Synthesis, DNA and Protein Binding and DNA Cleavage Activity of a Ferric (III) Complex Containing Novel TACN

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Abstract. A new Ferric(III) complex, [Fe(L)(Cl)\textsubscript{3}] (1) [L = N-(4-(Benzyloxy)benzyl)-1,4,7-Triazacyclononane], has been synthesized and characterized by IR, elemental analysis and ESI-MS. The electrospray mass spectrum of 1 in solution indicates that mononuclear ion [Fe(L)Cl\textsubscript{2}]\textsuperscript{+} (2) is the active species. Fluorescence spectroscopy studies of 2 with calf thymus DNA (ct-DNA) show that 2 partially intercalates to ct-DNA. 2 with bovine serum albumin (BSA) protein investigated using fluorescence method indicated that there occurred strong binding of ferric complex to BSA over the ligand. Further, in the absence of external agents, supercoiled plasmid DNA cleavage by 2 was performed under aerobic condition.

Introduction

Enzymes (protein-based, as well as ribozymes) are used to promote phosphoryl transfer reactions in the biological arena. Low molecular weight metal complexes are attractive mimic reagents because of their inherently diverse electron structure, the studies can help us to understand and clarify the role of metal ions in natural nucleases, and more importantly, metal complexes which can efficiently bind and cleave DNA under physiological conditions are considered as potential candidates for use as therapeutic agents in medicinal applications and for genomic research. On the other hand, like DNA, proteins are also considered to be one of the main molecular targets in the action of anticancer agents. Interactions of complexes having rings are very important for protein stabilization and various regulatory processes. Most of the reported ferric-based complexes are not intrinsically fluorescent, so they need to be modified with a fluorescent tag in order to be visualized within the cells. Here, we report the complex [Fe(L)Cl\textsubscript{3}] (1) with a fluorescent ligand (Figure 1, \(L = N-(4-(Benzyloxy)benzyl)-1,4,7-Triazacyclononane\)). The ESI-MS indicates that 1 dissolves to give [Fe(L)Cl\textsubscript{2}]\textsuperscript{+} (2) in DMSO solution. The rare-cutting restriction endonuclease NotI contains a unique iron-binding fold that positions nearby protein elements for DNA recognition and serves as a structural role. Because DNA and protein are the main cellular targets for the anticancer activity, DNA cleavage, and DNA and protein binding studies of 2 by fluorescence spectroscopy have been carried out.

![Figure 1. Structure of the ligand.](image-url)

Experimental Section

Materials and Instrumentation

Ethidium bromide (EB), bovine serum albumin (BSA), calf thymus DNA (ct-DNA) and pBR322 plasmid DNA were from Sigma. Unless stated, all other reagents used in this research were obtained from commercial sources and used without further purification. Tris-HCl (tris(hydroxymethyl)aminomethane) buffer solution was prepared using deionized sonicated triple-distilled water. Solvents used in this research were purified by standard procedures. Solution
of the Fe(III) complex and other reagents used for strand scission were prepared freshly in triple-distilled water before use.

Elemental analyses for C, H, and N were obtained on a Perkin-Elmer analyzer model 240. Infrared spectroscopy on KBr pellets was performed on a Bruker Vector 22 FT-IR spectrophotometer in the 4000−400 cm\(^{-1}\) regions. ESI-Mass spectrum was recorded using a LCQ DECA XP Liquid Chromatography-Mass Spectrometer. The fluorescence spectral was recorded on a Cary 300 fluorescence spectrophotometer. The Gel Imaging and Documentation DigiDoc-It System (UVI, England) were assessed using Labworks Imaging and Analysis Software (UVI, England).

**Syntheses**

1,4,7-Triazacyclononane, \([9]_{\text{ane}}\text{N}_3\), was either prepared by published methods\(^{13}\) or purchased. The ligand, N-(4-(Benzyloxy)benzyl)-1,4,7-Triazacyclononane, were synthesized according to the procedure of Wieghardt et al.\(^{14,15}\)

\([\text{Fe}(\text{L})(\text{Cl})_3]\) (I)

To a solution of \(\text{FeCl}_3 \cdot 6\text{H}_2\text{O}\) (0.2 mmol) in 10 mL methanol was added dropwise 0.10 mol/L methanol of \(\text{L}\) (0.2 mmol, 2 mL). The red mixture was continuous stirring for 2 h at ambient temperature, and filtered. Evaporation of the resulting solution overnight provided red microcrystalline material, filtered and washed with diethyl ether (3×2 mL) and then dried in the oven for 0.5 hr. (yield 43%). Elemental analysis data for \(\text{C}_{20}\text{H}_{27}\text{Cl}_3\text{FeN}_3\text{O(}\text{I}\): C, 49.26; H, 5.58; N, 8.62%. FT-IR(KBr phase, \(v_{\text{max}}/\text{cm}^{-1}\)): 3384 br (OH), 2926 s (CH), 1603 s (NH), 1385 m, 1354 m, 1083 vs, 984 s, 857 s, 617 m, 539 s (br, broad; vs, very strong; s, strong; m, medium).

