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Efficient Plasmid DNA Cleavage by a Mononuclear Copper(II) Complex

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The Cu(II) complex of the ligand *all-cis*-2,4,6-triamino-1,3,5-trihydroxycyclohexane (TACI) is a very efficient catalyst of the cleavage of plasmid DNA in the absence of any added cofactor. The maximum rate of degradation of the supercoiled plasmid DNA form, obtained at pH 8.1 and 37 °C, in the presence of 48 μM TACI·Cu^{II}, is 2.3×10^{-3} s^{-1}, corresponding to a half-life time of only 5 min for the cleavage of form I (supercoiled) to form II (relaxed circular). The dependence of the rate of plasmid DNA cleavage from the TACI·Cu^{II} complex concentration follows an unusual and very narrow bell-like profile, which suggests an high DNA affinity of the complexes but also a great tendency to form unreactive dimers. The reactivity of the TACI·Cu^{II} complexes is not affected by the presence of several scavengers for reactive oxygen species or when measured under anaerobic conditions. Moreover, no degradation of the radical reporter Rhodamine B is observed in the presence of such complexes. These results are consistent with the operation of a prevailing hydrolytic pathway under the normal conditions used, although the failure to obtain enzymatic religation of the linearized DNA does not allow one to rule out the occurrence of a nonhydrolytic oxygen-independent cleavage. A concurrent oxidative mechanism becomes competitive upon addition of reductants or in the presence of high levels of molecular oxygen: under such conditions, in fact, a remarkable increase in the rate of DNA cleavage is observed.

Introduction

The cleavage of nucleic acids is an important enzymatic reaction involved in several biological processes as well as in biotechnological manipulation of genetic material. Artificial DNA cleaving agents could find applications in several fields, in nucleic acids structural studies, biotechnology, or drug development.[1,2]

DNA is very sensitive to oxidative cleavage, and it is not surprising that the great majority of the studies on artificial DNAses have been focused on molecules capable of cleaving DNA with an oxidative mechanism.[2] This effort has brought to development several efficient cleaving agents which involve free radicals or other reactive oxygen species (ROS) able to induce an oxidative pathway. In the case of one of the commonly used systems, $Fe(EDTA)^{2-}$ in the presence of hydrogen peroxide or molecular oxygen and a reducing agent, diffusible hydroxyl radicals produced via the Fenton reaction are responsible for the DNA cleavage.[3] On the other hand, in the case of Bleomycin, an antitumor antibiotic which requires the presence of Fe^{2+}, hydrogen peroxide or molecular oxygen, and a reducing agent, nondiffusible metal−oxene species are involved in the process.[3,4] A similar mechanism apparently operates also in the case of bis(1,-10-phenanthroline)copper(I) complex.[3] Other oxidative com-

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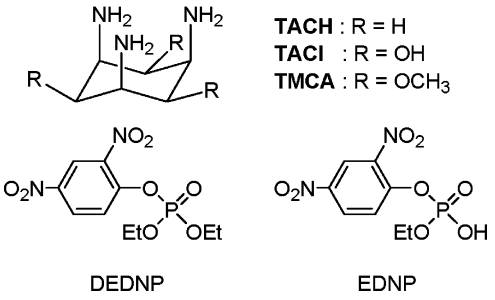
pounds require the presence of an activation step that can be granted by an oxidizing agent or light.[2] These systems have found application in DNA footprinting, as probes for different DNA structures and to properly localize DNA conformational variations as base mismatches or loops, and they have been proposed also as anticancer drugs.[2] However, despite their high efficiency and versatility, most of the oxidative cleavaging agents are subjected to severe limitations in some important applications. In fact, their activity usually depends on a cofactor such as light or an oxidizing or a reducing agent, and this hampers their use in vivo, especially in the case of therapeutic applications. Moreover, in some instances, the cleavage is mediated by diffusible free oxygen or hydroxyl radicals that may cause undesirable and uncontrolled damages as well as cytotoxic effects. Finally, they produce nonnatural fragments of DNA which are not suitable for further enzymatic manipulation like as labeling or ligation.[1a] As a consequence, their use as tools in molecular biology is limited. To overcome, at least partly, these limitations, the attention has recently been addressed to the production of "self-activating" oxidative agents which do not depend on cofactors, but the examples of systems of this type are still quite rare.[5]

