Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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To cite this Article Kang, Jingwan , Dong, Shuqing , Lu, Xiaoquan , Su, Biquan , Wu, Haixia and Sun, Kun(2006) 'Study on DNA Cleavage by the Hexaaza Macrocyclic Copper(II) Complex', Journal of Macromolecular Science, Part A, 43: 2, 279 — 288
To link to this Article: DOI: 10.1080/10601320500437177
URL: http://dx.doi.org/10.1080/10601320500437177

Journal of Macromolecular Science®, Part A: Pure and Applied Chemistry, 43:279–288, 2006

ISSN 1060-1325 print/1520-5738 online
DOI: 10.1080/10601320500437177

Study on DNA Cleavage by the Hexaaza Macrocyclic Copper(II) Complex

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The Hexaaza macrocyclic copper(II) complex (Cu(II)L-1,8-Dihydroxyethyl-1,3,6,8,10,13-hexaazacyclotetradecane copper(II) perchlorate monohydrate) was synthesized and purified. The interaction of this complex with calf thymus DNA has been explored by using absorption, emission, viscosity measurements, electrochemical studies and DNA cleavage. All of the experimental results indicate that the complex bind to DNA by non-classical or partial intercalative interaction. The complex has also been found to promote the cleavage plasmid pBR 322, in the presence of H_2O_2 and ascorbic acid. The cleaving mechanism for the Cu(II)L complex has been proposed.

Keywords Cu(II)L, macrocyclic complex, DNA cleavage, bind constant

Introduction

Deoxyribonucleic acid (DNA) is a molecule that acts as a form of memory storage for genetic information (1). DNA is usually the target of some anti-tumor reagents, these reagents react with DNA thereby stopping the replication of DNA and inhibiting the growth of the tumor cell (2). DNA offers the analytical chemist a powerful tool in the recognition and monitoring of many important molecules (3).

The interaction of transition metal complexes with DNA has been extensively studied in the past few years. Barton and co-workers (4, 5) have studied the interaction of enantiomers of $Ru(phen)_3^{3+}$ with various DNA, the results lead them to the conclusion that there were two modes of interaction, intercalative and electrostatic binding. Kharatishvili et al. (6) also reported the effect on DNA binding in the presence of a planar intercalating ligand such as quinoline for both mononuclear and dinuclear Pt complexes. Mahadeven and Palaniandvavr (7) have studied copper(II) complexes of bis(pyrid-2-yl)-di/trithia ligands bound to calf thymus DNA and found that the coordination geometry and the ligand donor atom type play a key role in deciding the mode and extent of binding of complexes to DNA. Lin and co-workers (8) have prepared

Received May 2005; Accepted June 2005.
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the 1:1 copper(II) complexes of certain N,N−dialkyl-1,10−phen anth roline-2,9-dimethanamine and studied the thermodynamic and kinetic properties of the complex-DNA binding. Recently, the investigation based on DNA interactions with small molecular compounds have great importance in understanding the action mechanism of some anti-tumor and anti-viral drugs and origins of some diseases and to design new DNA-targeted drugs and also to screen these drugs *in vitro*. Polyaza macrocyclic compounds with N-carboxylic acid groups were of current interest in particular because of their potential applications in various fields, such as medical imaging agents or supramolecular architectures (9, 10).

In this paper, we first synthesized Cu(II)L (the structure is in Scheme 1) which has a octahedral structure similar to some natural complexes, such as chlorophyll, hemoglobin, vitamin B_{12} and other bioenzymes, and studied its binding properties to calf thymus DNA by different spectroscopic methods and pBR322 DNA cleavage experiment. Our purpose was to understand the selectivity and efficiency of DNA recognized and cleaved by macrocyclic copper complex, and to develop new effective antitumor reagents or useful DNA probes.

