DNA-Binding and Cytotoxicity of the Cobalt(III) Ethylenediamine Pyrazole Complex \([\text{Co(en)}_2(\text{pyz})_2]^{3+}\)

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The complex of cobalt(III) ethylenediamine was synthesized, isolated and characterized by UV-Vis, IR, and \(^1\)H NMR spectral methods. The binding of the complex with calf thymus DNA was investigated by absorption and emission spectroscopy, viscosity measurements, DNA melting and DNA photocleavage. The spectroscopic studies together with the viscosity measurements and DNA melting studies indicated that the complex binds to calf thymus DNA in a nonintercalative mode. This complex is found to promote photocleavage of the DNA plasmid pBR322 and shows a cytotoxic effect against CHO cells.

**Keywords:** DNA-binding, Cobalt(III) complexes, Bioinorganic chemistry, Cytotoxic activity

**INTRODUCTION**

Transition metal complexes of 2,2-bipyridine (bpy), 1,10-phenanthroline or their modified variants have been widely employed in DNA studies due to their applicability in several areas of research, including bioinorganic and biomedical chemistry [1-10]. The metal complexes can bind to DNA in noncovalent modes such as electrostatic, intercalative and groove binding. The above applications require that the complex can bind to DNA through an intercalative mode wherein the planar aromatic heterocyclic group insert and stacks between the base pairs of DNA [11-15]. These inert chiral complexes \(\Delta\) and \(\Lambda\)-isomers, display different binding affinity for the right-handed B-DNA. This difference makes these complexes, possible structural probes to determine B-DNA and Z-DNA. Furthermore upon irradiation these complexes can promote DNA cleavage, and they also exhibit enantioselectivity in DNA cleavage [16].

These findings underscore the importance of an intimate association of the metal ion with the duplex. The high level of recognition of DNA conformation by these chiral inorganic complexes suggests their utility in the design of stereospecific DNA binding drugs [17].

In the present paper, we report the synthesis and characterization of the complex \([\text{Co(en)}_2(\text{pyz})_2]^{3+}\) (Fig. 1). The DNA-binding properties of this compound are studied by electronic absorption, fluorescence emission spectra, viscosity measurement and DNA melting temperature. The plasmid

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**Fig. 1.** Molecular structure of the \([\text{Co(en)}_2(\text{pyz})_2]^{3+}\) complex.
DNA cleavage of the complex is also demonstrated. The cytotoxicity studies of \( \text{cis-[Co(en)_2Cl}_2 \text{Cl} \) and \([\text{Co(en)}_2(pyz)_2\text{Br}_3 \) complexes are compared in this paper [18].

**EXPERIMENTAL**

**Materials**

All materials were purchased and used without further purification. Pyrazole ethylenediamine and calf thymus (CT) DNA were purchased from Aldrich. All experiments involving interaction of the complexes with DNA were carried out in BPE buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.2). A solution of CT DNA in this buffer gave a UV-Vis absorbance ratio of 1.8-1.9 at 260 and 280 nm, indicating that the DNA was satisfactorily free of protein [19]. The DNA concentration per nucleotide was determined by absorption spectroscopy using a molar absorption coefficient of 6600 m\(^{-1}\) cm\(^{-1}\) at 260 nm [20].

**Synthesis of [Co(en)_2(pyz)_2]Br_3**

The \( \text{cis-[Co(en)}_2\text{Cl}_2 \text{Cl} \) was prepared as previously reported [21]. \([\text{Co(en)}_2(pyz)_2\text{Br}_3 \) was synthesized as described earlier [22,23,24]. A mixture of \( \text{cis-[Co(en)}_2\text{Cl}_2 \text{Cl} \) (1.43 g) and pyrazole (1 g) was dissolved in ethanol (6 ml). A solution of sodium bromide (3.0 g) in water (5 ml) was added to the above solution and heated in a water bath (temperature 95 °C) until it turned dark yellow. It was then cooled on ice and the thick crystalline precipitate of \([\text{Co(en)}_2(pyz)_2\text{Br}^3 \) that formed was collected and recrystallized from water (30 ml). The yield by this method was about 80%, producing the following characteristics determined by various techniques: UV-Vis, 337 and 462 nm (Table 1); IR, 1458 (C=C), 1578 (C=N), 589 (Co-N (en)) and 490 cm\(^{-1}\) (Co-N (L)); \(^1\)H NMR (D\(_2\)O), (en)-CH\(_2\) 3.1, H-4 6.4 (t), H-3 7.9 (d) (J) 0.11, H-5 7.6 (d), (J) 0.12; Anal. Calcd. for C\(_{10}\)H\(_{24}\)Br\(_3\)N\(_8\)Co: C, 21.64; H, 4.36; N, 20.1; Found: C, 20; H, 4.1; N, 20.