An alternative approach in the development of cleaving agents which may be utilized in a wider range of applications, including in vivo applications, relies on systems capable to catalyze a hydrolytic DNA strand scission. Unfortunately, the hydrolysis of DNA is exceedingly slow and the actual challenge in this field is still the development of really efficient cleaving systems. With inspiration from the observation that in living beings nucleophilic displacement reactions on phosphate esters are mostly promoted by metalloenzymes,[6] attention is currently addressed to metal ion based hydrolytic catalysts.[1] Some of the systems so far realized have been proved very effective in the cleavage of model phosphates[7] and RNA,[8] but examples of application on DNA are still relatively rare. Lanthanide ions, especially Ce(IV), and their complexes have been reported to hydrolyze single- and double-strand DNA with very good accelerations.[1d,f] Dinuclear complexes of Er(III),[9] Co(III),[10] Zn(II),[11] and particularly Ce(IV)[12] and Fe(III)[13] cleave hydrolytically plasmid DNA with accelerations of over 10 million fold.[1g] On the other hand, only few examples of successful DNA hydrolytic cleavage by mononuclear complexes have been reported. Remarkable rate accelerations have been achieved with mononuclear Zn(II) complexes tethered to DNA intercalators[14,15] and with Co(III) complexes of polyamine ligands.[10,16] Also Cu(II) complexes with linear[17] or macrocyclic polyamines,[18] aminoglicosides,[19] and even with simple histidine[20] show a good nuclease activity. Among these the most effective are the Cu(II) complexes of kanamycin, neamine,[19] and *cis,cis*-1,3,5-triaminocyclohexane (TACH).[18b]

Chart 1. Ligands and Substrates



Recently, we reported a preliminary study on the cleavage of plasmid DNA catalyzed by the Zn(II) and Cu(II) complexes of *all-cis*-2,4,6-triamino-1,3,5-trihydroxycyclohexane (TACI; see Chart 1) and of *all-cis*-2,4,6-triamino-1,3,5-trimethoxycyclohexane (TMCA).[21] The most impressive reactivity was observed using the Cu(II) complex of

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TACI and, quite important, under "hydrolytic conditions" i.e., in the absence of any reducing agent. This paper is the full account of our studies of the reactivity of the Cu(II) complex of TACI as DNA cleaving agent, aimed at defining the parameters influencing its activity and at clarifying its mechanism of action.

Experimental Section

General Methods and Materials. UV−vis spectra and kinetic traces were recorded on a Perkin-Elmer Lambda 16 spectrophotometer equipped with a thermostated cell holder. NMR spectra were recorded using a Bruker AV300 (300 MHz) spectrometer; the operating frequency for ^{31}P experiments was 121.5 MHz. $Cu(NO_3)_2$ was an analytical grade product. Metal ion stock solutions were titrated against EDTA by following standard procedures. The buffer component, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma, pH 7.0−7.5), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPES, Sigma, pH 8.0−8.5), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES, Sigma, pH 9.0−9.5), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, Sigma, pH 10.0−10.5) were used as supplied by the manufacturer. The diethyl 2,4-dinitrophenyl phosphate (DEDNP) and the ethyl 2,4-dinitrophenyl phosphate (EDNP) lithium salt were prepared by following literature procedures.[22] Ligands TACH,[23] TACI,[24] and TMCA[30] were synthesized as reported.

Kinetic Measurements with Model Substrates. The kinetic traces were recorded on a Perkin-Elmer Lambda 16 spectrophotometer equipped with a thermostated multiple cell holder. Reaction temperature was maintained at 25 ± 0.1 °C. Stock metal complexes solutions (1 mM) were prepared by mixing equimolar amounts of the desired ligand and $Cu(NO_3)_2$ in water and adjusting the pH to a value close to 7 with 0.1 M NaOH. The reactions were started by adding 20 μL of a $(1-2) \times 10^{-3}$ M solution of DEDNP (in acetonitrile) or ENDP (in water) to a 2-mL solution of metal complex in the appropriate buffer and monitored by following the absorption of 2,4-dinitrophenolate at 400 nm. The initial concentration of substrate was $(1-2) \times 10^{-5}$ M, and the kinetics were in each case first order up to 90% of the reaction. The pseudo-first-order rate constants (k_ψ) were obtained by nonlinear regression analysis of the absorbance vs time data, and the fit error on the rate constant was always less than 1%. Cu(II) complex dimerization (K_{dim}) constants were obtained by nonlinear regression analysis of the pseudo-first-order rate constants vs total complex concentration (C_t) data according to the following equation: $k_\psi = k_2'(1/K_{dim})(-1/2 + 1/2(1/4 + 2C_tK_{dim})^{1/2})$, where k_2' is the apparent second-order rate constant of the reaction. The k_2' values were also obtained by the linear fit of the pseudo-first-order rate constants previously obtained at low Cu(II) complex concentrations ($1 \times 10^{-5}-1 \times 10^{-4}$ M). The rate constants k_2' obtained with the two methods were always identical within the experimental error. Kinetic K_a values and second-order rate constants k_2 were obtained by nonlinear regression analysis of the k_2' vs pH data according to the following equation: $k_2' = k_2K_a/(K_a + [H^+])$.

