Association of the Minor Groove Binding Drug Hoechst 33258 with d(CGCGAATTCGCG)$_2$: Volumetric, Calorimetric, and Spectroscopic Characterizations†

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ABSTRACT: We employed ultrasonic velocimetry, high-precision densimetry, circular dichroism and fluorescence spectroscopy, and isothermal titration calorimetry to characterize the binding of Hoechst 33258 to the d(CGCGAATTCGCG)$_2$ oligomeric duplex at 25 °C. We used this experimental combination to determine the full thermodynamic profile for the binding of Hoechst 33258 to the DNA. Specifically, we report changes in binding free energy, enthalpy, entropy, volume, and adiabatic compressibility accompanying the binding. We interpret our volumetric data in terms of hydration and evaluate the number of waters of hydration that become released to or taken up from the bulk. Our calorimetric data reveal that the drug−DNA binding event studied in this work is entropy-driven and proceeds with an unfavorable change in enthalpy. The favorable binding entropy predominantly results from hydration changes. In contrast to a large and positive change in hydrational entropy, the binding-induced change in configurational entropy is insignificant. The latter observation is consistent with the "lock-and-key" mode of minor groove binding.

Rational design of new therapeutic agents that bind to DNA in a sequence- and/or structure-specific manner is a field of considerable interest and urgency (*1*−*3*). Many small molecules that bind to DNA via noncovalent interactions have proven to be effective anticancer and antitumor agents, although the exact mode of binding and nature of thermodynamic forces that regulate drug−DNA interactions are often poorly understood. This lack of understanding hampers many efforts to rationally modify existing drugs and/or design new therapeutic agents that bind to target DNA sequences with predictable affinity and specificity. Characterization of the forces that govern drug−DNA interactions traditionally relies on detailed knowledge of the thermodynamic and structural properties of the drug, the DNA, and the complex (*3*−*5*).

We have started a program in which we routinely employ a combination of volumetric, calorimetric, and spectroscopic measurements to characterize the association of DNA-binding molecules with DNA. We focus on identifying and quantifying changes in hydration associated with ligand−DNA interactions and evaluating the energetic impact of these changes. In this work, we investigate the binding of Hoechst 33258 to the oligomeric d(CGCGAATTCGCG)$_2$ DNA duplex. Hoechst 33258 is a bisbenzimidazole derivative and a prototypical minor groove binder (see Figure 1). It is a brightly fluorescent chromosomal dye that is often used in microscope imaging (*6*). Hoechst 33258 preferably binds

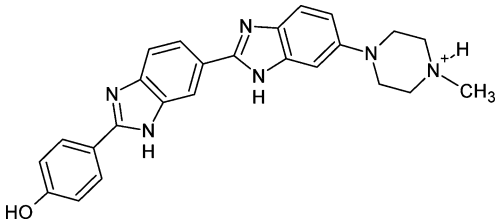

FIGURE 1: Chemical structure of Hoechst 33258.

to AT-rich domains of double-stranded DNA by deeply penetrating into its minor groove, a feature in common with other minor groove binding ligands, such as netropsin and distamycin (*7*−*9*). The Hoechst 33258−DNA complex is stabilized by a set of structurally well characterized electrostatic, van der Waals, hydrophobic, and hydrogen-bonding interactions between the drug and DNA groups lining the minor groove walls. The network of newly formed hydrogen bonds between the drug and DNA replaces previously existing solute−solvent hydrogen bonds in the minor groove (*10*, *11*). On the basis of their fluorescence data, Jin and Breslauer have proposed that the minor groove of the Hoechst 33258−DNA complex is highly nonpolar with a local dielectric constant of 20 D compared to the dielectric constant of bulk water of 80 D (*12*). The low dielectric constant environment of the minor groove increases the strength of drug−DNA hydrogen bonds relative to DNA−water hydrogen bonds, thereby providing an additional enthalpic driving force for the binding event (*12*).

Interactions of Hoechst 33258 and its analogues with DNA have been extensively studied by thermodynamic (*13*−*16*), structural (*8*, *10*, *17*−*21*), and footprinting (*22*) techniques. Structural and thermodynamic studies have revealed that

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hydration plays a key role in Hoechst 33258 association with DNA. However, the binding-induced change in hydration and its respective energetic contribution are yet to be identified and quantified. This deficiency is unfortunate since hydration often represents the dominant contributor to the binding energetics as emphasized by numerous results from our laboratory. Specifically, for a number of protein and nucleic acid recognition events, the energetic contribution of hydration has been found to be several times larger in magnitude than the net binding free energy (*23*−*26*).

In this paper, we combine volumetric, calorimetric, and spectroscopic measurements to characterize the binding of Hoechst 33258 to the d(CGCGAATTCGCG)$_2$ duplex at 25 °C. The main objective of this study is to quantify changes in hydration accompanying Hoechst 33258−DNA binding and to evaluate the contribution of these changes to the binding energetics.

MATERIALS AND METHODS

Materials. Hoechst 33258·3HCl [2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bis(1*H*-benzimidazole) trihydrochloride] was obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada). The palindromic d(CGCGAATTCGCG)$_2$ duplex was synthesized by ACGT Corp. (Toronto, Ontario, Canada). These reagents were of the highest grade commercially available and were used without further purification.

All measurements were performed in a pH 6.7 buffer consisting of 10 mM cacodylic acid/sodium cacodylate, 100 mM NaCl, and 1 mM Na_2EDTA. DNA samples were dissolved in the buffer and exhaustively dialyzed at 4 °C against the same buffer using a dialysis tubing with a molecular mass cutoff of 1000 Da (Spectrum, Houston, TX) for at least 48 h. The drug solution was prepared before each experiment by dissolving Hoechst 33258 in the dialysate.

Concentration Determinations. The concentrations of the DNA and the drug were determined spectrophotometrically at 25 °C. We used the following molar extinction coefficients when determining solute concentrations: Hoechst 33258, ϵ_{338} = 42000 M^{-1} cm^{-1}; and d(CGCGAATTCGCG)$_2$, ϵ_{260} = 183240 M^{-1} cm^{-1} (*14*). The spectrophotometric measurements were conducted using an AVIV model 14 DS UV/vis spectrophotometer (Aviv Associates, Lakewood, NJ).

Optical Spectroscopy. CD[1] spectra were recorded at 25 °C in a 1 mm path length cuvette using an AVIV model 62 DS spectropolarimeter (Aviv Associates). CD titration profiles were measured by adding aliquots of Hoechst 33258 to a known amount of DNA solution. The DNA concentrations were in the range of 30−50 μM.

