The influence of reducing agent and 1,10-phenanthroline concentration on DNA cleavage by phenanthroline + copper

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ABSTRACT

Copper in the presence of excess 1,10-phenanthroline, a reducing agent, and molecular oxygen causes cleavage of DNA with a preference for T-3',5'-A-steps, particularly in TAT triplets. The active molecular species is commonly thought to be the bis-(1,10-phenanthroline)Cu(I) complex, $(Phen)_2Cu(I)$, regardless of the reducing agent type. We have found that $(Phen)_2Cu(I)$ is not the predominant copper complex when 3-mercaptopropionic acid (MPA) or 2-mercaptoethanol are used as the reducing agents, but $(Phen)_2Cu(I)$ predominates when ascorbate is used as the reducing agent. Substitution of ascorbate for thiol significantly enhances the rate of DNA cleavage by 1,10-phenanthroline + copper, without altering the sequence selectivity. We show that $(Phen)_2Cu(I)$ is the complex responsible for DNA cleavage, regardless of reducing agent, and that 1,10-phenanthroline and MPA compete for copper coordination sites. DNA cleavage in the presence of ascorbate also occurs under conditions where the mono-(1,10-phenanthroline)Cu(I) complex predominates (1:1 phenanthroline:copper ratio), but preferential cleavage was observed at a CCGG sequence and not at TAT sequences. The second phenanthroline ring of the $(Phen)_2Cu(I)$ complex appears essential for determining the T-3',5'-A sequence preferences of phenanthroline + copper when phenanthroline is in excess.

INTRODUCTION

Copper plus excess 1,10-phenanthroline, in the presence of molecular oxygen and a reducing agent, acts as an effective DNAase on double stranded DNA (1,2) (reviewed in ref. 3–5). The presence of both a reducing agent and an oxidizing agent drives the Cu(I) <=> Cu(II) redox cycle which generates activated oxygen species. When the complex is bound to DNA, the activated oxo-species react with H1' or H4'-deoxyribose protons of nucleotides in the vicinity of the binding site. This reaction initiates a series of reactions culminating in DNA chain cleavage (6,7). The binding mode of the complex has not been resolved. A partial intercalation model has been proposed in which one phenanthroline ring intercalates from the minor groove side and the second, unintercalated phenanthroline ring of the complex makes favorable contacts within the minor groove (8–12). A second model invokes minor groove binding without intercalation (3,5).

The nuclease activity of copper + phenanthroline has been widely used to 'footprint' binding of other ligands to DNA (13–16), to construct synthetic restriction endonucleases (17–24), and to probe DNA conformation (8,9,13,23,25–34). Previously we showed that DNA cleavage by 1,10-phenanthroline-copper with mercaptopropionic acid (MPA) as the reducing agent occurs with a preference for T-3',5'-A steps, with attack at adenines of TAT triplets being most preferred (8,10).

Effective use of 1,10-phenanthroline-copper as a probe of DNA structure or the catalytic center of a synthetic restriction endonuclease requires optimization of the cleavage reaction and identification of the active species. It has been widely assumed that the bis-(1,10-phenanthroline)Cu(I) complex, $(Phen)_2Cu(I)$, is the active species when the phenanthroline to copper ratio is greater than 2:1, *regardless of the reducing agent*, and is responsible for determining the sequence preferences of DNA cleavage.

While examining the non-covalent DNA binding of $(Phen)_2Cu(I)$ (12), we noted that the absorption spectrum expected for the $(Phen)_2Cu(I)$ complex was not observed when MPA was used as a reducing agent instead of ascorbic acid. This observation raised the possibility that the sequence specificity observed for DNA cleavage in the 1,10-phenanthroline-copper-thiol systems used by us (8,10) and others (3,7,9,14,15,26–28) was due to some type of MPA:phenanthroline:Cu(I) complex rather than $(Phen)_2Cu(I)$. We also observed that the mono-phenanthroline Cu(I) complex bound to DNA. This complex

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could contribute to the DNA cleavage preferences observed, depending on the relative binding affinities and redox potentials of the species in the DNA-bound state.