Procedure for DNA Cleavage Experiments. Stock solutions of TACI and $Cu(NO_3)_2$ were prepared in water, and the pH was adjusted to 7−8 by addition of NaOH. DNA cleavage experiments were performed using pBR 322 (Gibco BRL) in 10 mM HEPES, pH 8.1. Reactions were performed incubating DNA (12 μM base pairs) at 35 °C in the presence/absence of increasing amount of metal complex for the indicated time. When required, the incubation was carried out in the same buffer but in the presence of proper amount of scavengers (1 mM NaN_3, 100 U/mL superoxide dismutase, 0.8 mM DMSO, or 0.8 mM *tert*-BuOH). To assess the presence of apurinic sites the reaction products were additionally incubated for 1 h at 35 °C in 0.1 M KCl, 50 mM HEPES, 10 mM MgCl, and 50 mM lysine.[25]

Reaction products were resolved on a 1% agarose gel in TAE buffer (40 mM TRIS base, 20 mM, acetic acid, 1 mM EDTA) containing 1% SDS to dissociate the ligands from DNA. The resolved bands were visualized by ethidium bromide staining and photographed. The relative amounts of different plasmid structures were quantified using a BioRad Gel Doc 1000 apparatus interfaced to a PC workstation. The intensity of the band relative to the plasmid supercoiled form has been multiplied for 1.43 to take account of its reduced affinity for ethidium bromide. Kinetic profiles were fitted with a two consecutive and independent first-order processes model with the program MicroMath Scientist.[26]

Procedure for DNA Ligation Experiments. After incubation of pBR 322 with increasing concentration of metal complex for 3 h at 35 °C, the cleavage reactions were purified on a G-25 column. The plasmid was then incubated for 17 h at 16 °C with 10 U of T4 ligase (Gibco BRL) in the buffer provided by the supplier. In control experiments, the T4 ligase reactions were carried out also on pBR322 previously linearized with ECO RI. The reaction products were resolved on a 1% agarose gel in TAE buffer (40 mM TRIS base, 20 mM, acetic acid, 1 mM EDTA) in the presence/absence of 2 μM ethidium bromide.

Anaerobic and Pure Oxygen Atmosphere Reactions. These experiments were performed by following the protocol reported by Burstyn and co-workers.[18a] Deoxygenated water was prepared by five freeze−pump−thaw cycles, and before each cycle the water was equilibrated with argon under sonication to favor the deoxygenation process. Deoxygenated water was used immediately after preparation. All anaerobic stock solutions were prepared in a nitrogen-filled glovebag. Reaction mixtures were immediately prepared in the same glovebag by addition of the appropriate amounts of stock solutions to the reactions tubes, which were subsequently sealed and incubated at 35 °C. All other conditions were the same as reported in the previous paragraph. Reactions under pure oxygen atmosphere were performed in the same way as the ones under anaerobic conditions except that water was equilibrated with oxygen instead of argon. Reaction mixtures were quickly prepared on the benchtop open to the atmosphere, and the reaction tubes were filled with oxygen before sealing and incubation. All other conditions were the same as reported in the previous paragraph.

Results

Model Studies. The reactivity of the Cu(II) complexes of TACI and, for comparison, of TACH and TMCA has been first studied toward the model phosphate triester DEDNP and diester EDNP (see Chart 1). The results obtained closely follow the behavior of other Cu(II) complexes reported[27] and, in the case of the TACH complexes, are similar to those previously reported by Fujii.[28] The products of the reactions,

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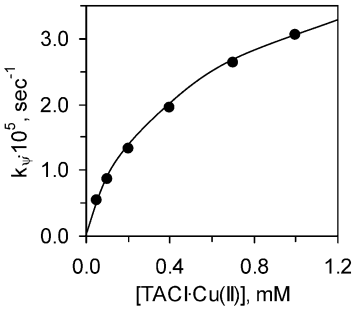

Figure 1. Complex concentration dependence of the pseudo-first-order rate constants (k_ψ) for the TACI·Cu^{II} promoted hydrolysis of DEDNP. (The line shows the fit of the data.) Conditions: pH 8.1 (0.05 mM HEPES buffer); 25 °C.

Table 1. Complex Formation (β_1) and Dimerization Constants (K_{dim}), Metal Coordinated Water Molecule Acidity Constants (pK_a), and Second-Order Rate Constants (k_2) for the Reaction of DEDNP and EDNP with the Cu(II) Complexes of TACH, TACI, and TMCA in Water at 25 °C with Literature Data in Parentheses

ligand	log β_1[a]	log K_{dim}	pK_a	k_2(DEDNP), M^{-1} s^{-1}	k_2(EDNP), M^{-1} s^{-1}
TACH	10.7		7.9 (7.7[c]−8.2[d])	1.1 (1.2)[e]	0.02
TACI	12.1	3.6 (4.0−4.8)[b]	8.0 (8.0−8.4)[b]	1.5	0.06
TMCA	14.4	2.9	8.0	1.1	0.05

[a] Data from ref 30. [b] From potentiometric titration.[29] [c] From potentiometric titration.[31] [d] From potentiometric titration.[28] [e] Data from ref 28.

identified by ^{1}H and ^{31}P NMR, were those expected for a hydrolytic mechanism, namely 2,4-dinitrophenolate and diethyl phosphate or ethyl phosphate. With both DEDNP and EDNP, the substrate dependence is first order in the concentration range explored, while the complex concentration dependence is first order in the case of TACH·Cu^{II} complex and half-order in the case of TACI·Cu^{II} and TMCA·Cu^{II} complexes (Figure 1). As clearly demonstrated by the work of Burstyn and co-workers on Cu(II) complexes of related tripodal poliaminic ligands, such behavior indicates the dimerization of the mononuclear complexes to form unreactive μ-hydroxo-bridged systems.[27] In the case of TACI·Cu^{II} complexes, the existence of such dimers have been also postulated by Hegetschweiler and co-workers on the basis of potentiometric experiments.[29] Dimerization constants (K_{dim}) obtained from kinetic experiments (see Experimental Section) are reported in Table 1 and correlates well with those reported for the TACI·Cu^{II} complexes.

