Zinc complexes for hydrolytic cleavage of DNA

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Artificial metallonucleases [Zn^II(CysGly)(HisAla)] (1) and [Zn^II(CysGly)(HisLeu)] (2) have been prepared, characterized and their DNA cleavage abilities probed. UV, viscosity and fluorescence investigations show that the 1:1 complexes bind to CT-DNA through an intercalation. Gel electrophoresis studies reveal that these complexes cleave the plasmid pBR 322 DNA (form I) through nicked (form II) to linear (form III) forms under physiological conditions (37°C, H_2O, pH 7.5). DNA hydrolytic cleavage rate constants for complexes 1 and 2 at 37°C have been determined to be 0.55 h^-1 and 0.49 h^-1, respectively. Since redox-active metals can also account for the DNA cleavage, the cleavage by non-redox metal like zinc assumes importance.

Artificial metallonucleases are efficient tools for the foot printing and sequence specific targeting of nucleic acids\(^1\)\(^-\)\(^2\). The development of peptide based metal complexes, which cleave nucleic acids hydrolytically under biological conditions, is of great interest in the nucleic acid chemistry\(^3\)\(^-\)\(^5\). Most of the earlier reports\(^6\)\(^-\)\(^9\) on DNA cleavage activity are confined to oxidative cleavage, where it requires the addition of an external agent (e.g., light or hydrogen peroxide) to initiate cleavage and are thus restricted to \textit{in vitro} applications. Since hydrolytic cleavage agents do not require co-reactants, their usage has been highlighted recently\(^10\)\(^-\)\(^12\). Metal complexes that promote the hydrolytic cleavage of DNA, therefore, could be useful not only in molecular biology and drug design but also in elucidating the precise role of metal ions in enzyme catalysis. Imidazole and thiol groups are efficient catalysts for ester hydrolysis at neutral pH and histidine and cysteine residues are often involved in natural hydrolytic metallo-enzymes active centers\(^13\)\(^-\)\(^15\) and have potential to bind DNA. Therefore, we have chosen zinc with cysteine and histidine containing dipeptides, cysteinylglycine, histidylalanine and histidylleucine for our study. The synthesis, characterization, DNA binding and cleavage abilities of these complexes have been probed by different physicochemical techniques under physiological conditions.

Materials and Methods

The ligands cysteinylglycine (CysGly), histidylalanine (HisAla), histidylleucine (HisLeu) and ethidium bromide (ETB) were obtained from Sigma (USA). The calf thymus DNA (CT DNA) was obtained from Fluka (Switzerland). pBR322 DNA was obtained from Bangalore Genei (India) and zinc chloride was of Analar grade (E-Merck, Germany).

Synthesis of metal complexes

The complexes [Zn^II(CysGly)(HisAla)](1) and [Zn^II(CysGly)(HisLeu)](2) were synthesised by mixing aqueous solutions containing equimolar ratios of CysGly (0.001 moles, 0.121 g) and HisAla (0.001 moles, 0.155 g), CysGly (0.001 moles, 0.121 g) and HisLeu (0.001 moles, 0.155 g), which were added simultaneously and independently to equimolar concentration of zinc chloride. The reaction mixture was refluxed for 2 h. pH of the solution mixture was changed using 0.1 mol dm\(^{-3}\) NaOH solution to precipitate the complex.

[Zn^II(CysGly)(HisAla)](1)—Anal. Caled for Zn_14H_28N_6O_6S; C, 35.97%; H, 4.71%; N, 17.95%, S, 6.84%; Zn, 14.02%; Found: C, 35.86%; H, 4.69%, N, 17.92%; S, 6.85%, Zn, 14.09%, IR (KBr disc); v (NH\(_3\)) / v (NH\(_2\)) : 3326 (asy), 3123 (sym), v COO\(^-\) : 1571(asy), 1457(sym), v(C=O):1685, v m/z (in DMSO): 14 ohm\(^{-1}\)cm\(^{-1}\)mol\(^{-1}\); FAB-MS: The molecular ion peak: M^+ m/z 466.

