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The Influence of Solutes on the Enthalpy/Entropy Change of the Actinomycin D Binding to DNA: Hydration, Energy Compensation and Long-Range Deformation on DNA

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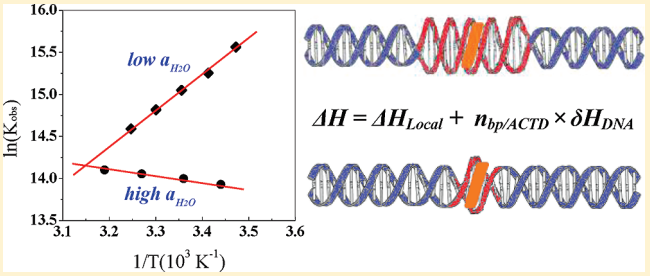
ABSTRACT: The effects of the changes in the temperature and in the water chemical potential on the energetic of the actinomycin D (ACTD) interaction with natural DNA are studied. At reduced water chemical potential, induced by the addition of neutral solute (sucrose), the ACTD-to-DNA binding isotherms show that the drug accesses two types of binding sites: strong and weak. The binding constants to the stronger sites are sensitive to changes in the temperature and in the water chemical potential, while the weak sites are practically insensitive to these changes. The van't Hoff analyses of the binding in different water chemical potential shows that the binding process to the more specific sites is endothermic in phosphate buffer ($\Delta H_{vH} \sim 1$ kcal/mol) and becomes exothermic when the water chemical potential decreases ($\Delta H_{vH} = -11$ kcal/mol in sucrose 30%). The number of water molecules released on the binding to the stronger sites, obtained from the slopes of linkage plots in different temperatures, increases with the decrease in the temperature. Ring closure reactions in the presence of neutral solutes have shown that the reduction in the water activity induces DNA unwinding. It was observed that both reduced water chemical potential and small ratios of daunomycin bound per base pairs have the same effects on the ACTD binding isotherms and consequently on the binding thermodynamic parameters. The results presented indicate that the ACTD binding to the recognition site is enthalpycally unfavorable, which should be compensated by the deformation in the DNA. This compensation would probably be the origin of the synergism observed for these two drugs.



INTRODUCTION

The anticancer drug actinomycin D (ACTD) interrupts RNA synthesis at very high DNA/drug ratios. Because of this biological action, this drug has been used as a potent antitumor agent in the treatment of some highly malignant tumors. Physical chemical and structural studies of ACTD binding to DNA have supported a complex binding mode: intercalation of its phenoxazone rings between two adjacent base pairs and insertion of its two cyclic pentapeptide chains into the DNA minor groove. These studies have revealed that the binding process of the ACTD is not common to other intercalators. Drug specificity, binding energetics, and gross structural changes in the host duplex are characteristics that seem to be unique for the ACTD binding.

The molecular recognition of DNA sequences by ACTD involves at least four bases with preference for the central 5′GC3′ base pair. Takusagawa et al.[1] showed that ACTD did not form the intercalated structure with $d(GC)_2$. These results suggest that, besides the requirement for the central 5′GC3′, the flanking base pairs are very important in the molecular recognition process. The sequence preference of the ACTD is TGCA > CGCG > AGCT ≫ GGCC, as revealed by Chen et al., Bailey et al. and Goodisman et al.[2−5]

Structures of ACTD−oligonucleotides complexes, with high resolution, have shown that the molecular recognition process involves van der Waals contact and hydrogen bonds.[6−8] The lactone threonines and the guanine amino groups are hydrogen bonded, and they could be responsible for the requirement of guanine in the central pair. These studies also revealed that the central bases in the intercalation site are unstacked and unwound. The helix is wound asymmetrically at the binding site: loosened at one end and tightly wound at the other end. The minor groove is distorted, and this perturbation spreads out through the whole oligonucleotide. This asymmetrical distortion favors tight van der Waals contacts of the lateral chains in the minor groove.[6,7] Nuclease digestion experiments of the complex ACTD oligonucleotides indicate also that the distortion on the double helix extends beyond the four base pairs that are in physical contact with the drug.[9,10]

The binding of the ACTD to the DNA is enthalpically unfavorable. The intercalation process was observed to be endothermic. The enthalpy values measured are slightly positive

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($\Delta H \sim 1$ kcal/mol).[11,12] Otherwise, the binding of other intercalators is in general enthalpically driven ($\Delta H \sim -9$ kcal/mol). Calorimetric measurements by Quadrifoglio et al. (1976)[13] compared the binding enthalpies of ACTD and its analogue actinomine. The absence of the cyclic pentapeptide side chains rendered the binding of the actinomine enthalpically favorable. These results suggested that the origin of the entropically driven process, observed in the ACTD to DNA binding, is the interaction of the drug pentapeptide side chain with the bases in the minor groove. These studies also suggested that the driving force in the binding process is the release of water molecules in the contacts with the minor groove.