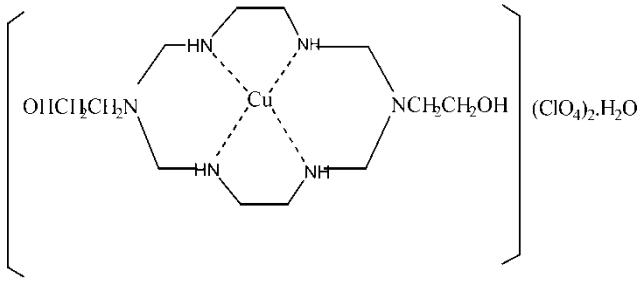
Experimental

Materials and Methods

The Cu(II)L complex was synthesized by methods previously described in our laboratory (11). All reagents and solvents were purchased commercially and used without further purification unless otherwise noted. All experiments were carried out at pH 7.6 in Tris-HCl buffer (10 mM Tris-HCl, 50 mM NaCl). A solution of calf thymus DNA (Sino-American Biotechnology Co.) in the buffer gave a ratio of UV absorbance at 260 and 280 nm of about 1.8−1.9 : 1, indicating that the DNA was sufficiently free of protein. The DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient ($6600\,M^{-1}\,cm^{-1}$) at 260 nm (12). pBR322 (0.50 μg/μL) was also obtained from Sino-American Biotechnology Co. (Beijing, China).

Physical Measurements

UV-Visible absorption spectra was recorded on a UV Agilent 8453 spectrophotometer (Hitachi, Japan). Fluorescence determination was performed on RF-540 spectrofluorophotometer (Hitachi).



Scheme 1. The structure of 1,8-Dihydroxyethyl-1,3,6,8,10,13-hexaazacyclotetradecane copper(II) perchlorate monohydrate(Cu(II)L).

Cyclic Voltammetry was carried out by a CHI660 electrochemical workstation (CH Instruments Ltd. Co., USA). A standard three-electrode system comprising a glassy carbon working electrode, platinum-wire auxiliary electrode and Ag/AgCl reference electrode was used. The supporting electrolyte was 50 mM NaCl/10 mM Tris-HCl buffer (pH 7.6). Solutions were deoxygenated by purging with N_2 prior to measurements. All the experiments were carried out at the laboratory temperature.

Viscosity experiments were carried on an Ubbelodhe viscometer, immersed in a thermostatic water-bath maintained at a constant temperature at 20°C. Calf thymus DNA samples approximately 200 base pairs in average length were prepared by sonication in order to minimize complexities arising from DNA flexibility (13).

For the gel electrophoresis experiments, supercoiled pBR 322 DNA (0.5 μg/mL) in Tris-HCl buffer was treated with different concentrations Cu(II)L; and in the presence of peroxide hydrogen; followed by the addition of ascorbic acid in the reaction solution. The samples were incubated for 2 h at 37°C, a loading buffer containing 25% bromophenol blue (2 μL) was added and electrophoresis was performed at 80 V for 2 h in TAE buffer using 0.8% agarose gel containing 1.0 μg/mL ethidium bromide. Bands were visualized by UV light and photographed on a capturing system (Gelprinter plus TDI).

Results and Discussion

Electronic Absorption Spectra

The absorption spectra of the complex in the absence and presence of calf thymus DNA is shown in Figure 1. In the UV region, all the present macrocyclic copper(II) complexes exhibit an intense absorption band around 250 nm which is attributed to the ligand

