Calorimetric Investigation of Ethidium and Netropsin Binding to Chicken Erythrocyte Chromatin†

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Received November 5, 1997; Revised Manuscript Received February 2, 1998

ABSTRACT: We have investigated the thermodynamic aspects of the ligand binding to chromatin, using isothermal titration calorimetry. Two classical DNA ligands were used: an intercalator, ethidium bromide, and a minor groove binder, netropsin. Stoichiometry, affinity constant, and thermodynamic parameters were determined at various salt concentrations and different temperatures. The effect of ionic strength was analyzed according to the Record theory applied to chromatin. We also compared the binding parameters on naked DNA, H1/H5-depleted chromatin, and chromatin. We demonstrated that the presence of histones on DNA still allows the ligand binding that takes place according to a simple one single-site model. For both ligand types, the thermodynamic driving force is enthalpic and the association is characterized by a somewhat weaker affinity and more scattered ligand distribution than on naked DNA. The ligand affinity is weakly altered by the salt-induced compaction of the chromatin and the binding is accompanied by a release of one counterion per ligand molecule. The temperature-dependent studies revealed the existence of a small heat capacity change associated with ligand binding to chromatin, together with an enthalpy−entropy compensation that maintains the free energy constant over the investigated temperature range.

Over the past decades, the interaction of small ligands, such as drugs, to nucleic acids has been extensively studied using various biophysical techniques. Much interest has come from the potential ability of these molecules to interfere with the gene regulation, and therefore for some of them to act as chemotherapeutic agents.

Two main modes of binding of such ligands to nucleic acids are distinguished: the intercalation between base-pairs and the insertion into the minor groove of the double helix. For example, ethidium bromide is known as a classic intercalator (*1*, *2*), while netropsin is judged to be a classic minor groove binder (*3*, *4*). Ethidium is widely used as a fluorescent stain for DNA, and it is famous for its antibactericid and carcinogenic properties. Netropsin shows an antibiotic activity against a wide range of bacteria, fungi, and viruses, and its dicationic structure mimics an oligopeptide (*5*).

Molecular modeling and X-ray crystallography have provided insights into the energetic and structural basis of the observed DNA specificity (*6*−*8*). Complementary biophysical studies have helped to elucidate the mode of binding to the double helix, the sequence specificity, the stoichiometry, and the kinetics of the association of the drugs to DNA. However, most of the studies have focused on natural DNAs and poly- or oligonucleotides (*9*, *10*).

It has recently become clear that it is also of importance to examine how the presence of the histone structure proteins might affect the affinity and stoichiometry of the ligand binding (*11*). Indeed, few studies have investigated the binding properties of ligands on chromatin in solution, which is known to model more closely the arrangement of DNA in vivo (*12*−*14*). Thus, we report here a thermodynamic investigation of ethidium and netropsin binding to chromatin, using isothermal titration calorimetry. We first investigated the influence of the presence of nucleosome core histones and of H1/H5 histones on the binding. We also looked at the influence of salt-induced chromatin condensation and of temperature on the ligand−nucleic acid association.

MATERIALS AND METHODS

Chemicals and DNA. Netropsin was purchased from Serva (Heidelberg, Germany) and ethidium bromide from Sigma Chemical Co. Both were used without further purification. Calf thymus DNA (Sigma Type I) was purified to reach a residual protein content smaller than 1%.

Chicken Erythrocyte Nuclei Preparation. Blood was collected from freshly killed chickens and diluted in two volumes of citrate buffer (0.76% sodium citrate, 1% *d*-glucose, 0.5 mM PMSF at pH 7.4). All extracting steps were carried out at 4 °C. After centrifugation, the pellet was used to extract the nuclei and to prepare long soluble chromatin fibers, according to the preparation procedure developed by Bordas (*15*). The nuclei were washed several times with STM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM PMSF at pH 7.5) and then washed with STM buffer containing 0.5% of Nonidet P4O until getting a totally white pellet. These nuclei were usually resuspended in STM containing 50% (v/v) of glycerol + 3 mM $MgCl_2$ and kept for several months at −18 °C.

† This work was supported by the Actions de Recherches Concertées Contract 95/00-193.

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S0006-2960(97)02735-9 CCC: $15.00
Published on Web 05/30/1998

Chicken Erythrocyte Chromatin and H1/H5-Depleted Chromatin Preparation. Nuclei were carefully thawed up to 4 °C and all following steps were conducted at this temperature, unless specified. Nuclei were centrifuged at 700*g* for 15 min, washed with STMN, and resuspended in 30 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM Tris-HCl, and 0.1 mM PMSF at pH 7.5 in a potter; the suspension concentration was evaluated from the absorbance of an aliquot diluted 50 times into denaturating NaOH 1 M. The chromatin was digested by 15 units of micrococcal nuclease (Pharmacia Biotech)/mg of DNA at 37 °C until reaching a 2% content of acidosoluble residues. The digestion was stopped by adding 10% (v/v) of EDTA 0.1 M, and the pellet was washed several times with digestion buffer/EDTA to get rid of the nuclease. The nuclei were lysed by dialysis against a buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, and 0.1 mM PMSF at pH 7.5. After 15 min centrifugation at 20000*g*, the soluble chromatin was characterized by circular dichroism and by the ratios A^{260}/A^{230} and A^{260}/A^{280} close to 1.5 and 1.8, respectively. The preparation was shown to remain stable for about 10 days at 4 °C. H1 and H5 histones were depleted by adding drop by drop NaCl 4 M to the chromatin solution up to 650 mM. H1/H5-depleted chromatin was isolated by ultracentrifugation on a 5%−25% (w/w) sucrose gradient in 650 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA (16 h, 24000 rpm, SW27 Beckman rotor at 4 °C). The collected fractions were then dialyzed against 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA.