Aiming to estimate the differential number of water molecules released from the drug and the DNA upon complexation and its effect on the drug affinity, we have studied the ACTD binding to DNA in the presence of neutral solutes.[14] The neutral solutes: ethylene glycol, glycerol, betaine, and sucrose were added to adjust the water activity of the solution reaction. The binding isotherms obtained, at reduced water activity, are biphasic, suggesting the presence of two different classes of binding sites. In pure phosphate, buffer the binding isotherms evidence the existence of only one class of binding site. These studies have revealed also that the stronger sites are very sensitive to the reduction of the water activity. Both the binding constants to the stronger sites and the number of base pairs apparently involved in the binding to these sites increase with the reduction in the water activity. For the stronger sites, a linear dependence of the drug binding free energy on the water chemical potential was observed. The analysis of this dependence through a linkage relation ($\partial\Delta G/\partial\Delta\mu_w$) provided the differential number of water molecules released on the binding. It was also observed that the number of base pairs involved in the binding, or disturbed by the bound drug to the stronger sites, is linearly dependent on the water chemical potential. In this way, changes in the binding free energy were linearly correlated with the increase in the number of base pairs disturbed by the bound drug to the stronger sites. As the binding becomes more favorable, the number of base pairs involved in the binding becomes larger.

Our working hypothesis to explain the coupling of the binding free energy and the number of base pairs disturbed by the bound drug is that, on its binding, the deformation applied by the ACTD on the base pairs adjacent to the binding site should be transmitted over several base pairs. The resistance to this deformation by the flanking base pairs is an energetic penalty for the binding. The reduction of the water activity decreases this energetic barrier to the drug binding. This decrease in the energetic barrier is probably related to the effect of dehydration on the DNA elastic properties. The reduction of the water activity has been shown to unwind the double helix (Lee et al. 1981 and Ruggiero Neto et al. 2001).[15,16] The twist energetic parameter also decreases on DNA dehydration.[16] These effects of dehydration would decrease the resistance to the transmission of the deformation imposed by the bound drug over several base pairs. We have proposed that the release of water molecules from the DNA induces an intermediate conformation on the double helix. This long-range conformation would regulate the ACTD access to the stronger sites.[14] On the basis of this working hypothesis, the linkage between the binding free energy and the number of base pairs perturbed by the bound drug have allowed decomposing the changes in the binding free energy in two components. One component that is a local contribution and takes into account the binding free energy to the recognition sequence. The second one is a nonlocal contribution. This term represents the free energy associated with the transmission of the distortion imposed by the bound drug through the base pairs flanking the recognition tetranucleotide.

In this work, we present a study of the effect of the reduction of the water chemical potential on the energetics of the ACTD binding to natural DNA. In this study we have used sucrose to adjust the water activity. The main purpose was to correlate the changes in the thermodynamic parameters of the ACTD binding to higher specific sites with both the number of water molecules released and the DNA deformation imposed by the binding.

MATERIALS AND METHODS

ACTD from Calbiochem was used without any further purification, as well as sonicated and phenol extracted calf thymus DNA from Pharmacia. Sucrose used as neutral cosolvent was analytical grade. Both DNA and drug were prepared in 10 mM phosphate buffer pH7.5, containing 150 mM NaCl and 1 mM EDTA. Different concentrations of sucrose were added to the buffer in the drug titrations.

ACTD binding to DNA, in the presence and absence of sucrose, has been studied by spectrophotometric titration. Small aliquots of drug were added to a DNA solution, in a cuvette of high optical path length (10 cm). In very low drug/DNA ratio, the absorption signal was measured by very high optical path length (32 or 46 cm) using a liquid core waveguide device.[17] Drug absorbance at 440 nm was measured 30−60 min after each drug addition depending on the experiment temperature. The actinomycin concentration was determined using a molar extinction coefficient $\varepsilon_F = 24800\ M^{-1}\ cm^{-1}$ at 440 nm. The DNA solutions in the presence or absence of sucrose were filtered two or three times through a Millipore 0.25 μm filter, before determining DNA concentration, which was ~40 μM. Aggregation was not observed after this procedure. The extinction coefficient of the bound drug was obtained by extrapolation of the drug absorption in the presence of a large excess of binding sites to an infinite amount of DNA, and the value obtained was $\varepsilon_B = 11900\ M^{-1}\ cm^{-1}$ both in the presence and in the absence of the sucrose. The extinction coefficient of free ACTD was found to be the same in the presence as in the absence of sucrose in the buffer. The total ACTD concentration ($[ACTD]_T$) used in these titrations were maintained less than 5 μM to avoid drug aggregation. With this procedure, free and bound drug concentrations were obtained, neglecting any drug associative process. The bound drug concentrations, $[ACTD]_B$, were determined as in Crothers and Miller:[18]

$$[ACTD]_B = \frac{\left([ACTD]_T \times \varepsilon_F - \frac{A_i}{\ell}\right)}{\varepsilon_F - \varepsilon_B} \qquad (1)$$

where A_i is the optical density in 440 nm at the ith actinomycin addition and ℓ is the optical pathlength used to measure A_i. The free drug concentrations $[ACTD]_F$ were given by

$$[ACTD]_F = [ACTD]_T - [ACTD]_B \qquad (2)$$

The binding data were analyzed through Scatchard plots: $r/[ACTD]_F$ versus r (= $[ACTD]_B/[DNA]$), where [ACTD] is the ACTD concentration and F and B subscripts refer to free and bound drug. The binding constants were obtained from the slope of these plots when the binding isotherm is monophasic. Alternatively, biphasic isotherms were analyzed by nonlinear

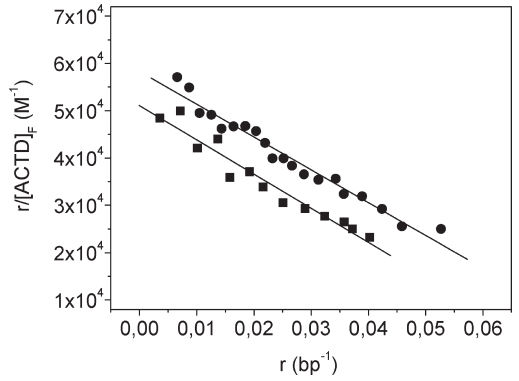

Figure 1. Scatchard plots of ACTD-to-DNA binding in 10 mM phosphate buffer, 150 mM NaCl, and 0.1 mM EDTA, pH 7.5. (■) 15 °C and (●) 25 °C.