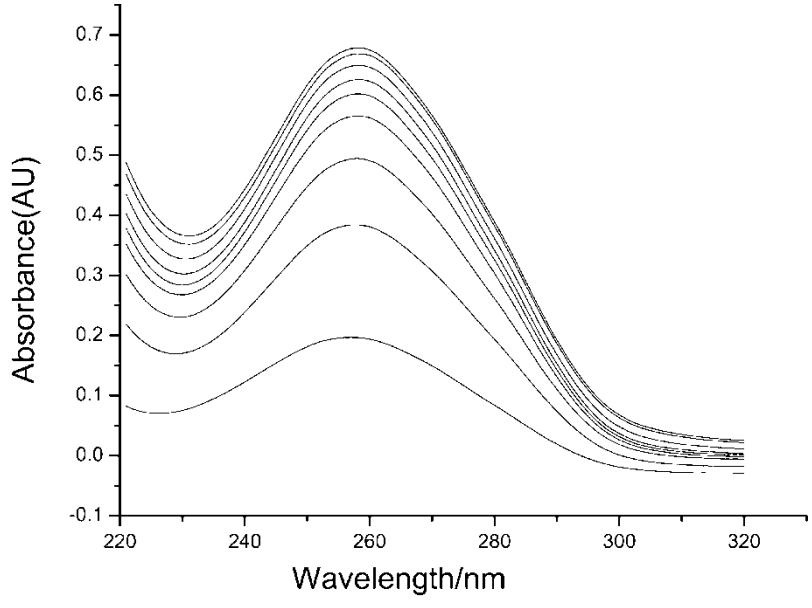


Figure 1. Absorption spectra of Cu(II)L, in the absence and presence of increasing amounts of DNA, $[Cu(II)L] = 5 \times 10^{-5}$. Arrows show the absorbance changes upon increasing DNA concentration.

of metal charge-transfer absorption (MLCT). With the addition of calf thymus DNA, the complex shows an increase of the MLCT absorption band, indicating its binding to DNA (14).

In order to study intensely the binding strength of the copper(II) complex, the intrinsic binding constant K of the complex with CT-DNA is obtained by monitoring the change in the intraligand band of 258, with increasing concentration of DNA using the following function equation (15):

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K(\varepsilon_b - \varepsilon_f)$$

Where ε_a, ε_f and ε_b correspond to $A_{obsd}/[Cu]$, the extinction coefficient for the free copper complex, and the extinction coefficient for the free copper complex in the fully bound form, respectively. In the plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ vs. [DNA] (Figure 2), K is given by the ratio of slope to the intercept.

The binding constant obtained for the Cu(II)L complex is $(2.4 \pm 0.02) \times 10^4$. The K value is lower than those observed for typical classical intercalators (ethidium-DNA, 1.4×10^6 in 25 mM Tris-HCl/40 mM NaCl buffer, pH = 7.9) (16). This is indicative of binding of the complex with DNA with an affinity less than the classical intercalators. It is implied that partial intercalative interaction could be ruled out for the complex.

Fluorescence Spectroscopic Studies

Ethidium bromide (EB) emits intense fluoresence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs. It was previously reported that the enhanced fluoresence could be quenched by the addition of a second molecule (17, 18). The quenching extent of fluorescence of EB bound to DNA is used to determine the extent of binding between the second molecule and DNA.

The emission spectra of EB bound to DNA in the absence and presence of the copper (II) complex is given in Figure 3. The addition of the complex to DNA pretreated with ethidium bromide causes appreciable reduction in emission intensity, indicating that the complex compete with ethidium bromide in binding to DNA.

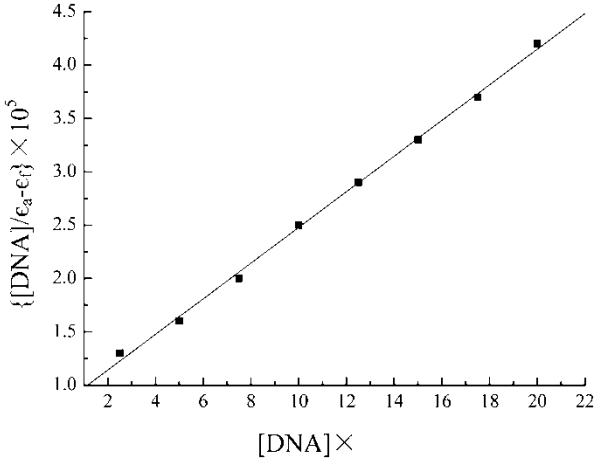


Figure 2. Plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ vs. [DNA] for absorption titration of CT DNA with Cu(II)L.

