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Oxidative cleavage of DNA by homo- and heteronuclear Cu(II)-Mn(II) complexes of an oxime-type ligand

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Abstract

Novel homodinuclear Cu(II) (K1), heterodinuclear Cu(II)-Mn(II) (K2) and homotrinuclear Cu(II) (K3) complexes with a novel oxime-type ligand have been prepared and their nucleolytic activities on pCYTEXP were established by neutral agarose gel electrophoresis. The analyses of the cleavage products obtained electrophoretically indicate that although the examined complexes induces very similar conformational changes on supercoiled DNA by converting supercoiled form to nicked form than linear form in a sequential manner as the complex concentration or reaction period is increased, K3 is less effective than the two others. The oxime complexes were nucleolytically active at physiological pH values but the activities of K1 or K2 were diminished by increasing the pH of the reaction mixture. In contrast, K3 makes dominantly single strand nicking by producing nicked circles on DNA at almost all the applied pH values. Metal complex induced DNA cleavage was also tested for inhibition by various radical scavengers as superoxide dismutase (SOD), azide, thiourea and potassium iodide. The antioxidants inhibited the nucleolytic acitivities of the oxime complexes but SOD afforded no protection indicating that the nucleolytic mechanism involves of copper and/or manganese complex-mediated reactive oxygen species such as hydroxyl radicals being responsible for the oxidative DNA cleavage.

Introduction

In the recent years, the interaction of transition metal complexes with nucleic acids has gained much more attention (Barton 1986; Sigman & Chen 1990; Papavassiliou 1995). These interactions are of great importance for the understanding of the requirements for designing new chemotherapeutic agents and developing tools or probes for the study of nucleic acid structure. Of these, the hydrolytic cleavage of nucleic acids is of fundamental chemical and biochemical significance. However, it is well known that nucleic acids are generally not reactive upon hydrolysis in the absence of an appropriate nucleolytic enzyme under mild conditions. In the last years, designing an effective chemical nuclease has been the focus of investigation of metal-mediated nucleic acid hydrolysis since these redox active compounds cleave the phosphodiester backbone of DNA molecules under physiological pH and temperature (Sigman *et al.* 1979; Pope & Sigman 1984).

Nucleolytic activities of several copper complexes with synthetic or natural ligands have been studied extensively. The first synthetic complex possessing nucleic acid cleavage activity was the bis(1,10-phenanthroline)-copper(II) (Pope & Sigman 1984; Travers 1993). Several other compounds such as salen- (Gravert & Griffin 1993; Sato *et al.* 1994; Mandal *et al.* 1996; Routier *et al.* 1996), porphyrin- (Groves & Farrell 1989), semicarbazone- (Reddy *et al.*, 2000a, b), pyrrole- (Borah *et al.* 1998; Asad *et al.* 1999), thioether-(Dülger *et al.* 2000; Athar *et al.* 2001), and polyamine-type ligands (McLachlan *et al.* 1996) which were complexed with copper ions were also shown to have DNA-relaxation activities.

It has been reported that these nuclease mimics induce DNA cleavage via metal mediated processes, and their activities involve reversible formation of weak or strong complexes with DNA followed by the scisson reaction (Sigman *et al.* 1979; Hertzberg & Dervan 1982; Travers 1993). Induction of nucleic acid cleavage by various transition metal complexes (Dervan 1992; Pratviel *et al.* 1993; Gravert & Griffin 1993; Woodson *et al.* 1993; Papavassiliou 1995; Sargeson 1996; Ross *et al.* 1999) has not only stimulated investigations of their clinical applications especially in cancer chemotherapy (Sherman & Lippard 1987; Veal & Rill 1988; Groves & Farrell 1989) but also provided tools for studying nucleic acid structure and function. Therefore, the development of novel metal complexes which interact and cleave nucleic acids and the understanding of their nature of interaction with DNA would provide more effective utilization of metal complexes for diverse purposes such as in molecular biology, pharmacology and gene therapy (Sigman 1986; Corey *et al.* 1990; Sigman *et al.* 1993; Mandal *et al.* 1996; Ross *et al.* 1999) and in the development of anticancer agents (Gonzalez *et al.* 1996; Criado *et al.* 1999; Peti *et al.* 1999).

The considerable interest developed to copper complexes as nuclease mimics in the recent years prompted us to synthesize homodinuclear copper(II) and heterodinuclear copper(II)-manganese(II) and homotrinuclear copper(II) complexes of a novel oxime-type ligand (Serbest *et al.* 2001), and to characterize their nuclease efficiencies by agarose gel electrophoresis.