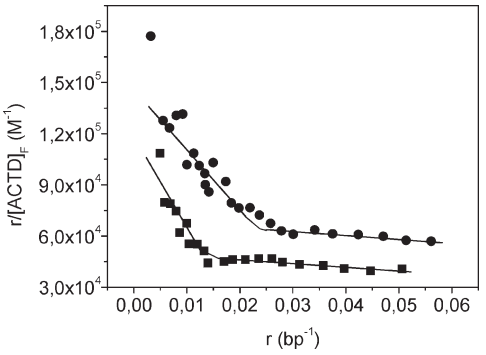

Figure 2. Scatchard plots of ACTD-to-DNA binding in 10 mM phosphate buffer, 150 mM NaCl ,and 0.1 mM EDTA, pH 7.5, in the presence of 23% (w/v) sucrose. (■) 15 °C and (●) 25 °C.

least-squares fitting, using a two independent binding-site model:

$$\frac{r}{[ACTD]_F} = \frac{K_1 n_1}{1 + K_1[ACTD]_F} + \frac{K_2 n_2}{1 + K_2[ACTD]_F} \quad (3)$$

where r is the ratio of bound drug per base pair, K_1 and K_2 are the binding constants, and n_1 and n_2 are the number of base pairs occupied upon drug binding.

The binding of the actinomycin to the DNA was studied in the presence of daunomycin. In these titrations, both cuvettes (sample and reference) initially contained DNA and a small ratio of daunomycin bound per base pair. Each volume of ACTD added to the sample cuvette was followed by the addition of the same amount of phosphate buffer in the reference cuvette, and the difference spectrum was registered after 30 min of each addition. These ratios (ν) were calculated with the values of the binding constants determined by Xodo et al.[19] In the calculations of the binding data, the possible displacement of bound daunomycin by the binding of ACTD was not considered. The binding isotherms were analyzed using the two binding site model.

The binding enthalpies in the presence and absence of sucrose were determined from the binding isotherms in different temperatures (10, 18, 25, 33, and 40 °C). The binding constants were displayed as van't Hoff plots d ln(K) versus $1/T$ and the enthalpy values were determined from the slope of these plots:

$$d\ ln\ K_{obs}/d(1/T) = -\Delta H/R \quad (4)$$

where R is the gas constant. The entropy values at 25 °C were obtained from the difference between Gibbs free energy change at this temperature and the van'tHoff enthalpy:

$$\Delta S = -(\Delta G - \Delta H)/T \quad (5)$$

RESULTS AND DISCUSSION

Representative Scatchard isotherms of ACTD to calf thymus DNA in two different temperatures in the absence and in the presence of sucrose 23% (w/v) are shown in Figures 1 and 2, respectively. In the absence of sucrose, the linear dependence of $r/[ACTD]_F$ versus r observed at any temperature probed indicates that ACTD binds to a single class of binding site. The apparent association constant, given by the slopes of the Scatchard plots (Figure 1), increases slightly with the increase in the temperature. This reveals that the binding of ACTD to DNA in pure phosphate buffer is endothermic. The van't Hoff analyses of these data result in a slightly positive heat change, $\Delta H \sim 1$ kcal/mol. This enthalpy value is in good agreement with those determined by Quadrifoglio et al. and Marky et al.[11,12] The sequence of calf thymus DNA helix carries a large number of ACTD binding sequences. Studies using short synthetic DNA oligomers have shown that ACTD makes direct interactions with a tetrameric helix sequence with a preference for central 5′G-C3′ (2−5). The calf thymus sequence contains 42 mol % GC, and there are 16 different 5′XGCY3′ sequences, where X and Y represent the four different nucleotides. Footprinting experiments have shown that ActD may bind to 14 different sequences in the 139 bp fragment of pBR322, with binding constants distributed between 1×10^5 to 6×10^6 M^{-1}.[27] Despite that, Figure 1 shows that the interaction of ACTD with calf thymus DNA is well described by a single macroscopic constant with a value of about $(9 \pm 1) \times 10^5$. This value is within the range measured by footprinting. The linear Scatchard plot suggests that the ACTD binds independently to different site sequences. The lack of discrimination between ACTD binding to a weaker and to a stronger affinity site sequence, as observed in buffer without sucrose (Figure 1), may be a consequence of the large number of sites with affinities whose values are fairly close to each other, as indicated by footprinting experiments.[27] However, changes in solution conditions such as increase in Na^+ concentration[18] or the presence of osmolytes,[16,17] favor the discrimination of ACTD binding to weak and strong sites in natural DNA, as well as change the energetics of binding (see below).