These possibilities were investigated by examining influences of the choice of reducing agent and 1,10-phenanthroline to copper stoichiometry on the cleavage of DNA restriction fragments. Although the *rate* of DNA cleavage was strongly dependent on the reducing agent and concentration of phenanthroline, relative to copper, the sequence preferences of cleavage were not significantly influenced provided the phenanthroline/copper ratio was modestly greater than the 2:1 stoichiometry required for forming $(Phen)_2Cu(I)$. A preference for C-3',5'-G steps, rather than T-3',5'-A steps was observed at a phenanthroline to copper ratio of 1:1, which is expected to strongly favor formation of the mono-phenanthroline Cu(I) complex. Additional features of the interaction of mercaptopropionic acid and 2-mercaptoethanol with the $(Phen)_2Cu(I)$ complex were investigated by absorption and proton NMR spectroscopy.

MATERIALS AND METHODS

Standard anaerobic solutions of ACS reagent grade phenanthroline, reagent grade copper sulfate and ascorbic acid were prepared as described previously (12). Absorption spectra were obtained on Cary 2200 or Cary 19 UV/Vis spectrophotometers. Proton NMR spectra were obtained at 500 MHz on a Varian VXR500 spectrometer. Samples of phenanthroline:copper(I) complexes were prepared for NMR studies in ^2H-DMSO or ^2H-DMSO/^2H_2O mixtures to enhance solubility.

The effect of reducing agent type on the absorbance spectrum of reduced copper in the presence of excess phenanthroline was examined by preparing three identical cuvettes (1 cm) containing 66 μM $CuSO_4$, 167 μM phenanthroline, 20 mM PIPES (adjusted to pH 7.0 with NaOH), and 20% ethanol. Samples were saturated with N_2, baselines were recorded, then ascorbate (1 mM), mercaptopropionic acid (1 mM or 2 mM), or 2-mercaptoethanol (1 mM) were added to individual cuvettes without introduction of air. The visible absorption spectra were recorded after 10 min. of mixing.

Proton NMR spectra of phenanthroline and phenanthroline:copper (I) complexes were obtained on a Varian VXR500 spectrometer in 32K data points using 45° pulses, 16 to 64 acquisitions, and a delay of 5 sec. between acquisitions. All spectra were taken at ambient temperature (22°C) in 60:40 (v/v) DMSO:D_2O using quadrature detection with the carrier frequency set on the HOD resonance. The nitrate salt of $(Phen)_2Cu(I)$ was prepared and precipitated from ethanol. Samples of 5 mM $(Phen)_2Cu(I)$ in 60:40 DMSO:D_2O containing 100 mM ascorbate or MPA were prepared by weighing the solid black nitrate salt into NMR tubes, adding ascorbic acid or MPA, then adding solvent previously degassed with argon. Samples were dissolved and further degassed for 20–30 min. by bubbling with argon. A sample containing 1:1 phenanthroline and copper (I) with MPA in excess was prepared by dissolving solid 1,10-phenanthroline and $CuSO_4$ and liquid MPA in deaerated solvent to yield final concentrations of 5 mM Phen and Cu, and 100 mM MPA.

The 329 base pair EcoR1/BstEII fragment of the sea urchin *S. purpuratus* early H3 histone gene (cloned in plasmid pUC9) was prepared, labeled with ^{32}P on the EcoR1 3'-end, and analyzed for cleavage by $(Phen)_2Cu(I)$ essentially as described previously (8,10). Cleavage reactions were conducted with 5 μM Cu(I) at 27°C in 200 mM KCl, 5 mM Hepes, 9.8% w/v ethanol (pH 7.5, adjusted with NaOH). These conditions were utilized in prior studies to examine the non-covalent DNA binding of $(Phen)_2Cu(I)$ (12). The reducing agent (MPA or ascorbate) and concentrations of phenanthroline were varied in individual experiments as indicated in the text and figure legends.