Materials and methods

Chemicals

pCYTEXP was a gift from Dr J.E.G. McCarthy (Biomolecular Sciences, UMIST, UK). The plasmid was grown in *E. coli* JM101 cells in LB media for overnight (Belev *et al.* 1991) and purified by the Promega Corporation Wizard Plus SV Minipreps DNA Purification Systems (Madison, USA). Other commercial reagents were of reagent quality and used without further purification.

Preparation of the ligand, its homodinuclear copper(II) (K1), heterodinuclear copper(II)-manganese(II) (K2) and homotrinuclear copper(II) complexes (K3)

3-{2-[2-(2-hydroxyimino-1-methyl-propyldeneamino)-ethylamino] -ethylimino}-butane-2-on oxime, homodinuclear copper(II) (K1), heterodinuclear copper(II)-manganese(II) (K2) and homotrinuclear copper(II) complexes (K3) (Figure 1) were prepared as reported (Serbest *et al.* 2001).

Reaction of the complexes with DNA

The complexes were dissolved in 100 μl of dimethylsulfoxide and then diluted to 1 ml with Milli-Q water as a 10 mM stock solution just prior to assay. pCYTEXP ($\sim$40 μg ml) was incubated in a 10 μl reaction mixture containing individual metal complexes (K1, K2, and K3) in the absence or presence of 1 mM magnesium monoperoxyphthalate (MMPP) at various conditions. Nucleolytic efficiencies of the complexes were assayed with 0.1–1,000 μM of K1, K2 or K3 in a pH range of 6.0–7.5 in 50 mM potassium phosphate buffer or pH 8.0–10.0 in 20 mM tris-acetate buffer. Reaction mixtures were incubated for 5, 10, 20, 30, 40, 50, 60, 120 or 360 min at 37 °C. Reactions were initiated with or without the addition of MMPP and terminated by the addition of 5 μl of a terminating agent containing 10 mM β-mercaptoethanol, 20% glycerol, 25 mM EDTA and 0.05% bromophenol blue:xylene cyanol (1:1) after an appropriate incubation period as described previously (Dülger *et al.* 2000). DNA strand breaks were measured by converting circular double-stranded supercoiled DNA into nicked circular and linear forms. Preliminary experiments have shown that 10 min of incubation with metal complexes causes appreciable DNA cleavage. DNA strand cleavage was estimated on 0.7% neutral agarose jel including 0.5 mg ml ethidium bromide by conversion of the supercoiled plasmid (form I) DNA initially to the nicked (form II) and finially to linear (form III) plasmids. DNA bands were visualized by UV light and photographed. The relaxed plasmid (form II) is electrophoretically less mobile than forms I and III, and this is readily detected. Quantitation of cleavage products, supercoiled, linear and nicked forms of plasmid DNA, generated as a result of treatment DNA with K1, K2 and K3 was performed by Molecular Analyst/PC Windows software for Bio-Rad's Image Analysis Systems, Version 1.4 (Bio-Rad Laboratories, USA).

