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Dedicated to the memory of
Professor Ecaterina Ciorănescu-Nenitzescu (1909–2000)

THE INFLUENCE OF IONIC STRENGTH ON THE BINDING OF ANTITUMOR DRUG ACTINOMYCIN D TO DOUBLE STRANDED DNA

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The interaction of actinomycin D with double stranded DNA has been investigated at different ionic strength and the results were interpreted within the framework of polyelectrolyte theory, dividing the binding free energy into its non-electrostatic and polyelectrolyte contributions. Such a partition provides deep insight into the molecular interactions that stabilize the drug-DNA complex. The results indicate that for uncharged actinomycin D molecule, the electrostatic contribution to the binding free energy is almost negligible (maximum 11%) in comparison with nonelectrostatic contribution. The nonelectrostatic contribution represents the energy contribution from hydrogen bonds, hydrophobic and van der Waals stacking interactions and the results indicate a major role of such interactions in stabilizing the actinomycin D – DNA complex.

INTRODUCTION

A detailed investigation of the DNA binding properties of different compounds with clinical utility is essential for understanding their mode of action, the nature of the interactions and for the rational design of new DNA binding agents with enhanced selective activity. Improved drug binding affinity and the ability to discriminate larger DNA sequences can allow targeting unique sites in the genome.[1]

Actinomycin D is an antitumor drug that contains a 2-aminophenoxazin3-one chromophore and two cyclic pentapeptide lactones (Fig. 1). The drug has been used clinically for the treatment of highly malignant tumors, like Wilms' tumor[2] and gestational choriocarcinoma,[3] and in combination with other antitumor agents to treat high-risk tumors.[4] The biological activity of actinomycin D is believed to be the consequence of its binding to double-stranded DNA (dsDNA), which results in the inhibition of transcription elongation by a blockage of RNA polymerase.[5] It is well known that actinomycin D binds to dsDNA by intercalation of the planar chromophore, preferably at the GpC sequence, with the two pentapeptide rings resting on the minor groove. A model of dsDNA-actinomycin D complex has been generally accepted in which the phenoxazone ring is intercalated between the G·C and C·G base pairs, forming strong hydrogen bonds in the minor groove between the guanine 2-amino groups and the carbonyl oxygen atoms of the L-threonine residues of the cyclic peptides.[6,7] Additional stabilizations are derived from hydrophobic interactions between groups on the peptides and sugar residues, and from other specific weaker hydrogen bonds.[8,9] Also, actinomycin D may also bind strongly via some non-classic modes, such as to single-stranded DNA (ssDNA)[10-13] and to some DNA sequences containing no GC site.[14,15]

The binding of actinomycin D to dsDNA and ssDNA was discussed previously in the frame of MM^+ molecular mechanics and AM1 semiempirical method. Also, these complexes were analyzed using HBexplore program,[16] based on

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geometrical criteria and SHB_interactions program,[17] based on quantum-chemical criteria (Mulliken overlap populations). The results outline the contribution of specific hydrogen bonding as well as C-H···O(N) and other atom-atom intermolecular interactions to the stabilization of the actinomycin D-DNA complexes.[18,19]

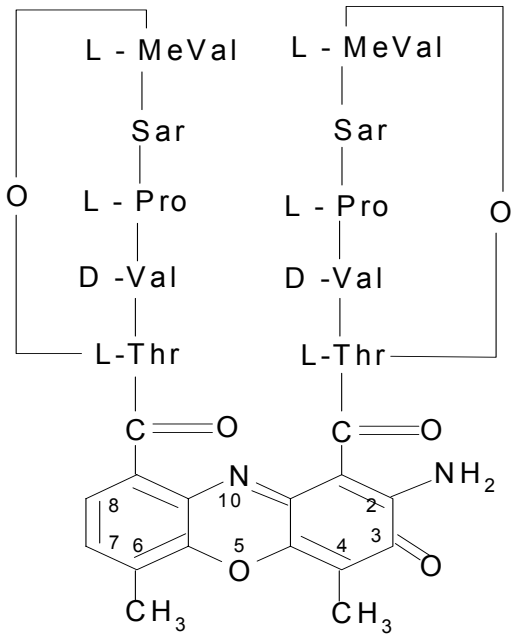

Fig. 1 – The chemical structure of actinomycin D.