RESULTS

Disruption of $(Phen)_2Cu(I)$ by mercaptopropionic acid or 2-mercaptoethanol

Previous attempts to prepare $(Phen)_2Cu(I)$ from 1,10-phenanthroline and copper using a large molar excess of MPA as the reducing agent did not yield a complex with the expected visible absorption spectrum (12). Substitution of ascorbate for MPA as reducing agent yielded a complex with the spectrum, wavelength maximum, and molar extinction coefficient at this wavelength consistent with literature values for $(Phen)_2Cu(I)$ (λ_{max} = 435 nm, ϵ = 7,200–7,400 $M^{-1}cm^{-1}$) (35–37). This phenomenon was examined further by preparing identical cuvettes containing 1,10-phenanthroline and copper(II) at a 2.5:1 ratio, then reducing the copper with ascorbate, mercaptoethanol, or MPA. It should be noted that higher concentrations of copper and phenanthroline were used here than for DNA binding studies (12) to more accurately assess the effect of reducing agent. These concentrations necessitated the addition of ethanol to prevent dimerization of $(Phen)_2Cu(I)$. Extensive dimerization/aggregation of $(Phen)_2Cu(I)$ in aqueous solutions at concentrations >10 μM is a long standing observation which has been overlooked in some recent studies of the complex.

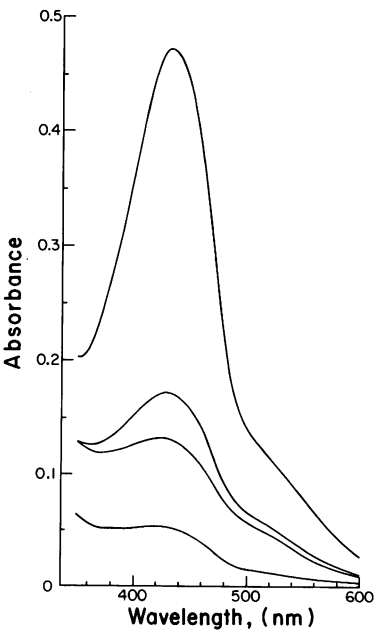

Figure 1. The visible absorption spectrum of 1,10-phenanthroline + copper (≈2.5:1) in the presence of (top to bottom): 1 mM ascorbate, 1 mM mercaptopropionic acid, 2 mM mercaptopropionic acid, and 1 mM 2-mercaptoethanol. All samples contained identical concentrations of $CuSO_4$ (66 μM) and 1,10-phenanthroline (167 μM), in PIPES buffer (pH 7.0) and 20% ethanol.

Reduction of copper with MPA or mercaptoethanol was not effective for forming the $(Phen)_2Cu(I)$ complex, as indicated by the low magnitude of the characteristic $(Phen)_2Cu(I)$ visible absorption band at 435 nm compared to that observed with ascorbate (Fig. 1). Mercaptoethanol appeared somewhat more effective in suppressing the $(Phen)_2Cu(I)$ spectrum than MPA at equal concentrations. Increasing the MPA concentration further decreased the 435 nm absorption of $(Phen)_2Cu(I)$. Similar results were obtained when phenanthroline/copper solutions were titrated with MPA in the presence of 1 mM ascorbate (data not shown). These results eliminate the possibility that failure to produce the expected spectrum was due to incomplete reduction of copper(II) by MPA, and suggest instead that MPA competes with phenanthroline for copper binding.

More careful examination of the copper equilibria with 1,10-phenanthroline suggests that addition of MPA or 2-mercaptoethanol may yield a mixed phenanthroline-thiol-copper complex. Formation of $(Phen)_2Cu(I)$ is described by the following equilibria.

$$Cu(I) + Phen = (Phen)Cu(I) \qquad K_1 \approx 10^{10.3} \qquad (1)$$

$$(Phen)Cu(I) + Phen = (Phen)_2Cu(I) \qquad K_2 \approx 10^{5.5} \qquad (2)$$

Overall,

$$Cu(I) + 2\ Phen = (Phen)_2Cu(I) \qquad K = 10^{15.8} \text{ (ref. (38))} \qquad (3)$$