[Zn^II(CysGly)(HisLeu)](2)—Anal. Caled for Zn_14H_28N_6O_6S; C, 40.07%; H, 5.48%; N, 16.50%, S, 6.28%; Zn, 12.84%; Found: C, 40.09%; H, 5.48%, N, 16.49%; S, 6.85%, Zn, 12.91%, IR (KBr disc); v (NH\(_3\)) / v (NH\(_2\)) : 3327 (asy), 3171 (sym), v COO\(^-\) :
1576(asy), 1460(sym), \( v(C=O): 1686 \), \( \text{vimz in plane: } 1052 \), \( v(M-N): 480 \), \( v(M-S): 394 \).

**AM** (in DMSO): 13 ohm \( \cdot \) cm \( \cdot \) lmo/\[; \] FAB-MS: The molecular ion peak: \( M^+ \) /1/ z 508.

**Elemental analysis and conductivity data**

Carbon, hydrogen and nitrogen analyses were obtained from micro analytical Heraeus Carlo Etba 1108 elemental analyser. Chloride analysis was done by Mohrs method. The metal contents were estimated from these solutions on atomic absorption spectrometer Perkin-Elmer 23380. The conductivity of metal complexes was measured in freshly prepared DMSO solutions using Digisun digital conductivity bridge (model: DI-909) and a dip type cell calibrated with KCl solution.

**Spectral analysis**

The IR spectra were recorded (in KBr discs) using Shimadzu IR-435, and Perkin-Elmer FTIR in the region 4000-400 cm\(^{-1}\). The reflectance spectra of the complexes were recorded (in KBr discs) on Shimadzu UV-160 spectrophotometer. FAB mass spectra of the complexes were recorded using a JEOL SX-120 instrument.

\(^1\)H and \(^13\)C NMR spectra of individual ligands and ligands together in the absence and presence of zinc were recorded at room temperature (25°C) on Varian Gemini 200 MHz pulsed FT-NMR spectrometer in D\(_2\)O. The concentration of Zn\(^{11}\) dipeptide complexes was maintained at 5\(\times\)10\(^{-5}\) mol.cm\(^{-3}\) and \(pH \sim 7.5\). The internal standard for \(^1\)H NMR spectra was 1,4-dioxane with a chemical shift of 66.5 ppm.

**Thermogravimetric analysis**

The absence of coordinated water was established for Zn\(^{11}\) complexes by TGA which was carried out using Perkin-Elmer model TGS-2 instrument.

**Preparation of DNA solution**

Concentrated CT DNA solution was prepared in 5 mM tris-HCl/50 mM NaCl in water (\(pH = 7.5\)). Its concentration was determined by UV absorption spectrum at 260 nm (molar absorption coefficient 6600 M\(^{-1}\)cm\(^{-1}\) as per ref. 16). The solutions were stored at 4°C and used with in a week. Purity of DNA was checked by measuring the UV absorption ratio (\(A_{260}/A_{280} \sim 1.8-1.9\)), which is an indication for protein free DNA (ref. 17). Concentration of ethidium bromide was determined spectrophotometrically using the extinction coefficient of 5680 M\(^{-1}\)cm\(^{-1}\) at 480 nm.

**DNA binding studies**

Absorption spectra were recorded with Shimadzu UV-160A UV-Vis spectrophotometer using microcuvettes with a path length of 1 cm. Increasing known amounts of free zinc, free peptides, complexes 1 and 2 were added to CT DNA ([\(\lambda_{max} = 260\) nm, OD = 0.540], until the ratio of these with DNA reached \(\sim 2.0\). The experiments were carried out in 5 mM tris-HCl/50 mM NaCl (\(pH = 7.5\)), phosphate buffer [1 mM] and 10 mM NaCl. After each addition, the mixture was shaken and kept for \(\sim 5\) min and the absorbance recorded. The binding constants of DNA with complexes 1 and 2 were determined based on the differences in their UV profile.

Absorption spectra were also recorded for free ETB (\(\lambda_{max} \sim 480\) nm) and DNA with ETB in a stoichiometric ratio of 1:1.5 (\(\lambda_{max} \sim 485\)nm). To this solution, increasing known amounts of 1 and 2 were added until the ratio of these with DNA reached \(\sim 1.5\). The
experiments were carried out in 5 mM tris-HCl/50 mM NaCl (pH = 7.5) at 25°C.