Fluorescence intensity measurements were performed in a 1 cm path length cuvette using an AVIV model ATF 105 spectrofluorometer (Aviv Associates) with a bandwidth adjusted to 2 nm. For the fluorescence measurements, the excitation, λ_{ex}, and emission, λ_{em}, wavelengths were 355 and 456 nm, respectively.

The fluorescence titrations were performed at 25 °C by adding aliquots of DNA to a known amount of the drug (~10 nM). The resulting drug−DNA binding profiles were fit by a simple independent binding site model which is mathematically represented by the relationship:

$$\frac{F}{F^0} = 1 + \frac{1}{F^0[\mathrm{L}]}(F^b - F^0)\left(\frac{[\mathrm{L}]}{2} + \frac{[\mathrm{DNA}]}{2} + \frac{1}{2K_b} - \sqrt{\frac{[\mathrm{L}]^2}{4} + \frac{[\mathrm{DNA}]^2}{4} + \frac{1}{4K_b^2} + \frac{[\mathrm{L}]}{2K_b} + \frac{[\mathrm{DNA}]}{2K_b} - \frac{[\mathrm{L}][\mathrm{DNA}]}{2}}\right) \quad (1)$$

where F is the fluorescence intensity at each point of titration, F^b is the molar fluorescence intensity of the bound ligand, F^0 is the molar fluorescence intensity of the free ligand, [L] is the total concentration of Hoechst 33258, [DNA] is the total concentration of DNA, and K_b is the binding constant (*14*, *27*, *28*).

Equation 1 does not take into account the site exclusion effect that arises when a ligand of a finite size, such as Hoechst 33258, binds to a long polymeric structure, such as poly(dAdT)poly(dAdT) or poly(dA)poly(dT). However, since d(CGCGAATTCGCG)$_2$ has only a single binding site (AATT/TTAA), this limitation should not result in any appreciable error in K_b determination.

Isothermal Titration Calorimetry (ITC). Calorimetric data on drug−DNA binding were obtained using a CSC 4200 isothermal titration calorimeter (Calorimetric Sciences Corp., Lindon, UT) by titrating aliquots of Hoechst 33258 into an initial DNA solution. The calorimeter was routinely calibrated electrically with 500 μJ electrical pulses, as well as chemically by measuring the enthalpy of binding of $BaCl_2$ to 18-Crown-6 as described in the instrument's manual. When calculating the binding enthalpy, we corrected the primary ITC data for the heat of ligand dilution.

The ITC experiments were performed at 25 °C. The binding enthalpy and stoichiometry for Hoechst 33258 association with d(CGCGAATTCGCG)$_2$ were determined using an independent binding site model (*29*). In this model, the total heat of binding, Q, is given by the equation (*29*−*31*):

$$Q = n\alpha[\mathrm{DNA}]V\Delta H \quad (2)$$

where α is the fractional saturation, n is the number of independent binding sites, V is the sample volume, and ΔH is the binding enthalpy. The fractional saturation, α, can be obtained as a root of the quadratic equation:

$$\alpha^2 - \alpha\left(1 + \frac{[\mathrm{L}]}{n[\mathrm{DNA}]} + \frac{1}{nK_b[\mathrm{DNA}]}\right) + \frac{[\mathrm{L}]}{n[\mathrm{DNA}]} = 0 \quad (3)$$

The ITC data were fit by a combination of eqs 2 and 3.

High-Precision Densimetry and Ultrasonic Velocimetry. All densities were measured by an Anton Paar Model DMA 5000 vibrating tube densimeter (Anton Paar, Graz, Austria) with a precision of $\pm 1.5 \times 10^{-6}$ g cm^{-3}. The partial molar volumes, $V°$, of the ligand, free nucleic acids, and drug−DNA complexes were calculated from the relationship (*32*):

$$V° = M/\rho_0 - (\rho - \rho_0)/(\rho_0 C) \quad (4)$$

where ρ and ρ_0 are the densities of the solution and neat solvent, respectively, C is the solute concentration, and M is the molecular weight of a solute.

[1] Abbreviations: CD, circular dichroism; ITC, isothermal titration calorimetry.

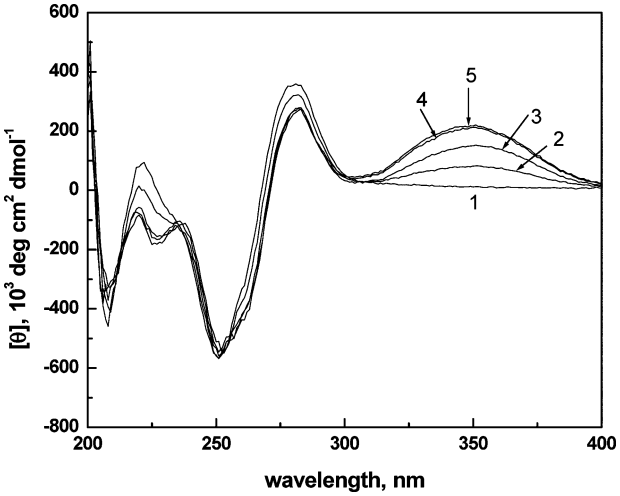

FIGURE 2: CD spectra of d(CGCGAATTCGCG)$_2$ in the presence and absence of Hoechst 33258. Curves 1, 2, 3, 4, and 5 correspond to drug-to-DNA ratios of 0, 0.292, 0.584, 0.974, and 1.169.

Solution sound velocity measurements were carried out at 7.2 MHz with a relative precision of ±1.5 mm s^{-1} by analyzing the amplitude−frequency characteristics of an ultrasonic resonator (*33*−*36*). The analysis of the frequency characteristics of the resonator was performed by an Agilent Technologies Model E5100A network/spectrum analyzer (Mississauga, Ontario, Canada). For our measurements, we used previously described ultrasonic resonator cells equipped with lithium niobate piezotransducers and requiring a minimum sample volume of 0.8 mL. The particular resonator cells used in our experiments have been designed and manufactured in collaboration with Dr. Wladimir Urbach at the University of Paris V.

The key characteristic of a solute directly derived from ultrasonic velocimetric measurements is its relative molar sound velocity increment, [U]:

$$[\mathrm{U}] = (U - U_0)/(U_0 C) \quad (5)$$

where U and U_0 are the sound velocities in the solution and neat solvent, respectively.

Differential densimetric and ultrasonic velocimetric titration experiments were performed at 25 °C by adding equal aliquots of Hoechst 33258 solution to both the sample and reference cells as previously described (*37*). The values of the relative molar sound velocity increment, [U], were used in conjunction with the measured partial molar volume data, $V°$, to calculate the partial molar adiabatic compressibility, $K°_S$, of the solutes using the relationship (*38*, *39*):

$$K°_S = \beta_{S0}(2V° - 2[\mathrm{U}] - M/\rho_0) \quad (6)$$

where β_{S0} is the coefficient of adiabatic compressibility of the solvent. The densimetric and ultrasonic velocimetric experiments were performed at least three times with the average values of [U] and $V°$ being used in eq 6 for calculating $K°_S$.