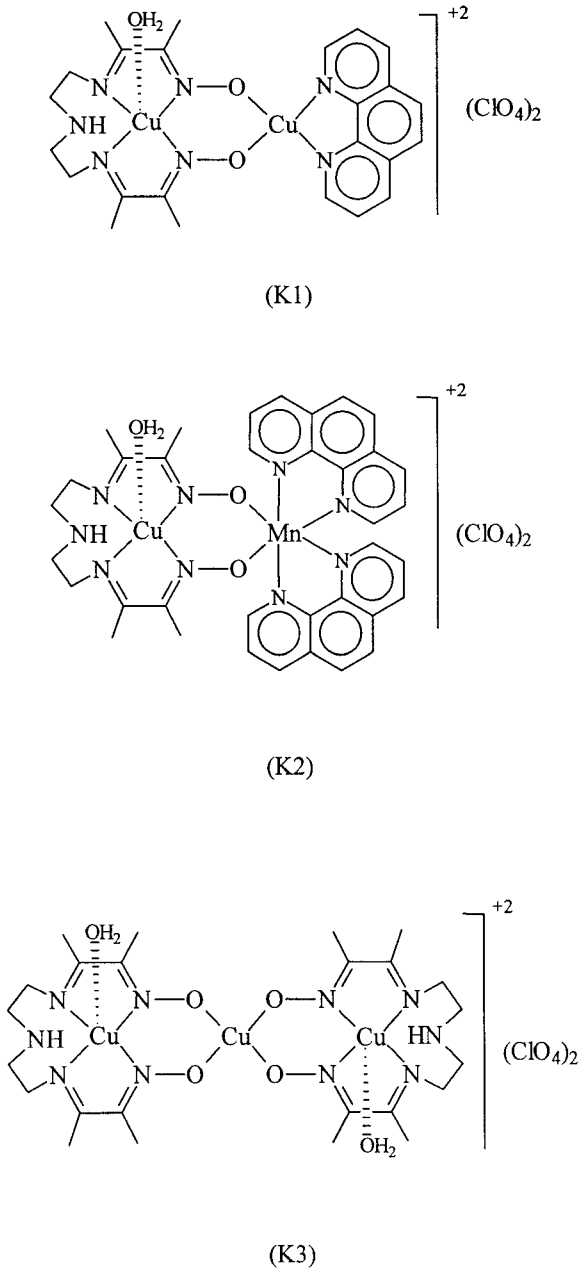

Fig. 1. Proposed structures for the dinuclear copper (K1), dinuclear copper/manganese (K2) and trinuclear copper (K3) complexes of the oxime ligand.

The effect of radical scavengers on DNA breakage

The nucleolytic properties of K1, K2 and K3 on pCYTEXP in the presence of various radical scavengers were assayed electroforetically. pCYTEXP (~40 μg ml) was incubated in a reaction mixture (10 μl) containing effective concentrations of individual metal complexes (K1, K2, and K3) and various radical scavengers as sodium azide, potassium iodide, thiourea and superoxide dismutase (SOD) in the absence or presence of 1 mM MMPP in 50 mM potassium phosphate buffer, pH 7.5 at 37 °C. Reactions were terminated by the addition of 5 μl of a terminating agent containing 20% glycerol, 25 mM EDTA, 10 mM β-mercaptoethanol and 0.05% bromophenol blue:xylene cyanol (1:1) after 1 h of incubation. DNA strand cleavage was estimated on 0.7% neutral agarose jel including 0.5 mg/ml ethidium bromide. Quantitation of cleavage products were performed by Molecular Analyst/Windows software for Bio-Rad's Image Analysis Systems, version 1.4.

Results and discussion

The interactions of homodinuclear copper(II) (K1), heterodinuclear copper(II)-manganese(II) (K2) and homotrinuclear copper(II) (K3) complexes (Figure 1) of the oxime ligand with DNA in the absence or presence of magnesium monoperoxyphthalate (MMPP) as cooxidant were electrophoretically investigated using supercoiled form of pCYTEXP (5 kb). In some experiments supercoiled plasmid DNA concentrations were either low or contaminated with nicked DNA. Upon addition of lower amount of either complexes, some of the nicked forms are modified and therefore, split into two bands one of which has slower mobility and most probably structurally different than the reference form II.

Control experiments carried out in the presence of MMPP together with DNA and in the absence of the complexes showed no background cleavage, and chlorates of copper(II) or manganese(II) at concentrations where K1, K2 and K3 showed cleavage of DNA were ineffective. The preliminary data observed by neutral agarose gel electrophoresis experiments confirm that the examined complexes perturb conformational changes on the DNA and therefore supercoiled form of DNA (form I) is converted to nicked (form II), linear (form III) and/or smaller fragments. The differences in electrophoretic mobilities of these forms were clearly observed and their relative intensities indicating the predominant DNA form under various conditions such as complex concentration, incubation period of the complexes with DNA and pH of the reaction mixture were taken as the type of nucleolytic effectiveness. At low concentrations of either complex, one additional

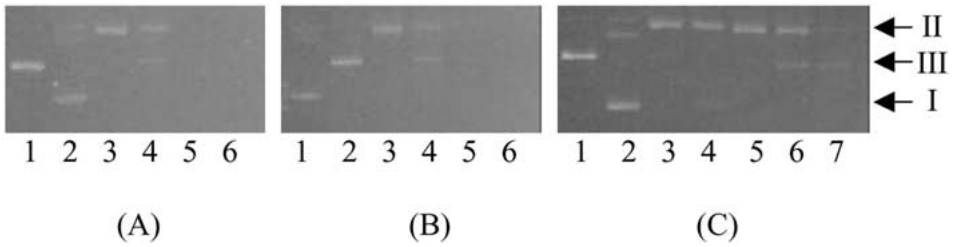

Fig. 2. DNA cleavage as a function of increasing concentrations of oxime complexes in the presence of 1 mM cooxidant MMPP for 60 min of reaction period as described under Experimental Section. Lanes in (A) are **1**, DNA-*Eco*RI; **2**, DNA only; **3**, 0.01 μM K1; **4**, 0.1 μM K1; **5**, 1 μM K1; **6**, 10 μM K1. Lanes in (B) are **1**, DNA only; **2**, DNA-*Eco*RI; **3**, 0.01 μM K2; **4**, 0.1 μM K2; **5**, 1 μM K2; **6**, 10 μM K2. Lanes in (C) are **1**, DNA-*Eco*RI; **2**, DNA only; **3**, 1 μM K3; **4**, 10 μM K3; **5**, 50 μM K3; **6**, 100 μM K3; **7**, 300 μM K3.