**Physical Measurements**

UV-Vis spectra were recorded on an Elico Biospectrophotometer model BL198. Emission spectra were recorded on a Shimadzu RF-2000 luminescence spectrometer at room temperature. IR spectra were recorded (KBr) with a Perkin-Elmer FTIR-1605 spectrophotometer. \(^1\)H NMR spectra were measured on a Varian XL-300 MHz spectrometer with D\(_2\)O as a solvent at room temperature and tetramethylsilane (TMS) as the internal standard. Microanalyses (C, H, N) were carried out on a Perkin-Elmer 240 elemental analyzer.

Spectrophotometric titrations were carried out at room temperature to determine the binding affinity between the DNA and the complex. Initially, solutions (3.0 ml) of each, containing buffer (blank) and the cobalt(III) complex (20 \( \mu \)M), were placed in the reference and sample cuvettes (1 cm path length), respectively. The spectrum was then recorded in the range of 200-600 nm. During the titration, small aliquots (1-10 \( \mu \)l) of buffered DNA solution (5 to 10 mM per nucleotide) were added to each cuvette to eliminate the absorbance of the DNA itself. The solutions were mixed for \( \sim 5 \) min; the absorption spectra were then recorded. This titration procedure was continued until there was no change in the spectra, indicating that binding saturation had been attained.

Emission measurements were carried out using a Hitachi F-4500 spectrofluorimeter with Tris buffer as the blank. The excitation wavelength was fixed and the emission range was adjusted before measurements. All measurements were made at 25 °C in a thermostated cuvette holder with entrance and exit slit widths of 5 nm. Emission titration experiments were performed at a fixed concentration of the metal complex (20 \( \mu \)M) to which increasing amounts of CT DNA over a range of 0-160 \( \mu \)M were added. The emission enhancement factors were measured by comparing the intensities at 559 nm in the absence and presence of CT DNA.

The viscosity was determined using an Ostwald viscometer.

**Table 1. Absorption Peaks*, \( T_m \) and IC\(_{50}\) Values of the Complexes**

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Absorption peaks at T(_m) (ºC)</th>
<th>IC(_{50}) (nM) Mean ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl(_2)6H(_2)O</td>
<td>227 and 492 nm</td>
<td>-</td>
</tr>
<tr>
<td>( \text{cis-[Co(en)}_2\text{Cl}_2 \text{Cl} )</td>
<td>247 and 592 nm</td>
<td>90 ± 2 nM</td>
</tr>
<tr>
<td>([\text{Co(en)}_2(pyz)_2\text{Br}_3 )</td>
<td>337 and 462 nm</td>
<td>110 ± 1 nM</td>
</tr>
</tbody>
</table>

*The absorption peaks of the complexes show additional metal-to-ligand charge transfer bands.
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maintained at 30.0 ± 0.1 °C in a thermostatic water-bath. CT DNA samples, averaging approximately 200 base pairs, were prepared using sonication in order to minimize the complexities arising from DNA flexibility [25]. Data were analyzed as \((\eta/\eta_0)^{1/3}\) vs. the concentration of Co(III) complex, where \(\eta\) is the viscosity of DNA in presence of complex and \(\eta_0\) is the viscosity of DNA alone. Viscosity values were calculated from the observed flow time of DNA-containing solution \((t > 100\ s)\) corrected for flow time of buffer alone \((t_0)\), \(\eta = (t - t_0)/t_0\) [26].

DNA denaturation was carried out by incubating the complex with CT DNA as the temperature was raised from 25 °C to 85 °C over a period of 1 h. The OD was monitored at 260 nm using an Elico Bio-spectrophotometer model BL198 coupled with a temperature-controlled circulating bath; pH measurements were also carried out before and after heating the complex [33]. Absorbance was monitored at various temperatures in the absence and presence of the complex.

To analyze this compound’s ability to induce photocleavage, supercoiled pBR322 DNA (100 µM) was treated with different concentrations of the Co(III) complex in 50 mM Tris-HCl and 18 mM NaCl buffer at pH 7.2. The solutions were then irradiated at room temperature for 1 h at 302 nm using an ultraviolet lamp (10 W). The samples and controls were analyzed by gel electrophoresis for 2.5 h at 40 V on a 0.8% agarose gel in Tris-acetic acid-EDTA buffer at pH 7.2. The gel was stained with 1 µg ml⁻¹ ethidium bromide and then photographed under UV light [27].