Apparent second-order rate constants (k_2') for the reaction between the metal complexes and the substrate were obtained at low complex concentrations at different pH values. The pH dependence of the second-order rate constants follows in each case a sigmoidal profile (Figure 2): such behavior is diagnostic of the deprotonation of a metal-coordinated water molecule to form the active nucleophile. Fitting of the reactivity versus pH data yielded the pK_a values of the water molecule and the second-order rate constants (k_2) for the reaction between the hydroxo complex and the substrates (Table 1). The results obtained compare well with the data

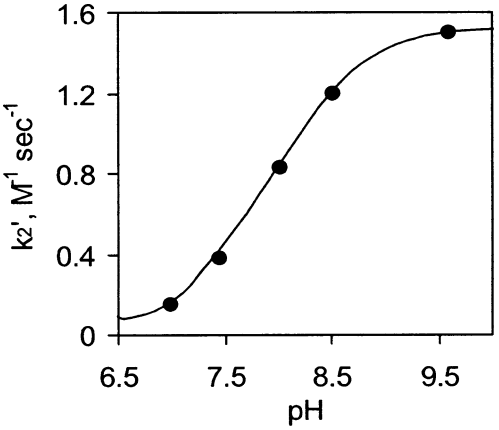

Figure 2. pH dependence of the apparent second-order rate constants (k_2') for the TACI·Cu^{II}-promoted hydrolysis of DEDNP. (The line shows the fit of the data.) Conditions: [buffer] = 0.05 mM; 25 °C.

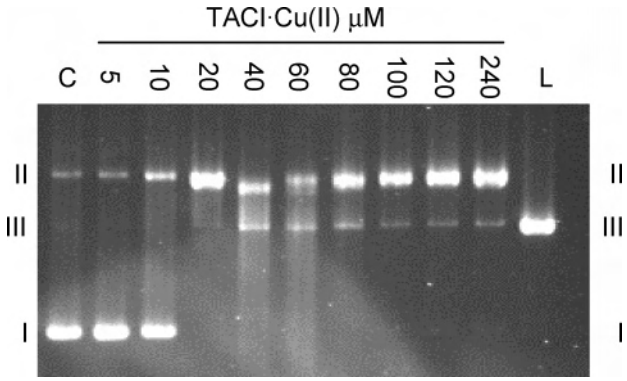

Figure 3. Agarose gel of pBR 322 (12 μM in bp) incubated for 3 h at pH 8.1 (10 mM HEPES) and 35 °C with increasing concentrations of TACI·Cu^{II}: lane C, control DNA; lane L, plasmid DNA linearized with ECO RI.

available in the literature, and in particular, the kinetic pK_a values are in good agreement with the thermodynamic ones obtained by potentiometric titrations. This result further confirms the identification of the metal bound hydroxide as the nucleophile promoting the substrate cleavage.

Inspection of Table 1 indicates that there are not significant differences in the reactivity of the three complexes toward phosphate esters. This may be reasonably explained by considering that the substitution on the cyclohexane ring strongly influences the formation constant of the complexes but not the acidity of the metal ion-coordinated water molecule. In fact the log β_1 values increase from 10.3 to 14.6 on going from TACH to TMCA[30] while the pK_a values of the metal-coordinated water molecule are close to 8 for all the three complexes. Therefore, the nucleophilicity of the coordinated hydroxide anion is little influenced by the ligand structure at least in the series here considered.

Cleavage of Plasmid DNA. Incubation of plasmid DNA pBR 322 with the Cu(II) complex of TACI at pH 8.1 for 3 h at 35 °C results in extensive cleavage of DNA as shown in Figure 3. The supercoiled form I is first degraded to form II (relaxed circular) and then to form III (linear). The cleavage is really efficient, and form I is totally transformed into form II in the presence of 20 μM TACI·Cu^{II} complex