RESULTS

Circular Dichroism Spectra. Figure 2 presents the CD spectra of d(CGCGAATTCGCG)$_2$ in the presence and absence of Hoechst 33258. Neither free Hoechst 33258 nor free DNA exhibits CD signal between 300 and 400 nm. However, inspection of Figure 2 reveals that the binding of Hoechst 33258 to DNA is accompanied by CD spectral changes between 200 and 300 nm (the DNA region) and between 300 and 400 nm. In the latter range, a strong binding-induced CD band comes to existence upon an addition of the drug to DNA. Isoelliptic points that are observed at 235 and 300 nm suggest a single mode of binding. Figure 3 presents the CD-monitored profile of the drug−DNA association. Inspection of Figure 3 reveals that the binding of Hoechst 33258 to d(CGCGAATTCGCG)$_2$ is characterized by a 1:1 binding stoichiometry.

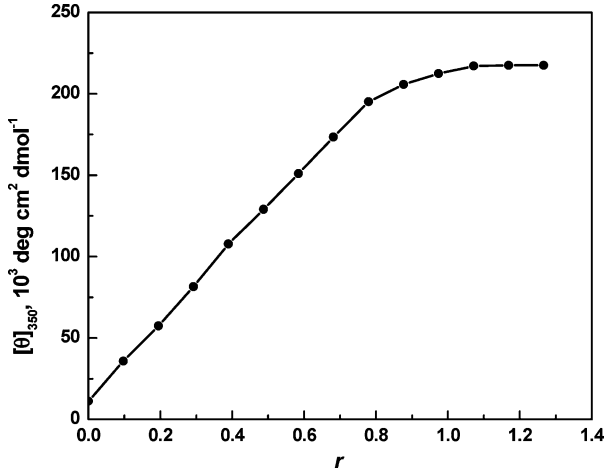

FIGURE 3: Hoechst 33258−d(CGCGAATTCGCG)$_2$ binding profile monitored by CD ellipticity at 350 nm.

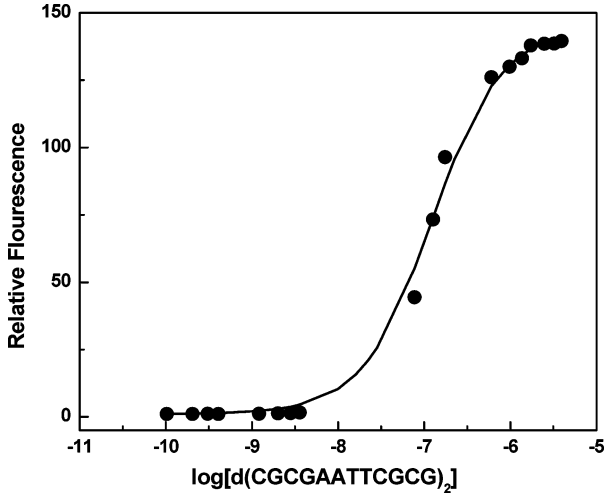

FIGURE 4: Hoechst 33258−DNA binding profile monitored by fluorescence intensity. The emission, λ_{em}, and excitation, λ_{ex}, wavelengths are 456 and 355 nm, respectively. The concentration of Hoechst 33258 is ∼40 nM.

Fluorescence Titration. Figure 4 shows the drug-to-DNA binding profile as monitored by fluorescence intensity. This binding profile was fit by eq 1. Our determined binding constant, K_b, of (43 ± 3.4) × 10^7 M^{-1} is in good agreement with 32.0 × 10^7 M^{-1}, the value reported by Loontiens et al. (*14*) (see Table 1).

Isothermal Titration Calorimetry. Figure 5 shows our primary ITC data (panel a) and integrated heats (panel b)

Table 1: Thermodynamic Parameters for Hoechst 33258 Association with d(CGCGAATTCGCG)$_2$ at 25 °C

K_b, 10^7 M^{-1}	43 ± 3.4 (32.0)[a]
ΔG_b, kcal mol^{-1}	-11.8 ± 0.1
ΔH_b, kcal mol^{-1}	10.0 ± 0.1
ΔS_b, cal mol^{-1} K^{-1}	73.1 ± 0.5
ΔV_b, cm^3 mol^{-1}	-48 ± 11
ΔK_{Sb}, $\times 10^{-4}$ cm^3 mol^{-1} bar^{-1}	-26.0 ± 13.9

[a] Reference *14* (at 100 mM NaCl and 25 °C).

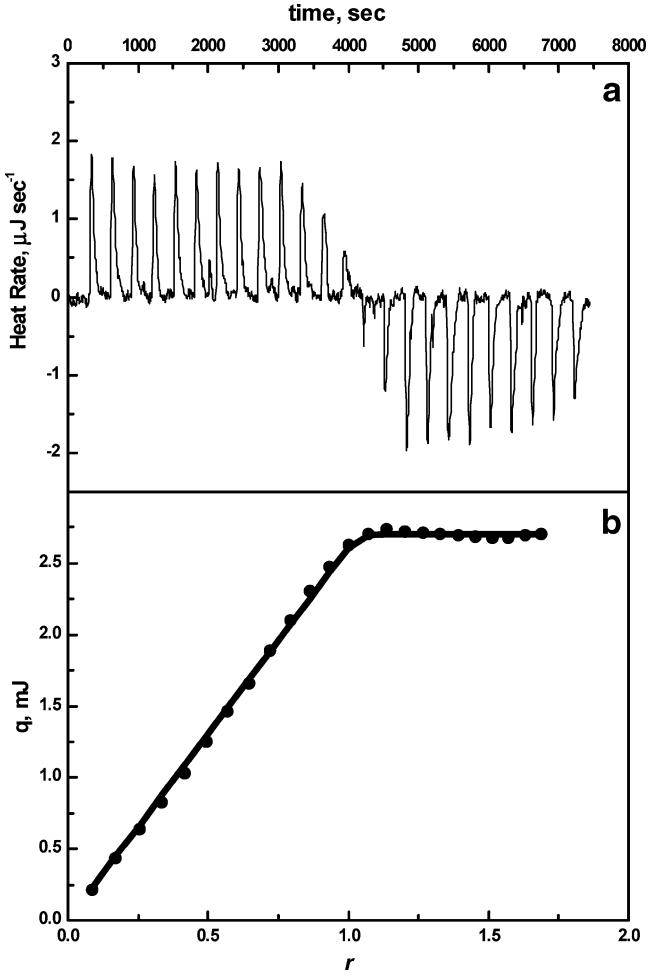

FIGURE 5: (a) Primary ITC data for Hoechst 33258 association with d(CGCGAATTCGCG)$_2$. 10 μL aliquots of 0.54 mM Hoechst 33258 were titrated into 1.0 mL of 0.078 mM DNA. (b) Integrated heat of binding plotted versus the drug-to-DNA binding ratio, *r*. The heat of dilution has been subtracted from the original data. The solid line represents the best least-squares fit of the data (see text).