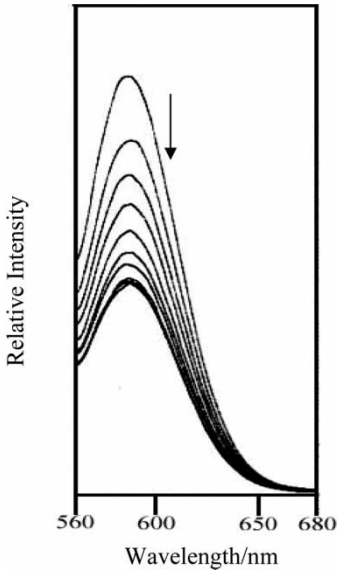


Figure 3. Emission spectra of EB bound to DNA in the absence and presence of the Cu(II)L. [Cu(II)L] = 5×10^{-5} M, EB = 1×10^{-5} M, [Cu(II)L]/[DNA] = 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7.

According to the classical Stern–Volmer equation (17):

$$I_0/I = 1 + Kr$$

where I_0 and I are the fluorescence intensities in the absence and presence of complex, respectively. K is a linear Stern–Volmer quenching constant dependent on the ratio of r_{EB} (the ratio of the bound concentration of ethidium bromide to the concentration of DNA). r is the ratio of total concentration of complex to that of DNA.

The fluorescence quenching curves of EB bound to DNA by the copper (II) complex are shown in Figure 4. The quenching plots illustrate that the quenching of EB bound to DNA by the Cu(II)L complex is in good agreement with the linear Stern–Volmer equation, which also proves that the complex can bind to DNA. In the plot of I/I_0 vs. [complex]/[DNA], K is given by the ratio of the slope to intercept. The K value for the complex is 1.14, which is lower than those observed for classical intercalators. The result suggests that the Cu(II)L can bind to DNA, but the affinity is not strong, which is consistent with the above absorption spectral result.

Viscosity Measurements

As a means for further clarifying the binding of the copper complex, viscosity measurements are carried out on CT DNA by varying the concentration of the added complex. Hydrodynamic measurements which are sensitive to length increase (for example, viscosity, sedimentation) are regarded as the most critical tests of binding in solution in the absence of crystallographic structure data (18). A classical intercalative mode causes a significant increase in viscosity of DNA solution due to an increase in separation of base pairs at intercalation sites and hence, an increase in overall DNA length. By contrast, complexes that bind exclusively in the DNA grooves by partial

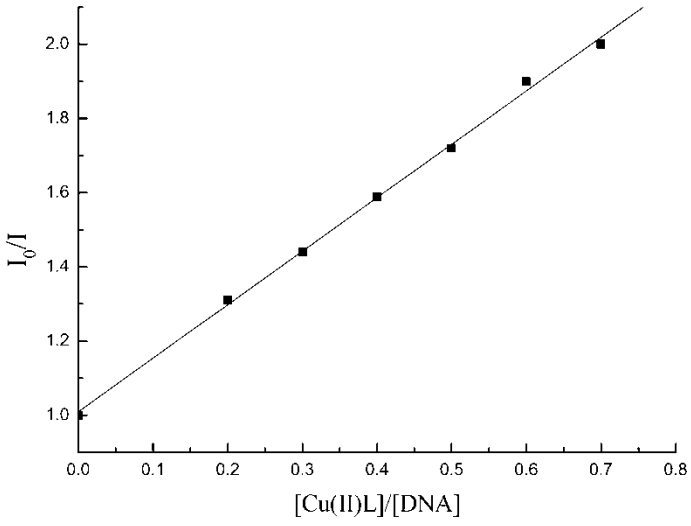


Figure 4. Fluorescence quenching curve of EB bound to DNA by Cu(II)L, [DNA] = 10 mM, [Cu(II)L]/[DNA] = 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7.