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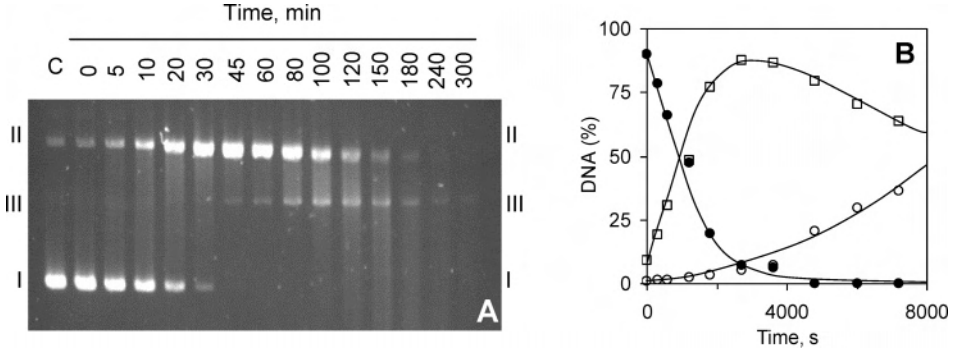

Figure 4. Time course of pBR 322 plasmid DNA (12 μM bp) cleavage by TACI·Cu^{II} 60 μM at pH 8.1 (10 mM HEPES) and 35 °C. (A) Agarose gel of the reaction products: lane C, plasmid DNA incubated for 300 min in the absence of metal complex. (B) Quantification of pBR 322 plasmid forms: supercoiled (●); nicked (□); linear (○); fitting of the data (lines).

only. Form III is detectable only after all the supercoiled plasmid has been nicked, whereas no DNA cleavage was observed under identical experimental conditions in the absence of either Cu(II) or TACI (data not shown).

The activity remarkably increases with the concentration of metal ion complex up to a maximum at approximately 60 μM. At this concentration, the bands corresponding to form II and III are ill-defined and a considerable amount of smearing, resulting from the generation of smaller fragments of DNA, is visible. By further increase of the complex concentration, the reaction slows down and the amount of DNA damage decreases substantially.

To better define the behavior of this system, we investigated the kinetics of DNA cleavage by the TACI·Cu^{II} complexes. Reactions were followed by gel electrophoresis in the presence of a constant concentration of plasmid DNA and increasing concentrations of the metal ion complex. Figure 4a shows a typical agarose gel generated by incubating the pBR 322 (12 mM in bp) plasmid DNA in the presence of 60 μM TACI·Cu^{II}.

Densitometric quantification of the different products allowed the evaluation of the kinetic parameters describing the decrease of form I and the increases of form II and III, as shown in Figure 4b. These curves have been analyzed using two simplified kinetic models: (i) two consecutive and independent first-order processes, in which form II is first produced from form I (k^1) and then is cleaved to form III (k^2); (ii) three independent first order processes, in which form III can be produced either directly from form I (k^3) or from form II (k^2), which can in turn be formed by degradation of form I (k^1). The first model involves the occurrence of sole single-strand scission events: as a consequence, double-strand cleavage is observed only when two cuts randomly occur sufficiently close on the two strands. The second model implies the possibility of nonrandom double-strand scission events. Fitting of the data gave satisfactory results only with the first model (lines of Figure 4b), ruling out any substantial direct conversion of form I to form III and, as the consequence, the occurrence of nonrandom double-strand scissions. This is in agreement with the results obtained by applying the statistical test developed by Povirk and co-workers.[32] Such analysis assumes a Poisson distribution of the strands cuts and calculates the average number of single (n_1) and double (n_2) strands cuts from the fraction of supercoiled and linear DNA present after the reaction. The amount of double strand scissions experimentally observed (n_2) is not significantly greater than the theoretical amount (n') calculated from the Freifelder−Tumbo relationship[33] for a completely random process.

Table 2. First-Order Kinetic Constants for the Degradation of Form I (k_1) and Form II (k_2) of PBR 322 Plasimd DNA in the Presence of TACI·Cu^{II} Complexes[a]

[TACI·Cu^{2+}], μM	k^1, s^{-1}	k^2, s^{-1}
12	8.5×10^{-5}	4.8×10^{-6}
24	1.3×10^{-3}	5.6×10^{-5}
48	2.3×10^{-3}	6.2×10^{-5}
60	7.2×10^{-4}	6.8×10^{-5}
120	3.1×10^{-4}	1.6×10^{-5}

[a] $[DNA]_{bp} = 12$ μM, 35 °C, pH 8.1.

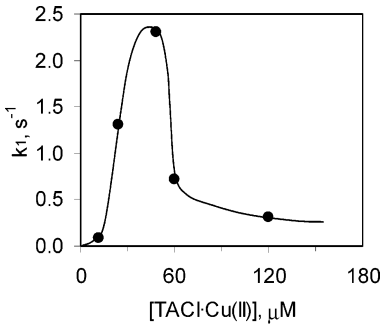

Figure 5. First-order rate constant versus the metal complex concentration for the nicking process of supercoiled pBR 322 at pH 8.1 (10.0 mM HEPES) and 35 °C by Cu(II) complexes of TACI.

The values for the kinetic rate constant k^1 and k^2 that can thus be estimated at different TACI·Cu^{II} concentrations are reported in Table 2. The k^2 values obtained from this kinetic analysis, as expected, are lower than those of corresponding k^1 by factors ranging from 10 to 40, depending on the complex concentration.