The IR spectra of the complex clearly exhibit bands at 1458 cm⁻¹ and at 1578-1590 cm⁻¹, which respectively correspond to the C=C and C=N of the pyrazole ring. Bands around 589 cm⁻¹ and 490 cm⁻¹ corresponding to the Co-N(en) and Co-N of NH₂(en). The UV-Vis spectra of the complex (\(\lambda_{max}\)) show peaks at 400-500 nm [19], as shown in Table 1. In the \(^1\)H NMR spectra of the Co(III) complex, the peaks due to various protons of pyrazole are shifted downfield compared to those of the free ligand, suggesting complexation. As expected, the signal for pyrazole appeared between 6.4 to 7.9, and the CH₂ of ethylenediamine gave peaks at 3.1 (Br, 4 H, CH₂(en)).

Cell Viability MTT Assay

All cell culture reagents and media were purchased from Sigma-Aldrich and used without further purification unless otherwise stated. Cytotoxicity assays were performed using Chinese hamster ovary (CHO) cells in order to assess the cytotoxicity of the complex. The cells were grown as monolayers in Eagle’s minimum medium, supplemented with 2 mM L-glutamine and Earle’s balanced salt solution, containing 1.5 g dm⁻³ sodium hydrogen carbonate, 0.1 mM amino acids, 1.0 mM sodium pyruvate, 100 mg l⁻¹ penicillin and 60 mg l⁻¹ streptomycin. All cells were grown to exponential phase at 37 °C in a humidified atmosphere in the presence of 5% CO₂.

Cytotoxicity was assessed using an MTT assay. Cells (100 µl) were seeded at a density of 5 × 10⁴ cells cm⁻² into sterile 96-well flat-bottomed plates (Falcon Plastics, Becton-Dickinson) and grown using the abovementioned conditions. Test compounds were dissolved in culture media. Each drug solution (100 µl) was added to replicate wells at concentrations in the range of 0.1-100 µM and then incubated for 72 h.

A miniaturized viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was carried out according to the method described by Mosmann [28]. The IC₅₀ value, defined as the drug concentration causing a 50% reduction in cellular viability, was calculated for each drug. Viability was calculated as a percentage of the solvent-treated control cells. The significance of any reduction in cellular viability was determined using one-way ANOVA (analysis of variance). A probability of 0.05 or less was deemed statistically significant.

RESULTS AND DISCUSSION

Absorption Spectral Studies

The application of electronic absorption spectroscopy in DNA-binding studies is one of the most useful techniques [29]. The absorption spectra of complex in the absence and presence of CT DNA at a constant concentration of complex ([Co] = 20 µM) is shown in Fig. 2. As the concentration of DNA increases, the MLCT transition bands of the complex at 462 nm exhibit hypochromism. The spectra indicate that there are some interactions between the complex and DNA. To quantitatively determine the binding strength of the complex, the intrinsic binding constant \(Kₘ\) of the complex with CT-DNA was obtained by monitoring the changes in absorbance of the
complex at 462 nm with increasing DNA concentrations according to Eq. (1):

$$\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_f)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{(K_b(\varepsilon_b - \varepsilon_f))} \tag{1}$$

where $[\text{DNA}]$ is the concentration of DNA in nucleotides, and $\varepsilon_a$, $\varepsilon_f$, and $\varepsilon_b$ are the extinction coefficients of the apparent, free, and bound metal complexes, respectively. On plotting $[\text{DNA}]/(\varepsilon_a - \varepsilon_f)$ vs. $[\text{DNA}]$, $K_b$ is determined from the ratio of the slope to the intercept. The intrinsic binding constant $K_b$ obtained is approximately $4.8 \pm 0.2 \times 10^3$.

**Emission Studies**

In the absence of DNA, the complex is fluorescent in Tris buffer at ambient temperature, with a fluorescence maximum appearing at 559 nm. Upon addition of CT DNA, the emission intensity of the complex increases relative to the intensity of complex alone (Fig. 3). This implies that the complex strongly interacts with DNA and can be protected by DNA efficiently, since the hydrophobic environment inside the DNA helix reduces the accessibility of the solvent water molecules to the duplex. The complex mobility is restricted at the binding site, leading to a decrease in the vibrational modes of relaxation, and thus to higher emission intensity.

This observation is further supported by the emission quenching experiment using $[\text{Fe(CN)}_6]^{3-}$ as a quencher. The ion $[\text{Fe(CN)}_6]^{3-}$ distinguishes between DNA bound Co(III) species and the positively charged free complex ion. The complex bound to DNA can be protected from the quencher, because highly negatively-charged $[\text{Fe(CN)}_6]^{3-}$ would be repelled by the negative charge of the DNA phosphate backbone, which would hinder the quenching of the emission of the bound complex. The ferro-cyanide quenching curves for this complex in the presence and absence of CT DNA are shown in Fig. 4. The results obtained indicate binding of complex to DNA.

