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THE ROLE OF SUPEROXIDE AND HYDROXYL RADICALS IN THE DEGRADATION OF DNA AND DEOXYRIBOSE INDUCED BY A COPPER-PHENANTHROLINE COMPLEX

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Abstract—DNA degradation by a copper(II)-phenanthroline complex was studied in the presence of NADH, 2-mercaptoethanol or a mixture of hypoxanthine and xanthine oxidase, which generates the superoxide radical, $O_2^{\cdot-}$. In all cases degradation was prevented by catalase but not by scavengers of the hydroxyl radical, $OH^{\cdot}$. It remains possible, however, that $OH^{\cdot}$ was generated in close association with DNA so that the scavengers could not remove it before it reacted. Superoxide dismutase inhibited DNA degradation at low copper(II) phenanthroline concentrations in the presence of NADH or hypoxanthine–xanthine oxidase, but not at higher complex concentrations. Superoxide dismutase had little effect on DNA degradation in the presence of 2-mercaptoethanol. The role of oxygen radicals in the DNA degradation induced by copper(II) phenanthroline is discussed.

A complex between the chelating agent 1,10 phenanthroline and copper(II) ions is able to induce the degradation of DNA in the presence of a reducing agent [1–6]. This reaction is of interest firstly because it may well explain why phenanthroline inhibits the action of DNA and RNA polymerases *in vivo* [1–6] and secondly because its mechanism may represent a simple model for the DNA scission produced *in vivo* by anti-tumour antibiotics such as bleomycin and tallsomycin, which require metal ions for their action [1–9].

The reducing agents employed *in vitro* to facilitate DNA degradation by the Cu-phenanthroline complex have included ascorbate [6], thiols [1, 2, 4, 5], NADH [3] and systems generating the superoxide radical, $O_2^{\cdot-}$ [3, 6]. Superoxide is formed in almost all aerobic cells, which employ superoxide dismutase enzymes to protect against it [10, 11]. These enzymes are specific for $O_2^{\cdot-}$ as substrate [17]. The damage done by $O_2^{\cdot-}$ *in vivo* has often been attributed to the formation in a complex series of reactions of the highly reactive hydroxyl radical, $OH^{\cdot}$ [11, 12]. Indeed, $OH^{\cdot}$ has been suggested to be responsible for the DNA damage induced by $O_2^{\cdot-}$-generating systems both in the presence and in the absence of Cu-phenanthroline [6, 13].

However, the role of oxygen radicals in DNA damage induced by Cu-phenanthroline in the presence of reducing agents such as NADH, ascorbate and thiols is not clear. There have been reports that superoxide dismutase inhibits DNA degradation under these conditions [2, 3] and other reports that it does not [5, 6]. In the presence of copper ions and either ascorbate or thiols, great care must be taken to avoid nonspecific inhibitions by added proteins [1, 14–16]. Bearing this in mind, we have carried out a detailed study of the role of $O_2^{\cdot-}$ and $OH^{\cdot}$ in DNA degradation induced by Cu-phenanthroline in the presence of several reducing agents. DNA degradation may be conveniently followed by the formation of thiobarbituric-acid-reactive products from the deoxyribose moiety [18, 19].

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MATERIALS AND METHODS

Reagents

Superoxide dismutase (bovine erythrocyte, sp. act. 2900 units/mg protein as defined using the cytochrome *c* assay [22]), catalase (bovine liver, thymol free, sp. act. 25,000 μmoles H_2O_2 decomposed per min using the assay conditions in the Sigma catalogue), albumin (human, fatty-acid-free), allopurinol, NADH, 2-deoxyribose, calf-thymus DNA type I, xanthine oxidase, hypoxanthine and 1,10-phenanthroline were obtained from Sigma Chemical Co. (London, U.K.). H_2O_2 (Aristar) was from BDH Chemicals (Poole, U.K.) and desferrioxamine (as 'Desferal') from CIBA-Geigy (Horsham, U.K.). All other reagents were of the highest quality available from BDH Chemicals Ltd. The superoxide dismutase contained no catalase activity, nor did the catalase contain significant superoxide dismutase activity [25].

Measurement of DNA degradation

Reaction mixtures contained, in a final volume of 1 ml, the following reagents at the final concentrations stated. A stock solution of DNA was prepared as 1 mg/ml in 0.15 M NaCl. All solutions were made up in Chelex-treated water.

Hypoxanthine–xanthine oxidase. DNA (0.3 mg/ml), NaCl (45 mM), *o*-phenanthroline (0.2 mM),

$CuCl_2$ (2 or 100 μM), hypoxanthine (0.3 mM) and xanthine oxidase (100 μl of a 1 in 40 dilution of the stock enzyme as purchased). Tubes were incubated at 37° for 1 hr. Reaction was stopped by addition of 1.0 ml thiobarbituric acid (1% w/v in 0.05 M NaOH) followed by 1.0 ml 28% (w/v) trichloroacetic acid. After heating for 10 min at 100°, fluorescence was measured against a rhodamine B standard as in [18].