Isothermal Titration Calorimetry (ITC). Isothermal titrations were performed on a Microcal MCS titration calorimeter (Microcal, Inc., Amherst, MA) at 20 °C (or other specified temperature) and data analyzed according to a single set of identical sites model. In a typical drug−DNA interaction experiment, 1.33 mL of a DNA duplex or chromatin at a 0.6 mM mononucleotide concentration was titrated with a 0.8 to 1.5 mM ligand solution in 0.1 mM EDTA, 10 mM Tris-HCl buffer (pH 7.5) and specified salt concentration by ∼25 injections of 10 μL each, using a 250 μL syringe rotating at 400 rpm. The duration of each injection was about 20 s, and the delay between injections was 5 min. The reference cell of the calorimeter was filled with water, and the instrument was calibrated by means of a known standard electrical pulse. For each injection, the area under the resulting peak was proportional to the heat of interaction. A blank was performed under the same conditions by injections of the drug stock solution into the sample buffer and subtracted from the experimental data to take into account heats of dilution. After normalization to the titrant solution concentration, the total molar reaction heat $Q(i)$ in a solution volume V_0 after the i^{th} injection is proportional to the binding constant K_b and the stoichiometry of the reaction n (in number of ligand per phosphate) and the binding enthalpy ΔH_b (*16*).

$$Q(i) = \frac{nP\Delta H_b V_0}{2}\left[1 + \frac{D(i)}{nP} + \frac{1}{nK_bP} - \sqrt{\left(1 + \frac{D(i)}{nP} + \frac{1}{nK_bP}\right)^2 - \frac{4D(i)}{nP}}\right]$$

The data analysis program Origin provided by MicroCal performs the fitting of experimental points expressed in molar heat released at the i^{th} injection $\Delta Q(i)$ versus $D(i)/P$, taking into account a correction for displaced volume dV_i :

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left(\frac{Q(i) + Q(i-1)}{2}\right) - Q(i-1)$$

This enables the K_b, the n, and the binding enthalpy ΔH_b to be determined directly. Having established these parameters for a given interaction, other thermodynamic quantities such as the standard binding free energy $\Delta G°$ and the standard binding entropy $\Delta S°$ were obtained from the relationship $-RT \ln K_b = \Delta G° = \Delta H_b - T\Delta S°$. The site size length N, defined in number of base-pairs per ligand molecule, was equal to $1/(2n)$.

Resolution of Manning Equations. The solutions of the Manning equations (*17*) were computed numerically using a modified Marquardt−Levenberg algorithm in Mathcad 4.0 (*18*). The Manning equation used contained the fraction of phosphate neutralized by the histones θ_H, and the unknown fraction of phosphate neutralized by the sodium ions θ:

$$-2(1-\theta-\theta_H)\xi \ln\left(1 - \exp\left(-b\sqrt{\frac{2000N_A q^2([Na^+] + (1-\theta)[P])}{\epsilon_0\epsilon_r kT}}\right)\right) = 1 + \ln\left[\frac{\theta}{V([Na^+] + (1-\theta)[P])}\right]$$

where $\xi = q^2/(4\pi\epsilon_o\epsilon_r kTb)$ is the charge density parameter (4.182 for DNA), N_A the Avogadro number, q the electron charge, ϵ_0 the permittivity of a vacuum, ϵ_r the medium relative dielectric constant (80 for water at 20 °C), T the absolute temperature in K, b the spacing between phosphate charges along the array (1.7×10^{-10} m), V the cylindrical volume around the DNA within which counterions are considered as bound (6.43×10^{-4} m^3 mol^{-1} of mononucleotide for DNA at 20 °C), $[Na^+]$ the sodium concentration, and [P] the phosphate concentration in the chromatin solution equal to 6×10^{-4} M. The above equation was solved for the three different domains in chromatin characterized by a distinct θ_H, and an average fraction of phosphate $\theta_{I,\ II,\ or\ III}$ neutralized by the sodium ions on this range of salt concentration was obtained for each domain (see Results and Discussion).