**Viscosity Studies**

The bonding between the complex and DNA was further elucidated by viscosity measurements. Further clarification of the interaction between the complex and DNA was carried out by viscosity measurements. Viscosity experiments give valuable information regarding mode of binding metal complex with DNA. Otherwise X-ray crystallographic or
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Fig. 3. Emission spectra of the complex in Tris-HCl buffer. Fluorescence intensity increases upon increasing CT DNA concentrations (5, 10, 15 and 20 μl of DNA). Insert: plots of relative integrated emission intensity vs. [DNA]/[Co].

Fig. 4. Ferrocyanide induced emission quenching of [Co(en)$_2$(pyz)$_3$]$^{3+}$, free in 0.8 μM (a) or bound to CT DNA at [DNA]/[Co] = 40 (b).

NMR technique are to be used to know the mode of binding [28]. When a metal complex intercalate between the base pairs of DNA length of DNA increases which lead to the increase in viscosity. On the other hand, a partial and/or non-classical intercalation of the ligand with the DNA helix reduces its effective length and concomitantly its viscosity [31]. Effects of the complex on the viscosity of rod-like DNA is shown in Fig. 5. The viscosity of the DNA-bound complex does not increase with the increase in the concentration of the complex. This pattern is not similar to that of the proven DNA

Fig. 5. Effect of increasing amount of complex on the relative viscosities of CT DNA at 25 ± 0.1 °C: (a) EtBr and (b) pyrazole complex.
intercalator, ethidium bromide (EtBr). Based on the viscosity results, the complex binds with DNA through electrostatic groove binding.

**DNA Melting Studies**

As intercalation is known to cause stabilize base stacking, thereby raising the melting temperature of double-stranded DNA, this DNA melting experiment is useful in establishing whether or not intercalation is the type of binding and, if so, the extent of intercalation [32]. In the present study, the presence of monophasic melting curves with no change in pH, and small changes in $\Delta T_m$ (3 °C) indicate a non-intercalation behavior (Fig. 6). The $\Delta T_m$ value shows that this binding is not in between the DNA base pairs, which further supports the results of the viscosity experiments.

**Photocleavage of pBR322 DNA by the Co(III) Complex**

There has been considerable interest in DNA endonucleolytic photocleavage reactions that are activated by metal ions [35]. The delivery of high concentrations of metal complex to the helix, locally generating oxygen or hydroxide radicals, yields an efficient DNA photocleavage reaction. DNA photocleavage was monitored by conversion of supercoiled circular pBR322 (form I) into nicked circular (form II) and linear (form III). When this circular DNA plasmid is subjected to electrophoresis, relatively fast migration is observed for the supercoiled form (form I). If scission occurs on one strand (nicking), the supercoils will relax to generate an electrophoretically slower-moving open circular form (form II) [36]. If both strands are cleaved, a linear form (III) will be generated that migrates between forms I and II. Fig. 7 shows the gel electrophoresis pattern of the pBR322 DNA after incubation with the Co(III) complex and irradiation at 302 nm. The conversion of form I to II occurs after 60 min of irradiation in the presence of varying concentrations of [Co(en)$_2$(pyz)$_2$]$^{3+}$. It can be seen that, by increasing the concentration of the complex, form II increases progressively while form I diminishes; however, at higher concentrations of complex, form II and form III molecules result. This supports the conclusion that an increase in bound complex leads to a more extensive cleavage. Furthermore, the unirradiated control solution confirmed that the complexes did cause photosensitized cleavage.

**Cytotoxicity Studies**

The ability of the cobalt(III) complexes [Co(en)$_2$(pyz)$_2$]Br$_3$ and [Co(en)$_2$Cl$_2$]Cl to induce cytotoxicity were investigated using CHO cells and a standard MTT bioassay. Cells were continuously exposed to each test agent for 72 h, and their effects on cellular viability were evaluated. In order to identify those derivatives with cytotoxicity potential, cell viability vs. complex concentration is illustrated in Fig. 8, from which the IC$_{50}$ values for each derivative were determined (Table 1). The relative potency of each test complex displayed a concentration dependent cytotoxic profile. The plots indicate that the cytotoxic effect of [Co(en)$_2$(pyz)$_2$]Br$_3$ is greater than that of cis-[Co(en)$_2$(Cl)$_2$]Cl.

**CONCLUSIONS**

While reports on the interactions of Co(III) complexes with DNA are numerous in the literature, in this study we have attempted to unravel the DNA interaction with the ethylenediamine pyrazole Co(III) complex. The binding behavior of this complex with DNA was characterized by absorption titration, fluorescence quenching and viscosity measurements. The experimental results indicate that the complex binds to DNA by a nonintercalative mode. The results of the present study show that the complex containing...
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pyrazole as a ligand has better cytotoxicity than cis-
[Co(en)2Cl2]. The efficacies of these complexes on various
cancer cell lines are being examined in our laboratory.

REFERENCES

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