Douglas, 1989). In the absence of Cu(II) ions thiols were not effective cleavers of DNA under the conditions used (see Fig. 1); similarly Cu(II) ions in the absence of thiols were ineffective. At higher concentrations and with extended incubation periods clearly Cu(II) ions or thiols in the absence of each other can give substantial backgrounds.

The Cu(II)-thiol DNA-cleavage system is very powerful. Strand cleavage can be detected readily at thiol concentrations as low as 10 μ M in the presence of excess Cu(II) ions (see Fig. 3a). The rate of cleavage of supercoiled DNA is markedly dependent on the temperature (Fig. 1); even mildly raised temperatures enhanced the cleavage potential of the Cu(II)-GSH system. The reaction is strongly dependent on the amount of NaCl present, this most probably being an effect on the stability/conformation of the (supercoiled) double helix. The optimal concentration of NaCl apparently differs for the Fe(II)-EDTA and Cu(II)-thiol systems. A broader optimum occurs for the methidiumpropyl-Fe(II)-EDTA system (Hertzberg & Dervan, 1984) than for the Cu(II)-thiol system. The former reagent binds to DNA to effect its cleavage, but it is likely that the Cu(II)-thiol 'reagent' does not bind to DNA before the cleavage, an aspect that is discussed below.

We found no evidence of specificity, at a gross level, for any of the plasmids used, indicating that the Cu(II)-GSH system does not specifically target structural features such as Z-DNA, cruciform structures or a left-handed but non-Z sequence.

Interestingly, thiol cleavage of linear fragments of DNA can show apparent sequence preference. The linear 160 bp *tyrT* fragment from pKMA98 plasmid DNA was cleaved with preference at five sites with the consensus (3'→5') sequence pur-pur-pyr-pur-pur (pur = purine base; pyr = pyrimidine base). The size of this consensus sequence (about a half-turn of a B-type helix) and the independence of the cleavage site of thiol size, structure and stereochemistry were interpreted as indicating that a common species (probably free radical) was produced by all the thiol systems and that this reactive species was then highlighting local conformation/dynamics in the *tyrT* chain (John & Douglas, 1989). There is no evidence so far of such sequence effects for plasmid DNA. A random single-strand cut of supercoiled DNA leads to relaxed (open circular) duplexes. Further cleavage, also single-strand, occurs at random positions also. Linear duplex DNA will only be produced when two single-strand nicks have occurred on opposite strands but sufficiently close in base-sequence position to allow local duplex 'melting' between and permit strand separation. Such cuts would have to be within perhaps a dozen or so base-pairs of each other. Statistically continued random cuts are increasingly likely to occur opposite (or nearly opposite) an existing cut, leading to smaller fragments of duplex DNA that appear as a smear on ethidium bromide-stained gels. On the basis of studies such as are shown in Fig. 5 there is no evidence for specificity of cleavage at any stage in this series. The resolution of the technique used in this study on plasmid DNA is inherently much less than that used in the study of *tyrT* linear DNA (John & Douglas, 1989).

There is considerable base damage before extensive backbone cleavage. However, when backbone nicking occurs some of the products have 5'-hydroxy and 3'-hydroxy ends. Under the conditions used we would not have detected 3'-phosphate and 5'-phosphate termini; fuller analysis of termini structures remains to be undertaken. Malondialdehyde is also formed, but in low yield relative to the bleomycin reaction.

Nature of the cleaving species

The cleavage of plasmid DNA by Cu(II)-thiol is much diminished in N_2 -scrubbed media, strongly indicating O_2 -derived species as intermediates. Active O_2 -derived species occur in Cu(II)-ion-catalysed thiol oxidations near neutrality, e.g. superoxide radical ion ($O_2^{\cdot-}$) and H_2O_2 (Barron *et al.*, 1947; Cavallini *et al.*, 1968; Hanaki & Kamide, 1975; Yee & Shipe, 1982; Gerweck *et al.*, 1984; Starkebaum & Root, 1985). We were surprised to find that iron salts were ineffective catalysts of the DNA-cleavage reaction (Reed & Douglas, 1989), although $HO^{\cdot}$ radicals have been reported in Fe(II)-cysteine autooxidation (Searle & Tomasi, 1982).

The major possibilities for the DNA-cleaving species for this system are $HO^{\cdot}$ or H_2O_2 , as $O_2^{\cdot-}$ is relatively unreactive towards DNA (Hutchinson, 1985). The $HO^{\cdot}$ radical reacts with DNA, attacking both sugars and bases, essentially at diffusion-controlled rates (Hutchinson, 1985), and is regarded as being responsible for the Fe(II)-EDTA-based DNA cleavers (Hertzberg & Dervan, 1984). $HO^{\cdot}$ scavengers (N_3^- , glycerol) protected DNA against Cu(II)-GSH cleavage (Table 1), although mannitol failed even at 0.1 M. The protection by catalase presumably indicates that H_2O_2 is involved. H_2O_2 causes strand breaks in the presence of micromolar concentrations of Cu(II) ions (see below). Catalase is inhibited by GSH (Sun & Oberley, 1989), but at the concentrations used here the effective GSH concentration would not have been perturbed even if the catalase were saturated with GSH. Titration with Ellman's reagent showed that GSH was not removed by catalase. As native and denatured superoxide dismutase were equally effective protectors, it is possible that, as in the case of bleomycin cleavage of DNA, protection is from

binding of superoxide dismutase to DNA (Galvin *et al.*, 1981).