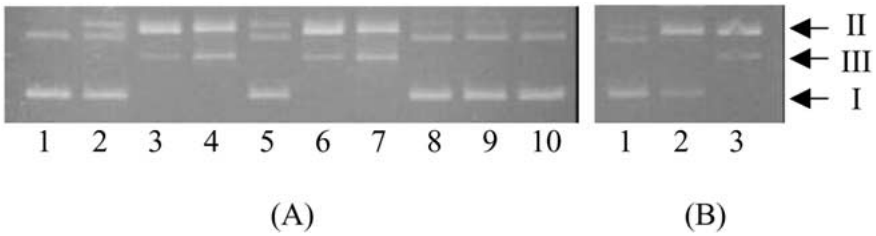

Fig. 3. DNA cleavage as a function of increasing concentrations of oxime complexes in the presence of 1 mM cooxidant MMPP for 10 min of reaction period as described under Experimental Section. Lanes in (A) are **1**, DNA only; **2**, 0.01 μM K1; **3**, 0.1 μM K1; **4**, 1 μM K1; **5**, 0.01 μM K2; **6**, 0.1 μM K2; **7**, 1 μM K2; **8**, 0.01 μM K3; **9**, 0.1 μM K3; **10**, 1 μM K3. Lanes in (B) are **1**, 10 μM K3; **2**, 100 μM K3; **3**, 1000 μM K3.

band having slower mobility than the form II observed in the was observed.

Concentration dependency of nucleolytic efficiencies of the complexes were examined at their concentrations between 0.01–1,000 μM for 60 min of reaction period using 34.9 μg ml DNA in phosphate buffer, pH 7.0 (Figure 2). In the presence of MMPP and within the 1 h-incubation period, 0.01 μM K1 or K2 completely induces single strand cleavage of pCYTEXP and converts the circular supercoiled DNA (form I) to nicked DNA (form II). At 0.1 μM concentration of either K1 (Figure 2A) or K2 (Figure 2B), a small fragment of form II is converted to form III. At greater concentrations, both complexes completely degraded DNA into smaller fragments. The same profile was observed for K3, but greater than 100 μM concentrations of the complex is needed for complete DNA degradation (Figure 2C).

The nucleolytic effect of each metal complexes for shorter periods (10 min) of reaction time was also investigated for the complex concentration ranging 0.01 μM-1,000 μM in phosphate buffer (pH 7) (Figure 3). Up to 0.1 μM of K1 or K2, percentage of the supercoiled form of DNA was declined while levels of nicked form was elevated (Figure 4A). At 0.1 μM or greater concentrations of either complex, supercoiled form is converted to nicked and linear forms of DNA. The amount of linear forms increases when the concentration of the complex is increased. Under these conditions there is an obvious decrease in the levels of nicked form of DNA. The percent ratio of linear form to nicked circles was greater when either complex concentration was increased from 0.1 μM to 1 μM. Furthermore, K1 above 1 μM succeded complete degradation of DNA into smaller fragments within 60 min at 37 °C since no ethidium bromide staining was observable. Although very similar results were also observed for K2 (Figure 4B), K3 has shown different nucleolytic behaviour. The supercoiled form dominates up to 10 μM of K3 and even at that concentration no linear form was observed (Figure 4C). In order to complete the conversion of supercoiled form into nicked or linear forms, greater concentrations of K3 are required and at 1 mM K3, conversion of the supercoiled form into nicked form (78%) and linear form (22%) is completed. These results indicate that although the examined complexes induces very similar conformational changes on supercoiled DNA as conversion of supercoiled form to nicked form than linear form in a sequential manner but K3 is less effective than the two others.