Viscosity was determined using an Ostwald-type viscometer. The flow rate of the buffer and known amounts of CT DNA in the presence and absence of free peptides and complexes 1 and 2 was measured with manually operated timer. Experiments were repeated till the concurrent values with an error of 0.1 sec were obtained. The relative specific viscosity was calculated according to the relation 1 = (t - t₀)/t₀, where t₀ is the flow time for the buffer and t is the observed flow time for CT DNA in the presence and absence of free ligands and zinc complexes. All the measurements were carried out at 25°C.

Fluorescence spectra were recorded with SPEX-Fluorolog 0.22m fluorimeter equipped with a 450 W xenon lamp. The slit widths were 2x2x2x2 and the emission spectral range 560-700 nm. All fluorescence titrations were carried out in 5mM tris-HCl/50 mM NaCl (pH = 7.5, 25°C). Solutions containing DNA and ethidium bromide were titrated with varying concentrations of complexes 1 and 2. The solutions were excited at 540 nm and fluorescence emission corresponding to 606 nm was recorded. The samples were shaken and kept for 2-3 min for equilibrium before recording the spectra. The DNA concentration was always 78 μM DNA-phosphate. The concentration of the complexes 1 and 2 was in the range of 5-80 μM and ETB concentration was 50 μM.

Fluorescence spectra were used to obtain Scatchard plots. For this, titrations of DNA against ETB in the absence and presence of zinc complexes were performed. Initial concentration of DNA in 5mM tris-HCl/50 mM NaCl was 53 μM and ETB was 100 μM. After each addition of ETB to the solutions containing DNA and zinc complexes, the emission spectra was recorded from 550 to 650 nm with 540 nm excitation at 25°C. Corrections were made to the data for the volume changes during the course of titrations. The data was analyzed by the method of Lepec and Paoletti to obtain bound (c₀) and free (c₁) concentration of ETB. Scatchard plots were obtained by plotting rcr versus r (where r = c₀/conc. of DNA).

Electrophoresis experiments were performed with pBR322 DNA. The cleavage of pBR322 by complexes 1 and 2 was accomplished by mixing (in order) 16 μL of 5 mM tris HCl (pH 7.5) containing 5 mM NaCl buffer, varying concentrations of 1 and 2 and 2 μL of pBR322 (0.35 μg/μL; 10 mM tris-HCl, pH 8.0 and 1 mM EDTA). Initial concentration of DNA was kept at 34.3 nM or 147 mM. After mixing, the DNA solutions were incubated at 37°C for 4 h. The reactions were quenched by the addition of bromophenol blue and the mixtures were analyzed by gel electrophoresis. The % agarose gels were run at 50 V for 5 h in TAE following electrophoresis. The gels were stained with ETB solution at 0.5 μg/mL to observe the cleaved DNA products. The extent of DNA cleavage was determined by using the volume quantification method in UVI Doc Mw version 99.03. The relative amounts of the different forms of DNA were determined by dividing the fluorescence intensity for a particular band by the sum of the fluorescence intensities for each band in that lane. In order to examine if hydroxyl radicals were present, hydroxyl radical scavenger such as DMSO was introduced. However, kinetic studies were performed at a fixed concentration of (0.96 mM) of 1 and 2 with different incubation time.

Results and Discussion

The solid complexes (1) and (2) are quite stable to air and moisture. They decompose at high temperatures (above 250°C). The complexes are amorphous in nature and colourless. The analytical data corresponding to the above complexes (Table 1) indicate that the complexes are in 1:1:1 ratio. No evidence was found for the presence of chloride ions in the coordination sphere of the complexes. The conductivity values (Table 1) in DMSO/H₂O show