In previous absorption and emission experiments, the Scatchard plots for the binding of actinomycin D to dsDNA and ssDNA indicate three different processes: I – a cooperative process at high r values in the range 0.6 – 0.06, corresponding to low values of the ratio between the polymer and drug concentration (P/D), assigned to the external binding of actinomycin D to nucleic acids; II – a non-cooperative process at intermediate r values (0.06 – 0.03) corresponding to the intercalation of the phenoxazone chromophore of the drug between the base pairs of the nucleic acids; III – a cooperative process at low r values (0.03 – 0.018; high P/D ratios) tentatively assigned to the interaction of the actinomycin D-DNA intercalation complexes with another ssDNA or dsDNA sequence.[13,18-21]

Electrostatic interactions play a crucial role in molecular interactions involving DNA, the free energy of the binding of different ligands to DNA depends strongly on salt concentration. Studies of the salt dependency of drug-DNA binding constants provide data that can be interpreted in the framework of polyelectrolyte theory,[22,23] which allows to divide the binding free energy into its non-electrostatic and electrostatic contributions. Such a partition furnishes considerable insight into the molecular interactions that stabilize the drug-DNA complexes.[24]

Therefore, the object of the present paper is to analyse the binding of actinomycin D to double stranded DNA over a range of NaCl concentrations in the frame of the polyelectrolyte theory, to divide the DNA binding free energy into its nonelectrostatic and polyelectrolyte contributions.

MATERIALS AND METHODS

Actinomycin D was purchased from Fluka and calf thymus DNA was obtained from Sigma. All solutions were prepared in sodium phosphate buffer (pH 7.4). The concentrations of the solutions were determined spectrophotometrically by using molar extinction coefficients of ε_{260} = 6 600 $M^{-1}cm^{-1}$ (per nucleotide) for calf thymus DNA and ε_{440} = 24 400 $M^{-1}cm^{-1}$ for actinomycin D.[25] Absorption spectra were measured with an UNICAM-UV 4 HELIOS spectrophotometer. Spectral titrations were carried out at 20 – 25^{0}C in phosphate buffer with NaCl added to give the various concentrations indicated in the text. After each DNA addition, an incubation time of 15 minutes was allowed prior to the absorption measurements, due to the slow rate of interaction.

RESULTS AND DISCUSSION

Intermolecular interactions between DNA (structure with regularly placed negative charges along its length due to the ionisable phosphate groups in the backbone) and charged ligands are sensitive to cation-dependent electrostatic effects. Positive ions are condensed around the polyanionic DNA helix such that they form a mobile "cloud" of charge around the backbone. Binding of a positively charged ligand has the effect of releasing a cation as the charge on the bound ligand provides competing backbone neutralization. Thus, DNA-ligand and DNA-cation binding are thermodynamically linked events. The results of the salt dependency of DNA-ligand binding constant experiments can be interpreted according to the polyelectrolyte theory described by Manning[22] and Record.[23]

Theory predicts that log K shows a linear dependence on log $[M^+]$ as:

$$\frac{\partial \log K}{\partial \log[M^+]} = -Z\psi \qquad (1)$$

where K is the ligand binding constant, ψ is the fraction of monovalent cation associated per DNA phosphate group (ψ = 0.88 for B-DNA), M^+ is the monovalent cation concentration and Z is the charge of the ligand.