Because of the differences between K_1 and K_2, micromolar concentrations of copper and 1,10-phenanthroline are sufficient to guarantee that $\geq$ 99% of the copper is complexed with phenanthroline, but considerably higher concentrations are required to drive all the copper into the $(Phen)_2Cu(I)$ form. For example, under the conditions of the above experiments (66 μM Cu(I), 167 μM 1,10-phenanthroline), approximately 93% of the copper is expected to be complexed as $(Phen)_2Cu(I)$, and 7% as the (Phen)Cu(I) complex. Since thiols are generally good ligands of transition metals, and copper binding of the second phenanthroline is relatively weak, it is reasonable to assume that MPA and 2-mercaptoethanol can complete with phenanthroline for binding to the two remaining coordination sites in (Phen)Cu(I). The binding constant for the thiols need not be large to effectively minimize formation of $(Phen)_2Cu(I)$ under the conditions of experiments using the combination of copper, 1,10-phenanthroline and thiols to produce nuclease activity. MPA (1 mM) in 30-fold excess over the free phenanthroline (nominally 35 μM) appeared insufficient to completely displace all of the $(Phen)_2Cu(I)$ complex in the above experiments, as indicated by the residual peak at 435 nm (Fig. 1), suggesting that the association constant for MPA is less than 10^4 M^{-1}. (More precise estimation of this association constant would require knowledge of the stoichiometry of the putative mixed phen-MPA-Cu(I) complex.)

The formation of a mixed phenanthroline-MPA-copper(I) complex was also suggested by changes in the phenanthroline proton NMR chemical shifts in the presence of copper and ascorbate or MPA (Fig. 2). The 500 MHz proton NMR spectrum of phenanthroline (10 mM) in 60:40 DMSO:D_2O is characterized by four low field resonances--a singlet (b), quartet (a), and two doublets of doublets (b,d), assigned as indicated in Fig. 2A according to ref. (39). Three of the four resonances were shifted downfield by $\approx$0.18$-$0.26 ppm in the spectrum of $(Phen)_2Cu(I)$ (5 mM) in the presence of ascorbate (100 mM) (Fig. 2B). The fourth resonance (d), corresponding to protons closest to the copper-coordinating nitrogens, was shifted upfield $\approx$0.17 ppm. When $(Phen)_2Cu(I)$ (5 mM) was dissolved in 60:40 DMSO:D_2O containing MPA, resonances b and d were

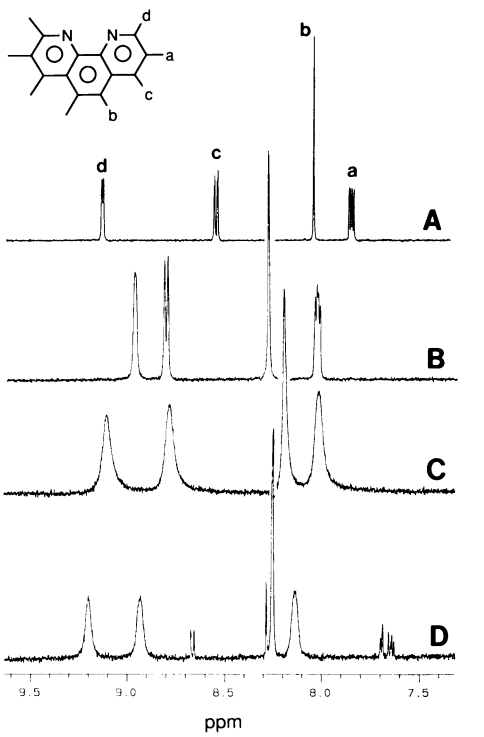

Figure 2. The effects of copper (I) and reducing agent (ascorbate or 3-mercaptopropionic acid) on the 500 MHz proton NMR spectrum of 1,10-phenanthroline. Spectra are shown for (A) phenanthroline alone (10 mM); (B,C) 2:1 phenanthroline:$CuSO_4$ (5 mM Cu) in the presence of (B) 100 mM ascorbate and (C) 100 mM MPA and 100 mM ascorbate, and (D) 1:1 phenanthroline:copper (5 mM) in the presence of 100 mM MPA.

