Interactions of chromomycin A₃ and mithramycin with the sequence 
d(TAGCTAGCTA)₂

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Anti-cancer antibiotics, chromomycin A₃ (CHR) and mithramycin (MTR) inhibit DNA directed RNA synthesis in vivo by binding reversibly to template DNA in the minor groove with GC base specificity, in the presence of divalent cations like Mg²⁺. Under physiological conditions, (drug)₂Mg²⁺ complexes formed by the antibiotics are the potential DNA binding ligands. Structures of CHR and MTR differ in their saccharide residues. Scrutiny of the DNA binding properties reveal significant differences in their sequence selectivity, orientation and stoichiometry of binding. Here, we have analyzed binding and thermodynamic parameters for the interaction of the antibiotics with a model oligonucleotide sequence, d(TAGCTAGCTA)₂ to understand the role of sugars. The oligomer contains two potential binding sites (GpC) for the ligands. The study illustrates that the drugs bind differently to the sequence. (MTR)₂Mg²⁺ binds to both sites whereas (CHR)₂Mg²⁺ binds to a single site. UV melting profiles for the decanucleotide saturated with the ligands show that MTR bound oligomer is highly stabilized and melts symmetrically. In contrast, with CHR, loss of symmetry in the oligomer following its association with a single (CHR)₂Mg²⁺ complex molecule leads to a biphasic melting curve. Results have been interpreted in the light of saccharide dependent differences in ligand flexibility between the two antibiotics.

Introduction

Chromomycin A₃ (CHR) and Mithramycin (MTR) are two anti-cancer antibiotics of the aureolic acid group that potentially inhibit DNA directed RNA synthesis, in vivo²,³ (Fig. 1). They do so by binding reversibly to template DNA in the minor groove with GC base specificity⁴-⁷. At and above physiological pH the above association takes place only in the presence of divalent cations⁵, like Mg²⁺. We have earlier shown that in the absence of DNA, the antibiotics have the potential to bind to Mg²⁺, to form two different types of drug-Mg²⁺ complexes. The stoichiometries of the complexes formed are 1:1 (Complex I) and 2:1 (Complex II) in terms of drug:Mg²⁺ ratio⁸,¹¹. These complexes bind to DNA at and above physiological pH. Studies from different laboratories including ours have shown that under normal physiological conditions, in the presence of millimolar concentration of the metal ion, (drug)₂Mg²⁺ complex or complex II is the DNA binding ligand⁹,¹². Structurally, the two antibiotics are closely related, each consisting of a planar aglycone, the chromomycinone moiety and five hexapyranoses attached to it (Fig. 1). The antibiotics differ only in a small number of substitutions in the saccharide residues. Unlike CHR, MTR does not contain acetoxyl or methoxy substituents on its sugar residues. These differences lead to significant dissimilarities in their chemical properties. As for example, MTR has a pKₐ of 5.0 whereas for CHR, the value¹³ is 7.0.

Gross structural similarities between the two antibiotics lead to GC base specific association for both the (drug)₂Mg²⁺ complexes with DNA. The association originates from ligand induced alteration in the base specific groove width dimension, finally leading to a local distortion of the B-DNA double helix to a A-type conformation. However, a closer scrutiny of the DNA binding properties also indicate significant differences which are primarily the result of chemically distinctive sugar residues. Footprinting studies with the drug-DNA complexes show that the two antibiotics exhibit differences in their sequence selectivity⁶,¹⁴, though the minimum requirement of two contiguous GC base pairs for association is observed for both antibiotics⁶. NMR studies indicate a difference in the geometry and orientation of the two antibiotics in their complex with oligonucleotides¹²,¹⁵,¹⁶. This leads to differences in their binding geometry and binding stoichiometry with the same DNA sequence. However, the role of
sugar residues in the two antibiotics leading to different DNA binding characteristics of the antibiotic is still not clearly understood. It is necessary to understand this aspect in order to explain their pharmacological properties which are attributed to the DNA binding potentials, e.g. in terms of transcription inhibition potential under in vivo conditions, CHR is found to be more effective than MTR.