and/or nonclassical intercalation, under the same conditions, typically cause negative or no change in DNA solution viscosity (19). The values of $(\eta/\eta_0)^{1/3}$, where η and η_0 are the specific viscosities of DNA in the presence and absence of the complex, respectively are plotted against [Cu(II)L]/[DNA] (Figure 5). For the Cu(II)L complex, the viscosity of DNA decreases with an increase in concentration of the added complex. The results reveal that the presence of the complex has an obvious effect on the relative viscosity of CT DNA. The decreased relative viscosity of DNA may be explained by a binding mode, which produced bends or kinks in the DNA, and thus reduced its effective

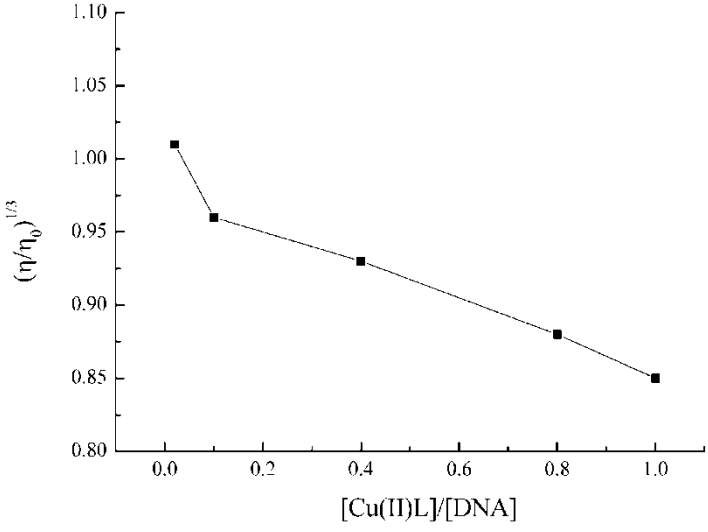


Figure 5. Effect of increasing amounts of [Cu(II)L] on the relative viscosities of CT DNA.

length and concomitantly its viscosity. On the other hand, the Cu(II)L proposes to be bound to DNA by partial intercalation (20), which is consistent with the above results.

Redox Studies

A Cyclic Voltammetric technique has been employed to study the interaction of the present redox active copper(II) complex with DNA with a view to further explore the DNA binding modes assessed from the above spectral and viscometric studies.

Typical cyclic voltammetric (CV) behavior of 5×10^{-5} M Cu(II)L in the absence and presence of CT DNA is shown in Figure 6. The Cyclic Voltammogram of the copper(II) complex in the absence of DNA features a reduction of +2 to the +1 form at a cathodic peak potential, E_{pc} of 0.659 V vs. Ag/AgCl. Reoxidation of +1, +2 occurs, upon scan reversal, at 0.605V. The separation of the anodic and cathodic peak potentials, $\Delta Ep = 54$ mV, the values of $i_{pa}/i_{pc} = 1.44$. The Cyclic Voltammograms of the complex reveal that the redox tends to be complicated by adsorption of copper(II) species (21). The formal potential $E^{0'}$(or voltammetric $E_{1/2}$), taken as the average of E_{pc} and E_{pa}, is 0.634V in the absence of DNA.

Upon addition of CT DNA, both the cathodic and anodic peak currents decrease remarkably and the peaks nearly disappear. The drop of the voltammetric currents in the presence of CT DNA can be attributed to diffusion of the metal complex bound to the large, slowly diffusing DNA (22). No redox peaks observed may result from kinetic complications during electron transfer, uncompensated solution resistance, etc. The more pronounced the decrease of the peak currents upon addition of CT DNA, the stronger the binding affinity of the complex to DNA; partial intercalation mode, as deduced above, can account for this (23). The shift in $E_{1/2}$ for Cu(II)L complex on binding to DNA suggests that both Cu(II) and Cu(I) forms bind to DNA but to different extents.