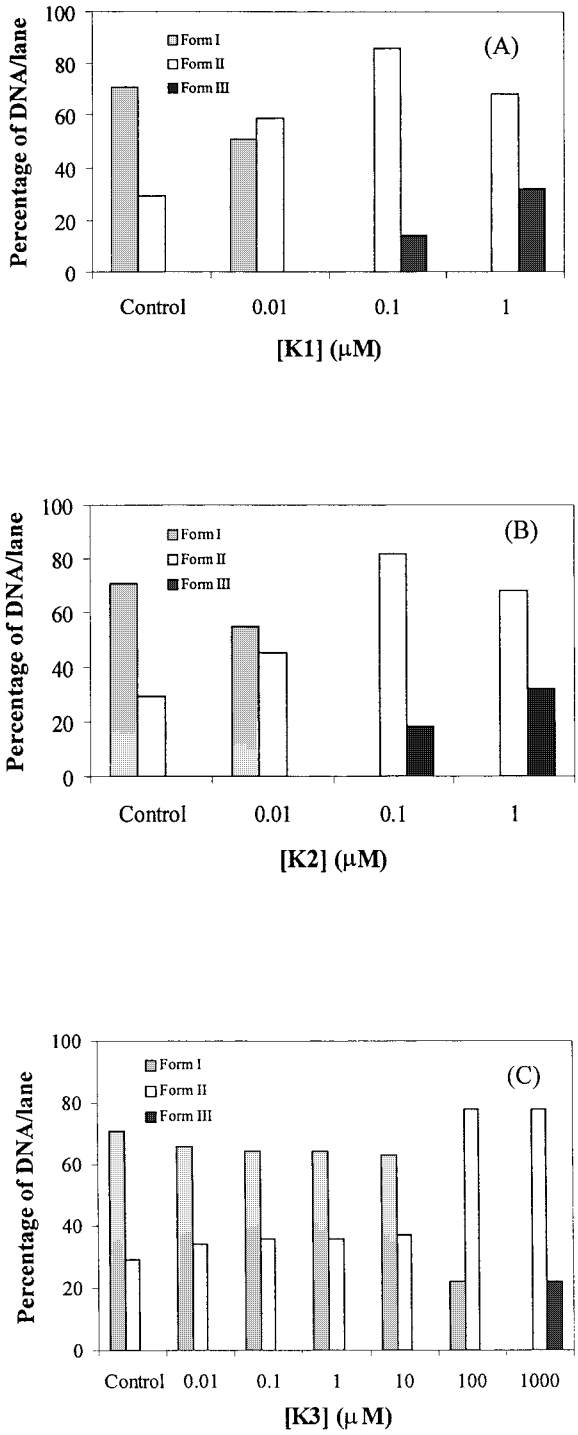

Fig. 4. A plot of the concentrations of the oxime complexes versus the percentage of the DNA for 10 min of reaction period as described under Experimental Section. The amount of forms I, II, and III produced in the presence of the oxime complexes (A) K1 (B) K2 (C) K3 were determined by Molecular Analyst/Windows software for Bio-Rad's Image Analysis Systems, Version 1.4.

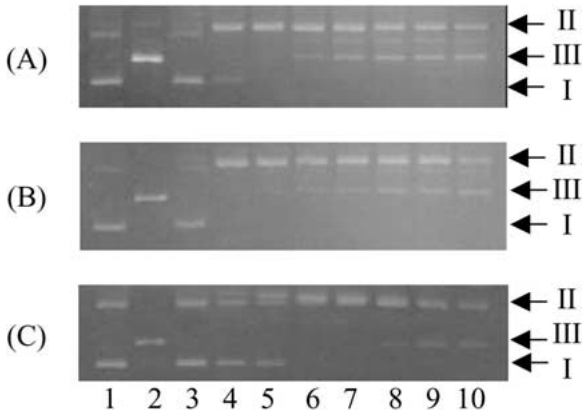

Fig. 5. DNA cleavage as a function of increasing period of reaction between the oxime complexes and pCYTEXP in the presence of 1 mM cooxidant MMPP. The reactions were carried out with 19.1 μg ml DNA in phosphate buffer, pH 7.0. (A) is for 0.1 μM K1 with 33.2 μg ml DNA, (B) for 0.1 μM K2 with 19.1 μg ml DNA, and (C) for 100 μM K3 with 47.9 μg ml DNA. Lanes are **1**, DNA only; 2, DNA-*Eco*RI; **3**, at the start of the reaction; **4**, 5 min; **5**, 10 min; **6**, 20 min; **7**, 30 min; **8**, 40 min; **9**, 50 min; **10**, 60 min.

Time dependencies of the interaction of the complexes with plasmid DNA were electrophoretically monitored just after the mixing of the reagents and then at 10 min intervals. The fragments produced in the presence of each complex were analyzed by electrophoresis (Figure 5) and a plot of the incubation time versus the percentage of DNA form was obtaiend by using the electrophoretic data (Figure 6). The reactions were performed with 19.1 μg ml DNA and 0.1 μM K1, 0.1 μM K2 or 100 μM K3 in the presence of MMPP at 25 °C for various period of incubation time in phosphate buffer (pH 7.0).