In the presence of sucrose, otherwise, the thermal properties of the drug interaction change dramatically. Isotherms obtained in the presence of 23% (w/v) sucrose (shown in the Figure 2) at different temperatures are biphasic. This behavior evidences the binding to two classes of different sites: strong and weak. Inspection of the Scatchard plots shows that the binding constant to the strong binding sites, at lower r values, decreases with the increase of temperature. These results suggest that, in the presence of sucrose, the binding of ACTD to the stronger sites is exothermic. We also note that the binding affinity of ACTD to the weak sites on DNA (higher r-values) decreases either with added sucrose or increase in the temperature. The analysis of the binding isotherms through nonlinear least-squares fitting using two independent binding sites (eq 3) confirm these qualitative conclusions. Therefore, the decrease in the sucrose concentration and the increase in the temperature induce opposite changes on the binding characteristics of ACTD to the strong in relation to the weak binding sites on DNA. However, the interaction of

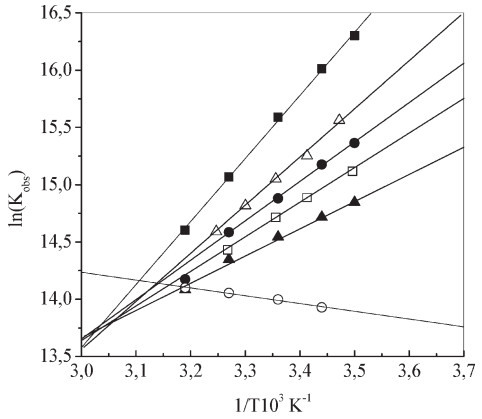

Figure 3. van't Hoff plots: $\ln(K_{strong})$ versus $1/T$ for the actinomycin binding to the DNA in phosphate buffer 10 mM, NaCl 150 mM, and EDTA 0.1 mM (○) and in the presence of 7% (▲), 10% (□), 15% (●), 23% (△) and 30% (■) (w/v) sucrose. The binding constant values K_{strong} were obtained from the nonlinear least-squares fitting of the Scatchard plots using the two binding site model.

Table 1. van't Hoff Enthalpy for Actinomycin Binding to DNA under Different Conditions

condition	ln (a_w)	a_w	ΔH (kcal/mol)
no sucrose	0	1	1.3 (±0.1)
7% sucrose	−0.0041	0.99596	−4.2 (±0.4)
10% sucrose	−0.0061	0.99397	−5.6 (±0.5)
15% sucrose	−0.0098	0.99025	−7.2 (±0.1)
20% sucrose	−0.0142	0.98590	−8.2 (±0.3)
23% sucrose	−0.0175	0.98265	−8.4 (±0.3)
30% sucrose	−0.0256	0.97472	−11.0 (±0.4)

ACTD with the strong binding site is the most affected by changes in temperature and solution conditions. Thus, hereafter, we will focus our analysis on the effect of sucrose and temperature on the binding parameters related to the stronger sites.

The changes in the binding constants with the temperature, measured in the absence and in the presence of 0, 7, 10, 15, 23 and 30% (w/v) sucrose are displayed as the van't Hoff plots shown in Figure 3. The van't Hoff enthapies, ΔH_{vH}, determined from the slope of these plots, are tabulated in Table 1. As anticipated by the isotherms shown in Figures 1 and 2, the reduction in the water activity reversed the thermal character of the drug binding. In the presence of sucrose, the binding to the specific sites changes from endothermic to exothermic. While in the absence of sucrose ΔH_{vH} is about +1 kcal/mol, in the presence of 30% sucrose ΔH_{vH} reaches the value of −11 kcal/mol. Despite the significant changes observed in the binding enthalpy (∼−12 kcal/mol), only a slight change is observed in the free energy (∼1 kcal/mol), indicating that the enthalpy change is compensated by an entropic contribution.

The average values of the free energy, enthalpy, and entropy changes associated with ACTD binding to the stronger DNA sites, determined at 25 °C and different sucrose concentrations, are given in Table 2. The experimental method used does not inform which sequences are accessed. Bailey et al. and Snyder et al.[4,20] obtained the enthalpy and entropy values for the ACTD binding to the specific sequences. They showed that the binding

Table 2. Thermodynamic Results for Actinomycin Binding to DNA under Different Conditions

condition	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (e.u.)
phosphate buffer	−8.3 (±0.9)	1.3 (±0.1)	32.5 (±0.9)
7% sucrose	−8.6 (±0.7)	−4.2 (±0.4)	14.9 (±0.8)
10% sucrose	−8.7 (±1.3)	−5.6 (±0.5)	10.2 (±1.4)
15% sucrose	−8.8 (±1.1)	−7.2 (±0.1)	5.4 (±1.1)
20% sucrose	−8.9 (±1.6)	−8.2 (±0.3)	2.5 (±1.6)
23% sucrose	−9.0 (±1.6)	−8.4 (±0.3)	−2.1 (±6.4)
30% sucrose	−9.2 (±1.4)	−11.0 (±0.4)	−6.0 (±1.5)
0.015 daunomycin/pb	−8.5 (±0.7)	−2.8 (±0.9)	19.0 (±1.1)
0.030 daunomycin/pb	−8.8 (±1.0)	−6.7 (±1.5)	7.1 (±1.8)