Figure 5 shows the dependence of the rate of disappearance of form I (k^1) from the [TACI·Cu^{II}] concentration. The profile describes a very narrow bell-shaped curve with the maximum centered at a metal ion complex concentration of 48 μM, which corresponds to a [TACI·Cu^{II}]/[DNA] ratio of 4. At

(31) Childers, R. F.; Wentworth, R. A. D.; Zompa, L. J. *Inorg. Chem.* **1971**, *10*, 302−306.
(32) Povirk, L. F.; Wubker, W.; Kohnlein, W.; Hutchinson, F. *Nucleic Acids Res.* **1977**, *4*, 3573−3580.
(33) Freifelder, D.; Trumbo, B. *Biopolymers* **1969**, *7*, 681−693.

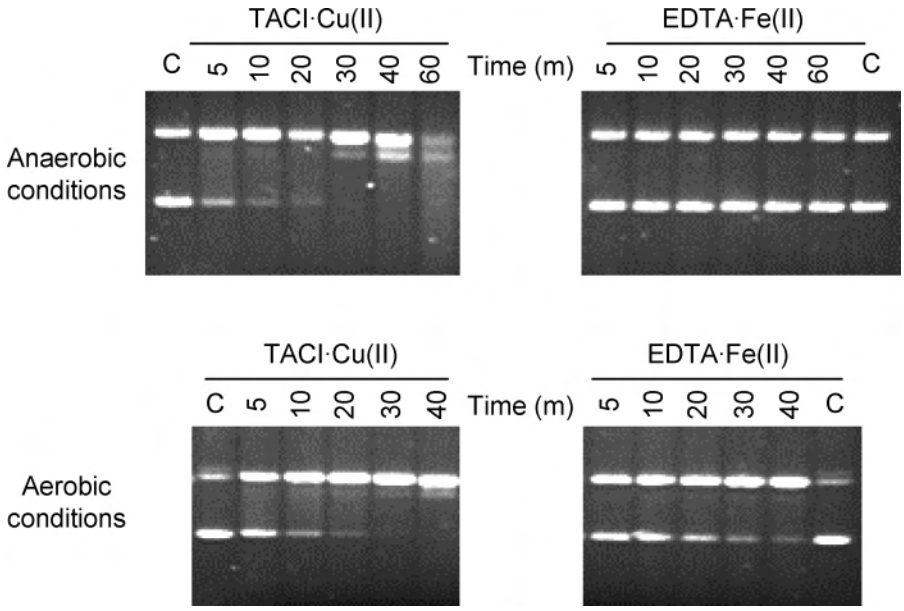

Figure 6. Agarose gel of pBR 322 (12 μM in bp) incubated with 60 μM TACI·Cu^{II} or 100 μM Fe^{II}·EDTA in 10 mM HEPES, pH 8.1, at room temperature for increasing time (0−60 min). Gels in the upper part are relative to reactions performed under nitrogen atmosphere using deaerated solutions; those in the lower part are relative to reactions performed with no modification in the atmosphere composition.

the point of maximum activity the k^1 value is 2.3×10^{-3} s^{-1}. Although a rigorous comparison is made difficult by the different experimental conditions employed, this value is one of the highest ever reported for simple mononuclear Cu(II) complexes of polyamine ligands in "hydrolytic conditions",[17−20] i.e., in the absence of added cofactors.[34]

Cleavage Mechanism. Copper complexes can cleave DNA both by hydrolytic and oxidative chemistry. In the second case, they have been shown to react with molecular oxygen or hydrogen peroxide to produce a variety of active oxidative intermediates (reactive oxygen species, ROS), including diffusible hydroxyl radicals and nondiffusible copper−oxene species.[2] Normally, a reducing agent (a thiol or ascorbic acid) is required to initiate and sustain the radical reaction, but particularly with the employment of DNA derived from biological sources, the presence of adventitious reducing agent is always possible.

To verify if reactive oxygen species are, at least in part, responsible for the cleavage of DNA promoted by the TACI·Cu^{II} complex, reactions were performed in the presence of scavengers for singlet oxygen (NaN_3, 1 mM), for superoxide (superoxide dismutase, SOD, 100 U/mL), and for hydroxyl radical (0.8 mM DMSO and 0.8 mM *tert*-BuOH). None of the above additives has any effect on the rate of plasmid DNA cleavage by the TACI·Cu^{II} complex ruling out the involvement of these reactive oxygen species, at least in a free and diffusible form. The presence of apurinic sites on the cleaved DNA, which are easily formed in an oxidative cleavage process, was also investigated. After the reaction with the TACI·Cu^{II} complexes, the DNA samples containing the cleaved products were incubated in the presence of lysine, followed by electrophoresis and quantitation. No increase in the amount of nicked and linear forms of plasmid DNA, diagnostic of the presence of apurinic sites, was detected after the treatment with lysine.