These experiments support that all of the complexes make conformational changes on plasmid DNA by making single strand nicking therefore converting supercoiled form to nicked form. When the reaction period is increased the nicks are made in opposite strands occuring on nearby sites to produce double strand fragments of linear DNA. These nucleolytic strand nicking activities of the complexes are obviously both time and complex concentration-dependent. These results also confirm that conversion of form II to form III in the case of K1 and K2 takes lesser reaction periods than that of K3 (Figure 6).

pH dependencies of the interaction of the complexes with plasmid DNA were performed in appropriate buffers. pH was varied over a pH range of 6.0–7.5 in 50 mM phosphate buffer and pH 8.0–10.0 in 20 mM tris-acetate buffer. The effect of complexes on supercoiled form of DNA between pH 6.0–10.0 at the most

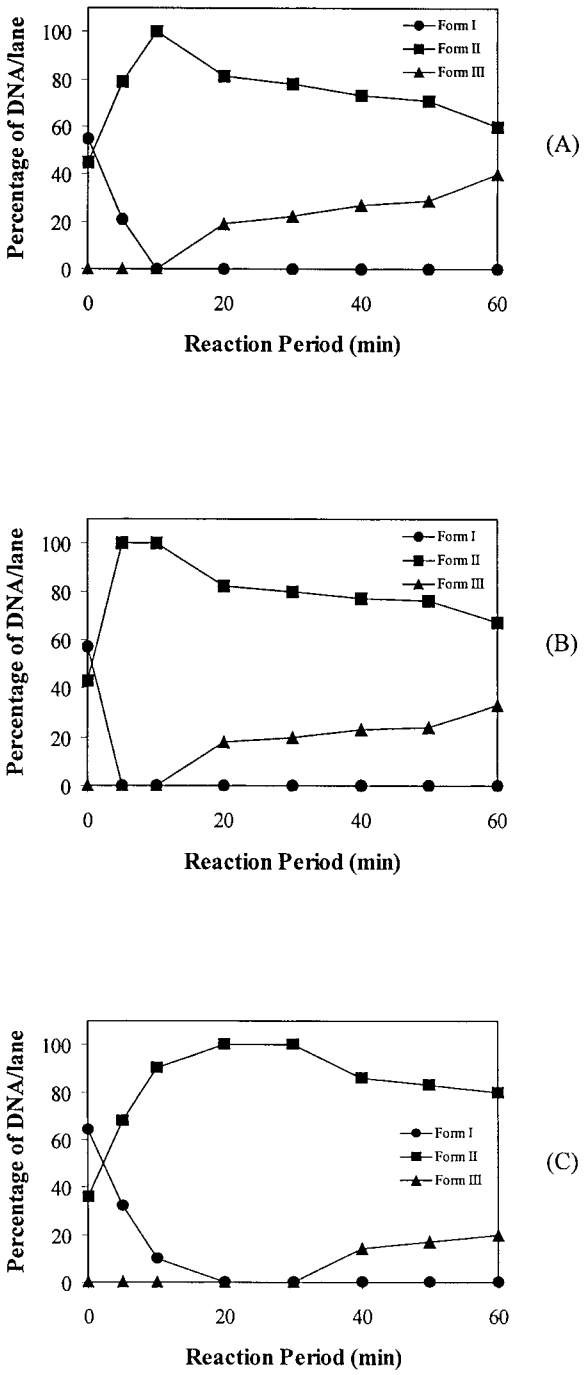

Fig. 6. A plot of the incubation time versus the percentage of DNA. The amount of forms I, II, and III produced in the presence of the oxime complexes (A) K1 (B) K2 (C) K3 were determined by Molecular Analyst/Windows software for Bio-Rad's Image Analysis Systems, Version 1.4.

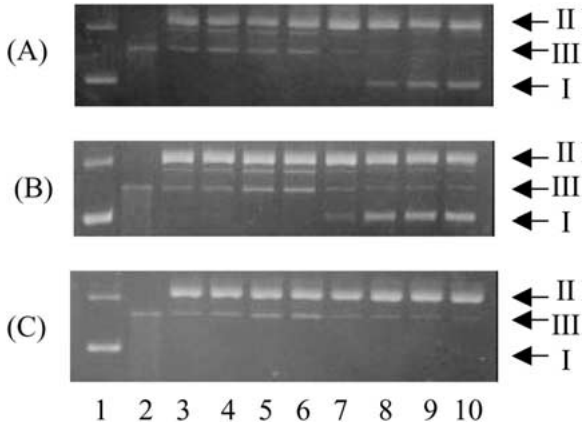

Fig. 7. DNA cleavage as a function of pH of the reaction mixture in the presence of the oxime complexes. The reactions were carried out with 74.5 μg ml DNA in 50 mM phosphate buffer, pH range of 6.0–7.5 and tris-acetate buffer, pH range of 8.0–10.0 for 1 h of incubation. (A) is for 0.1 μM K1, (B) for 0.1 μM K2, and (C) is for 100 μM K3. Lanes are **1**, DNA only; **2**, DNA-*Eco*RI; **3**, pH 6.0; **4**, pH 6.5; **5**, pH 7.0; **6**, pH 7.5; **7**, pH 8.0; **8**, pH 8.5; **9**, pH 9.0; **10**, pH 10.0.

effective complex concentration was observed electrophoretically by monitoring the DNA forms to be produced (Figure 7). The data were analyzed by plotting pH changes versus percentage DNA form present per lane (Figure 8). Both K1 and K2 were nucleolytically active at physiological pH values. However, their activities were diminished by increasing the pH of the reaction mixture (Figure 8A and B). pH dependency of the nucleolytic activity in the presence of 100 μM K3 was very different from those of K1 and K2. In contrast, K3 makes dominantly single strand nicking by producing nicked circles on plasmid DNA at almost all the applied pH values, and a very small fragment (less than 20%) of form III was also observed (Figure 8C).