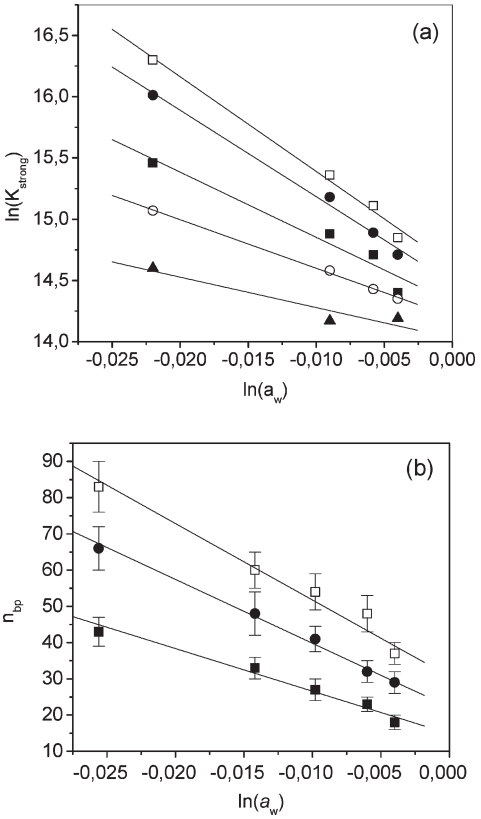

Figure 4. (a) Linkage plots $\ln(K_{strong})$ versus $\ln(a_w)$ at 13 °C (□), 17 °C (●), 25 °C (■), 33 °C (○), and 40 °C (▲).The binding constant values K_{strong} were obtained from the nonlinear least-squares fitting of the Scatchard plots using a two binding site model. $\ln(a_w) = -Osm/55.5$, where Osm is the solution osmolarity. (b) Number of base pairs involved in the binding (n_{pb}) versus $\ln(a_w)$ at 13 °C (□), 17 °C (●), 25 °C (■).

to these sequences is characterized by a small gain in enthalpy or in some cases it was observed to be an endothermic process. In the case of specific sequences as well as the values obtained in the present work, the small gain in binding energy is complemented by a large positive entropy change. In the presence of sucrose, the changes in enthalpy and entropy seems to be compensated.

The dependence of binding free energy on the water chemical potential at different temperatures is emphasized in the plots: $\ln(K)$ on $\ln(a_w)$ shown in Figure 4a. As can be seen, at each temperature, the binding affinity decreases linearly with $\ln(a_w)$.

These linkage plots[21,22] show how changes in water activity modulate the binding free energy via the difference in hydration, Δn_w, between the free reactants and the complex.[22] The linkage equation that accounts for this hydration change is given by

$$\frac{d\ ln(K)}{d\ ln(a_w)} = \Delta n_w = (n_w^{complex} - n_w^{ACTD} - n_w^{DNA}) \qquad (6)$$

The slope of $\ln(K)$ versus $\ln(a_w)$ and, therefore, the differential number of water molecules released per bound drug, decreases with the increase of temperature. At 13 °C, for instance, the number of water molecules released per bound drug, Δn_w, is 64 ± 4 and at 25 °C 39 ± 2, suggesting that the higher the affinity of the site accessed, the larger the number of water molecules released. These results suggest that the thermal effect induced by the reduction of the water activity has a strong contribution due to the transference of Δn_w water molecules to the bulk solution.

The number of base pairs apparently involved in the binding to the stronger sites is also dependent on both the water activity and on temperature, as can be seen in Figure 4b. The number of base pairs increases with the decrease in the water chemical potential. This increase is higher for lower temperatures. The effect of the changes in the water chemical potential observed on both the drug binding free energy and on the number of bases involved in the binding (n_{bp}) suggest a parallel increase in both Δn_w and n_{bp}. The increase in the number of water molecules released allows higher binding levels, excluding a larger number of base pairs. As a consequence of the linear correlation observed for both $\ln(K)$ and n_{pb} with the water chemical potential, a linear dependence between these two parameters would be expected. In a previous study we have observed, in fact, a linear dependence of the binding free energy on the number of base pairs excluded at each temperature. We have interpreted the linear correlation between these two parameters as a consequence of the deformation imposed in the double helix by the bound drug.[14] In that work, we proposed that the drug binding free energy could be decomposed into two contributions. A local contribution (ΔG_{local}) due to the binding to the recognition sequence and a nonlocal contribution (δG_{DNA}) due to the propagation of the deformation through the sequences flanking the recognition site.

Effects induced in the double helix by the addition of sucrose can be better understood through experiments of ring closure reactions with T4 DNA ligase of linearized plasmids (pUC18) in the presence of sucrose.[16] These experiments revealed that this reaction generates more negatively supercoilled plasmids compared with the topoisomers generated in the ligase buffer alone. The values of the twist angles obtained from these experiments indicate that the lower the water activity, the more underwound are the topoisomers generated. The changes in the DNA torsional energy due to the reduction in the water activity, obtained with the addition of sucrose, are also linearly dependent on the variations in the water chemical potential.[16] Consequently, when one compare the results of these two independent experiments (ACTD titrations and ring closure reaction), both in the presence of sucrose, one can observe a linear dependence between the changes in the drug binding free energy and the DNA torsional energy.[16]

These results suggest that the increase in the drug affinity to the stronger sites are modulated by changes in both the base pair twist angle (and consequently in the DNA torsional free energy)