The general progress can be described by a square scheme (shown below) similar to that proposed previously by Bard et al. (21). The ratio of the equilibrium constants

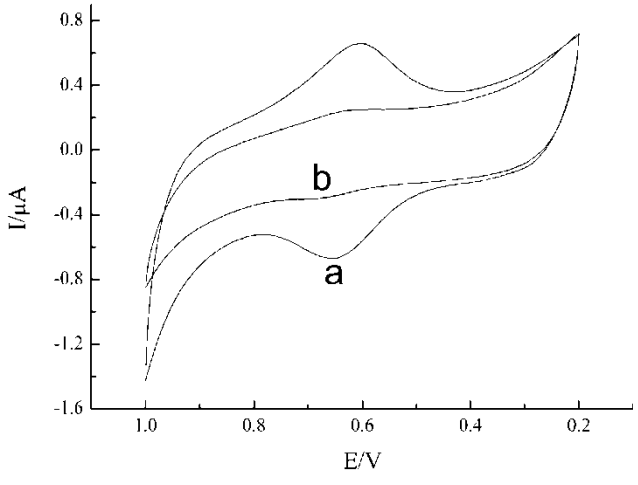


Figure 6. Cyclic voltammogram of 5×10^{-5} MCu(II)L in the absence (a) and presence (b) of DNA. scan rate: 50 mV s^{-1}.

for binding of the Cu(II) and Cu(I) species to DNA has been estimated from the net shift in $E_{1/2}$ on the addition of DNA using the equation,

$$E_b^{0'} - E_f^{0'} = 0.059 log(K_+/K_{2+})$$

where $E_b^{0'}$ and $E_f^{0'}$ are the formal potentials of the Cu(II)/Cu(I) couple in the free and bound forms, respectively, and K_+ and K_{2+} the corresponding binding constants for the binding of +1 and +2/species to DNA, respectively, the K_+/K_{2+} value is collected 3.35.

$$\begin{array}{ccc} Cu(II)L + e^- & \rightarrow & Cu(I)L^+ \\ K_{2+} \downarrow & & \downarrow K_+ \\ Cu(II)L - DNA & \rightarrow & Cu(I)L^+ - DNA \end{array}$$

The complex has been suggested to bind DNA by partial intercalation, with the reduced Cu(I) form interacting more strongly than the oxidized Cu(II) form (K_+/K_{2+}, 3.35).

Agarose Gel Electrophoresis

There has been considerable interest in DNA endonucleolytic cleavage reactions that are activated by transition metal complex (24, 25). The delivery of high concentrations of metal ion to the helix, in locally generating oxygen or hydroxide radicals, yields an efficient DNA cleavage reaction. DNA cleavage is monitored by the relation of supercoiled circular pBR 322 DNA(scDNA) (Form I) into nicked circular (Form II) and linear (Form III).

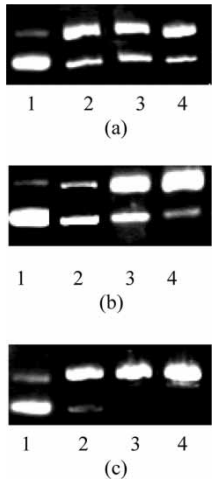


Figure 7. Agarose gel electrophoresis of pBR322 DNA treated with copper(II) complex in the presence of H_2O_2 or H_2O_2 and ascorbate in a buffer containing 10 mM Tris and 50 mM NaCl at 37°C. (a) (1) DNA alone; (2–4) in the presence of a different concentration of Cu(II)L, (2) 20 μM, (3) 40 μM, (4) 60 μM; (b) (1) DNA + 0.5 mM H_2O_2; (2–4) in the presence of 0.5 mM H_2O_2 and different concentration of Cu(II)L, (2) 20 μM, (3) 40 μM, (4) 60 μM; (b) (1) DNA + 0.5 mM H_2O_2 + 1 mM ascorbic acid; (2–4) in the presence of 0.5 mM H_2O_2 and 1 mM ascorbic acid and different concentration of Cu(II)L, (2) 20 μM, (3) 40 μM, (4) 60 μM.