Metal complex induced DNA cleavage was also tested for inhibition by various radical scavengers as superoxide dismutase, azide, thiourea and potassium iodide. Sodium azide is a singlet oxygen scavenger, superoxide dismutase (SOD) remove superoxide anion, and potassium iodide and thiourea eliminate hydroxyl radicals. All the antioxidants inhibited the nucleolytic acitivities of the examined complexes but SOD afforded no protection (Figure 9) indicating the involvement of reactive oxygen species such as hydroxyl radical in the mechanism for DNA cleavage by the examined complexes (Ahsan & Hadi 1998). It can be evaluated that these radicals are produced at a very close proximity to the interaction site upon binding of the metal complexes with DNA (Pryor 1988).

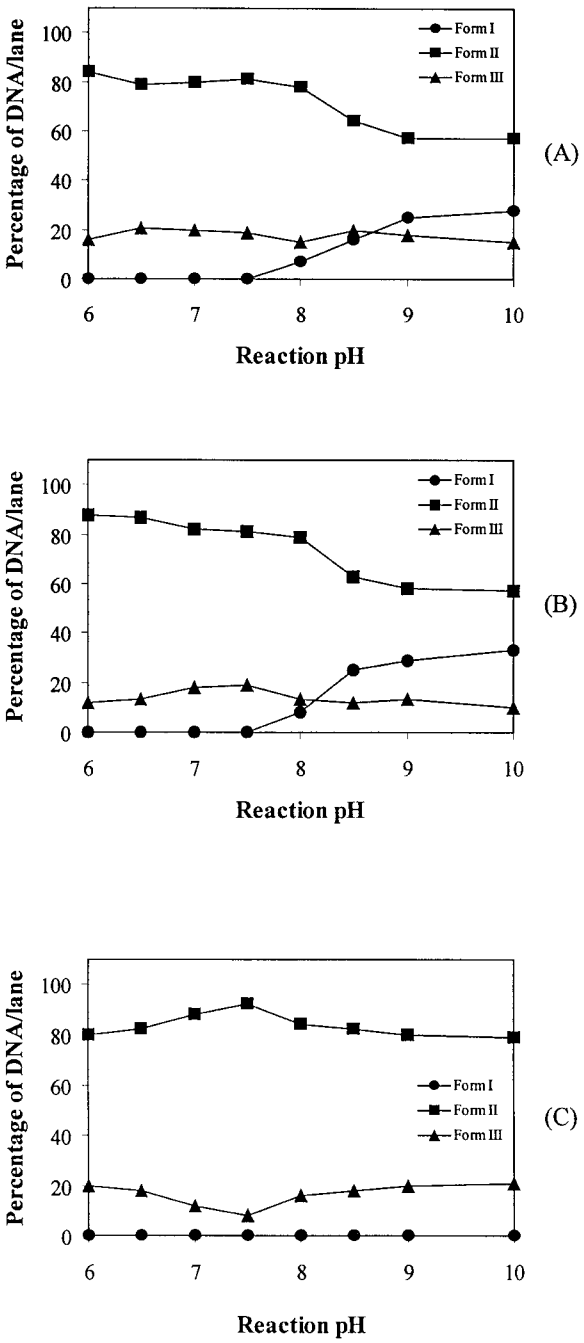

Fig. 8. A plot of the reaction pH versus the percentage of DNA. The amount of forms I, II, and III produced in the presence of the oxime complexes (A) K1 (B) K2 (C) K3 were determined by Molecular Analyst/Windows software for Bio-Rad's Image Analysis Systems, Version 1.4.

Therefore, the nucleolytic process performed by the oxime complexes should involve copper and/or manganese complex-mediated hydroxyl radicals which are responsible for the oxidative DNA cleavage (Imlay & Linn 1988; Halliwell & Aruoma 1991; Dizdaroglu 1992; Halliwell 1995; Tsou & Yang 1996; Asad *et al.* 1999; Liu *et al.* 1999). Superoxide radicals may not participate in the nucleolytic reaction since DNA strand cleavage was enhanced by SOD as observed by electrophoresis. The increased induction of DNA breakage by the oxime complexes at pH above 6 is consistent with previous results indicating the occurence of hydroxyl radical production in neutral and/or alkaline solutions (Shi *et al.* 1993). It can be concluded from this result that hydroxyl radicals generated by the studied copper complexes produce oxidative damage to DNA through a Fenton-like reaction (Detmer *et al.* 1996).