for Hoechst 33258 binding to d(CGCGAATTCGCG)$_2$. The experimental data shown in Figure 5b were analytically fit using eqs 2 and 3. The best fit corresponds to the binding enthalpy, ΔH_b, of 10.0 ± 0.1 kcal/mol, the binding constant, K_b, of $1.0 \pm 0.1 \times 10^7$ M^{-1}, and the binding stoichiometry, *n*, of 1.1 ± 0.1.

Volumetric Properties. Table 2 shows the relative molar sound velocity increment, [U], partial molar volume, $V°$, and partial molar adiabatic compressibility, $K°_S$, of d(CGCGAATTCGCG)$_2$ and Hoechst 33258·3HCl (in 10 mM cacodylic acid/sodium cacodylate buffer at pH 6.7). Figure 6 shows changes in the relative molar sound velocity increment, $\Delta[U]_b$ (panel a), volume, ΔV_b (panel b), and adiabatic compressibility, ΔK_{Sb} (panel c), associated with the binding of Hoechst 33258 to the oligomer.

Table 2: Relative Molar Sound Velocity Increments, [U], Partial Molar Volumes, $V°$, and Partial Molar Adiabatic Compressibilities, $K°_S$, of d(CGCGAATTCGCG)$_2$ and Hoechst 33258·3HCl at 25 °C

compound	[U], cm^3 mol^{-1}	$V°$, cm^3 mol^{-1}	$K°_S$, $\times 10^{-4}$ cm^3 mol^{-1} bar^{-1}
DNA	1828 ± 19	4128 ± 36	-1206 ± 37
drug	162.5 ± 1.1	379.9 ± 3.2	-43.3 ± 3.0

DISCUSSION

Binding Energetics. Table 1 lists the thermodynamic parameters of Hoechst 33258−DNA binding at 25 °C including the binding constant, K_b, free energy, $\Delta G_b = -RT \ln K_b$, enthalpy, ΔH_b, and entropy, $\Delta S_b = (\Delta H_b - \Delta G_b)/T$. When analyzing Hoechst 33258 association with d(CGCGAATTCGCG)$_2$, we used the binding constant, K_b, which was determined from our fluorescence measurements (43×10^7 M^{-1}) rather than that obtained from the ITC measurements (1×10^7 M^{-1}). The discrepancy between the two values reflects the fact that ITC provides reliable determination of the binding constant, K_b, when the value of K_b is 1−1000 times greater than the total concentration of DNA (*29*, *40*). For relatively tight binding (as for Hoechst 33258−DNA binding), this requirement implies that the optimal range of DNA concentrations should be between 2 nM and 2 μM. However, such a low concentration would not permit accurate determination of the binding enthalpy, ΔH_b. Thus, at our experimental concentration of DNA on the order of ~100 μM, ITC measurements provide a good accuracy of ΔH_b determination with error of K_b evaluation being rather high. Consequently, we use below the value of K_b determined from our fluorescence titration experiments that provide a better accuracy.

Inspection of data in Table 1 reveals that Hoechst 33258 binding to DNA is an entropy-driven process that proceeds with an unfavorable change in enthalpy at 25 °C. This observation is in agreement with Haq et al. (*15*), who have used ITC measurements to determine a change in enthalpy accompanying the binding of Hoechst 33258 to the d(CGCAAATTTGCG)$_2$ duplex. By contrast, Loontiens et al. (*14*), who have used the van't Hoff approach, measured a favorable change in enthalpy accompanying Hoechst 33258 association with the polymeric poly(dAdT)poly(dAdT) and poly(dA)-poly(dT) duplexes. It should be noted, however, that our isothermal titration calorimetric data on the binding of Hoechst 33258 with poly(dAdT)poly(dAdT) and poly(dA)-poly(dT) reveal positive (unfavorable) changes in enthalpy for both polymers (data not shown). The discrepancy between the calorimetric and van't Hoff enthalpies may be related to a significantly negative change in heat capacity, ΔC_{pb}, accompanying Hoechst 33258−DNA binding that has not been accounted for in the van't Hoff analysis (*15*, *41*).

Differential Volumetric Properties of the Drug−DNA Complex and Free DNA. Inspection of Figure 6 reveals that the binding of Hoechst 33258 to the d(CGCGAATTCGCG)$_2$ oligomeric duplex is accompanied by decreases in volume (panel b) and compressibility (panel c). Our measured changes in volume, ΔV_b, and adiabatic compressibility, ΔK_{Sb}, accompanying the binding of Hoechst 33258 to d(CGCGAATTCGCG)$_2$ are presented in Table 1.