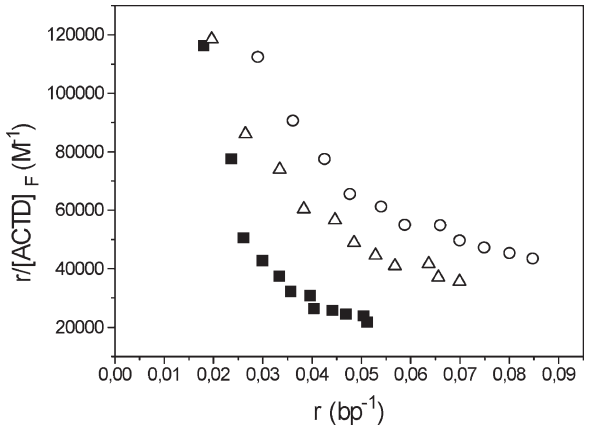

Figure 5. Scatchard plots of the ACTD interaction with DNA in 10 mM phosphate buffer, 150 mM NaCl, and 0.1 mM EDTA, pH 7.5, in the presence of r = 0.03 daunomycin bound per base pair at 16 °C (■), 20 °C (△) and 25 °C (○).

and water release. Our hypothesis is that the release of water molecules from the DNA would induce a structural change in the double helix, and would act as an allosteric regulation for the drug binding.[14] This new secondary structure would be unwound, with a wider minor groove, when compared with the B conformation. One way to test this hypothesis is by inducing double helix unwinding by another factor and by measuring the changes in the binding levels of actinomycin. This hypothesis was tested through ACTD-to-DNA titrations, in different temperatures and in the presence of small concentrations of daunomycin. In these titrations daunomycin was previously bound to the DNA. The daunomycin is known to untwist the double helix by 11°.[23] When ν daunomycin molecules are bound per base pair, the untwist angle between two adjacent base pairs is given by $\Delta\varphi = \nu \times 11°$. In these titrations, we choose two different ν values so that $\Delta\varphi$ due to the daunomycin will be in the range of untwist angles observed from the ring closure experiments in the presence of sucrose. The $\Delta\varphi$ values obtained from the circularization reaction of pUC18 in the presence of sucrose ranged from ~0.1 to 0.37.[16] The concentrations of daunomycin chosen were such that $\Delta\varphi$ was 0.165 and 0.33.

The titrations of ACTD in the presence of daunomycin were carried out in the absence of neutral solutes, so that the changes in the DNA twist angle were uniquely due to the daunomycin intercalation. The fractions of bound daunomycin were estimated using the values of the binding constants and enthalpy determined by Xodo et al.[19] The Scatchard isotherms for the ACTD obtained in these conditions, and in different temperatures (Figure 5), also showed the presence of two binding sites, as observed in the presence of neutral solutes. Both the binding constants to the stronger sites and the number of base pairs excluded by the bound drug are dependent on the temperature, as observed in the presence of sucrose. The binding constants to the stronger sites and the number of base pairs increase with the decrease in the temperature. The weaker sites, however, showed affinities that are practically constant with the temperature. The values of the binding constant of actinomycin to the stronger sites, obtained at $\nu \sim 0.03$ and $\nu \sim 0.015$ of daunomycin bound per base pair (or $\Delta\varphi \sim 0.33$ and $\Delta\varphi \sim 0.16$) are in the same range of those obtained in the presence of sucrose. The increase in the ACTD access to the stronger sites observed in the presence of small fractions of daunomycin bound to DNA are in accord

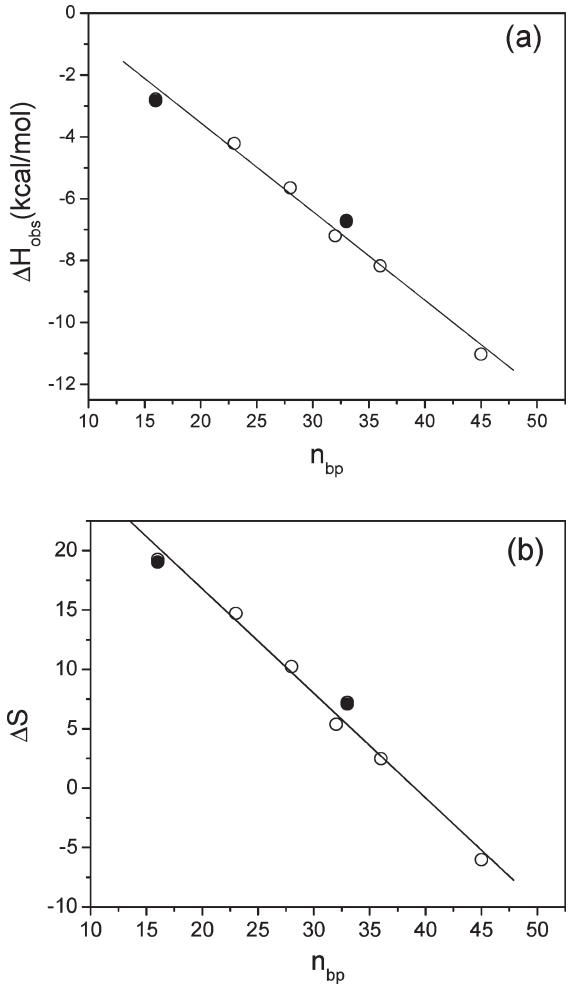

Figure 6. (a) Plot of the van't Hoff binding enthalpy (ΔH_{vH}) as a function of the average number of base pairs disturbed by the ACTD binding. (b) Plot of the calculated entropy change obtained from the difference between free energy at 298 K and the van't Hoff enthalpy. Open circle ACTD binding in the presence of different amounts of sucrose, extracted from Figure 3. Solid circle ACTD binding in the presence of two different concentrations of daunomycin (0.15 and 0.3) bound per base pair.

with the footprinting results reported by Ridge et al.[24] Analyzing the 7-azido-ACTD binding to DNA fragments in the presence of daunomycin, these authors have concluded that the structural deformations of the double helix induced by daunomycin were responsible for the changes in ACTD specificity.