Mercaptoethanol. DNA (0.3 mg/ml), NaCl (45 mM), *o*-phenanthroline (0.2 mM), $CuCl_2$ (2 or 100 μM) and mercaptoethanol (1 mM). Tubes were incubated and developed as above.

NADH/H_2O_2. DNA (0.3 mg/ml), NaCl (45 mM), *o*-phenanthroline (0.2 mM), $CuCl_2$ (2 or 100 μM), NADH (100 μM) and H_2O_2 (0.5 mM). Tubes were incubated and developed as above.

Measurement of 2-deoxyribose degradation

Reaction mixtures were as described above, except that 2-deoxyribose at a final concentration of 0.5 mM replaced DNA in NaCl, and 2.8% (w/v) trichloroacetic acid was used in chromogen development instead of 28%.

RESULTS

DNA degradation by Cu-phenanthroline and a hypoxanthine–xanthine oxidase system

Table 1 shows the effect of various reagents upon DNA degradation by a hypoxanthine–xanthine oxidase system in the presence of two different concentrations of Cu-phenanthroline. All components of the reaction mixture were required for degradation, and it was inhibited by the xanthine oxidase inhibitor allopurinol. High concentrations of EDTA, which remove copper from phenanthroline and form a complex unreactive with oxygen radicals [20], also inhibited DNA degradation. In contrast, desferrioxamine, a specific chelator of iron(III) and an inhibitor of iron-dependent radical reactions [19, 21], had little effect. Catalase inhibited DNA degradation at both concentrations of copper complex, showing that H_2O_2 is involved. This was not a nonspecific effect of protein [14–16] since heat-denatured catalase or human serum albumin at equal or greater protein concentrations had little effect. By contrast, superoxide dismutase only inhibited significantly at low complex concentrations, which means that $O_2^{\cdot-}$ is required for degradation at low concentrations of Cu-phenanthroline but not at higher concentrations.

Since it has been suggested that $OH^{\cdot}$ might be responsible for the DNA degradation, we examined the action of the $OH^{\cdot}$ scavengers mannitol, sodium formate and ethanol. At concentrations up to 100 mM they produced no marked inhibitory effect.

DNA degradation by Cu-phenanthroline plus 2-mercaptoethanol

Table 2 shows a related series of experiments in which a xanthine–xanthine oxidase system was replaced by 2-mercaptoethanol as reducing agent in the presence of 2 or 100 μM Cu-phenanthroline. All components of the reaction mixture were necessary for degradation to occur and, as above, it was

Table 1. Formation of thiobarbituric-acid-reactive products during DNA degradation by Cu-phenanthroline and hypoxanthine–xanthine oxidase

Addition to reaction mixture	TBA-reactive products (as fluorescence units) formed from DNA	
	2 μM Cu-phen	100 μM Cu-phen
None	116	119
None (omit xanthine oxidase)	3	7
None (omit hypoxanthine)	6	9
None (omit Cu-phen)	13	17
None (omit phen)	13	3
EDTA (0.2 mM)	56	112
(10 mM)	13	13
Desferrioxamine (0.2 mM)	109	116
Ethanol (5 mM)	133	137
(50 mM)	123	136
Mannitol (10 mM)	109	125
(50 mM)	122	125
(100 mM)	118	134
Ammonium formate (10 mM)	116	129
(50 mM)	103	131
(100 mM)	93	103
Superoxide dismutase (50 μg/ml)	17	116
Superoxide dismutase (omit hypoxanthine)	5	5
Catalase (50 μg/ml)	7	10
Human serum albumin (100 μg/ml)	109	122
Allopurinol (0.2 mM)	13	23
Boiled catalase (50 μg/ml)	112	125

A typical set of results is presented, but they were highly reproducible. Reagents were added to give the final concentrations stated in the reaction mixture. Specific activities of enzymes are given in Materials and Methods. Where indicated, catalase was heated at 100° for 10 min before use.