<table>
<thead>
<tr>
<th>Complex</th>
<th>Carbon%</th>
<th>Hydrogen%</th>
<th>Nitrogen%</th>
<th>Sulphur%</th>
<th>Metal%</th>
<th>ohm⁻¹cm⁻¹mol⁻¹ (in DMSO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Zn(CysGly)(HisAla)]</td>
<td>35.86</td>
<td>4.69</td>
<td>17.92</td>
<td>6.85</td>
<td>14.09</td>
<td>14</td>
</tr>
<tr>
<td>[Zn(CysGly)(HisLeu)]</td>
<td>40.09</td>
<td>5.48</td>
<td>16.49</td>
<td>6.27</td>
<td>12.91</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 1—Analytical and conductivity data of the complexes 1 and 2

Note: The conductivity values were calculated by using the method of Leppe and Paoletti. The complexes are all usually amorphous in nature and colorless, and the analytical data (Table 1) indicate that the complexes are in 1:1:1 ratio. No evidence was found for the presence of chloride ions in the coordination sphere of the complexes. The conductivity values (Table 1) in DMSO/H₂O.
The dipeptides (CysGly, HisAla and HisLeu) exist as Zwitter ions both in solution and solid-state. The IR spectra of CysGly, HisAla and HisLeu exhibited significant features in $\nu$ NH: $\nu$ COO regions, Table 2.

In dipeptides (CysGly, HisAla and HisLeu), the peaks observed in the range of 3233-3100 cm$^{-1}$ were assigned to $\nu$NH asymmetric and symmetric stretching vibrations. The peaks observed in the range of 2956-2936 cm$^{-1}$ were assigned to $\nu$CH stretching frequency. The asymmetric and symmetric vibrations of carboxylate group of dipeptides were observed in the range of 1589-1549 cm$^{-1}$ and 1464-1400 cm$^{-1}$, respectively. The peak due to S-H was observed at 2537 cm$^{-1}$. The peaks due to $\nu$imidazole in planar were observed in the range of 974-946 cm$^{-1}$. The peaks due to NH$_3^+$ twisting and rocking and COO wagging frequencies were observed in the range 1200-600 cm$^{-1}$. The carbonyl group of the peptides link was observed in the range 1690-1677 cm$^{-1}$. IR spectra of the metal complexes showed characteristic band positions, band shifts and band intensities which can be correlated to bidentate chelation with metal ion (Table 3).

IR spectra exhibited significant features in $\nu$NH$_2$ and COO$^-$ regions. It is known that the free dipeptides exist as Zwitter ions (NH$_2^+$, COO$^-$) and the IR spectra of these cannot be compared entirely with those of metal complexes as dipeptides in metal complexes do not exist as Zwitter ions. Particularly, free dipeptides with NH$_2^+$ function show $\nu$NH$^+$ in the range of 3200-3050 cm$^{-1}$. In the complexes, NH$_2^+$ gets deprotonated and binds to metal through neutral NH$_2$ group. The transformation of NH$_2^+$ to NH$_2$ must result in the upward shift in $\nu$NH$^+$ compared to the free dipeptides. At isoelectric point, they must show $\nu$NH$_2$ in the range of 3500-3300 cm$^{-1}$. In the present complexes, the IR spectra showed characteristic bands in the region 3350-3000 cm$^{-1}$ which is lower as compared to free $\nu$NH$_2$. Hence, it may be concluded that the nitrogen of the amino group was involved in metal coordination. The spectra also showed shifting of $\nu$imidazole in plane from $\sim$950 cm$^{-1}$ to $\sim$1050 cm$^{-1}$ indicating the coordination of imidazole nitrogen with metal. The peak due to $\nu$ S-H was lost in the spectra of mixed ligand complexes due to the deprotonation of the S-H group on binding with metal ion. The other low intensity bands observed in far IR region in the
range of 450-500 cm⁻¹ were assigned to v(M-N) stretching and 385 cm⁻¹ and 375 cm⁻¹ to v(M-S) stretching vibrations. Based on the IR spectra of the mixed ligand complexes, it was assumed that the cysteinyglycine binds to metal ion through thiol sulfur and amino nitrogen and histidylalanine/histidyleucine bind to metal ion with imidazole and amino nitrogens.