**Protein Binding Studies**

The protein binding study was performed by tryptophan fluorescence quenching experiments using bovine serum albumin stock solution (BSA, 1.5 mM) in 50 mM phosphate buffer (pH = 7.4) and stored in the dark at 4 °C. The fluorescence spectra were recorded at room temperature with excitation wavelength of BSA at 280 nm and the emission range was adjusted before measurements. Fluorescence intensities at 602 nm were measured at different complex concentrations.

**DNA Binding Experiments**

By the fluorescence spectral method, the relative binding of the complexes to ct-DNA was studied with an EB-bound ct-DNA solution in 5 mM Tris-HCl/50mM NaCl buffer (pH 7.4). The excitation wavelength was fixed at 510 nm and the emission range was adjusted before measurements. Fluorescence intensities at 602 nm were measured at different complex concentrations. The experiments were carried out by titrating complexes into EB-DNA solution containing 5 × 10\(^{-5}\) M EB and 5 × 10\(^{-5}\) M DNA.

**pBR 322 DNA Cleavage Assay**

Cleavage of supercoiled (SC) pBR322 DNA by the complex was studied by agarose gel electrophoresis. The reaction was carried out prepared following a literature method. Bands were visualized by UV light and photographed. The extent of cleavage of the SC DNA was determined by measuring the intensities of the bands using Gel Documentation System. Supercoiled plasmid DNA values were corrected by a factor 1.22, based on average literature estimate of lowered binding of EB.
Results and Discussion

Synthesis and Spectroscopic Measurements

While we investigated the coordination chemistry of the ligand $L$ with metal salts $FeCl_3$, the complex $[Fe(L)(Cl)_3]$ was obtained. Ligand $L$ is a very feasible ligand in that it has three imine N atoms, which are potential donors to act as a metal acceptor. In $I$, each $L$ serves as a tridentate ligand (N$_3$) and coordinates to Fe$^{3+}$ cation as a terminal ligand. Each Fe$^{3+}$ cation is in a six-coordination provided by an N$_3$Cl$_3$ donor set including three terminal chloride ions and three N atoms, as shown in Figure 2. $I$ was synthesized in good yields and characterized by satisfactory elemental analyses.

FTIR spectra are being dominated by a broad band at 3384 cm$^{-1}$ assigned to the stretching vibrations of water molecules ($\nu_{O-H}$). The (C–N) of TACN ligand for $I$ is a shoulder involving a split sharp peak at 1603 cm$^{-1}$, while (C–H) of the benzene group is a split sharp peak at 2926 cm$^{-1}$. Additionally middle strong absorption, 1354, 1385 cm$^{-1}$, strong absorption, 539, 857, 984, 1083 cm$^{-1}$ are observed, indicating the benzene groups belonging of the ligand.

Figure 2. Structure of the complex $I$.

Solution Properties of $I$

$I$ exhibits a good solubility in DMSO, CH$_2$Cl$_2$, CHCl$_3$ etc hydrophobic solvent, which facilitates to investigate its behavior in solutions. The species distribution of $I$ in DMSO solution was measured by the ESI-MS method. The electrospray mass spectrum in positive mode (ESI$^+$) gives a peak at $m/z$ 451.088 corresponding to $[Fe(L)Cl_2]^+$ species (Figure 3).

Protein Binding Studies

Fluorescence Quenching Studies

The interactions between serum albumin and complex have attracted increasing research interest in recent years, since serum albumin constitutes ~55% of the total protein in blood plasma and it plays a pivotal role in drug transport and drug metabolism. Bovine serum albumin (BSA) is the most extensively studied serum albumin, due to its structural homology with human serum albumin (HSA) and their ability to transport amino acids and drug molecules, etc. Fluorescence quenching measurements have been widely used to study the interaction of metal complexes or small molecules with proteins.

The interaction of bovine serum albumin with $I$ was studied by fluorescence measurement at room temperature. A solution of BSA (2.8 µM) was titrated with various concentrations of $I$ (0–133 µM), as shown in Figure 2. Fluorescence spectra of the samples were recorded in the wavelength range of 300–450 nm upon excitation at 280 nm. Upon addition of an increasing amount of $I$ to
BSA, hypochromism of 31.6 % and blue-shift of 4 nm for 2 was observed at 348 nm maximal peak, which suggests intercalation between 2 and BSA. The observed blue shift is mainly due to the fact that the active site in the protein is buried in a hydrophobic environment. The quenching constants ($K_q$) at room temperature were calculated to be $3.5 \times 10^4 \text{M}^{-1}$ for 2, was calculated from the plot of $I_0/I$ versus [Q] (Figure 4 Insert figure).

Figure 4. The emission spectrum of bovine serum albumin (BSA) (2.8 µM; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 345$ nm) in the presence of increasing amounts of 2 (0–133 µM). The arrow shows that the emission intensity decreases upon the increase in concentration of 2. Scatchard plots of the fluorescence titration of 2 with BSA (Insert figure).