RESULTS

Influence of the Presence of Histones on the Ligand−DNA Interaction. Nucleic acids are compacted in the nucleus by means of basic proteins−DNA interactions that organize them into fundamental structural units, the nucleosomes. The repetition of these nucleosomes along the DNA fiber composes the chromatin. When the DNA is wrapped in 1.75 left-handed superhelical turns around the core histone octamer and the resulting nucleosome core particles locked by the presence of linker histones such as H1 and/or H5, the structure of its grooves and the base-pairs stacking are modified compared to the naked nucleic acid (*19*). Moreover, the highly positively charged tails of the linker histones are known to be involved in the folding of chromatin into

higher order structure. Although chromatin is a very dynamic structure, these protein−DNA interactions and the compaction of the chromatin are likely to affect ligand−DNA association, by enhanced steric hindrance, by partial neutralization of the nucleotide negative charges, or by modification of the helicoidal structure. To verify this, calorimetric titrations of our two ligands were performed on protein-free DNA, H1/H5-depleted chromatin and on solubilized whole chromatin, at a sodium concentration of 80 mM, where the H1/H5-containing chromatin starts to precipitate and where the effect of salt-induced condensation is the more pronounced.

Figure 1 shows typical titration curves for ethidium bromide. It is to be noted that we have never observed any curve that should be analyzed in terms of two distinct binding sites model, although the concomitant presence of free linker DNA and protein-bound DNA could have resulted in a minority of high-affinity sites on the former and in a majority of weaker affinity sites on the later.

The thermodynamic data displayed in Table 1 indicate that the presence of histones modifies in the same way an intercalative process and a groove insertion. Indeed, the average stoichiometry and the binding constants for the netropsin and the ethidium bromide are affected in the sense of a weaker interaction and a larger ligand distribution on base-pairs of the chromatin as compared to naked DNA. These parameters gradually change from histone-free DNA to H1/H5-free chromatin and chromatin. This supports the view that ligand association is affected by the incorporation of DNA into nucleosomal structures (that are already found in H1/H5-depleted chromatin) as well as by the reduced DNA accessibility resulting from condensation in H1/H5-containing chromatin.

The binding constant K_b of the netropsin varies with increased histone content from 10^5 to 7.2×10^4 and the intercalation constant of ethidium bromide from 1.6×10^5 to 5.6×10^4, so for both processes, the binding affinity changes by less than 1 order of magnitude. On the other hand, the apparent binding site size N is very sensitive to the presence of the nucleosomal structure: it lengthens from 4 base-pairs to 7 and from 2.2 to 4 for netropsin and ethidium, respectively. That is to say that in naked DNA, every bound netropsin is juxtaposed to the neighboring one, since N corresponds to the actual number of base-pairs covered by a netropsin moiety, as revealed by crystallography (*6*). In chromatin, the global site size determined from these experiments is obviously larger than the region actually covered by a single molecule. In the same way, the usual neighboring site exclusion model is suitable to describe the interaction of ethidium bromide on naked DNA, while a more scattered intercalation over a wider base-pairs range takes place on chromatin. So, histones−DNA interactions are strong enough to dictate the position of the ligand on the superstructure rather than being weakened upon drug binding. Both ligand interactions with DNA or chromatin are exothermic, and both enthalpy values exhibit a 20−25% increase when the double helix is constrained into nucleosomal structures. The change in binding enthalpy is mostly compensated by the entropy component variation, since the binding free energy $\Delta G°$, as the affinity constant, are not much influenced by the nature of the macromolecular support.