Reflectance spectra
The reflectance spectra of the complexes 1 and 2 showed multiple bands at 28820, 32790 and 35210 cm⁻¹, which were assigned to charge transfer bands in the complexes.

Thermogravimetric analysis
Water molecules in complexes are generally of two types — lattice water and coordinated water. The lattice water will be lost at low temperatures (60-120°C) where the loss of coordinated water molecule is observed at high temperatures (150-200°C). In the thermograms of DTA and TGA of complexes 1 and 2, there was a sudden weight loss at 340 and 360°C, respectively, which indicates the absence of water molecules in coordination sphere of the complexes. The weight loss of the complexes varied from 44-54%.

'H and 13C NMR
The 'H and 13C NMR spectra were utilized to identify the mode of binding in these complexes. The spectral assignments of free ligands were made based on correlation spectra and literature. The spectra of individual ligands and ligands together in the absence and presence of zinc ion both for binary and ternary systems were recorded to identify the effect of the metal ion on the chemical shifts of the ligands listed in Tables 4-7.

<table>
<thead>
<tr>
<th>Table 4—1H-NMR chemical shifts of free CysGly, HisAla and HisLeu ligands and ligands together</th>
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<tr>
<td></td>
</tr>
<tr>
<td>CH(α)</td>
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<tr>
<td>CH(β)</td>
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<tr>
<td>CH(δ)</td>
</tr>
<tr>
<td>CH(ε)</td>
</tr>
<tr>
<td>CH(α')</td>
</tr>
<tr>
<td>CH(β')</td>
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<tr>
<td>CH(γ')</td>
</tr>
<tr>
<td>CHδ(δ)</td>
</tr>
<tr>
<td>CH(α)</td>
</tr>
<tr>
<td>CH(β)</td>
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<tr>
<td>CH(δ)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5—1H-NMR chemical shifts in binary (1:1) Zn²⁺-CysGly, Zn²⁺-HisAla, Zn²⁺-HisLeu and ternary (1:1:1) Zn²⁺-CysGly-HisAla and Zn²⁺-CysGly-HisLeu systems</th>
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<tr>
<td></td>
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<tr>
<td>CH(α)</td>
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<tr>
<td>CH(β)</td>
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<tr>
<td>CH(δ)</td>
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<tr>
<td>CHδ(δ)</td>
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<td>CH(α')</td>
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<td>CH(β')</td>
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<td>CHδ(δ)</td>
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<td>CH(α)</td>
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<tr>
<td>CH(β)</td>
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<tr>
<td>CH(δ)</td>
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</table>
There is no change in the $^1$H and $^{13}$C NMR spectra of free ligands compared to the spectra recorded for mixture of ligands (Tables 4 and 6). NMR spectra for binary (1:1) and ternary (1:1:1) complexes of zinc are given in Tables 5 and 7. The data clearly reveal that there is considerable interaction between zinc and these ligands.

All compounds that contain imidazole exhibit resonances due to CH(O) around $\delta$ 7.0 ppm in D$_2$O. The resonances of this proton would be expected to shift downfield upon binding with a metal ion. Consistent with this, the CH(δ) resonances were shifted to downfield on the addition of zinc for all the complexes investigated. In addition to this, the resonances of CH(ε) of imidazole ring was also shifted to downfield by $\delta$ 0.81 ppm in histidylalanine and $\delta$ 0.73 ppm histidylleucine in the corresponding zinc complexes. The CH(α) and CH(β) were also shifted to downfield in these complexes. In cysteinylglycine, the resonances of CH(α') shifted to downfield and CH(α) resonances were shifted to upfield on the addition of zinc. The resonances of CH$_2$(α'), CH$_2$(α') in cysteinylglycine CH(α), CH(β) in histidylalanine and CH(α'), CH(β),
CH(γ), CH(δ) in histidylleucine did not significantly change in the presence of zinc ion. Accordingly, it was assumed that the alanine and leucine moieties were not involved in binding. This suggests a bidentate coordination of these ligands with zinc.