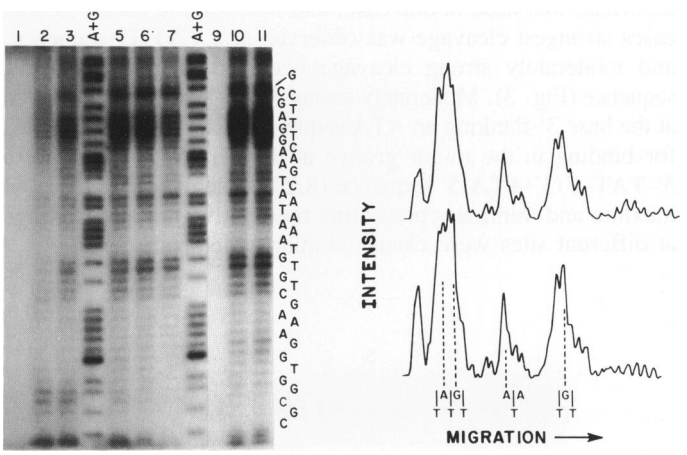

Figure 3. The influence of reducing agent (ascorbate or MPA) on the patterns of DNA cleavages by phenanthroline:copper. All reaction mixtures contained 5.2 μM $CuSO_4$, 157 μM DNA (nucleotides), and 0.5 mM ascorbate (lanes 1$-$7) or MPA (lanes 9$-$11) and were incubated for 75 sec. at room temperature; phenanthroline concentrations were varied. LEFT: autoradiogram of sequencing gel. Lane 1, control; lanes 2$-$3, 5.3 and 10.6 μM phenanthroline (ascorbate reductant); lane 4, A+G sequencing reaction; lanes 5$-$7, 21.1, 52.8, and 104 μM phenanthroline (ascorbate reductant); lane 8, A+G reaction; lanes 9$-$11, 21.1, 52.3, and 104 μM phenanthroline (reductant = MPA). RIGHT: Densitometer scans of lane 6 (lower scan, ascorbate reductant) and lane 10 (upper scan, MPA reductant) from the autoradiograms. Note the strong cleavage at TATGT, ATA, and TGT sequences in both cases.

shifted upfield and downfield, respectively, relative to $(Phen)_2Cu(I)$ in ascorbate, but resonances a and c were shifted only slightly upfield (Fig. 2C). These shifts were in the expected directions, but were not quantitatively consistent with averaging of the free phenanthroline and $(Phen)_2Cu(I)$ spectra by rapid chemical exchange.

We also obtained the spectrum of phenanthroline plus copper in a 1:1 ratio (5 mM) and excess MPA (100 mM) (Fig. 2D). Comparison of this spectrum to that of $(Phen)_2Cu(I)$ in ascorbate (Fig. 2B) showed that resonances (a) and (c) were shifted by $\approx 0.12-0.13$ ppm further downfield (≈ 0.3 to 0.4 ppm with respect to free phenanthroline), while resonance b was only slightly shifted. By contrast to $(Phen)_2Cu(I)$, resonance (d) occurred ≈ 0.1 ppm *downfield* relative to (d) in free phenanthroline. The spectrum observed for the 2:1 phenanthroline:copper mixture in the presence of excess MPA and ascorbate is consistent, therefore, with exchange averaging of the spectra for free phenanthroline, $(Phen)_2Cu(I)$, and some $(Phen)(MPA)_nCu(I)$ complex. (We have assumed that Cu(I) will not complex MPA without displacement of phenanthroline, as such an occurrence would require that Cu(I) be penta-coordinate. Interestingly, although five-coordinate Cu(I) species are strongly disfavored, they do occur and provide an alternative, though unusual, explanation for the alterations in the $(Phen)_2Cu(I)$ spectrum caused by MPA.)

The effects of mercaptopropionic acid on the specificity of DNA cleavage in the phenanthroline/copper system

The finding that MPA disrupts or alters the $(Phen)_2Cu(I)$ complex raises the possibility that the sequence preferences of DNA cleavage by phenanthroline:copper(I) observed previously were not determined by $(Phen)_2Cu(I)$, as assumed (8). This possibility was tested by cleaving a DNA restriction fragment containing preferred TAT and TGT sequences under two conditions that were identical excepting the reducing agent--ascorbate was used in one case, and MPA in the other. In both cases strongest cleavage was observed at a TATGT sequence, and moderately strong cleavage occurred at a second TGT sequence (Fig. 3). Moderately strong cleavage was also observed at the base 3'-flanking an ATA sequence. This result is expected for binding in the minor groove at the T-3',5'-A-3' step of a 5'-TAT-3'/3'-ATA-5' sequence (8,27). The overall patterns of maxima and minima representing the relative rates of cleavage at different sites were clearly similar under both conditions.