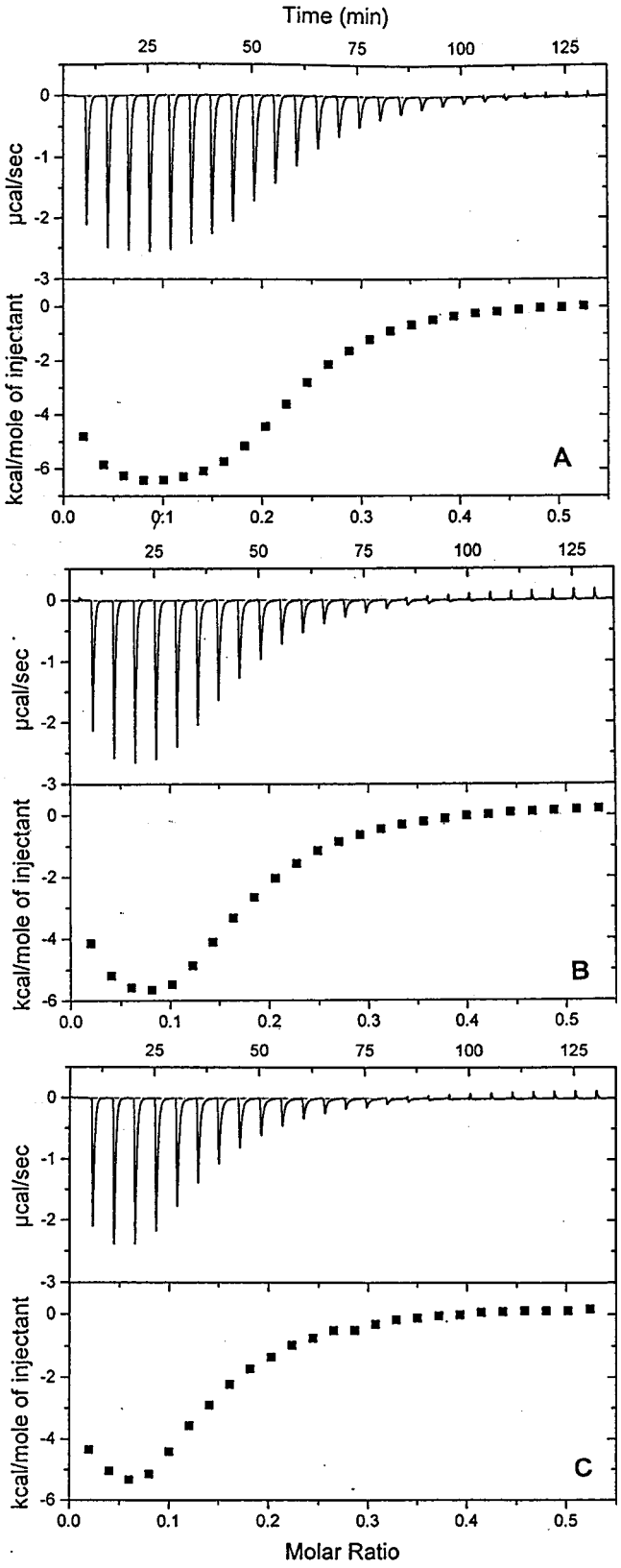

FIGURE 1: Raw calorimetric data for ethidium bromide titration of (A) calf thymus DNA, (B) chicken erythrocyte H1/H5-depleted chromatin, (C) chicken erythrocyte chromatin. All measurements were made at 20 °C in 10 mM Tris, 80 mM NaCl, 0.1 mM EDTA, pH 7.5.

Salt Dependence of the Binding Parameters on Chromatin. The results of the ITC experiments performed at different concentrations are listed in Table 2. The maximum salt

Table 1: Effect of the Presence of Histones on the Ligand Binding Parameters Derived from the Fitting Analysis of Calorimetric Isotherms[a]

	n^b (±1%)	K_b (±4%)	$\Delta H_b{}^c$ (±3%)	$\Delta G°{}^c$ (±0.4%)	$\Delta S°{}^d$ (±10%)	N^e (±1%)
			Netropsin			
CT DNA	0.12	1.0×10^5	−9200	−6700	−8.5	4.2
H1/H5-depleted chromatin	0.09	8.4×10^4	−10200	−6600	−12.3	5.8
CE chromatin	0.07	7.2×10^4	−11400	−6500	−16.7	7.3
			Ethidium			
CT DNA	0.23	1.6×10^5	−7400	−7000	−1.5	2.2
H1/H5-depleted chromatin	0.16	8.6×10^4	−8100	−6600	−5.1	3.0
CE chromatin	0.12	5.6×10^4	−9000	−6400	−9.2	4.0

[a] All values in 10 mM Tris buffer, 80 mM NaCl, 0.1 mM EDTA at pH 7.5 and 20 °C. [b] *n* is the average number of bound ligands per phosphate. [c] ΔH_b and $\Delta G°$ in cal/mol ligand. [d] $\Delta S°$ in cal/K^{-1} mol·ligand. [e] *N* is the average number of base-pairs per binding site.

Table 2: Thermodynamic Parameters Derived from the Fitting Analysis of Calorimetric Ligand Binding Isotherms on Chromatin at Various Sodium Chloride Concentrations[a]

	n^b (±1%)	K_b (±5%)	$\Delta H_b{}^c$ (±1.5%)	$\Delta G°{}^c$ (±0.5%)	$\Delta S°{}^d$ (±6%)	N^e (±1%)
			Netropsin			
0 mM NaCl	0.13	1.9×10^5	−8200	−7100	−4.0	3.9
20 mM NaCl	0.09	1.6×10^5	−9500	−7000	−8.5	5.3
40 mM NaCl	0.09	1.2×10^5	−9000	−6800	−7.3	5.3
60 mM NaCl	0.10	1.0×10^5	−8500	−6700	−6.2	5.0
			Ethidium			
0 mM NaCl	0.18	1.5×10^5	−8800	−6900	−6.3	2.7
25 mM NaCl	0.17	1.1×10^5	−7500	−6800	−2.4	3.1
50 mM NaCl	0.16	8.9×10^4	−7500	−6600	−2.8	3.2
80 mM NaCl	0.12	5.6×10^4	−9100	−6400	−9.2	4.0

[a] All values in 10 mM Tris buffer, 0.1 mM EDTA at pH 7.5 and 20 °C. [b] *n* is the average number of bound ligands per phosphate. [c] ΔH_b and $\Delta G°$ in cal/mol ligand. [d] $\Delta S°$ in cal/K·mol ligand. [e] *N* is the average number of base-pairs per binding site.

concentration attainable is limited by the chromatin precipitation. Figure 2 shows the dependence of K_b on salt concentration, presented in double logarithmic plots of log K_b versus log [Na^+]. It appears from this graph that the binding constants decrease with increasing salt concentration. This can be due to two distinct phenomena: (i) a reduced contribution of electrostatic interactions to the binding of a charged ligand at higher ionic strength; (ii) an alteration of the binding affinity by the progressive transition from a 10 nm "beads-on-a-string" filament structure of the chromatin into a 30 nm more compact solenoidal filament structure.