In 13C NMR spectra, notable shifts were observed in the case of C(α), C(δ), C(γ) and C(ε) of histidylalanine, histidylleucine and C(α), C(β), C(δ) of cysteinylglycine. However, no changes in the resonances of C(α'), C(β') of alanine and C(α'), C(β'), C(δ') of leucine portions of the complexes were observed. This confirmed the non-involvement of carboxylate, amide groups and involvement of thiol of alanine and leucine moieties were not involved in binding. This suggests a bidentate coordination of these ligands with zinc.

FAB mass spectra
As an additional support for the above conclusions, the FAB mass spectra of the complexes were recorded. In the FAB mass spectrum of complex 1, the molecular ion peak was observed at m/z 466, which was in agreement with the molecular weight of the proposed structure. The spectrum showed a peak at m/z 307 which was assigned to meta nitro benzyl alcohol. The molecular ion loses CH₃ group resulting in an ion at m/z 453. The ion further loses two COO⁻ and two CH groups to give an ion at m/z 329.

In the FAB mass spectrum of complex 2, the molecular ion peak was observed at m/z 508, which was in agreement with the molecular weight of the proposed structure. The molecular ion further loses CH₃, CH₂ and CH groups giving an ion at m/z 450. The ion further loses 2COO⁻ and CH₂ giving an ion at m/z 341.

TGA provide information regarding the absence of water molecules in the co-ordination sphere of the complexes. Further, IR and NMR spectral data suggest a tetra co-ordination around zinc for these complexes. Geometry of the complexes was established based on the reflectance spectral data. The bands observed at 28820, 32790 and 35210 cm⁻¹ were indicative of a typical tetrahedral geometry26-28. Therefore, a tetrahedral geometry around the zinc ion was proposed (1 and 2).

DNA binding studies
Absorption titrations
The UV profile of CT DNA at a fixed concentration and increasing known quantities of Zn¹¹ dipeptide complexes was monitored at 260 nm (OD = 0.540) in 5 mM tris-HCl/50 mM NaCl in water (pH =7.5). No significant absorbance was observed at this wave length for free Zn¹¹ and dipeptides. However, on addition of increasing [Zn¹¹ dipeptide complexes], there was gradual decrease in absorbance, which is indicative of the interaction between the DNA and [Zn¹¹(CysGly)(HisAla)] (1)/[Zn¹¹(CysGly)(HisLeu)] (2). The decrease was gradual in all the cases and the saturation was reached. When the complex concentration was matched with that of DNA (complex/CT DNA = 1). The slight increase in OD with zinc ion may be due to the counter ion effect. The hypochromicity changes were utilized to calculate the association constants with the help of Scatchard plot29 from the following relation.

\[ K_d = \frac{[C-nX][D-X]}{[X]} \] ... (1)

On rearranging Eq. (1), we get:

\[ [C] = \frac{K_d}{[X]} + n \frac{[C]}{[D-X]} \] ... (2)

where \( K_d \) is the equilibrium dissociation constant, \( [C] \) and \( [D] \) denote the initial concentration of complexes 1 and 2 and DNA, respectively, \( [X] \) is the concentration of complex formed. The number of binding sites on DNA is n. The decrease in OD was used to determine free DNA [D-X] present and by difference, the bound DNA [X] as a function of added complexes. 1/[D-X] versus [C]/[X] was plotted, from which \( K_d \) and n were evaluated using Eq. (2). The two different slopes (\( K_d \)) and intercepts (n) indicate two types of binding processes that are independent of each other. One is a strong interaction with \( K_d (g) \) values varying from 2.3-7.6x10⁵ M⁻¹ and another a

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### Table 8—Association constants and viscometric properties of CT DNA on interaction with 1 and 2 at 25°C (pH 7.5)