Molecular oxygen is the essential coreactant in oxidative processes. To probe its role in the TACI·Cu^{II} cleavage of plasmid DNA, we performed reactions under strictly anaerobic condition. Furthermore, to assess the effective elimination of oxygen under the conditions used, we investigated also the reactivity of the well-known oxidative system $Fe(EDTA)^{2-}$/DTT which, in the presence of molecular oxygen, rapidly degrades plasmid DNA by producing diffusible hydroxyl radicals via the Fenton reaction.[3,18a] The results of parallel experiments with the same deoxygenation protocol (see Experimental Section) using the TACI·Cu^{II} complex and the $Fe(EDTA)^{2-}$/DTT system as cleaving agents are shown in Figure 6. While in the case of the $Fe(EDTA)^{2-}$/DTT system the cleavage of DNA is completely suppressed under the anaerobic condition used, no effect is observed with the TACI·Cu^{II} complex and the reaction proceeds at comparable rates both under aerobic and anaerobic conditions. Therefore, molecular oxygen is not required in the DNA cleavage process mediated by the TACI·Cu^{II}. The enzymatic religation of the linearized plasmid DNA with T4 DNA ligase was also attempted, but we were unable to evidentiate religation products.

The formation of hydroxyl radicals due to TACI·Cu^{II} complexes was also ruled out by using the Rhodamine B dye as reporter molecule.[19,35] In a typical experiment, Rhodamine B (10 μM) was incubated for 1 h in the presence of TACI·Cu^{II} (0.5 mM) monitoring the absorbance at 552 nm. The degradation of the dye provides a direct measure of the concentrations of hydroxyl radicals in the reaction mixture. The absorbance versus time profiles are reported

(34) With other metal ions a similar or higher reactivity has been reported only for Ce(IV) and Fe(III) bimetallic complexes; see refs 12b and 13b.

(35) Detmer, C. A., III; Pamatong, F. V.; Bocarsly, J. R. *Inorg. Chem.* **1996**, *35*, 6292−6298.

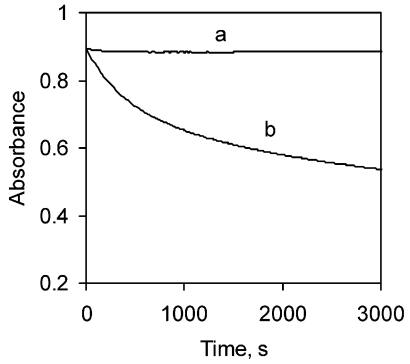

Figure 7. Rhodamine B degradation followed by decrease of the absorbance at 552 nm at pH 8.1 in 10 mM phosphate buffer: (a) in the presence of 0.1 mM TACI·Cu^{II}; (b) in the presence of of 0.1 mM TACI·Cu^{II}, 10 mM H_2O_2, 1 mM ascorbic acid.

in Figure 7 and show the absence of any variation of the absorbance of the dye in the presence of the TACI·Cu^{II}. As a counterproof, under the same conditions but after the addition of ascorbic acid as a reducing agent, we observed a remarkable degradation of Rhodamine B. The last observation suggests that ROS can be produced by the TACI·Cu^{II} complex under redox conditions.

Thus, we carried out experiments aimed at characterizing the reactivity of the TACI·Cu^{II} complexes under redox conditions. On one hand, we added to the reaction mixture 2-propanol, which is known to initiate and to propagate the radical chain, and as a result, we observed a large increase in the reaction rate so that, after the usual time of incubation, extended cleavage occurs and only smearing is visible on the electrophoretic gel. On the other hand, a high level of molecular oxygen also seems to drive the reaction mechanism toward oxidative pathways. Indeed, when the DNA cleavage experiment is performed using a buffer saturated with O_2, a fast and complete degradation of DNA is observed even at a stoichiometric DNA/metal complex ratio.

Discussion

The dependence of the rate of plasmid DNA cleavage on the TACI·Cu^{II} complex concentration follows an unusually narrow bell-like profile. The steep increase of the reactivity in the first part of the curve could indicate a very high affinity of the complex for the DNA backbone, although the shape of the same curve does not allow the evaluation of the affinity constant for the DNA backbone. On the other hand, the comparably fast decrease that follows suggests the concentration-dependent formation of an unreactive species. A similar behavior has been reported by Burstyn and coworkers for the plasmid DNA hydrolysis promoted by Cu(II) complexes of 1,4,7-triazacyclononane and *N*,*N*′,*N*″-trisopropyl-1,4,7-triazacyclononane[18d] and is attributed to the formation of unreactive μ-hydroxo dimers that may compete with the reactive monomeric complexes for the binding to the substrate.[36] According with such hypothesis, both the results obtained by the kinetic experiments with model phosphate esters and the potentiometric titrations reported by Hegetschweiler and co-workers[29] have highlighted that TACI·Cu^{II} complexes have a high tendency to form such dimers (log $K_{dim} = 3.6$), while the corresponding TACH complex does not show any appreciable presence of dimeric species up to millimolar concentration. This different tendency of the two complexes to form μ-hydroxo dimers correlates very well with their different reactivity profiles with DNA. In fact, in the case of the TACH·Cu^{II} complexes,[18b] a saturation profile is observed which is diagnostic of the sole binding of the complexes to the DNA backbone without any appreciable dimer formation.