Thiyl radicals (RS[•]) are unlikely to cause cleavage if plasmid and linear DNA are cleaved similarly. Linear B-DNA cleavage exhibits apparent sequence-specificity for *tyrT* DNA (John & Douglas, 1989), but the consensus cleavage (covering about 6 bp, much larger than any of the thiols used) was independent of the size, chirality and stereochemistry of the thiol used. This indicates a common reactive intermediate generated by the Cu(II)–thiol systems.

Thiols, and other reducing agents, in the absence of Cu(II) ions cause DNA cleavage (Bode, 1967; Rosenkrantz & Rosenkrantz, 1971; Morgan *et al.*, 1975; Lown, 1979), but at concentrations generally very much higher (millimolar or greater) than required in the presence of Cu(II) ions (Reed & Douglas, 1989). Ehrenfeld *et al.* (1985), studying the cleavage of PM-2 DNA by Cu(II) ions and dithiothreitol as controls in their studies of Cu–bleomycin, found little or no cleavage with 20 μ M-Cu(II) ion plus 5 μ M-dithiothreitol at pH 7.0, but incubation times were short.

Although high concentrations of Cu(II) ions of themselves do not lead to significant cleavage of DNA (Sagripanti & Kraemer, 1989; the present work), addition of H₂O₂ (10 μ M) leads to strand breaks even at 1 μ M-Cu(II) ion (Sagripanti & Kraemer, 1989). As for the Cu(II)–thiol system, the Cu(II)–H₂O₂ system was inhibited by metal-ion chelators, catalase or high concentrations of radical scavengers (e.g. N₃[−], mannitol). Superoxide dismutase protected DNA against the Cu(II)–thiol system but not against the Cu(II)/H₂O₂ system. This difference may only be apparent, as superoxide dismutase protected pSP64 plasmid DNA even after denaturation by boiling, indicating that protection may arise from a mechanism other than removal of O₂^{•−}. The DNA damage caused by the Cu(II)–H₂O₂ system was shown to be lesions at sequences of two or more adjacent G residues (Sagripanti & Kraemer, 1989), confirming an earlier report of attack by the Cu(II)–H₂O₂ system on poly(dG–dC) but not on poly(dA–dT) (Feldberg *et al.*, 1985). In the present study, attack by the Cu(II)–thiol system leads to extensive base damage that does not necessarily result in strand cleavage.

The cleavage of DNA by a number of DNA-directed molecules has been found to be activated by Cu(II) ions, e.g. 4'-(acridin-9-ylamino)methanesulphon-*m*-aniside (Wong *et al.*, 1984), a synthetic bleomycin analogue (Hudson & Mascharak, 1989), and 2-thiobarbituric acid-reactive products have been detected from the Cu(II)-ion-promoted reaction of hydroquinone or benzene-1,2,4-triol with DNA (Rao & Pandya, 1989). In the case of the synthetic bleomycin analogue no 2-thiobarbituric acid-reactive species were formed on DNA cleavage.

There are two ways in which Cu(II)–thiol might cause DNA cleavage. The first is reaction of Cu(II) ions and thiol in **free solution** generating a reactive intermediate, most probably a form of HO[•] (or possibly even H₂O₂), as seen from other evidence in this paper. The second would involve Cu(II) ions already bound to DNA when the thiol component attacks to generate the cleavage species. Cu(II) ions bind to DNA in several ways, as discussed by Schweitz (1969) in terms of Cu(II)–H₂O₂ cleavage.

Implications of the work

The Cu(II)–thiol system provides powerful chemical cleavage of supercoiled DNA, a simple observation with a number of apparent implications. The most obvious is a toxicological enigma. Almost all cells contain millimolar, or greater, concentrations of GSH, and Cu(II) ions are a normal cellular component, vital to the structure and function of a range of

crucial enzymes. Clearly, if nuclear or mitochondrial GSH were to encounter significant amounts of free Cu(II) ions, DNA lesions would pose potentially serious problems. Obvious lines of defence against this potential cytotoxicity of GSH (!) include protection by catalase (and possibly superoxide dismutase), Cu(II) ions and GSH being separated by cellular compartmentation, and/or the concentration of free Cu(II) ions in the cell being very low. Many proposals concerning the mechanism of cellular copper toxicity involve activated oxygen leading to inactivation of essential enzymes or to lipid peroxidation deleterious to membranes and organelles. In copper-resistant cell lines, elevated concentrations of metallothionein were found, whereas of the enzymes of reactive oxygen management glutathione peroxidase alone was found to be elevated, suggesting a role for H₂O₂ in cellular copper toxicity (Freedman *et al.*, 1989). Our studies suggest a further potential source of copper toxicity attack on DNA, or possibly RNA. In this vein, copper–thiol complexes have been reported to be strongly virucidal to T_{odd} and ϕ X174 phages with efficacy according to the concentration and structure of the thiol (Edebo *et al.*, 1967).

At a chemical level, the ready availability of the reagents makes the Cu(II)–thiol system a potential alternative to Fe(II)–EDTA as a footprinting system for DNA-binding ligand studies.

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