At this point it is important to reinforce that there is good evidence that sucrose does not bind either to DNA or to the drug. If there were sucrose bound to DNA, it would compete with the ACTD, probably decreasing the drug affinity to the DNA. We observed the opposite effect: in the presence of sucrose, ACTD reaches higher binding levels. Thermal denaturation experiments of natural and synthetic DNA provide more evidence for the absence of sucrose binding to DNA.[25] These authors observed a universal effect of the water activity on the inverse of the melting temperature, independent of the type of the neutral solute used to adjust water activity, suggesting that these neutral solutes do not bind to DNA. In this way, although sucrose and daunomycin have the same effect on the ACTD to DNA binding, the origins of these effects are different. Sucrose induces water release from DNA and consequently unwinds the double helix. Daunomycin intercalates between two adjacent base pairs and also unwinds the DNA. The energetic barrier for the actinomycin binding is reduced when the double helix is unwound.

The van't Hoff analysis of the ACTD binding in the presence of daunomycin reveals that the binding process to the stronger sites is exothermic, similar to that observed in the presence of sucrose. The binding process to the weaker sites, in the presence of daunomycin, involves only a small enthalpy change ($\Delta H_{vH} \sim 0$), suggesting that the binding to the stronger sites is modulated by the release of water molecules and DNA unwinding. The comparison of the enthalpy values obtained in the presence of sucrose or of daunomycin using the base pair untwist angle is not simple. We have not checked whether the fraction of daunomycin bound to DNA has changed in the course of the ACTD titrations. The base untwist angle, consequently, could be changed during the titration due to the competition between these drugs. In this way, we have used the average number of base pairs involved or perturbed in the ACTD binding to compare the van't Hoff enthalpies obtained in the two conditions. Each van't Hoff plot involves five different values of the number of base pairs perturbed in the binding. We have considered the arithmetic average value of these numbers ($\langle n_{bp} \rangle$) to construct the plot ΔH_{vH} versus $\langle n_{bp} \rangle$. Figure 6a shows the comparison of the van't Hoff enthalpies of the actinomycin binding to the DNA in the presence of sucrose and in the two ratios of daunomycin previously bound to DNA. The changes in the van't Hoff enthalpy obtained in these two conditions are well correlated with the average number of base pairs excluded, and all the values could be fitted by a unique straight line. Although we have used only two daunomycin concentrations, we observed that these values were very representatives. The concentrations of daunomycin used were such that the untwist angles $\Delta\varphi$ (0.16 and 0.33) represent the almost complete range of $\Delta\varphi$ obtained from the circularization of pUC18 in the presence of sucrose (0.1 and 0.37). The same could be said about the number of base pairs perturbed by the ACTD in the presence of these two concentrations of daunomycin.

Linearity observed in the plot of Figure 6 can be analyzed by taking in account the local and nonlocal contributions to the enthalpy as proposed previously Ruggiero Neto and Colombo.[14] The local contribution accounts for the contact of the actinomycin with the recognition sequence, while the nonlocal for the deformation in the double helix. The linear regression of this plot provides the values of the local and nonlocal contributions. We have obtained a positive value for the local contribution of the enthalpy $\Delta H_{local} = +2190 \pm 520$ cal/mol. This result suggests that the drug contact with the DNA is unfavorable. On the other hand, the value obtained for the nonlocal contribution is negative: $\delta H_{DNA} = -286 \pm 16$ cal/mol./bp. This result indicates that this term contributes to make the process exothermic. In this way, the presence of sucrose or daunomycin helps the propagation of the deformation through the double helix contributing to make the binding process enthalpically favorable.

In a similar way, one can estimate the rates of the changes in the entropy with the double helical deformation. In this analysis, the entropy values were calculated using the free energy obtained at 25 °C in the different sucrose and daunomycin concentrations. In Figure 6b, the values of the changes in the entropy for the binding of actinomycin to DNA in sucrose and in daunomycin is plotted as a function of the number of base pairs involved in the complex. The linear dependence of the entropy changes with the

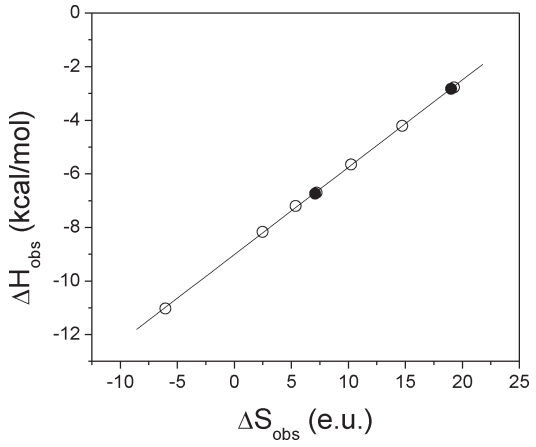

Figure 7. Compensation plot. Enthalpy versus entropy changes for the ACTD binding to DNA. Open circles: ACTD binding in the presence of different amounts of sucrose, extracted from Figure 3. Solid circles: ACTD binding in the presence of two different concentrations of daunomycin (0.15 and 0.3) bound per base pair.

extension of the binding allows a similar analysis used in the case of enthalpy. The local entropy change determined from the linear regression is $\Delta S_{local} = 34.4 \pm 1.7$ e.u., while the entropy contribution to the propagation of the deformation is $\delta S_{DNA} = -0.88 \pm 0.05$ e.u./bp. Taking into account the local and nonlocal changes in the enthalpy and entropy, one can estimate the corresponding free energy changes at 25 °C.