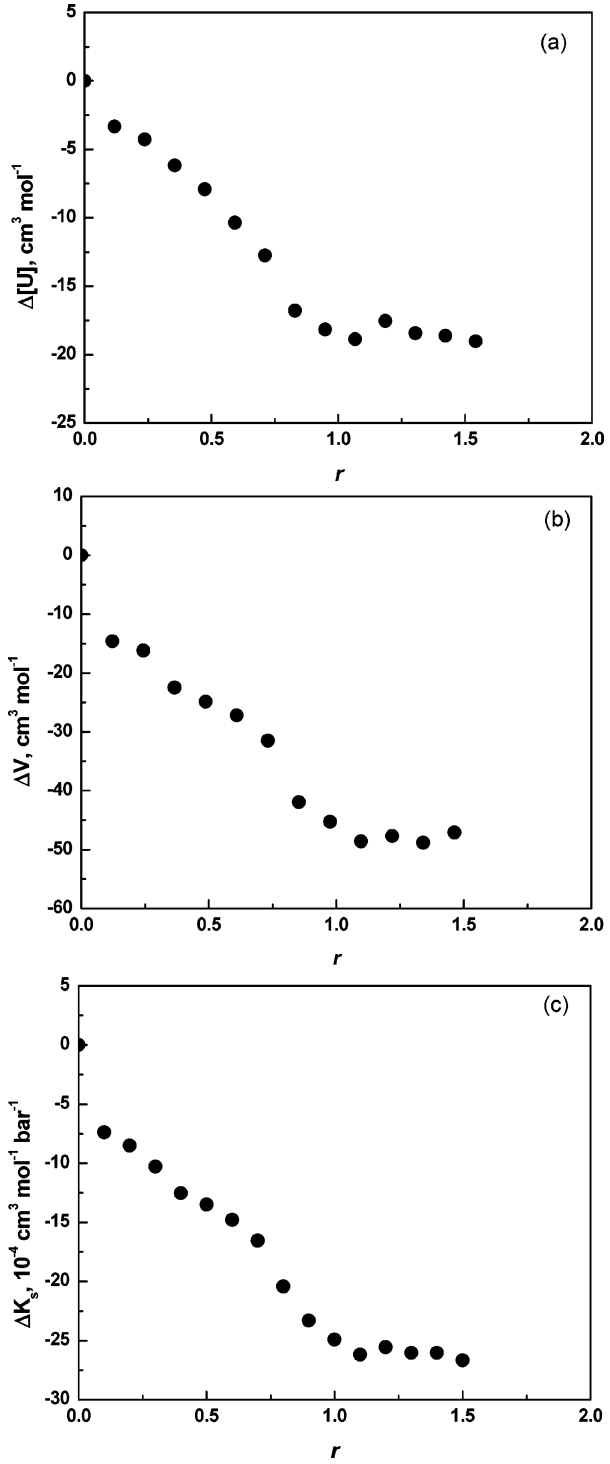

FIGURE 6: Changes in the relative molar sound velocity increment, Δ[U] (a), volume, ΔV (b), and adiabatic compressibility, ΔK_S (c), accompanying Hoechst 33258 association with d(CGCGAATTCGCG)$_2$ plotted versus the drug-to-DNA ratio, r. Errors in Δ[U], ΔV, and ΔK_S are ±6 cm^3 mol^{-1}, ±8 cm^3 mol^{-1}, and ±10 × 10^{-4} cm^3 mol^{-1} bar^{-1}, respectively.

Owing to their polyanionic nature, DNAs and RNAs are surrounded by a cloud of condensed counterions that reduce charge−charge repulsion between negatively charged phosphate groups (*42*, *43*). DNA association with charged ligands brings about a release of condensed counterions to the bulk (*44*). To maintain electroneutrality, when a ligand carrying a single positive charge binds to DNA, one sodium ion per binding site needs to be released to the bulk. In agreement with this expectation, Hoechst 33258 binding to poly(dAdT)-poly(dAdT) and d(CGCAAATTTGCG)$_2$ causes a release of 0.9 and 0.99 Na^+ ions, respectively (*14*, *15*, *45*). As a first approximation, it is reasonable to assume that Hoechst 33258 association with d(CGCGAATTCGCG)$_2$ also causes a release of a single Na^+ ion. Thus, the Hoechst 33258−DNA binding reaction can be expressed as

$$\text{drug}^+ + \text{DNA} \leftrightarrows \text{complex} + \text{Na}^+ \qquad (7)$$

On the basis of reaction 7, changes in volume, ΔV_b, and compressibility, ΔK_{Sb}, accompanying Hoechst 33258 association with DNA can be presented by the relationships:

$$\Delta V_b = V°(\text{complex}) - V°(\text{DNA}) + V°(\text{Na}^+) - V°(\text{drug}^+) \qquad (8)$$

$$\Delta K_{Sb} = K°_S(\text{complex}) - K°_S(\text{DNA}) + K°_S(\text{Na}^+) - K°_S(\text{drug}^+) \qquad (9)$$

where $V°$(complex), $V°$(DNA), $V°$(Na^+), and $V°$(drug$^+$) are the partial molar volumes of the drug−DNA complex, free DNA, Na^+ ion, and the monoprotonated form of Hoechst 33258, respectively, and $K°_S$(complex), $K°_S$(DNA), $K°_S$(Na^+), and $K°_S$(drug$^+$) are the partial molar adiabatic compressibility of the drug−DNA complex, free DNA, Na^+ ion, and the monoprotonated form of Hoechst 33258, respectively.

At 25 °C, the values of $V°$(Na^+) and $K°_S$(Na^+) are −6.9 cm^3 mol^{-1} and −33.5 × 10^{-4} cm^3 mol^{-1} bar^{-1}, respectively (*46*, *47*). At neutral pH, Hoechst 33258 is monoprotonated (Hoechst$^+$). Consequently, the apparent partial molar volume, $V°$, and adiabatic compressibility, $K°_S$, of Hoechst 33258·3HCl determined in cacodylic buffer at pH 6.7 correspond to the sums:

$$V° = V°(\text{Hoechst}^+) + 2\Delta V_{ion} + 3V°(\text{Cl}^-) \qquad (10)$$

$$K°_S = K°_S(\text{Hoechst}^+) + 2\Delta K_{S\ ion} + 3K°_S(\text{Cl}^-) \qquad (11)$$

where $V°$(Hoechst$^+$) and $K°_S$(Hoechst$^+$) are respectively the partial molar volume and adiabatic compressibility of the monoprotonated form of Hoechst 33258, ΔV_{ion} and Δ$K_{S\ ion}$ are respectively the changes in volume and adiabatic compressibility accompanying ionization of cacodylic acid, and $V°$(Cl^-) and $K°_S$(Cl^-) are respectively the partial molar volume and adiabatic compressibility of a chloride ion. The values of $V°$(Cl^-) and $K°_S$(Cl^-) equal 22.5 cm^3 mol^{-1} and −17.0 × 10^{-4} cm^3 mol^{-1} bar^{-1}, respectively (*46*, *47*). To determine the values of ΔV_{ion} and Δ$K_{S\ ion}$, we measured changes in sound velocity and density accompanying titration of sodium cacodylate with HCl (data not shown). The measured values of ΔV_{ion} and Δ$K_{S\ ion}$ are 12.8 ± 0.5 cm^3 mol^{-1} and (28.5 ± 0.8) × 10^{-4} cm^3 mol^{-1} bar^{-1}, respectively. Armed with the values of ΔV_{ion} and Δ$K_{S\ ion}$, we use the partial molar volume, $V°$, of 379.9 ± 3.2 cm^3 mol^{-1} and adiabatic compressibility, $K°_S$, of −(43.3 ± 3.0) × 10^{-4} cm^3 mol^{-1} bar^{-1} of Hoechst 33258·3HCl (see Table 2) in conjunction with eqs 10 and 11 to calculate $V°$(Hoechst$^+$) and $K°_S$(Hoechst$^+$). The calculated values of $V°$(Hoechst$^+$) and $K°_S$(Hoechst$^+$) equal 286.8 ± 3.2 cm^3 mol^{-1} and −(49.3 ± 3.1) × 10^{-4} cm^3 mol^{-1} bar^{-1}, respectively. With these quantities, we now use eqs 8 and 9 and measured values of