Moreover, the chemical environment around the central metal ions and their geometric structures may also effect the nucleolytic efficiency of the oxime complexes (Liu *et al.* 1999). In the dinuclear complexes (K1 and K2), in which the first copper(II) was complexed with nitrogen atoms of the oxime and imine groups in a square-planar coordination geometry. The second metal ion -either copper(II) or manganese(II)- is ligated with dianionic oxygen atoms of the oxime groups and are linked to the phenanthroline nitrogen atoms. The trinuclear copper(II) complex (K3), however, was formed by coordination of the third copper ion with dianionic oxygen atoms of each of two molecules of the mononuclear copper(II) complexes of the oxime ligand (Serbest *et al.* 2001). Therefore, the difference in the DNA cleavage activities of the oxime complexes may be attributed to their proximity to the DNA on binding since the phenanthroline units present in K1 and K2 may provide much more effective binding than K3 which has no such structural units. This may also imply that the binding of K1 or K2 to DNA makes metal ions more approachable to the DNA backbone than those in K3. Therefore, the difference in the cleavage behaviour of K3 is consistent with a distinct oxidative cleavage pathway. These observations suggest that the coordination environment of the central metal ions in the oxime complexes not only governs DNA binding but also determines the nucleolytic action.

The data obtained by electrophoresis may also suggest that DNA cleavage by the oxime complexes in the presence of MMPP is typical of hydroxyl radical-induced chain reactions (Pryor 1988; Detmer *et al.*

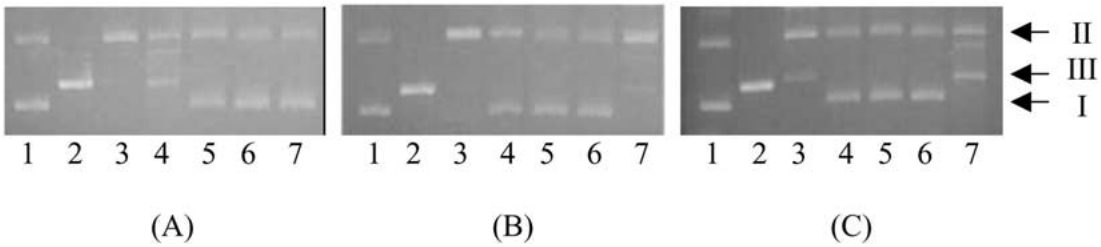

Fig. 9. Effect of various radical scavengers on DNA cleavage induced by the oxime complexes with 40 μg ml DNA in phosphate buffer, pH 7.5. The final concentrations of all the scavengers; sodium azide (SA), potassium iodide (PI) and thiourea (TU), were 50 mM except that SOD was 0.1 mg ml. Lanes in (A) are **1**, DNA only; **2**, DNA-*Eco*RI; **3**, DNA+0.1 μM K1; **4**, 0.1 μM K1+SOD; **5**, DNA+0.1 μM K1+SA; 6, DNA+0.1 μM K1+PI; **7**, DNA+0.1 μM K1+TU. Lanes in (B) are **1**, DNA only; **2**, DNA-*Eco*RI; **3**, DNA+0.1 μM K2; **4**, DNA+0.1 μM K2+SA; **5**, DNA+0.1 μM K2+PI; **6**, DNA+0.1 μM K2+TU; **7**, DNA+0.1 μM K2+SOD. Lanes in (C) are **1**, DNA only; **2**, DNA-*Eco*RI; **3**, DNA+100 μM K3; **4**, DNA+100 μM K3+SA; **5**, DNA+100 μM K3+PI; **6**, DNA+100 μM K3+TU; **7**, DNA+100 μM K3+SOD.

1996; Asad *et al.* 1998) and proceeds by an ordered mechanism in which oxime complex first binds to DNA via phenanthroline units and a DNA-oxime complex is formed. Hydroxyl radicals produced in close proximity of the DNA strands by metal ion-induction attack the DNA and lead to DNA cleavage (Pryor 1988; Detmer *et al.* 1996).

In conclusion, the electrophoresis experiments have showed that the binding of the oxime complexes to plasmid DNA produces reactive oxygen species, particularly hydroxyl radical, and the interaction of the complexes with DNA causes strand breakage which is most probably mediated by these reactive oxygen species.

Acknowledgements

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