Although the *relative* rates of nucleotide cleavages were very similar with both reducing agents, the *absolute* rate of DNA cleavage was much faster with ascorbate as the reductant. This difference was evident from the rapid rate of loss of the parent DNA band when ascorbate was substituted for MPA as the reducing agent (Fig. 4). The observation that MPA caused a decrease in overall DNA cleavage rate, while not significantly influencing the sequence preferences, suggests that $(Phen)_2Cu(I)$ is, in fact, the active species and determinant of the observed preferences, independent of reducing agent. Traces of $(Phen)_2Cu(I)$ are expected even in the presence of a large excess of MPA, and formation of $(Phen)_2Cu(I)$ will be driven by complexation with DNA if this DNA complex is more stable than the $(Phen)(MPA)_nCu(I)$ complex with DNA. If $(Phen)_2Cu(I)$ is the active species and phenanthroline and MPA are in competition for copper coordination sites, then increasing the concentration of phenanthroline while holding the concentrations of copper and MPA constant should also increase the overall rate of DNA cleavage. Enhanced DNA reactivity was observed when the phenanthroline concentration was increased from 20 μM to 50 or 100 μM in the presence of 5 μM Cu and 500 μM MPA (Fig. 5).

The effects of phenanthroline concentration on the specificity of DNA cleavage in the phenanthroline/copper/ascorbate system

Viscosity measurements indicated that the mono-phenanthroline Cu(I) complex binds to DNA in a similar manner to $(Phen)_2Cu(I)$ (12). If (Phen)Cu(I) is active in a redox equilibrium analogous to $(Phen)_2Cu(I)$ and produces activated oxygen species, then the observed DNA cleavage preferences could also be due partly or exclusively to this species. Furthermore, free phenanthroline binds DNA weakly in the presence of $(Phen)_2Cu(I)$ (12) and could influence the cleavage preferences.

These possibilities were tested by carrying out DNA cleavage reactions with ascorbate as the reducing agent and ratios of phenanthroline to copper varying from 1:1 to 20:1. Because the ratio of K_1/K_2 for the association of the first and second phenanthrolines with Cu(I) is approximately 10^5, copper must exist predominantly as the mono-phenanthroline complex when the phenanthroline:copper ratio is limited to 1:1. A phenanthroline to copper ratio of 2:1 was sufficient to generate preferences for TAT and TGT sequences as noted previously (Fig. 6). These

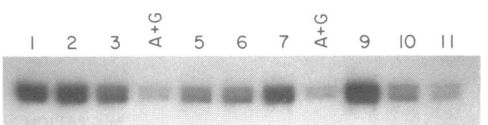

Figure 4. Dependence of the rate of overall DNA cleavage by $(Phen)_2Cu(I)$ on reducing agent and phenanthroline concentration. Samples were prepared as described in Figure 3 and contained 0.5 mM ascorbate (lanes 1–7) or MPA (lanes 9–11) as reductant; phenanthroline concentrations were varied. The autoradiogram was slightly exposed to show the parent bands. Lane 1, control; lanes 2–3, 5.3 and 10.6 μM phenanthroline (ascorbate reductant); lane 4, A+G sequencing reaction; lanes 5–7, 21.1, 52.8, and 104 μM phenanthroline (ascorbate reductant); lane 8, A+G reaction; lanes 9–11, 21.1, 52.3, and 104 μM phenanthroline (reductant = MPA). Note that at equal concentrations of phenanthroline (e.g. 21 μM, lanes 5 & 9) the parent bands are degraded much more slowly with MPA as reductant. (The parent fragment in this experiment was partially contaminated with a longer fragment.)