Interestingly, the reactivity of the TACI·Cu^{II} complexes is much greater than that of the closely related TACH and TMCA complexes.[18b,21] The experiments performed with model phosphate esters indicate that this reactivity order cannot be related to any chemical-physical property of the complexes. Hence, a key role is apparently played in the case of TACI by the three hydroxyl groups that could increase the complex affinity for the DNA. In fact, there are several indications, and among them the high DNA affinity of the neamine·Cu^{II} complexes,[19] that suggest that a sugar or aminoglicoside-type structure may lead to a strong recognition of nucleic acids.

Several mononuclear Cu(II) complexes of polyamine ligands show a DNA cleaving reactivity which has been attributed to a hydrolytic[17−20] mechanism, and both the structure and the reactivity of the TACI·Cu^{II} complexes closely resemble those of some of these systems. In fact, the DNA cleaving reactivity of TACI·Cu^{II} complexes does not decrease under anaerobic conditions, while oxidative DNA cleaving agents need molecular oxygen or hydrogen peroxide to form the reactive oxygen species. This observation points toward the occurrence of a hydrolytic process in the TACI·Cu^{II}-promoted DNA cleavage at least in the absence of added reducing agents. Such hypothesis is also supported by the failure of the Rhodamine B test in detecting any radical formation. Moreover, the negative results of the experiment of DNA cleavage in the presence of the different radical scavengers indicate that diffusible ROS are not formed and the absence of apurinic sites indicates that no oxidative mechanism, involving the attack of the reactive species to the DNA nucleobases, is at play.

Unfortunately, all the above strong evidence for a hydrolytic mechanism is not further substantiated by successful enzymatic religation of the linearized DNA. Such behavior is not unprecedented in the case of Cu(II)-based hydrolytic systems,[17] as this type of experiment may fail for many other different reasons beside the presence of nonnatural fragments derived from oxidative damages. As a matter of fact, the failure may be due to an exceedingly large number of breaks on the DNA chain that in the present case may be justified by the high reactivity of the system, to the moderate double strand cleavage efficiency, or to the formation of fragments, produced in a hydrolytic process, which do not end with the required 5′-phosphate and 3′-OH (ribose) termini. However, also the occurrence of an oxygen-independent oxidative

(36) Bimetallic complexes are known to have a higher affinity for the negatively charged phosphate backbone of DNA; see for example: Kesicki, E. A.; DeRosch, M. A.; Freeman, L. H.; Walton, C. L.; Harvey, D. F.; Trogler, W. C. *Inorg. Chem.* **1993**, *32*, 5851−5867.

cleavage, even if unprecedented, cannot be completely ruled out on the basis of the present results.

On the other hand, an undoubtedly oxidative and much more efficient DNA cleavage can be induced with TACI·Cu^{II} complexes under proper conditions, i.e., by the addition of a reducing agent or of molecular oxygen at high concentrations. The Rhodamine B test clearly indicates that, under these conditions, the increase of the cleavage reactivity is related to the presence of reactive radicals.

The observations so far discussed suggest that with the TACI·Cu^{II} complexes two different cleaving mechanisms are possible, depending on the operative conditions employed: one O_2-independent and probably hydrolytic and the other O_2-dependent and oxidative. A similar behavior has been reported for other Cu(II) complexes.[18a,37] However, molecular oxygen appears to be the limiting reagent in the O_2-dependent pathway at such extent that, under normal aerobic conditions, this mechanism adds no detectable contribution to the overall reactivity.

Conclusions

The results here reported demonstrate that the TACI·Cu^{II} complex is extremely efficient in promoting the cleavage of plasmid DNA under hydrolytic conditions, i.e., in the absence of oxygen and added reductants. The maximum rate of degradation of the supercoiled plasmid DNA form, obtained in the presence of 48 μM TACI·Cu^{II}, is 2.3×10^{-3} s^{-1}: a value which corresponds to a half-life time for the form I of only 5 min. However, at variance with other highly efficient systems[12b,19b] the systems lack the highly desirable requirement of activity at low concentrations, i.e., in the presence of an excess of DNA. The results of a number of experiments aimed at defining the mode of action of the complex point to a hydrolytic pathway as the main mechanism under "normal" aerobic conditions, although a nonhydrolytic oxygen-independent cleavage cannot be completely ruled out. On the other hand, a clearly oxidative pathway becomes highly competitive under high levels of molecular oxygen or in the presence of reducing agents.

Beside the mechanistic speculations, which need to be supported by further investigations, the main feature of the present system is the very high reactivity in the absence of cofactors which makes the TACI·Cu^{II} complex a very promising candidate for biological applications in vivo, especially in the therapeutic area, and for all those applications which are not compatible with the use of cofactor activated DNA-cleaving agents.

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(37) Zhu, L.; dos Santos, O.; Koo, M. Rybstein, C. W.; Pape, L.; Canary, J. W. *Inorg. Chem.* **2003**, *42*, 7912−7920.