As a sequel to our studies to understand the role of sugar residues in the base specific recognition of the antibiotics with their prime cellular target DNA, we have examined the effects of ligand flexibility and concomitant DNA distortion upon the association process using the model oligonucleotide sequence d(TAGCTAGCTA)₂. While the GpC base step in the sequence is sufficient for the association of the (drug)₂Mg²⁺ complexes with it, the characteristic feature of the oligomer is the presence of two GpC binding sites separated by one TpA base step. Since the binding site per ligand molecule is approximately around 6-7 bases, the two potential binding sites are partially overlapping. Spectroscopic techniques such as absorbance and fluorescence have been used to monitor the interaction because they permit us to work at the micromolar range of antibiotic concentration (10-50 μM) where aggregation of the free ligand is absent as detected by the optical spectroscopic tools. The interactions were further characterized from analysis of DNA melting studies and thermodynamic parameters associated with the interactions. The results have been compared with structural information known from previous NMR and optical spectroscopic studies.

Materials and Methods

Materials
Chromomycin A₃, mithramycin, Tris and magnesium chloride solution (4.9 M) were from Sigma Chemical Company, USA. The oligonucleotide, d(TAGCTAGCTA)₂ was synthesized using Gene Assembler Special from Pharmacia Biotech Ltd., Sweden, using the phosphoramidite method. Purity of the oligomer was checked from the appearance of a single band in 25% native polyacrylamide gel electrophoresis and a single peak in reverse phase HPLC column chromatography using ProRPC HR 5/10 column. Concentrations of the antibiotics were determined from their known molar extinction coefficients. Concentration of the oligomer was determined by phosphate estimation. Buffers used were 20 mM Tris-Cl buffer, plus 10 mM MgCl₂, pH 8.0 for binding studies and 5 mM Hepes buffer, plus 10 mM MgCl₂, pH 8.0 for UV DNA melting experiments. Buffers were prepared in deionized, all quartz distilled water.

Binding studies
Interaction of ligand with oligomer was studied by pre-incubating the antibiotic(s) with Mg²⁺ for an hour at the desired temperature to ensure complete complex formation. Small aliquots of oligomer were then added to the complex and the equilibrium
spectrum recorded. Absorption and fluorescence spectra were recorded with Hitachi U-2000 spectrophotometer and Shimadzu RF-540 spectrophotometer, respectively.

**UV Melting studies**

Ultraviolet DNA melting curves were determined using Hitachi U-3300 spectrophotometer, equipped with a programmable thermoelectric temperature controller. Oligonucleotide samples, pre-incubated with 10 mM MgCl₂ were used for the study, in 5 mM Hepes buffer, plus 10 mM MgCl₂, pH 8.0. To a final concentration of 100 µM of the oligomer, 40 µM (in terms of antibiotic) of (drug)₂Mg²⁺ complexes were added to ensure saturation of the DNA molecule with the ligand. The samples were then further incubated for 30 min for equilibrium to be established. During melting experiment, the samples were heated rapidly till 26°C at the rate of 3°C per min. Further heating was carried out slowly, at the rate of 1°C per min, while continuously monitoring the absorbance at 260 nm. Reversibility of the melting profile was checked by slow cooling of the melted sample.

**Analysis of binding data**

All spectrophotometric and spectrofluorometric titrations were carried out at least 10°C below the melting temperature of the oligomers. Results from fluorometric titrations were analyzed by the following methods. Apparent binding constant ($K_{ap}$) was determined using non-linear curve fitting analysis vide equations (1) and (2). All experimental points for binding isotherms were fitted by least-square analysis.

$$K_{ap} = \frac{[C_0](\Delta F/\Delta F_{max})C_p}{[C_p](\Delta F/\Delta F_{max})C_0} \times \frac{[C_0]}{[C_p]}$$

$$C_0(\Delta F/\Delta F_{max})^2 - (C_0+C_p+K_0)(\Delta F/\Delta F_{max}) + C_p = 0$$

where, $\Delta F$ is the change in fluorescence emission intensity at 540 nm ($\lambda_{ex} = 470$ nm) for each point of titration curve, $\Delta F_{max}$ is the same parameter when the ligand is totally bound to oligomer, $C_p$ is the concentration of the oligomer and $C_0$ is the initial concentration of the antibiotic. Double reciprocal plot was used for determination of $\Delta F_{max}$ using equation (3).

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \frac{1}{[K_{ap}\Delta F_{max}(C_p-C_0)]}$$

A linear plot of $1/\Delta F$ against $1/(C_p-C_0)$ is extrapolated to the ordinate and the value of $\Delta F_{max}$ obtained from the intercept. The approach is based on the assumption that emission intensity is proportional to the concentration of the ligand. This is found to be true for the concentration range of 10-50 µM of the ligands employed and under the condition $C_p >> C_0$ which was followed by keeping at least eight-fold excess of oligomer with respect to antibiotic concentration.