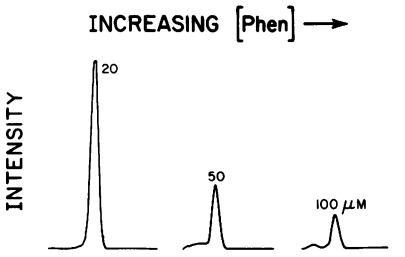

Figure 5. Dependence of the rate of overall DNA cleavage by $(Phen)_2Cu(I)$ on the concentration of phenanthroline in the presence of MPA as reducing agent. Samples were prepared as in Figure 3. The portions of scans of lanes on an autoradiogram corresponding to the band of the uncleaved parent 329 base pair DNA fragment are shown. All reactions were performed using 5 μM $CuSO_4$, 0.5 mM MPA, and 20, 50, or 100 μM phenanthroline, as indicated.

preferences were more pronounced at a ratio of 5:1, and were not further influenced by additional excess phenanthroline. Reducing the phenanthroline to copper ratio to 1:1 decreased the cleavages at TAT and TGT sequences to near the average, and significantly enhanced the reactivity of a 5'-CCGGG-3' sequence (Fig. 6).

By contrast with the behavior when MPA was used as the reductant, in the presence of ascorbate the *overall* rate of DNA cleavage was reduced both when the total phenanthroline concentration was low, and when the phenanthroline concentration was high, with respect to copper. The cleavage rate was maximal when the phenanthroline:copper ratio was 4:1 (Fig. 7). These results are consistent with previous observations that attainment of the maximal concentration of $(Phen)_2Cu(I)$ in micromolar copper solutions requires a modest excess of phenanthroline, but that a larger excess of phenanthroline inhibits DNA binding by $(Phen)_2Cu(I)$ (12).

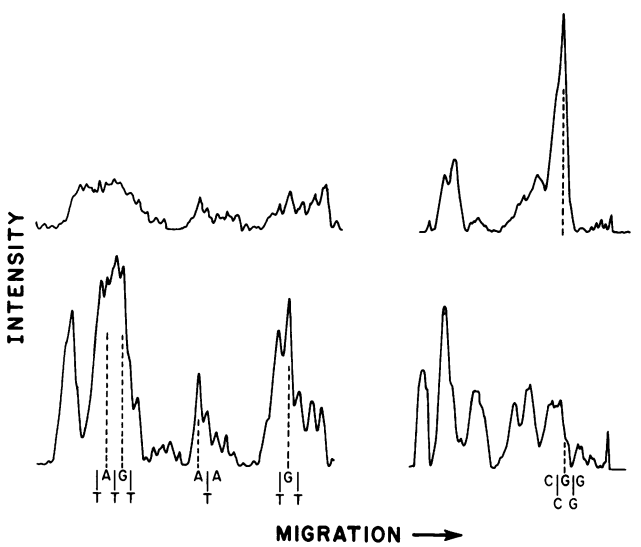

Figure 6. Comparison of the sequence preferences of DNA cleavage by the mono-(1,10-phenanthroline)Cu(I) and bis-(1,10-phenanthroline)Cu(I) complexes. Reactions were performed with phenanthroline:copper ratios of 1:1 (top scan) or 5:1 (bottom scan) using ascorbate as reducing agent as described in Figure 3. Two different sequences from the same fragment are shown. Note that a CCGGG sequence was preferred at a 1:1 phenanthroline:copper ratio, while TAT and TGT sequences were preferred at a 5:1 phenanthroline:copper ratio and not at the lower ratio.

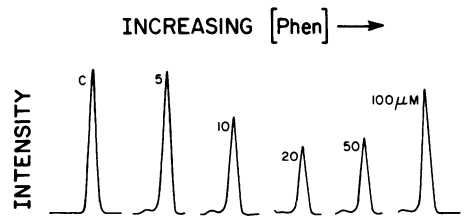

Figure 7. Dependence of the rate of overall DNA cleavage by phenanthroline:copper on the phenanthroline concentration using ascorbate as reducing agent. The portions of scans of lanes on an autoradiogram corresponding to the band of the uncleaved parent 329 base pair DNA fragment are shown. All reactions were performed using 5 μM $CuSO_4$, 1 mM ascorbate, and 0, 5, 10, 20, 50, or 100 μM phenanthroline, as indicated.