The first aspect of the problem can be analyzed in terms of counterions release using the polyelectrolyte theory of Record (*20*). According to this theory, if the salt-induced chromatin condensation is neglected, the slopes of the straight lines in Figure 2 should be equal to the following

$$\frac{\delta \log K_b}{\delta \log[Na^+]} = -\Psi Z$$

where Ψ is the apparent fraction of sodium counterions associated with each DNA phosphate in chromatin. *Z* is the number of sodium ions released upon ligand binding and corresponds to the number of ligand charges actually involved in the interaction with the macromolecular support.

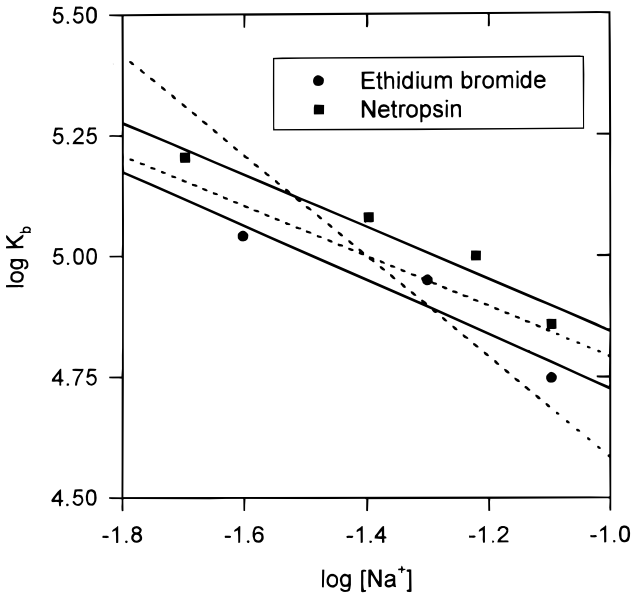

FIGURE 2: Log K_b vs log [Na^+] of ethidium bromide and of netropsin on chicken erythrocyte chromatin in 10 mM Tris, 0.1 mM EDTA, pH 7.5, 20 °C. The solid lines are the linear least-squares fits to the experimental data and the dashed lines represent the theoretical curves predicted from the Record theory for a monovalent ligand and a divalent ligand on chromatin.

Ψ has a value of 0.88 for double-stranded B form DNA (*21*). In chromatin, part of the phosphate charges are neutralized by positively charged histone tails which thus reduce the number of sodium ions condensed along the DNA phosphate backbone, leading to an average Ψ value of 0.52 for whole chromatin. This value was extracted directly from the Manning's polyelectrolyte theory (*17*) that establishes the real fraction of counterion condensed on the DNA, θ, which is related to Ψ by an additional term that includes the contribution of screening. This relationship had to be recalculated for the precise case of chromatin (see Discussion for more details).

We find slopes of −0.56 and −0.54 for ethidium bromide and for netropsin, respectively. A puzzling feature is that both ligands display a similar observed variance in the binding constant with salt concentration, although netropsin has two charges and the ethidium only one. The experimental slopes approach the value of 0.52 that is consistent with an electrostatic interaction involving a single charge for both ligands. Moreover, the concomitant compaction of the chromatin upon an increase of salt concentration was expected to further increase the slope, which is not found to be the case.

Temperature Dependence of the Binding Parameters for Chromatin. The data obtained from ITC experiments were used to examine the temperature dependence of the thermodynamic parameters. Table 3 reports the results, and Figure 3 plots the evolution of the binding constant and the binding thermodynamic values with temperature. At all temperatures investigated, the intercalation as well as the groove insertion into chromatin is enthalpically driven and entropically unfavorable. The slope of a linear least-squares fit of the enthalpy versus T yields the change in heat capacity (ΔC_p), that was found to be −75(±38) cal/mol·K for ethidium bromide and −113(±26) cal/mol·K for netropsin. While the negative enthalpy tends to become larger as the temperature

Table 3: Thermodynamic Parameters Derived from the Fitting Analysis of Calorimetric Ligand Binding Isotherms on Chromatin at Various Temperatures[a]

	n[b] (±1%)	K_b (±5%)	ΔH_b[c] (±1.5%)	$\Delta G°$ [c] (±0.5%)	$\Delta S°$ [d] (±6%)	N[e] (±1%)
			Netropsin			
10 °C	0.11	3.6×10^5	−8000	−7200	−2.9	4.6
20 °C	0.13	1.9×10^5	−8200	−7100	−4.0	3.9
30 °C	0.10	1.5×10^5	−9700	−7200	−8.2	4.8
37 °C	0.09	1.3×10^5	−11000	−7300	−11.9	5.5
			Ethidium			
10 °C	0.20	1.7×10^5	−7400	−6800	−2.1	2.5
20 °C	0.18	1.5×10^5	−8800	−7000	−6.3	2.8
30 °C	0.16	9.0×10^4	−10000	−6900	−10.5	3.1
37 °C	0.19	7.4×10^4	−9100	−6900	−7.0	2.6

[a] All values in 10 mM Tris buffer, 0.1 mM EDTA at pH 7.5. [b] n is the average number of bound ligands per phosphate. [c] ΔH_b and $\Delta G°$ in cal/mol ligand. [d] $\Delta S°$ in cal/°K·mol ligand. [e] N is the average number of base-pairs per binding site.

increases, we note that the resulting free energy remains quite constant, as it is commonly observed for biological interactions.