Table 3: Differential Volumetric and Hydration Properties of the Hoechst 33258−DNA Complex and Free DNA

$V°$(complex) − $V°$(DNA), $cm^3\,mol^{-1}$	246 ± 12
$K°_S$(complex) − $K°_S$(DNA), $10^{-4}\,cm^3\,mol^{-1}\,bar^{-1}$	−42 ± 14
n_h(complex) − n_h(DNA)	21 ± 7

ΔV and ΔK_S to calculate the differential partial molar volumes, [$V°$(complex) − $V°$(DNA)], and compressibilities, [$K°_S$(complex) − $K°_S$(DNA)], of ligand-free and ligand-bound DNA. These calculated differential volumetric parameters are shown in Table 3.

Interpretation of Volumetric Data in Terms of Hydration. The partial molar volume, $V°$, of a solute can be presented as the sum (*48*−*51*):

$$V° = V_M + V_T + V_I + \beta_{T0}RT \quad (12)$$

where V_M is the intrinsic volume which represents the geometric volume of a solute that is not penetrable by surrounding water molecules, V_T is the thermal volume which originates from thermally activated mutual vibrations of the solute and solvent molecules, V_I is the interaction volume which represents the solvent contraction around a solute due to hydrogen bonding or electrostriction, β_{T0} is the coefficient of isothermal compressibility of the solvent, R is the universal gas constant, and T is the absolute temperature.

The partial molar adiabatic compressibility, $K°_S$, of a solute can be represented by the sum of intrinsic, K_M, and hydration, ΔK_{Sh}, contributions (*52*−*55*):

$$K°_S = K_M + \Delta K_{Sh} = K_M + n_h(K_{Sh} - K_{S0}) \quad (13)$$

where K_M is the intrinsic compressibility of a solute, ΔK_{Sh} is the hydration-induced change in solvent compressibility, K_{Sh} and K_{S0} are the partial molar adiabatic compressibilities of water of solute hydration and bulk water, respectively, and n_h is the number of water molecules in the hydration shell of a solute. In this representation, the compressibility of the thermal volume is included in the hydration term, ΔK_{Sh}.

For low molecular weight compounds and nucleic acids, K_M is small and can be neglected in the analysis employed in this work (*56*, *57*). Consequently, for a volumetric analysis of drug−DNA complexation, eq 13 can be simplified to the form:

$$K°_S = n_h(K_{Sh} - K_{S0}) \quad (14)$$

Relationships for changes in volume, ΔV_b, and adiabatic compressibility, ΔK_{Sb}, accompanying ligand−DNA binding can be obtained by differentiating eqs 12 and 14:

$$\Delta V_b = \Delta V_M + \Delta V_T + \Delta V_I = \sum_i (\Delta V_{Mi} + \Delta V_{Ti} + \Delta V_{Ii}) \quad (15)$$

$$\Delta K_{Sb} = \Delta n_h(K_{Sh} - K_{S0}) = \sum_i \Delta[n_{hi}(K_{Shi} - K_{S0})] \quad (16)$$

where subscript i denotes the number of species participating in the reaction, and $\Delta V_{Ii} = \Delta[n_{hi}(V_{hi} - V_0)]$, where V_{hi} and V_0 are the partial molar volumes of water in the hydration shell of the ith component of the reaction and in the bulk state, respectively.

Changes in Volume. A change in intrinsic volume, ΔV_M, in eq 15 accompanying Hoechst 33258 association with a DNA duplex can be determined on the basis of structural information on the drug−DNA complex and the VOLBL computational algorithm developed by H. Edelsbrunner, P. Fu, and J. Liang (ftp://ftp.ncsa.uiuc.edu/visualization/alpha-shape). A change in thermal volume, ΔV_T, in eq 15 can be approximated by $(\Delta V_A - \Delta V_M)\delta/r_w$, where r_w is 1.4 Å, the radius of a water molecule; δ is 0.57 Å, the thickness of thermal volume for small molecules and, by extension, for DNA (*51*, *58*, *59*); ΔV_A is the change in excluded volume of DNA that represents the volume enclosed within the solvent-accessible surface of the molecule; and ΔV_M is the change in molecular volume as defined by Richards and Connolly (*60*−*62*). Armed with the values of ΔV_b (experimentally measured) and ΔV_M and ΔV_T (calculated from structure), one can determine a change in the interaction volume, $\Delta V_I = \Delta V_b - \Delta V_M - \Delta V_T$, thereby quantifying the release/uptake of water molecules from/by the hydration shells of the components of reaction 7. This is a viable approach that has been used for analyzing protein−protein and protein−ligand interactions (*23*, *24*, *26*, *63*). This approach requires high-resolution structural data that are available for the Hoechst 33258−dodecamer complex (*10*, *64*).

For calculating the values of ΔV_M and ΔV_T, we used X-ray crystallographic structures of the d(CGCGAATTCGCG)$_2$ duplex and its complex with Hoechst 33258 (NDB entries BDL001 and GDL006, respectively) (*10*, *64*). The calculated binding-induced changes in intrinsic, ΔV_M, and thermal, ΔV_T, volumes are equal to 342.9 and −36.4 cm^3/mol, respectively. Based on eq 15, a change in interaction volume, ΔV_I, equals −60.8 ± 11.5 cm^3/mol (245.7 − 342.9 + 36.4). The observed decrease in V_I is suggestive of enhanced hydration of the complex relative to the host DNA. To rationalize the observed change in V_I in terms of hydration, we make use of the relationship $\Delta V_I = [n_h(\text{complex}) - n_h(\text{DNA})](V_h - V_0)$, where $(V_h - V_0)$ is the average difference in partial molar volume between water of hydration and bulk water. The value of $(V_h - V_0)$ for DNA is ∼−1.8 $cm^3\,mol^{-1}$ (*65*). Thus, the value of [n_h(complex) − n_h(binding − site)] equals 34 ± 7 (60.8/1.8). This result indicates that the binding of Hoechst 33258 to d(CGCGAATTCGCG)$_2$ causes an uptake of ∼34 waters from the bulk.