DISCUSSION

DNA is cleaved by 1,10-phenanthroline + copper in the presence of both an oxidizing agent (usually O_2) and a reductant. $(Phen)_2Cu(I)$ has been generally presumed to be the complex responsible for both DNA cleavage and the observed sequence preferences. It is clear that phenanthroline is essential for efficient cleavage, and that DNA cleavage is distinctly non-random, hence at least one of several possible coordination complexes of copper with phenanthroline must be responsible for directing the active oxygen-producing center to preferred DNA sites.

We have shown that the predominant copper complex is not $(Phen)_2Cu(I)$ when excess thiol (mercaptopropionic acid or mercaptoethanol) is used as a reductant. Since phenanthroline is not totally released from the copper by thiol addition, a mixed phenanthroline/thiol/copper complex must be formed. The formation of a mixed complex is not surprising because the association constant for the second phenanthroline in $(Phen)_2Cu(I)$ is only about $10^{5.5}$ (40), and sulfur generally has a high affinity for copper. In addition, MPA is capable of forming a chelate complex with a favorable 5-membered ring via coordination of a carboxyl group oxygen as well as the sulfur. No direct evidence was obtained for this chelate, however, and it is possible that two MPA's are bound, each through the sulfur.

The $(phen)(MPA)_nCu(I)$ complex clearly was not as effective a DNA cleaving agent as $(Phen)_2Cu(I)$. Maintenance of the sequence specificity of DNA cleavage in the presence of MPA suggests that cleavage was solely due to the presence of $(Phen)_2Cu(I)$ in co-equilibrium. This suggestion was supported by the observation that increasing the phenanthroline concentration increased the rate of overall DNA cleavage, but did not affect the sequence selectivity. It would not be surprising if the $(phen)(MPA)_nCu(I)$ has little or no DNA affinity since the complex has no net positive charge, in contrast to the cationic $(Phen)_2Cu(I)$ and other typical DNA binding ligands such as ethidium and netropsin.

DNA was also cleaved when the phenanthroline:copper ratio was 1:1 (with ascorbate as reductant), though the rate of cleavage was less than at higher phenanthroline concentrations. Under these conditions the mono-(1,10-phenanthroline) Cu(I) complex is expected to predominate, but cleavage could be solely due to traces of $(Phen)_2Cu(I)$. The observed shift in the sequence preferences of cleavage from TA and TG sites to a CG site that is poorly cleaved in the presence of excess phenanthroline indicates, however, that the mono-phenanthroline Cu(I) complex was the active cleaving agent. A CG preference of the mono-phenanthroline Cu(I) complex is consistent with reports that N-alkyl-1,10-phenanthrolinium ions (41) and planar phenanthroline:platinum complexes (42) preferentially bind non-covalently to G+C-rich DNA.

We conclude that the $(Phen)_2Cu(I)$ complex is both the primary species responsible for DNA cleavage, and the species responsible for the TA cleavage preference by 1,10-phenanthroline+copper, provided phenanthroline is in at least two-fold molar excess over copper. As noted previously (10,12), this sequence preference is consistent with the steric requirements for a binding model in which one phenanthroline ring partially intercalates in a T-3',5'-A step, and the second ring resides in the minor groove with the ring plane near the positions occupied by the guanine N^2-amino groups in C-3',5'-G steps, which are poorly cleaved. If the partial intercalation binding model is correct, the non-intercalated phenanthroline ring may

play a role in determining the preference for TA steps that is positive via favorable interactions with neighboring bases and deoxyriboses at the binding site, or negative via repulsive interactions, e.g. with the guanine N^2-amino group. If the non-intercalated phenanthroline ring interacts favorably with DNA, then $(Phen)_2Cu(I)$ is best viewed as both a groove binder and an intercalator.

The rate, but not the specificity, of cleavage was decreased by a high excess of phenanthroline or use of MPA as the reducing agent. These observations are of practical significance to the use of phenanthroline-copper in synthetic nuclease systems and as a footprinting reagent. The overall DNA cleavage rate is approximately optimized using ascorbate as reducing agent and a phenanthroline/copper ratio of 4:1 if the copper concentration is near 5 μM.

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