The ability of the Cu(II)L complex in effecting DNA cleavage has been studied by gel electrophoresis using supercoiled pBR 322 DNA in 10 mM Tris-HCl/50 mM NaCl buffer pH 7.6. We incubate the Cu(II)L complex alone with pBR322 DNA and subsequent electrophoresis, Figure7(a) shows the gel electrophoretic separations of plasmid pBR322 DNA after incubation 2 h in the presence of varying concentrations of Cu(II)L complex alone. It can be seen that with increasing the concentration of Cu(II)L complex, Form II increases gradually, while Form I diminishs gradually, but the Form III does not occur. It is suggested that the Cu(II)L complex presents weak nuclease activity. As is well known, many copper complexes have been shown to cleave DNA in the presence of H_2O_2, which is the DNA cleaving species formed in the Fenton reaction (26). Under the same complex concentration containing H_2O_2, the electrophoresis experiments are carried out. From Figure 7(b), (lanes $2 \sim 4$), it is apparently clear that the scDNA is not also cleaved. This result indicates that the complex to damage DNA in the presence of H_2O_2 produced a small amount of hydroxyl radicals, and they are insufficient to cleave DNA, because without a reduction agent, it is difficult to produce the Cu(I) species to reduce H_2O_2. So under the same experimental conditions, adding an excess of reduction agent ascorbate (Figure 7(c)) (lanes $2 \sim 4$), ascorbate is added to reduce Cu(II) to more Cu(I), which then reacts with to produce more hydroxyl radicals (27). In Figure 7(c) (lane 4), it can be seen that even the scDNA can be converted to linDNA when the Cu(II)L concentration is increased. It is likely that the reduction is the important step leading to DNA cleavage. Control experiments using complex or H_2O_2 or H_2O_2 and ascorbic acid do not show any apparent cleavage of DNA ((a,b,c) lane1). From Figure 7, we can observe that increasing of the complex concentration also causes increasing DNA cleavage and the cleavage efficiency of the complex in the presence of H_2O_2 and ascorbate is found to be much better than that of only Cu(II)L or Cu(II)L and H_2O_2. Hence, it is clear that the Cu(II)L complex in the presence of H_2O_2 and ascorbate shows nuclease activity.

From the Agarose gel electrophoresis, the scDNA cannot be cleaved dramatically only by the Cu(II)L complex; the scDNA can be cleaved apparently in the presence of H_2O_2 and ascorbate, because there are more hydroxyl radicals. The process can be expressed as follows (26):

$$\mathrm{Cu(II)L} + \mathrm{e}^- \rightarrow \mathrm{Cu(I)L\ (reduction\ reaction)}$$
$$2\mathrm{Cu(I)L} + \mathrm{H_2O_2} \rightarrow \mathrm{OH}^- + \mathrm{OH} + 2\mathrm{Cu(II)L}$$
$$\mathrm{DNA} + \mathrm{OH}^- \rightarrow \mathrm{ocDNA} + \mathrm{LinDNA}$$

As mentioned above, the mechanism of the Cu(II)L complex cleavage reactions is the Fenton catalyst.

Conclusions

The Cu(II)L complex was synthesized and purified. Spectroscopic studies, together with viscosity experiments and electrochemical methods, support the fact that the complex binds to CT DNA by partial intercalation via its pyridine ring into the base pairs of DNA. The hydroxyl group of the complex on the side chain may lead to the weaker interaction with DNA, these observations suggest that the structure of the haxaaza macrocycle plays an important role in the binding mode affinity. Obviously, the complex has been found to cleave plasmid pBR322 DNA from the supercoiled form I to the open circular form II upon H_2O_2 and ascorbate, which may be taken as the potential DNA cleavage reagent.

Undoubtedly, further studies using various the metals complexes to evaluate the effect of intercalated ligand on the DNA binding and cleavage mechanisms are needed.

Acknowledgements

The present work was supported by a grant from the National Natural Science Foundation of China (No.20275031,20005030) and KJCXGC-01 from Northwest Normal University.

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