Changes in Adiabatic Compressibility. As mentioned above, a change in compressibility associated with a drug−DNA binding event can be assigned entirely to the binding-induced alteration in solute hydration. Consequently, the differential compressibilities presented in Table 3 reflect the differential hydration properties of ligand-bound and ligand-free DNA structures: $K°_S$(complex) − $K°_S$(DNA) = [n_h(complex) − n_h(DNA)](K_{Sh} − K_{S0}). On average, the differential partial molar adiabatic compressibility of water of DNA hydration and bulk water, $K_{Sh} - K_{S0} \approx -2 \times 10^{-4}$ $cm^3\,mol^{-1}\,bar^{-1}$ (*65*). By assuming, as a first approximation, that the values of ($K_{Sh} - K_{S0}$) are similar for ligand-free and ligand-bound DNA structures, the differential number of water molecules, [n_h(complex) − n_h(DNA)], solvating each DNA and its complex with Hoechst 33258 can be calculated by dividing [$K°_S$(complex) − $K°_S$(DNA)] by -2×10^{-4} $cm^3\,mol^{-1}\,bar^{-1}$ (*25*). The value of [n_h(complex) − n_h(DNA)] is equal to 21 ± 7 (shown in Table 3). This compressibility-

based estimate of the change in dodecamer hydration (21 ± 7 waters) is in qualitative agreement with the volume-based estimate (34 ± 7 waters). Thus, on the basis of our compressibility data, the Hoechst 33258−DNA complex contains in its hydration shell 21 more water molecules relative to its ligand-free counterpart. In the analysis below, we use the compressibility-based estimate (21 ± 7) of the differential hydration rather than the volume-based estimate (34 ± 7) since the former is less model-dependent.

The observed enhancement of hydration upon the binding of Hoechst 33258 to DNA may seem counterintuitive given a decrease in solvent-accessible surface area (ΔS_A = 142 Å) and release to the bulk of crystallographically ordered water molecules from the spine of hydration in the minor groove of the A-tract. It should be noted, however, that volumetric observables "sense" a much broader population of water molecules than crystallographically ordered water molecules. Analysis of patterns of DNA hydration reveals that double helical structures are prone to cooperative enhancement of hydration (*54*, *66*). Specifically, genomic DNA (salmon testes) and the poly(dAdT)poly(dAdT), poly(dGdC)poly(dGdC), and poly(dIdC)poly(dIdC) synthetic DNA duplexes possess in their hydration shells 36, 50, 72, and 92 waters per base pair (*54*, *65*, *66*). The large increase in hydration exhibited by periodic all-AT, all-GC, and, especially, all-IC DNA duplexes cannot be accounted for simply in terms of the number and chemical nature of solvent-exposed atomic groups (*54*, *66*). Instead, we have proposed that all-AT, all-GC, and all-IC rich structures form rigid scaffolds that facilitate a cooperative enhancement of duplex hydration (*54*, *66*). By analogy, as a possible explanation, we propose that the Hoechst−DNA complex may exhibit, relative to the host DNA, cooperatively enhanced hydration. It is difficult to reliably identify or even speculate about the structural origin of such a cooperative increase in hydration. At the moment, we put forward this "explanation" as a general hypothesis that needs to be either verified or refuted on the basis of future studies.

As a final note, the observed increase in hydration accompanying Hoechst 33258−DNA complexation correlates with results of one osmotic stress study in which hydration changes accompanying the binding of a netropsin analogue have been characterized (*67*). The authors have found that the binding results in an uptake of 50−60 water molecules (*67*). By contrast, our previous volumetric investigation of netropsin complexation with the poly(dA)poly(dT) and poly(dAdT)poly(dAdT) duplexes has revealed reduced hydration of the complexes relative to the host DNAs (*37*). Specifically, we have found that netropsin binding induces a release of approximately 22 waters from the hydration shell of the poly(dAdT)poly(dAdT) heteropolymeric duplex and approximately 40 waters from the hydration shell of the poly(dA)poly(dT) homopolymeric duplex (*37*). It is presently not clear why some minor groove binders cause an increase in hydration upon their binding to DNA, while others bring about dehydration. Further volumetric studies routinely combined with osmotic stress measurements and structural characterizations might clarify this important point.

Net Change in Hydration. The overall change in the number of water molecules in the hydration shells of the components of reaction 7, Δn_h, can be determined from the relationship:

$$\Delta n_h = \sum_i \Delta n_{hi} = n_h(\text{complex}) + n_h(Na^+) - n_h(\text{DNA}) - n_h(\text{Hoechst}^+) \quad (17)$$

where $n_h(Na^+)$ and $n_h(\text{Hoechst}^+)$ are the numbers of water molecules in the hydration shells of Na^+ and Hoechst^+, respectively.

Thus, in addition to [n_h(complex) − n_h(DNA)], the overall change in hydration involves dehydration of Hoechst^+ and hydration of Na^+. To estimate the number of water molecules in the hydration shell of the Hoechst^+ and Na^+ ions, we use our previously described two-state model of solute hydration (*65*). In the model, liquid water is presented as consisting of two structural species: the high-density/high-enthalpy species, structurally similar to ice III, and the low-density/low-enthalpy species, structurally similar to ice I. Water of solute hydration and bulk water are postulated to contain the same structural species. However, the fractional compositions of these species in water of solute hydration, f_{1h}, and bulk water, f_{1b}, are not the same. In fact, the differential thermodynamics of bulk and hydration water is assumed to result from the difference in f_{1h} and f_{1b}. The value of f_{1h} depends on the chemical nature of solute.

We have previously used the formalism described in ref *65* to determine the number of waters of hydration, n_h, and the value of f_{1h} for a Na^+ ion based on its interaction volume, V_I, and partial molar adiabatic compressibility, $K°_S$ (*25*). The values of $n_h(Na^+)$ and $f_{1h}(Na^+)$ are equal to 5.5 and 0.99, respectively (*25*). These values suggest that Na^+ ion has 5.5 water molecules in its hydration shell with the majority (99%) of these waters being in the high-density/high-enthalpy state.