The second method uses the Scatchard equation to estimate the intrinsic binding constant ($K_0$) and binding stoichiometry (n).

$$\frac{r}{C_p} = K_0(n-r)$$

$r/C_p$ was plotted against $r$ and the best-fit straight line of the experimental points was drawn. In the above equation, $r = C_p/C_p$, where $C_p$ is the concentration of the bound ligand and $C_p$ is the concentration of oligomer. $K_{ap}$ is given by $K_{ap}n$. The concentration of the bound ligand was determined from fluorometric titration using the expression, $C_b = (\Delta F/\Delta F_{max})C_0$

Binding stoichiometry determined from Scatchard equation gives the value of 'n' in terms of number of drug molecules bound per nucleotide bases. Binding stoichiometry was also determined from the intersection of the two straight lines in the plot of normalized increase in fluorescence against the ratio of the input concentrations of the oligomer and ligand.

**Analysis of thermodynamic parameters**

Thermodynamic parameters, $\Delta H$ (van't Hoff enthalpy), $\Delta S$ (entropy) and $\Delta G$ (free energy) were determined using the following equations:

$$\ln K_{ap} = -\Delta H/RT + \Delta S/R$$

$$\Delta G = \Delta H - T\Delta S$$

where $R$ and $T$ are the universal gas constant and absolute temperature respectively. $K_{ap}$ was determined at 15, 20 and 25°C, respectively to evaluate $\Delta H$ and $\Delta S$. These values were incorporated in equation (6) to obtain the value of $\Delta G$-van't Hoff $\Delta H$ values for the association were also calculated from the DNA melting curves. Enthalpy changes ($\Delta H$) for the following equilibria were considered,
2 single strands $\leftrightarrow$ duplex
(drug)$_2$Mg$^{2+}$+2 single strands $\leftrightarrow$
(drug)$_2$Mg$^{2+}$-duplex

Combining these two reactions, the binding reaction and its $\Delta H$ may be obtained:

(drug)$_2$Mg$^{2+}$ + duplex $\leftrightarrow$ (drug)$_2$Mg$^{2+}$-duplex

$\Delta H_1$ and $\Delta H_2$ were determined from the following equation:

$$\Delta H = -4.38 / [(1 / T_{\text{max}}) - (1 / T)]$$  \[7\]

where, $T_{\text{max}}$ is the temperature at the peak and $T$ is temperature corresponding to the half height of the peak on the higher temperature side, in the differential melting curve.

**Results**

As reported in our earlier results, association of the (drug)$_2$Mg$^{2+}$ complexes of CHR and MTR with d(TAGCTAGCTA)$_2$ is indicated from changes such as red shift and broadening of the band in the absorption spectra of the free ligand(s) upon addition of the oligonucleotide. Presence of a single isosbestic point supports an analysis in terms of free ligand and a single type of bound species. Increase in the fluorescence quantum yield of the ligands upon association with the oligomer was employed to monitor the binding quantitatively.

Apparent binding constant values were determined using non-linear curve fitting analysis. Representative example from fluorescence titration for CHR is shown in Fig. 2a. Both binding constant and binding stoichiometry were determined from Scatchard plot as well. Representative examples of Scatchard plots are shown in Fig. 2b. Stoichiometry of binding was also calculated directly from the binding isotherms according to our earlier method. The binding parameters are summarized in Table 1. Comparison of the binding parameters show that there is a difference in binding by the two ligands with the decameric sequence. The binding stoichiometry is significantly higher for CHR as compared to MTR. MTR covers ~5 bases per drug molecule whereas CHR covers ~10 bases per drug molecule. This indicates that (MTR)$_2$Mg$^{2+}$ complex binds to both the available sites in the decamer whereas, (CHR)$_2$Mg$^{2+}$ complex binds

only to a single site which makes its complex with the oligomer asymmetric in nature.

The thermodynamic parameters of association were determined using van't Hoff plot, shown in Fig. 2c. Binding enthalpy was also calculated from the differential DNA melting curves by van't Hoff's method as described under Materials and Methods. The calculated parameters are tabulated in Table 2. $\Delta H$ values from the two methods are consistent within the limits of experimental error, thereby validating the application of the two methods to understand the mode of association. They are higher for the association of (MTR)$_2$Mg$^{2+}$ as compared to
curves for the ligand bound oligomers, the amount of analysis of the melting curves of the oligomer in absence and presence of saturating concentration of CHR. Higher values of interaction of the antibiotics is obtained from a with the oligomer. Higher negative value of (CHR \cdot \text{Mg}^{2+})_{\text{complex}}$ gets kinked at the intermediate TpA base step in order to facilitate the approach of the two bulky (MTR)_{\text{Mg}^{2+}}$ ligands. The DNA is also distorted locally at the two individual GpC sites where a B→A
type transition in backbone geometry helps to accommodate the ligand. The two (MTR)_2Mg^{2+} ligands alter their conformations in order to fit into the groove without steric clash between them. Changes in the glycosidic linkage bonds in the C-D-E trisaccharide, in one of the monomers of the dimer-Mg^{2+} ligand, marks this change.