Table 2. Formation of thiobarbituric-acid-reactive products during DNA degradation by Cu-phenanthroline and 2-mercaptoethanol

Addition to reaction mixture	TBA-reactive products formed (as fluorescence units) from DNA	
	2 μM Cu-phen	100 μM Cu-phen
None	162	162
None (omit phen)	17	10
None (omit Cu-phen)	4	7
None (omit thiol)	3	7
EDTA (0.2 mM)	10	172
(10 mM)	7	26
Desferrioxamine (0.2 mM)	178	165
Mannitol (10 mM)	168	182
(50 mM)	162	162
(100 mM)	170	162
Ammonium formate (10 mM)	182	168
(50 mM)	159	143
(100 mM)	156	124
Superoxide dismutase (50 μg/ml)	116	162
Catalase (50 μg/ml)	13	30
Albumin (100 μg/ml)	158	162
Allopurinol (0.2 mM)	172	175
Boiled catalase (50 μg/ml)	124	168
Ethanol (5 mM)	185	165
(50 mM)	185	165

The concentrations stated are the final concentrations in each reaction mixture. For further details see the legend to Table 1. In a series of experiments superoxide dismutase produced a 20–30% inhibition in the presence of 2 μM Cu-phenanthroline, but had no effect in the presence of 100 μM Cu-phenanthroline.

inhibited by EDTA and catalase but not by albumin, heat-denatured catalase, desferrioxamine or $OH^{\cdot}$ scavengers. Allopurinol did not inhibit, which shows that its action in Table 1 is not due to some general radical scavenging ability. By contrast, superoxide dismutase inhibited DNA degradation only slightly (with 2 μM Cu-phenanthroline) or not at all (100 μM Cu-phenanthroline). It follows that $O_2^{\cdot-}$ plays little or no role in DNA degradation in the presence of this reducing agent, although H_2O_2 is required.

Table 3. Formation of thiobarbituric-acid-reactive products during DNA degradation by phenanthroline-Cu and NADH plus hydrogen peroxide

Addition to reaction mixtures	TBA-reactive products (as fluorescence units) formed from DNA	
	2 μM Cu-phen	100 μM Cu-phen
None	39	64
None (omit phen)	17	45
None (omit Cu-phen)	11	7
None (omit NADH and H_2O_2)	4	3
EDTA (0.2 mM)	11	22
(10 mM)	12	35
Mannitol (10 mM)	29	71
(50 mM)	35	67
(100 mM)	36	69
Ammonium formate (10 mM)	28	81
(50 mM)	32	62
(100 mM)	32	44
Superoxide dismutase (50 μg/ml)	17	74
Catalase (50 μg/ml)	12	10
Albumin (100 μg/ml)	33	73
Allopurinol (0.2 mM)	35	81
Boiled catalase (50 μg/ml)	33	78
Ethanol (5 mM)	37	76
(50 mM)	37	79

The concentrations stated are the final concentrations in each reaction mixture. For further details see the legend to Table 1.

Table 4. Formation of thiobarbituric-acid-reactive products during deoxyribose degradation by Cu-phenanthroline and mercaptoethanol

Addition to reaction mixture	TBA-reactive products (as fluorescence units) formed from deoxyribose 2 μM Cu-phen	100 μM Cu-phen
None	57	62
None (omit thiol)	5	18
None (omit Cu-phen)	4	6
None (omit phen)	8	12
EDTA (0.2 mM)	11	80
(10 mM)	4	14
Mannitol (10 mM)	35	48
(50 mM)	31	26
(100 mM)	29	20
Ammonium formate (10 mM)	27	40
(50 mM)	28	32
(100 mM)	19	24
Catalase (50 μg/ml)	8	34
Bovine serum albumin (100 μg/ml)	68	100
Allopurinol (0.2 mM)	70	112
Boiled catalase (50 μg/ml)	52	52
Superoxide dismutase (50 μg/ml)	52	52
Ethanol (5 mM)	52	40
(50 mM)	35	22
Urea (5 mM)	66	68
(50 mM)	72	82

Assays were carried out as described in the Materials and Methods section. The stimulatory effects of bovine serum albumin and allopurinol were reproducible but we have no explanation for them. However, the failure of these compounds to inhibit deoxyribose degradation suggests that the effects of the proteins and other reagents which do inhibit are not due to nonspecific actions.

DNA degradation by Cu-*phenanthroline plus NADH and* H_2O_2

It has been reported that a mixture of NADH and H_2O_2 will promote DNA degradation by Cu-phenanthroline [3]. The results in Table 3 confirm this statement, although considerable degradation was still seen if phenanthroline was omitted from the reaction mixture, i.e. it seems that copper ions, NADH and H_2O_2 can mediate significant DNA damage themselves, although the Cu-phenanthroline complex is more effective than is free copper ion. As previously observed catalase inhibited degradation at both Cu-phenanthroline concentrations whereas albumin, allopurinol, heat-denatured catalase and $OH^{\cdot}$ scavengers had little effect. Superoxide dismutase inhibited only at low Cu-phenanthroline concentrations, but not at higher concentrations.