<table>
<thead>
<tr>
<th>Zinc complex</th>
<th>( K_{a(5)} )</th>
<th>( K_{a(6)} )</th>
<th>Helix enhancement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Zn¹¹(CysGly)(HisAla)] (1)</td>
<td>2.24 x 10⁵</td>
<td>7.60 x 10³</td>
<td>26.5</td>
</tr>
<tr>
<td>[Zn¹¹(CysGly)(HisLeu)] (2)</td>
<td>8.24 x 10⁵</td>
<td>2.26 x 10³</td>
<td>31.0</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td></td>
<td></td>
<td>43.0</td>
</tr>
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</table>
weaker interaction with $K_{\text{obs}}$ values in the range of 2.0-8.0 x 10^2 M^{-1}, respectively. Similar assumptions were also made earlier. The constants are listed in Table 8. The values of $K_{\text{eq}}(1/K_\text{d})$ and $n_1$ are characteristic of the binding of zinc complexes at the DNA surface without base specificity. It is presumed that the primary binding is between the phosphate oxygen and imidazole (NH) through H-bonding. It is interesting to note that as the chain lengths of the peptides of zinc complexes increase, the stability also increases. This may be due to the increased hydrophobicity of zinc complexes, which increases in the order 1 < 2. The second binding step seems to involve the amino group of the zinc complexes and the G (N-7) of the DNA.

Since it has been established that the extent of hypochromicity is an accurate reflector of the extent of ordered secondary structure in DNA, these results clearly demonstrate the binding of zinc complexes to DNA.

**Viscosity measurements**

Since viscometric technique is a well-established method for investigating the extension of the DNA helix associated with intercalation, this technique was used in the present investigation also. In the major groove, the positions N-7 and O-6 (N-6) of purine bases, and O-4 (N-4) of pyrimidines, are quite accessible to chemical attack. Although the increased bond distances and helix diameter of the structure would presumably increase the flexibility of the duplex, zinc complexes by linking the bases, would tend to stabilize the helical structure. As the polynucleotide binds the zinc complex, the helix lengthens and stiffens which is reflected in the increasing specific viscosity of DNA. This has been regarded as a diagnostic feature of intercalation process. To further test the intercalating process, experiments were conducted with ETB, a well-known intercalator, which has also resulted in an increase in specific viscosity. Similar trends observed in both the systems confirm the intercalating binding of zinc complexes with DNA.

The percentage of helix enhancement was calculated from the following relation:

$$L = \left( \frac{\eta - \eta_0}{\eta_0} \right) \frac{L}{L_0}$$

where $L$ and $L_0$ are contour lengths of the DNA in the presence and absence of zinc complexes, respectively.

$\eta$ and $\eta_0$ represent corresponding values of the intrinsic viscosity (approximated to reduced viscosity) of the solution, $t_c$ the flow time of the complex, $t_a$ the flow time of free DNA, and $t_d$ the flow time of the buffer at a given volume in the viscometer. The influence of side chains of peptides were clearly observed in the viscosity studies also (Table 8).

The non-specific interactions have been further confirmed by competitive experiments with ETB. The ETB has been known to bind DNA in an intercalative fashion. The intercalative binding of complex 2 with DNA has been demonstrated through the visible absorption band of ETB at 480 nm. The band at 480 nm red shifted to 485 nm upon interaction with DNA at a ratio of [ETB]/[CT DNA] = 1.5 in 5 mM tris-HCl/50 mM NaCl (pH 7.5) with considerable hypochromicity. A gradual addition of complex 2 to intercalated ETB with DNA resulted in the blue shift of absorption band to 480 nm with noteworthy hyperchromicity. At 1:1 ratio of [DNA]/[2], the free ETB absorption profile was completely restored. This clearly demonstrated the complete displacement of bound ETB by 2 and its ability to intercalate with DNA. Similar results were also obtained for complex 1.

**Fluorescence spectral measurements**

Two mechanisms have been proposed to account for the quenching the replacement of molecular fluorophores and/or electron transfer. The quenching extent of the fluorescence of ETB bound DNA was utilized to determine the extent of binding between the second molecule and DNA. Accordingly, the fluorescence profile of DNA bound ETB in 5 mM tris-HCl/50 mM NaCl at pH 7.5 was monitored with increasing addition of complexes 1 and 2. Quenching of fluorescence of ETB bound DNA (Fig. 1) on addition of 2 demonstrates the binding of ZnII dipeptide complex with DNA. Similar results were also obtained with complex 1.