DISCUSSION

Positioning of Ligands on the Chromatin Fiber. It has been demonstrated in several studies that ethidium bromide induced the partial dissociation of the core particle at a critical concentration (*22*−*26*). This phenomenon, however, did not seem to perturb our calorimetric investigations since the titration curves exhibited a nice sigmoidal profile. Assuming that a major structural change actually happens, this would mean that the associated heat effect is not large enough or the process too slow to be detected during our calorimetric measurements. The overall lengthening of the apparent binding site size and the lower affinity for ethidium bromide as compared to naked DNA corroborates early biophysical studies including spectrophotometry and fluorimetry on ethidium interaction with chromatin (*14*, *27*). Previous studies of electric linear dichroism in our laboratory had also pointed out the reduced stoichiometry of ethidium- and proflavine-complexes to gel-forming chromatin (*28*, *29*).

The observed stoichiometry of the binding on chromatin are in the range of 2.7 to 4 base-pairs/binding site for ethidium and 3.9 to 5 for netropsin. This allows us to discard the model where total interference between histones and ligands forces the small molecules to bind solely to linker DNA. Indeed, as 22% of the base-pairs are found in this region, this situation would imply a stoichiometry of maximum 0.09 bound ligand per phosphate (minimum 5.6 base-pairs per intercalating site) if an ethidium molecule is found at every intercalating site. For a 4 base-pairs covering ligand such as netropsin, its stoichiometry should be in this case less than 0.022 ligand per phosphate, that is to say an apparent site length of 22 base-pairs per netropsin molecule.

So, it appears that the ligands are also scattered on the nucleosomal DNA. However, the widening of the apparent binding site compared to naked DNA claims for the existence of a partial competition between the ligands and the histone tails, that would hinder the binding sites to lie as close to each other as on naked DNA. Histone tails have been reported to play a major role in restricting transcription factor access to DNA. This competition effect toward regulatory molecules can be alleviated by core histones acetylation that might direct dissociation of the tails (*30*). We also think that there are some regions in the nucleosome (on either side of the dyad axis) containing sharp DNA bends that may not be able to accommodate a ligand molecule due to strong DNA distortion, as a consequence of a narrower minor groove (*31*−*33*). These severely deformed, kinked DNA regions were also revealed as being preferential targets for integration mediated by the HIV integrase, probably reflecting the enzyme preference for a wide major groove (*34*). Furthermore, although precise location of linker histones on the nucleosome core particle remains a subject of controversy (*35*), it is conceivable that the DNA regions simultaneously covered by the histone octamer and H1/H5 might be hardly accessible for ligand binding.

The determined stoichiometry for netropsin−chromatin complexes agrees well with DNAase I cleavage studies that reported that minor groove binders such as netropsin, distamycin, berenil together with the antibiotic echinomycin induced 180° rotational repositioning of DNA on the core particle (*13*, *36*, *37*). Indeed, if no rotation occurred, only half of the minor grooves on the core particle would be available for netropsin insertion. If we assume that a netropsin molecule covers 4 base-pairs, so $46/4 = 11.5$ molecules may be found on the internucleosomal DNA, while a maximum of $(166/2)/4 = 20.7$ molecules could lie on the nucleosome. This would result in a minimum apparent stoichiometry of $212/(11.5 + 20.7) = 6.6$ base-pairs per ligand. Hence, the experimental stoichiometry of 5 might be explained in the following way: during the binding of the netropsin molecules, a change in the rotational setting of the DNA takes place, so as to place some antibiotic occupied binding sites on the inward-facing surface of the DNA superhelix and thereby exposing some buried minor grooves.

Electrostatic Contributions to the Binding on Chromatin. To estimate the Ψ value for the chicken erythrocyte chromatin, we solved the Manning equations for three distinct domains of chromatin, as previously described, taking into account the contribution of histones to the neutralization of the phosphate negative charges. Domain I is the linker DNA, free of contact with histones and containing 92 phosphates in chicken erythrocyte chromatin (*38*); hence, the fraction neutralized by the histones in this region is $\theta_{H(I)} = 0$. Domain II is the part of chromatosome DNA containing 40 nucleotides that are partially neutralized by the basic histone H1 or H5, resulting in a reduction of the negative charge of the phosphates $\theta_{H(II)} = 0.58$ (*18*). Domain III is the core DNA, composed of 292 bases and has a fraction of phosphate charges neutralized by core histones $\theta_{H(III)} = 0.447$ (*39*). The degree of neutralization of DNA by sodium ions $\theta_{I,\ II,\ or\ III}$ was determined for each domain in the studied salt concentration range and was found to be equal to 0.76, 0.25, and 0.36 for the three domains, respectively. A global θ of 0.44 for whole chromatin was obtained by the weighted average of the θ of each region in chromatin, as follows:

$$\theta = \frac{92\ \theta_I + 40\ \theta_{II} + 292\ \theta_{III}}{424}$$

Similarly, a global fraction neutralized by the histones was calculated as the weighted average in each domain to give