The quantity Zψ is evaluated experimentally by measuring the ligand binding constant at different salt concentrations, and this value is used to evaluate the polyelectrolyte contribution (ΔG_{el}) to the binding free energy (ΔG_{obs}) in the relationship:

$$\Delta G_{el} = -Z\psi RT \ln[M^+] \qquad (2)$$

The binding free energy is calculated from the DNA binding constant (K) using the standard Gibbs equation:

$$\Delta G_{obs} = -RT\ln K \qquad (3)$$

The difference between the binding free energy and ΔG_{el} defines the nonelectrostatic contribution (ΔG_{ne}) to the binding free energy:

$$\Delta G_{ne} = \Delta G_{obs} - \Delta G_{el} \qquad (4)$$

For intercalators, there are two contributions to the polyelectrolyte effect that result in cation release, one from the binding of the charged ligand, and a second from the increased phosphate spacing resulting from the structural change in the DNA helix. For uncharged intercalators like actinomycin D, the salt dependence of the binding constant is expected to be mainly due to the second process, i.e. to the cation release arising from change in the phosphate spacing that results from intercalation of the drug.[26,27]

The equilibrium binding isotherms for the interaction of actinomycin D with calf thymus DNA at different ionic strengths are shown in the form of Scatchard plot (Fig. 2) using equation (5):

$$\frac{r}{C_f} = (n - r)K \qquad (5)$$

where r is the molar ratio of bound actinomycin D concentration (C_b) to the total DNA concentration, n – the number of binding sites per nucleotide, C_f – the free actinomycin D concentration and K – the binding constant.

The plots in Fig. 2 evidence three different processes for all investigated ionic strengths, similar with previous results.[13,21] The number of binding sites per nucleotide (n) is in the range 0.08 ÷ 0.11, indicating that actinomycin D molecule by pentapeptide rings covers about 5-6 base pairs, for all investigated ionic strengths. It can be observed that process II, corresponding to the intercalation of the phenoxazone chromophore of the drug between the base pairs of DNA, begins at lower r values as the NaCl concentration increases, probably because at high sodium ion concentration DNA adopts highly compact and bent interwound states[28] and the access of the drug molecule between base pairs of DNA is hindered. Also, the shape of the binding curve and the lower maximum value of r/C_f at higher NaCl concentrations (Fig. 2) indicate an increase of C_f at the same r value (*i.e.* a decrease of C_b), reflecting the restricted access of the drug to the binding sites. The increasing curvature of the binding data as the NaCl concentration decreases suggests that the cooperativity increases as the sodium ion concentration decreases.[29] As DNA flexibility and base pair orientation are affected by sodium ion concentration, this could indicate a role of these parameters in the cooperative binding of actinomycin D to DNA.[22]

The NaCl-dependent changes in binding constants were used, according to the polyelectrolyte theory,[22,23] to calculate the charge of the drug and the salt dependence of the binding constant using equations (1) and (2). The dependence of the binding constant for process II on NaCl concentration is shown in Fig. 3 and the results indicate that the binding constant changes slightly with NaCl concentration. The slope is $\partial \log K / \partial \log[NaCl] = -0.34 \pm 0.03$, which indicates that Z = 0.38, a charge value in agreement with uncharged actinomycin D molecule.