Deoxyribose degradation by Cu-*phenanthroline plus reducing agents*

The failure of $OH^{\cdot}$ scavengers to inhibit DNA degradation (Tables 1–3) might mean that $OH^{\cdot}$ are not involved, or alternatively that they are formed very close to the DNA by complexed Cu-phenanthroline so that the scavengers are not able to remove them before they react with the DNA. The TBA-reactive products formed during DNA degradation originate from the deoxyribose moiety. Table 4 shows the results of an investigation into the degradation of free deoxyribose by Cu-phenanthroline using mercaptoethanol as a reducing agent. Obviously, no intercalation of the complex is possible here. Most reagents tested affected deoxyribose degradation in exactly the same way as they affected that of DNA (compare Tables 2 and 4). However, the $OH^{\cdot}$ scavengers mannitol, ethanol and sodium formate produced significant inhibition of deoxyribose degradation whilst not affecting that of DNA at the concentrations used. Urea, which reacts only slowly with $OH^{\cdot}$, did not inhibit.

DISCUSSION

A copper(II)-phenanthroline complex in the presence of a reducing agent can degrade DNA to form products that react with thiobarbituric acid and produce a chromogen. The reducing agent probably functions to reduce the copper(II) complex to the +1 oxidation state [1–6], i.e.

$$Cu^{2+}\text{-phen} \xrightarrow{e^-} Cu^{+}\text{-phen} \qquad (1)$$

The Cu^{+}-complex then appears to interact with H_2O_2 in some way (hence the inhibition by catalase) to produce a species that attacks the DNA. One possible species is the hydroxyl radical, formed by the reaction

$$Cu^{+}\text{-phen} + H_2O_2 \longrightarrow Cu^{2+}\text{-phen} + OH^{\cdot} + OH^{-} \qquad (2)$$

However, since high concentrations of $OH^{\cdot}$ scavengers, which are known to protect DNA against damage by this species generated in free solution

[13], did not inhibit Cu-phenanthroline-dependent damage, it follows that free $OH^{\cdot}$ is not responsible. Either another active species attacks the DNA, or else $OH^{\cdot}$ is formed by Cu-phenanthroline so closely associated with the DNA that the scavengers cannot remove it before it attacks the deoxyribose rings. Consistent with the latter interpretation is the observation that degradation of free deoxyribose (Table 4) is partially inhibited by $OH^{\cdot}$ scavengers, although this does not, of course, constitute proof of the hypothesis.

Thiol compounds in aqueous solution autoxidise to produce $O_2^{\cdot-}$ and H_2O_2 [23]. Both $O_2^{\cdot-}$ and the thiol itself could conceivably reduce the Cu^{2+}-phenanthroline complex,

$$Cu^{2+}\text{-phen} + O_2^{\cdot-} \longrightarrow O_2 + Cu^{+}\text{-phen} \quad (3)$$

$$2Cu^{2+}\text{-phen} + 2R\text{-}SH \longrightarrow 2Cu^{+}\text{-phen} + R\text{-}S\text{-}S\text{-}R\text{-} + 2H^{+} \quad (4)$$

The predominant reaction would depend on the rate constants for reactions (3) and (4) and on the relative concentrations of $O_2^{\cdot-}$ and RSH. Reaction (3) would be inhibited by superoxide dismutase. The lack of effect of this enzyme reported in Table 2 strongly suggests that, using mercaptoethanol at 1 mM, reaction (4) was the major (at 2 μM Cu-phenanthroline) or the only (at 100 μM Cu-phenanthroline) process by which Cu^{+}-phen was formed.

NADH solutions also generate $O_2^{\cdot-}$ [3, 24, 26] so that in their presence reduction could be achieved by either reaction (3) or by a direct reaction, one possibility being

$$2Cu^{2+}\text{-phen} + NADH \longrightarrow 2Cu^{+}\text{-phen} + NAD^{+} + H^{+} \quad (5)$$

The effects of superoxide dismutase suggest that at low Cu^{2+}-phenanthroline concentrations reaction (3) makes a significant contribution to reduction, whereas at higher concentrations it does not, presumably because the rate of reactions such as that shown in equation (5) increase much more at higher complex concentrations than does the rate of reaction (3). The mechanism of NADH oxidation is complicated and probably does not follow simple rate laws [3, 24, 26].

When the reducing agent is $O_2^{\cdot-}$ produced by a hypoxanthine–xanthine oxidase system [22], one might expect superoxide dismutase to prevent DNA degradation. This is indeed observed using Cu^{2+}-phenanthroline at a concentration of 2 μM (Table 1). However, at a higher (100 μM) complex concentration, superoxide dismutase had essentially no effect even though in all other respects DNA degradation was similar and it was still dependent on xanthine oxidase activity as shown by the effect of allopurinol (Table 1) and the inhibition on omitting hypoxanthine from the reaction mixture. This means that free $O_2^{\cdot-}$ cannot be required to reduce the Cu^{2+}-phenanthroline complex under these reaction conditions. One possibility is that the xanthine oxidase itself could reduce this complex directly if it is presented at a sufficiently high concentration, perhaps using electrons supplied by hypoxanthine.

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