**Characteristics of Synchronous Fluorescence Spectra**

In synchronous fluorescence spectroscopy, the difference between excitation and emission wavelengths ($\Delta \lambda = \lambda_{em} - \lambda_{ex}$) reflects the spectra of a different nature of the chromophore. If the $\Delta \lambda$ value is 15 nm, the synchronous fluorescence of BSA is characteristic of tyrosine whereas a larger $\Delta \lambda$ value of 60 nm is characteristic of tryptophan.22-23 The synchronous fluorescence spectra of BSA with various concentrations of 2 were recorded at $\Delta \lambda = 15$ nm and $\Delta \lambda = 60$ nm and as shown in Figure 5 and 6. The arrow shows that the emission intensity decreases upon the increase in concentration of 2. In the synchronous fluorescence spectra of BSA at $\Delta \lambda = 15$, the addition of 2 to the solution of BSA resulted in a decrease of the fluorescence intensity of BSA at 292 nm, up to 61% of the initial fluorescence intensity of BSA for 2, and with no shift. On the other hand, in the case of synchronous fluorescence spectra of BSA at $\Delta \lambda = 60$, the addition of 2 to the solution of BSA resulted in a significant decrease of the fluorescence intensity at 286 nm, up to 69% of the initial fluorescence intensity of BSA accompanied by a red shift of 2 nm for 2. The above results suggested that the interaction of 2 with BSA affects the conformation of both tyrosine and tryptophan regions mainly due to the hydrophobic interactions.

![Synchronous spectra of BSA (2.8 µM) in the presence of increasing amounts of 2(0–256 µM) at a wavelength difference of $\Delta \lambda = 15$ nm.](image3)

![Synchronous spectra of BSA (2.8 µM) in the presence of increasing amounts of 2(0–256 µM) at a wavelength difference of $\Delta \lambda = 60$ nm.](image4)
DNA Binding Properties

As 2 is non-emissive, competitive binding studies with EB were carried out to gain support for the mode of binding of 2 with DNA. In the fluorescence spectra, the addition of 2 to the solution of EB-DNA induced a decrease in the fluorescence intensity at about 608 nm (Figure 7). This suggests that 2 displace DNA-bound EB and bind to DNA at the intercalation sites with almost the same affinity.\(^{24}\) The plot of \(I_0/I\) vs. the concentration of 2, where \(I_0\) and \(I\) represent the fluorescence intensities in the absence and presence of 2, respectively, the apparent binding constant (\(K_{\text{app}}\)) was calculated from the equation \(K_{\text{EB}}[\text{EB}] = K_{\text{app}}[\text{complex}]\), where the complex concentration was the value at a 50 % reduction of the fluorescence intensity of EB and \(K_{\text{EB}} = 1.0 \times 10^7 \text{ M}^{-1} ([\text{EB}] = 4.0 \mu\text{M}).\)\(^{25}\) The binding constant of the classical intercalator and metallointercalator was in the order of \(10^7 \text{ M}^{-1}.\)\(^{26}\) The apparent binding constants (\(K_{\text{app}}\)) at room temperature were calculated to be \(4.05 \times 10^3 \text{ M}^{-1}\) for 2, indicating that the interaction of the complex with DNA is a moderate intercalative mode. The value obtained suggests that EB molecule be replaced by 2, which could be the result of the intercalation of tacn ligand, which facilitate DNA binding.

![Figure 7](image)

Figure 7. Fluorescence quenching curves of ethidium bromide bound to DNA. [DNA] = 6.0 µM, [EB] = 4.0 µM, and [2] = 0–2.64 mM. Stern–Volmer plots of the fluorescence titrations of 2 (Insert figure).

DNA Cleavage Properties

To better define the behavior of the system, we carried out kinetic measurements of the DNA cleavage reaction using different concentrations of 2, as shown in Figure 8. A single cut or nick on a strand of supercoiled DNA relaxes the supercoiling and leads to Form II. A second cut on the complementary strand, within approximately 16 base pairs of the original cut site, linearizes the DNA to Form III.\(^{27}\) All supercoiled (form I) DNA was cleaved to form II in the concentration of 2 (27.5 µM) (Lane 2–3). At a slightly higher concentration of 2 (44 µM) the cleavage is found to be much more efficient, as is seen from the formation of linear form (form III) in lane 4–5. It is clear that the cleavage of pBR322 DNA is highly dependent on the concentration of the complex.

![Figure 8](image)

Figure 8. Cleavage of plasmid pBR322 DNA (40 µM, in base pairs) with varying concentration of 2 (incubation for 6 h at 37 °C) in Tris–HCl/NaCl buffer (pH 7.4); Lane 1, DNA control; lane 2-5: DNA + 2 (13.75, 27.5, 44, 110 µM).

Conclusion

In this manuscript, our attempt on the synthesis, structure characterization and biological interactions of a new Fe(III) TACN complex is presented. The binding ability of the active complex 2 with both DNA and BSA has been investigated by fluorescence emission measurements and an intercalative mode of binding between 2 and CT-DNA was established. Further, the supercoiled
DNA into a nicked circular form and linear form was facilitated by the active complex 2. From the protein binding studies, the mechanism of quenching of BSA was found that the complex 2 bind to BSA via hydrophobic interaction.

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