The local and nonlocal free energy changes can be estimated from the enthalpy and entropy changes. The values obtained for local free energy change are -8060 ± 100 cal/mol. The free energy change for the propagation of the deformation along the base sequences flanking the recognition site is 24 ± 3 cal/mol.bp. These values are nearly the same as those obtained previously for the actinomycin binding to DNA in the presence of different neutral solutes. These results indicate that the addition of neutral solutes, not only sucrose, to the binding reaction present the same effect on the ACTD affinity as the previous intercalation of daunomycin to the DNA.

It is noteworthy that despite the great changes observed in the van't Hoff enthalpy and in the entropy due to the reduction in the water chemical potential, the free energy values present only a small change. The van't Hoff enthalpy changes around 12 kcal/mol when the reacting molecules are transferred from the buffer to a solution containing buffer plus sucrose 30%. Despite this great change in the van't Hoff enthalpy, the free energy value decreased only 0.8 kcal/mol in this transference. This behavior suggests that the changes in the enthalpy and in the entropy have been compensated during the transference process. In fact, the linear dependence of both the enthalpy and the entropy with the number of base pairs deformed by the bound actinomycin points to a linear dependence between them. In Figure 7, the van't Hoff enthalpy values are plotted against the entropy for the binding in sucrose and in the presence of daunomycin. This plot shows that the gain in the enthalpy, due to the reduction in the water activity, is followed by a corresponding decrease in the entropy. The slope of this plot provides the temperature where the complete enthalpy−entropy compensation occurs. The temperature obtained is 325.7 K and is beyond that used as the reference to calculate the entropy changes (298 K). One would expect that if the enthalpy−entropy compensation were complete, then both temperatures would be the same. This is not occurring probably because the free energy is not maintained constant in the course of the transference process.

CONCLUSIONS

The factors that modulate the molecular recognition to DNA sequences could be classified as intrinsic and extrinsic. The intrinsic factors would account for the physical contacts between the ligand and the DNA. They are related to the short and long-range forces that appear during the complex formation. The extrinsic ones are related to the solution conditions such as the presence of ions, the amount of water, temperature, and so on modulating the binding. In the case of the actinomycin, the base sequences flanking the recognition site plays also an important role in its binding to DNA. The importance of these sequences came from different experimental approaches.

The results present in this paper provide strong evidence that both the entropic contribution to transfer water molecules from drug and DNA to the bulk and the DNA torsion energy modulate the recognition to specific sequences. The access to the stronger binding sites at very low drug/DNA ratios are in excellent accord with the results obtained by DNase I footprinting of the ACTD complex with DNA fragments used in kinetic experiments[26] and drug titrations.[27]

This is a strong evidence that the reversion observed in the binding enthalpy is due to the release of water molecules. These results reinforce the importance of the contacts of the drug pentapeptide chains and the bases in the minor groove in the recognition of the specific sequences as predicted by the microcalorimetric measurements Quadrifoglio et al.[13] and observed in the crystallographic and NMR structures of the complex of ACTD with oligonucleotides.[6−8]

The effect of the changes in the base twist angle, induced by DNA dehydration, in the drug binding energetics is similar to that observed in the titrations in the presence of small ratios of daunomycin bound to DNA. For a given $\Delta\varphi$ value, the reduction observed in the binding enthalpy and consequently in the binding energy is independent whether the $\Delta\varphi$ is induced by reduction in the water activity or by a small fraction of daunomycin bound. The alterations in the binding energy barrier would make some sequences favorable, while others would be unfavorable, so that sites accessed would be n_{bp} distant from each other. These results seems to stress proposition that the energy barrier for the ACTD access to the specific sites are modulated by water release and torsional perturbation of the double helix.

Although the present results were obtained at the equilibrium, they appear to be in accord with the shuffling hypothesis proposed by Waring and co-workers.[24,28] The DNase I footprinting of the 7-azido-ACTD complexed with different DNA fragments showed that initially the drug binds to nonspecific sequences in the DNA and subsequently migrates to locate the specific binding sequences.[28] Waring and co-workers[24] in a similar study carried out in the presence of daunomycin argued that structural changes, especially minor groove widening, induced in the double helix by the daunomycin could be responsible for the actinomycin recognition of the specific sites. Our results still indicate that release of water molecules and the structural adjust to some sequences induced by either daunomycin or dehydration, by addition of neutral solutes, result in a more favorable binding enthalpy.

The mechanism proposed for the increased recognition of the drug could be important in the DNA recognition by intercalating proteins such as ETS1, SRY, PurR and TBP.[29] The binding of these proteins to DNA present structural aspects very similar to the binding of ACTD to DNA.

The results shown in this work stress the contribution of water on the control of the thermodynamics of ligand−DNA interactions, and point out to the need to take water activity as a relevant variable in studies intending to mimic reactions in an intracellular medium.

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