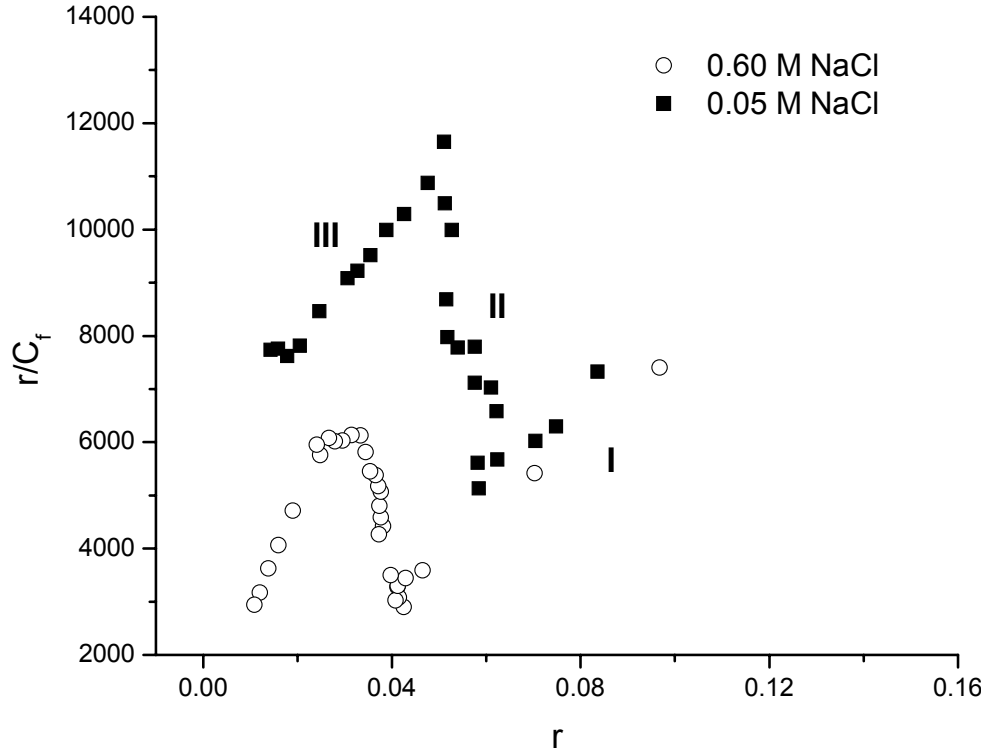

Fig. 2 – Scatchard plot for the binding of actinomycin D to DNA at two different NaCl concentrations, lower (0.05 M) and respectively higher (0.6 M) than the physiological ionic strength, 0.15 M.

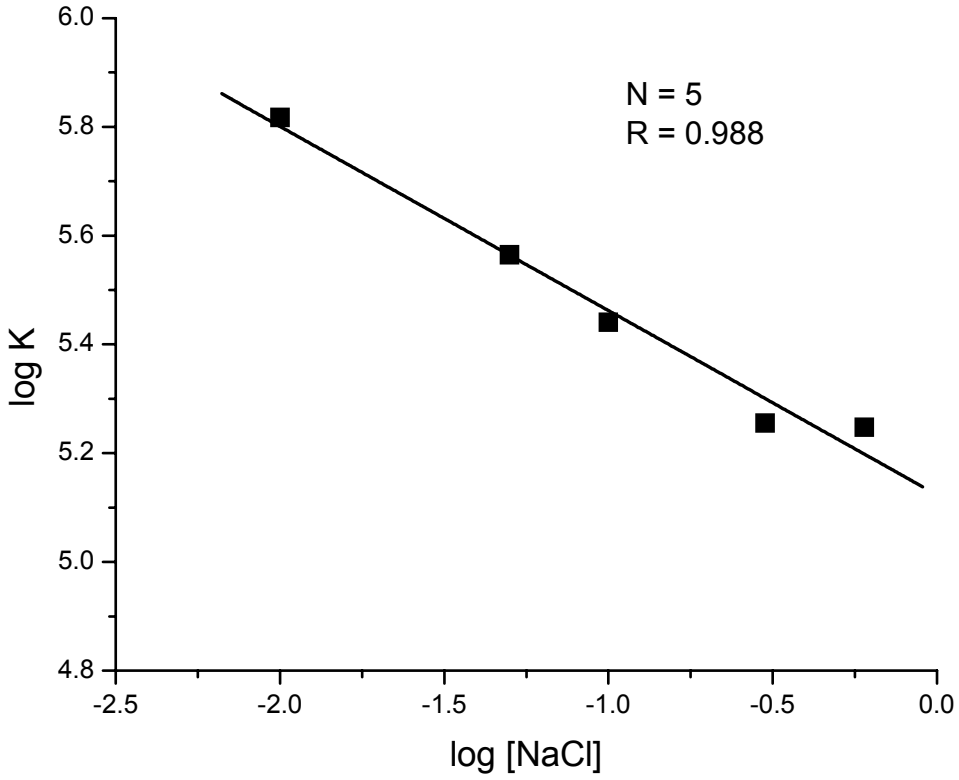

Fig. 3 – NaCl concentration dependency of the binding constant of actinomycin D to DNA for the process II. The linear least squares fit of the data yields to the slope, $\partial \log K / \partial \log\left[M^{+}\right] = -0.34 \pm 0.03$ and intercept = 5.12 ± 0.03.