Comparison of the binding stoichiometries for the association of (CHR)_2Mg^{2+} and (MTR)_2Mg^{2+} complexes with the decamer shows that CHR binds to one of the sites in the oligomer and the other site remains unoccupied. In contrast, MTR binds to both the sites. The (CHR)_2Mg^{2+}-oligomer complex is thus asymmetric as compared to the (MTR)_2Mg^{2+}-oligomer complex. The thermodynamic parameters for the association(s) also support a difference in the mode of interaction of the two drugs. As in our earlier reports we have analyzed the observed changes in the thermodynamic parameters as a sum of several contributing terms in the following manner:

\[ \Delta H_{\text{tot}} = \Delta H_{\text{bind}} + \Delta H_{\text{DNA}} + \Delta H_{\text{dimer}} \]

where, \( \Delta H_{\text{bind}} \) is the binding enthalpy, \( \Delta H_{\text{DNA}} \) and \( \Delta H_{\text{dimer}} \) are the enthalpy changes associated with structural alterations in the DNA molecule and the ligand molecule respectively, during their interaction. The \( \Delta H \) values show that binding by MTR involves a higher enthalpy change as compared to CHR (Table 2). The value for MTR is not double of that for CHR, as is expected if only binding enthalpies contribute to the total observed \( \Delta H \). Since association of two (MTR)_2Mg^{2+} ligands to the decamer involves considerable bending of the DNA molecule around the kink at the TpA base step along with local B \( \rightarrow \) A transitions at the two binding sites, there is a major contribution from the \( \Delta H_{\text{DNA}} \) term. This considerably reduces the negative value of observed \( \Delta H_{\text{tot}} \) for MTR. A higher negative value of \( \Delta S \) for MTR also suggests formation of a more structurally constrained complex.
ligand-DNA complex as compared to CHR. This is consistent with the NMR model of the (MTR)$_2$Mg$^{2+}$-oligomer complex (PDB ID 207d$^{18}$). ΔG values remain comparable for both types of ligands due to enthalpy-entropy compensation.

The observed difference in the binding geometry originates from the nature of the sugars, particularly the substituents present in the B and E sugars. The methoxy group present in the B sugar and acetoxy group in the B and E sugars of CHR impose constraints upon the free rotations about the glycosidic linkages in the disaccharide and trisaccharide chains in the ligand molecule. This affects the flexibility of (CHR)$_2$Mg$^{2+}$ complex which behaves as a rigid molecule. Thus, energetically permissible structural alterations in the ligand molecule like alterations in the glycosidic torsion angles of the C-D-E trisaccharide segment, defined as 'plasticity' of MTR, is absent in case of CHR$^{16}$. Earlier reports by Keniry et al., also implies higher flexibility in the (MTR)$_2$Mg$^{2+}$ complex during its association with an octameric sequence as compared to CHR$^{16}$.

Differences in the nature of the ligand-DNA complexes formed by the two drugs is reflected in the melting profiles of the ligand-oligomer complexes. Asymmetric association of (CHR)$_2$Mg$^{2+}$ gives rise to two distinct peaks in the melting profile of the decamer. The GpC site, where the (CHR)$_2$Mg$^{2+}$ ligand binds, is stabilized. Due to the rigidity of the ligand, considerable extent of DNA distortion is necessary for association at and around the binding site. This leads to partial unwinding and significant destabilization of the second GpC binding site once the first site is occupied. This is indicated from its melting at a lower $T_m$ as compared to the free duplex (Fig. 3). On the other hand, the melting profile for the (MTR)$_2$Mg$^{2+}$ bound decamer is characterized by a single peak, because the ligand binds symmetrically (Table 3). Asymmetric association of (CHR)$_2$Mg$^{2+}$ is schematically shown in Fig. 4.

Differences in extent of DNA distortion induced by MTR and CHR has also been reported earlier for their interaction with polymeric DNA$^{17}$. The present report succeeds in elucidating the molecular basis of the differential behaviour of the two ligands.

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**References**