The binding isotherms of ETB and DNA in the absence and presence of 2 were determined experimentally and presented in Fig. 2. The Scatchard plot in the presence of 2 (Fig. 2, curve 2) results in the decrease of slope, and slight decrease of intercept as compared to the complex free plot (Fig. 2, curve 1). Similar results were also obtained with 1. This confirms the intercalative binding of these complexes with CT DNA.
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Fig. 1—Fluorescence spectra of ethidium bromide (50 μM) intercalated to DNA (94 μM) on titration with 2 in 5 mM tris-HCl/50 mM NaCl (pH =7.5) at 25°C ([2] (1) 0 μM, (2) 15 μM (3) 40 μM (4) 76 μM (5) 96 μM).

Fig. 2—Fluorescence Scatchard plots of the binding of ETB to CT DNA: (1, ■) in the absence, and (2, ○) presence of [Zn(CysGly)(HisLeu)][2] (r = ratio of bound ETB to DNA and Cr concentration of free ETB).

Hydrolytic cleavage of double stranded DNA

After establishing the binding abilities of Zn11 dipeptide complexes with DNA, cleavage experiments were performed with pBR322 DNA. Figures 3 and 4 depict agarose gel electrophoresis patterns for the cleavage of pBR322 DNA. The conversion of form I (supercoiled) through form II (nicked) to form III (linearized) was observed with increase in concentrations of complexes 1 and 2. It was noted earlier that a single cut or nick on a strand of supercoiled DNA relaxes the supercoiling and leads to form II. A second cut or nick on the complementary strand, within approximately 12 base pairs of the original cut size, linearizes the DNA to form III48-50. Therefore, it is clear that 1 and 2 have cut the DNA at least twice to convert it from form I to form III. The extent of DNA cleavage was also quantified via fluoroimaging (Table 9).

Although, 1 and 2 did not require the addition of external agent, the possibility of DNA cleavage...
The possible explanation for the degradation of DNA is the formation of a three centered hydrogen bond involving the NH$_2$ group of guanine, the lone pair of electrons on pyrrole nitrogen of imidazole, and the COO$^-$ groups of HisAla/HisLeu and CysGly. Bruice et al.$^{31,52}$ in their study of bis-(2-carboxyphenyl) phosphate, found that carboxyl groups could participate in phosphodiester hydrolysis. Our findings support this. This is also consistent with the observations that metal ions are much more efficient in promoting hydrolysis than the organic buffers of similar pK$_a$ values.$^{53,54}$

The cleavage of pBR322 DNA by 1 and 2 has been kinetically characterized by quantification of supercoiled and nicked DNA. The observed distribution of supercoiled and nicked DNA in an agarose gel provides a measure of the extent of hydrolysis of phosphodiester bond in each plasmid DNA, and the data were used to perform simple kinetic analysis. Figures 5A and 5B are the time course plots for the decrease of form I and formation of form II during the reaction under mild conditions by 1 and 2, respectively. The decrease of form I fits well to a single exponential decay curve and the increase of form II also fits well to a single exponential curve. From these curve fits, the hydrolysis rate constants at 37°C and at complex concentration of 0.96 mM were determined to be 0.55 h$^{-1}$ for 1 ($R = 0.984$) and 0.49 h$^{-1}$ for 2 ($R = 0.987$).

Enhancement of rate constants in the range of 0.09-0.25 h$^{-1}$ for DNA hydrolysis by metal complexes are considered impressive$^{55,56}$ even though, they are still far from the rate enhancements produced by natural enzymes. The nuclease activities for 1 and 2 are 1.52×10$^7$ and 1.36×10$^7$ fold rate enhancement over unhydrolyzed double stranded DNA, respectively.

Since hydrolytic enzymes such as alkaline phosphatases$^{57}$ and phospholipase C$^{58}$, and the klenow fragment of DNA polymerase I$^{59}$, which are responsible for hydrolytic cleavage of the phosphodiester of DNA, contain Zn$^{2+}$ in their active site, the present findings assume importance.

It has been shown here that the stability of the complexes plays an important role in the hydrolytic cleavage of DNA. Therefore, the focus should be on the development of stable DNA binding complexes in particular with non-redox metals for efficient DNA cleavage.
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References