To determine the number of water molecules in the hydration shell of Hoechst^+, one needs to know, in addition to its partial molar adiabatic compressibility, $K°_S$, the interaction volume, V_I. Recall that the partial molar volume, $V°$, of monoprotonated Hoechst 33258 was calculated to be 286.8 ± 3.2 cm^3 mol^{-1}. According to eq 15, the interaction volume, V_I, of Hoechst^+ can be obtained by subtracting the sum ($V_M + V_T + \beta_{T0}RT$) from $V°$ (at 25 °C, $\beta_{T0}RT$ = 1.1 cm^3 mol^{-1}). We calculated the intrinsic volume, V_M, of 241.9 cm^3 mol^{-1} based on the atomic coordinates of the drug in the X-ray crystallographic structure of the dodecamer−Hoechst 33258 complex (*10*). The calculations were performed using the VOLBL algorithm and software. The thermal volume, V_T, of Hoechst^+ was evaluated to be 185.1 cm^3 mol^{-1} as described above (based on the calculated intrinsic and excluded volumes). Our calculated value of V_I for Hoechst^+ equals −141.3 ± 3.2 cm^3 mol^{-1} (286.8 − 241.9 − 185.1 − 1.1). Using the values of $K°_S$ and V_I in conjunction with the formalism described in ref *65*, we determine that Hoechst^+ contains in its hydration shell 81 ± 2 water molecules with the fractional composition, f_{1h}, of 0.74. It should be noted that the fractional composition 0.74 corresponds to $V_h - V_0 \approx -1.7$ cm^3 mol^{-1} and $K_{Sh} - K_{S0} \approx -0.5 \times 10^{-4}$ cm^3 mol^{-1} bar^{-1} (*65*). The number of water molecules within the first coordination layer of Hoechst 33258 can be obtained by dividing its solvent accessible surface area, S_A (695 Å^2), by 9 Å, the effective cross section of water molecule and equals 77 (695/9). This number is in good agreement with our volumetrically determined hydration number of 81.

Table 4: Net Change in Hydration, Δn_h, and Hydration, ΔS_{hyd}, and Configurational, ΔS_{conf}, Contributions to Entropy for Binding of Hoechst 33258 to d(CGCGAATTCGCG)$_2$

Δn_h	-55 ± 8
ΔS_{hyd}, cal mol^{-1} K^{-1}	71.2 ± 9.6
ΔS_{conf}, cal mol^{-1} K^{-1}	1.9 ± 9.6

The overall change in hydration, Δn_h, calculated with eq 17 is equal to -55 ± 8 ($21 + 5.5 - 81$) (this value is presented in Table 4). Thus, the binding of Hoechst 33258 to d(CGCGAATTCGCG)$_2$ causes a net release of 55 water molecules from the hydration shells of the interacting species to the bulk.

Entropic Contribution of Hydration to the Energetics of Hoechst 33258 Binding to DNA. A change in entropy, ΔS_b, accompanying a protein or nucleic acid binding event can be presented as the sum of intrinsic (configurational), ΔS_{conf}, hydration, ΔS_{hyd}, and rotational and translational, ΔS_{rt}, contributions (*68*):

$$\Delta S_b = \Delta S_{conf} + \Delta S_{hyd} + \Delta S_{rt} \quad (18)$$

The contribution of the ΔS_{rt} term is not large. For 1:1 binding stoichiometry, the value of ΔS_{rt} is roughly -8 cal K^{-1} mol^{-1} (*68*). In addition, a decrease in rotational/translational degrees of freedom due to association of the drug with DNA should be to some degree offset by an increase in these degrees of freedom due to dissociation of a Na^+ ion from DNA (*25*). Therefore, in the analysis below, we ignore the ΔS_{rt} contribution.

The hydration change in entropy, ΔS_{hyd}, can be estimated by multiplying the total number of water molecules released to the bulk, Δn_h, by $-(S_h - S_0)$, the average difference in partial molar entropy between water of solute hydration and bulk water. The data on hydration entropy for a large number of atomic groups suggest that, at 25 °C, the values of $(S_h - S_0)$ for water molecules solvating charged, polar, and hydrophobic groups are quite similar and approximately equal to -1.3 ± 0.4 cal mol^{-1} K^{-1} (*63*, *69*). This average value is in good agreement with -1.6 cal mol^{-1} K^{-1}, our estimate of the entropic penalty paid by water molecules solvating AT-rich DNA duplexes (*37*). Thus, at 25 °C, the differential partial molar entropy of water of hydration and bulk water does not appear to strongly depend on the chemical nature of solute. This notion is supported by theoretical calculations of Rashin and Bukatin (*70*, *71*).

Using $(S_h - S_0) = -1.3 \pm 0.4$ cal mol^{-1} K^{-1}, we calculate the hydration contribution, ΔS_{hyd}, for Hoechst 33258 association with d(CGCGAATTCGCG)$_2$ to be 71.2 ± 9.6 cal mol^{-1} K^{-1}. This result is shown in the Table 4. The value of ΔS_{hyd} is positive, suggesting a highly favorable contribution of hydration to the binding energetics. The hydration contribution to the binding free energy, $-T\Delta S_{hyd}$, is -21.2 ± 2.9 kcal mol^{-1}. This value is roughly twice as large in magnitude as the binding free energy, ΔG_b, presented in Table 1, an observation suggesting a dominant role of hydration in controlling the Hoechst 33258−DNA binding energetics.

The ΔS_{conf} term in eq 18 denotes binding-induced changes in configurational degrees of entropy of both the drug and the DNA. The value of ΔS_{conf} was calculated from eq 18 and is presented in Table 4. The configurational change in entropy, ΔS_{conf}, is insignificant (1.9 ± 9.6 cal mol^{-1} K^{-1}). The near-zero value of ΔS_{conf} is suggestive of the DNA and the drug undergoing only slight (if any) conformational changes upon their complexation. This notion is consistent with the "lock-and-key" mode of minor groove binding (*5*, *50*, *72*).

CONCLUDING REMARKS

We combined volumetric, calorimetric, and spectroscopic measurements to characterize the binding of Hoechst 33258 to the d(CGCGAATTCGCG)$_2$ duplex at 25 °C. For this drug−DNA binding event, we determined the full thermodynamic profile including changes in free energy, ΔG_b, enthalpy, ΔH_b, entropy, ΔS_b, volume, ΔV_b, and adiabatic compressibility, ΔK_{Sb}. At 25 °C, Hoechst 33258 association with d(CGCGAATTCGCG)$_2$ is entropy-driven and proceeds with a large and unfavorable change in enthalpy. We rationalized our volumetric data in terms of hydration and found that the binding of Hoechst to d(CGCGAATTCGCG)$_2$ involves a net release to the bulk of 55 ± 8 water molecules. We used this number to evaluate the hydration and configurational contributions to the binding entropy.

The hydration contribution, ΔS_{hyd}, to the binding entropy is 71.2 ± 9.6 cal mol^{-1} K^{-1}. With this estimate, the value of $-T\Delta S_{hyd}$ is roughly twice as large in magnitude as the net binding free energy. This observation underscores the overwhelming role of hydration in modulating the Hoechst 33258−DNA binding energetics. In contrast to ΔS_{hyd}, the change in configurational entropy, ΔS_{conf}, is insignificant (1.9 ± 9.6 cal mol^{-1} K^{-1}). In general, our work emphasizes the power of combining volumetric and calorimetric data for elucidating the relative role played by various inter- and intramolecular interactions in governing the energetics of molecular recognition.

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