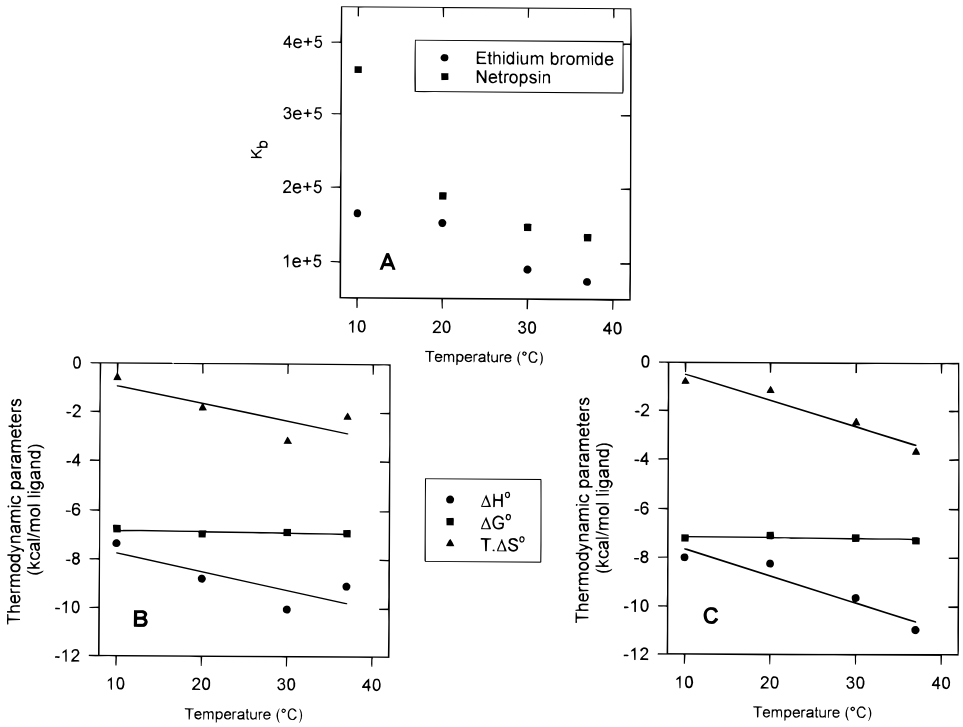

FIGURE 3: (A) Dependence of the ligand binding constant on the temperature in 10 mM Tris buffer, 0.1 mM EDTA at pH 7.5. (B) Dependence of observed thermodynamic quantities of ethidium binding to chromatin. (C) Dependence of observed thermodynamic quantities of netropsin binding to chromatin.

$\theta_H = 0.36$. The total fraction of phosphate charge neutralized on chromatin is then $\theta_T = \theta_H + \theta = 0.8$. For the specific case of chromatin, Ψ is related to θ according to $\Psi = \xi/2(1 - \theta_T)^2 + \theta = (4.182)(0.5)(0.2)^2 + 0.44 = 0.52$. This expression comes from the derivation of eq 7.7. in Record's paper (*20*) with regard to ln a_{Na^+}. For chromatin, the term containing the activity coefficients can be expressed, after suitable approximations, as $\ln(\gamma^0_A\,\gamma^0_B/\gamma^0_{AB}) = -Z(\xi/2)(1 - \theta_T)^2 \ln a_{Na^+} + Z(1 - \theta_T)^2\xi \ln \delta$. Assuming that $\ln a_{Na^+} \sim \ln[Na^+]$, differentiation of the whole eq 7.7. then gives $d \ln K_{obs}/d \ln [Na^+] = -Z(\xi/2)(1 - \theta_T)^2 - Z\theta = -Z\Psi$.

Our salt-dependent binding data reveal that, in agreement with the Record theory, the average number of counterions released upon ligand binding reaches a value of about one Na^+ ions per ligand molecule, whether the charge held by the ligand is one or two. Counterion release is essentially an entropically driven phenomenon and generally considered as an athermal process. However, the present thermodynamic study has shown that the high affinity of our ligands for chromatin is primarily due to enthalpy changes rather than to entropy changes. Consequently, the molecular origins for the observed enthalpy-driven binding affinity on chromatin must reside mainly in the hydrogen bonds, the van der Waals contacts, and/or the hydrophobic interactions instead of in electrostatic interactions.