Even if actinomycin D is an uncharged molecule, the binding constant for process II is slightly salt dependent, especially at lower NaCl concentration. The possibility of electrostatic binding of the drugs outside the helix is minor at the relatively high ionic strength (0.15 M, physiological ionic strength). According to the Manning-Record theory,[22,23] electrostatic interactions, relatively strong at low ionic strength, markedly decrease as the cations concentration increases. In the case of actinomycin D, the reason for this slight salt dependence of the binding constant on the NaCl concentration is that intercalators lengthen and unwind DNA, increasing the phosphate spacing along the helix axis. As a result, the charge density of the duplex decreases, releasing condensed counterions and providing an entropically favourable contribution to the binding free energy.[26]

From the dependence of the binding constant on salt concentration, the observed binding free energy is divided in the nonelectrostatic and polyelectrolyte components using equations (2-4). The results are presented in Table 1.

Table 1

Partition of the binding free energy (ΔG_{obs}) into its polyelectrolyte (ΔG_{el}) and non-polyelectrolyte (ΔG_{ne}) contributions

[NaCl], M	$K^{*}x10^{-5}$, M^{-1}	$-\Delta G_{obs}{}^{*}$, kcal/mol	$-\Delta G_{el}$, kcal/mol	$-\Delta G_{ne}$, kcal/mol
0.6	1.77	7.15	0.09	7.06
0.3	1.80	7.16	0.22	6.94
0.1	2.76	7.42	0.42	7.00
0.05	3.68	7.60	0.55	7.05
0.01	6.57	7.93	0.85	7.08

*The average errors are: K ±0.2 ($x10^5$) M^{-1}, ΔG_{obs} ± 0.05 kcal/mol, ΔG_{el} and ΔG_{ne} ±0.04.

It can be observed that for uncharged actinomycin D molecule, the electrostatic contribution (ΔG_{el}) to the binding free energy is almost negligible (maximum 11%) in comparison with the nonelectrostatic one, and increases with the decrease in NaCl concentration. The nonelectrostatic free energy contribution (ΔG_{ne}) is independent of salt concentration and represents the energy contribution from all other types of interactions, including hydrogen bond, hydrophobic and van der Waals stacking interactions, and the results indicate a major role of such interactions in stabilizing the actinomycin D – DNA complex. These results are in agreement with our previous theoretical analysis of actinomycin D-DNA model complexes using Mulliken overlap populations as quantum-chemical criterion, which have shown that the major contribution (~70%) to the stability of the drug-DNA complexes is due to classical hydrogen bonds interactions in the case of single-stranded DNA and to other atom-atom interactions for double-stranded DNA.[18] The magnitude of ΔG_{ne} for actinomycin D is greater than that found for the simpler intercalators like ethidium, propidium and quinacrine.[30]

CONCLUSIONS

The present paper analysed the influence of the ionic strength on the binding of actinomycin D to double stranded DNA over a range of NaCl concentrations around the physiological value, based on the polyelectrolyte theory. Partition of the DNA binding free energy into its nonelectrostatic and polyelectrolyte contributions was performed. The results indicate that for uncharged actinomycin D molecule, the electrostatic contribution to the binding free energy is almost negligible (maximum 11%) in comparison with nonelectrostatic contribution. The nonelectrostatic contribution for actinomycin D is greater than that found for the simpler intercalators like ethidium. A possible explanation is the structure of actinomycin D with two pentapeptide substituents, this drug being generally considered as a simple model for the protein-DNA interactions. His complex with DNA is stabilized by specific hydrogen bonds in addition to stacking and hydrophobic interactions, whereas the simpler intercalators lack hydrogen bonding interactions with DNA. These data have important implications for further rational design of drug analogues that should lead to DNA-drug complexes with a larger binding free energy.

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