The value of −0.54 for the doubly charged netropsin is much lower than the −1.05 theoretical value predicted from polyelectrolyte theory, assuming that every ligand charge contributes to electrostatic interactions. This discrepancy may be attributed to the fact that the oligopeptide would not be able to adopt the curvature of minor groove in higher ordered DNA in such a manner that solely a single charged end can interact with the negatively charged backbone. It must also be kept in mind that the theoretical formalism developed by Manning describes the binding of counterions on a polyelectrolyte chain as delocalized nonspecific binding of punctual charges. It has already been reported that the punctual charge of multivalent cations (polyamines, for example) had to be replaced by a lower effective charge resulting from the spatial dispersion of the charge on the whole molecule to reconcile the model with the experimental observations (*40*, *41*). Thus, it is not excluded that the binding behavior of the dication netropsin would be better explained by considering a reduced effective charge to account for its separated charges. This reduced effective charge could stem from the positioning of the two charges on a single molecular backbone of somewhat reduced flexibility: that constraint may to some extent prevent both charges from approaching the phosphate up to the ideal distance for complete neutralization and would lead to the formation of two dipoles (incomplete neutralization) that could still influence the neighboring monovalent sodium ions.

It is to be noted that similar studies on DNA duplexes have reflected the involvement of both charged ends of netropsin in the binding event, even when the body of the drug cannot penetrate deeply into the minor groove (*42*, *43*). Dickerson and co-workers' X-ray structure and Patel's NMR picture of the netropsin on a A_2T_2-containing oligomer revealed two intermolecular electrostatic interactions between the two netropsin charges and the phosphate groups (*6*, *44*). However, in a thermodynamic study of the netropsin interaction with the Dickerson oligomer, Rentzeperis and Marky also reported much lower slope values of the log K_b vs log $[Na^+]$ than the theoretical value of −1.76 for a dicationic ligand (*45*). Concerning ethidium bromide, two studies reported a release of one ion per intercalated molecule on DNA or on poly[d(A−T)]$_2$ (*43*, *46*), and binding on nonligated hairpins is accompanied by a range of released

counterions less than 1, this being attributed to a lower charge density in hairpins (*47*).

The other key point revealed by the salt-dependent thermodynamic studies is that the compaction of the chromatin fiber does not impede either minor groove insertion or intercalation between base-pairs, as a result of the dynamic feature of the 30 nm superstructure.

From the dependence of the binding constant on salt concentration, the "polyelectrolyte" contribution to the observed binding free energy may be calculated. The observed standard binding free energy may be partitioned into two contributions: $\Delta G°_{obs} = \Delta G°_{t} + \Delta G°_{pe}$ where $\Delta G°_{t}$ is the nonelectrostatic contribution to and $\Delta G°_{pe}$ is the polyelectrolyte contribution to $\Delta G°_{obs}$. Record and coworkers have shown that $\Delta G°_{pe}$ is equal to $\Delta G°_{pe} = -Z\Psi RT$ ln[NaCl] (*48*). The magnitude of $\Delta G°_{pe}$ for both ligands at 20−25 mM NaCl is about −1.2 kcal/mol, so representing around 18% of the overall binding Gibbs energy and indicating that nonelectrostatic forces play a significant role in the stabilization of the chromatin−ligand complexes.

Molecular Origins of the Thermodynamic Profile for Ligand Binding to Chromatin. The thermodynamic profile of a ligand binding results from various concomitant phenomena that all contribute differently to the enthalpy and the entropy terms of the global reaction (*42*, *49*, *50*). The exothermicity observed in both cases results essentially from van der Waals contacts in the minor groove and hydrogen bond formation for the netropsin, while ethidium bromide intercalation leads to enthalpically favored van der Waals stacking that dominates the unfavored hydrophobic interactions and the unstacking of base-pairs. The exalted enthalpy compared with free DNA as macromolecular support arises probably from the fact that the curved DNA conformation inside the superstructure displays a better arrangement for exothermic contacts and shows a reduced energy of stacking between adjacent base-pairs to be overcome upon intercalation. The unfavored negative entropy follows from the above-mentioned interactions. It seems unlikely that a loss of DNA flexibility would contribute an important part of the negative entropy, since the conformation of the DNA is already quite constrained within the chromatin structure. The counterion release, lower than what could be expected, is not able to compensate entropically for these effects. Similarly, the thermodynamic profile shows that the displacement of bound water is not a major aspect of the binding on chromatin, maybe reflecting a reduced degree of hydration of the supercoiled DNA.

CONCLUSION

We have investigated the interaction of two classical DNA ligands, an intercalator, and a minor groove binder with chromatin. First, we demonstrated that the presence of histones on DNA still allows the ligand binding that takes place according to a one single-site model. For both ligand types, the thermodynamic driving force is enthalpic and the association is characterized by a somewhat weaker affinity and more scattered ligand distribution than on naked DNA. Second, we have noticed that the ligand affinity is weakly altered by the salt-induced compaction of the chromatin, so the binding affinity decrease upon salt addition essentially results from modification of electrostatic interaction. Record's theory has shown that the binding is accompanied by one counterion release per ligand, equivalent to one single electrostatic interaction per ligand molecule. Third, the temperature-dependent studies have revealed the existence of a small heat capacity change associated with ligand binding on chromatin, together with an enthalpy−entropy compensation that maintains the free energy constant over the investigated temperature range.

ACKNOWLEDGMENT

Thanks are due to J.-C. Labie for technical assistance.

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BI972735V