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Thermodynamics of Cationic Lipid Binding to DNA and DNA Condensation: Roles of Electrostatics and Hydrophobicity

Daumantas Matulis, Ioulia Rouzina, and Victor A. Bloomfield*

Contribution from the Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, St. Paul, Minnesota 55108

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Abstract: Alkylammonium binding to DNA was studied by isothermal titration calorimetry. Experimental data, obtained as functions of alkyl chain length, salt concentration, DNA concentration, and temperature, provided a detailed thermodynamic description of lipid−DNA binding reactions leading to DNA condensation. Lipid binding, counterion displacement, and DNA condensation were highly cooperative processes, driven by a large increase in entropy and opposed by a relatively small endothermic enthalpy at room temperature. Large negative heat capacity change indicated a contribution from hydrophobic interactions between aliphatic tails.An approximation of lipid−DNA binding as dominated by two factors—ionic and hydrophobic interactions—yielded a model that was consistent with experimental data. Chemical group contributions to the energetics of binding were determined and could be used to predict energetics of other lipid binding to DNA. Electrostatic and hydrophobic contributions to Gibbs free energy, enthalpy, entropy, and heat capacity could be distinguished by applying additivity principles. Binding of lipids with two, three, and four aliphatic tails was investigated and compared to single-tailed lipid binding. Structurally, the model suggests that lipid cationic headgroups and aliphatic tails distribute evenly and lay down on DNA surface without the formation of micelles.

Introduction

The process of cationic lipid binding to DNA is of great importance for both basic science and biotechnological applications. However, neither the energetics of interaction nor the structure of the resultant complex is well understood despite many studies in the field. To clarify the energetics of this important but complex system, we investigated the thermodynamics of cationic lipidlike ligand binding to DNA by isothermal titration calorimetry. Extensive experimental data is presented as functions of lipid alkyl chain length, DNA concentration, ionic strength of solution, and temperature, yielding all thermodynamic parameters of the interaction process. We used model compounds that are not naturally occurring lipids, but are of the simplest chemical structure and bear two components of the naturally occurring lipids—cationic headgroup and hydrophobic aliphatic tail. By comparing the behavior of ligands with similar structures we obtained the contributions of chemical groups, such as CH_2, to the energetics of DNA−lipid binding.

We then developed a mathematical model of binding that accounts for all observed data in a global fashion. Similar models have been developed by others.[1] The model approximates DNA−lipid interaction as a sum of two processes, (i) ammonium headgroup electrostatic interaction with DNA phosphate by displacing sodium cation from counterion atmosphere and (ii) cooperative hydrophobic interaction between aliphatic tails that also brings multiple DNA molecules together. It appears that these two processes are sufficient to fit all experimental data.

DNA−cationic lipid interaction has been studied by a variety of methods using various DNAs and lipids, including lipids that are aggregated into micelles,[2] liposomes,[3,4] in organic solvents,[5] using fluorophore probes,[6] by potentiometric titration [1], fluorescence microscopy,[7] differential scanning calorimetry,[8] and isothermal titration calorimetry.[9] Investigation of closely related processes such as counterion release from DNA upon binding lipids[10] and DNA overcharging to a positively charged complex by a lipid has received considerable attention.[11,12] Modeling of electrostatic, hydrophobic, and hydration forces in such interac-

* Corresponding author. Telephone: 612-625-2268. Fax: 612-625-5780. E-mail: victor@umn.edu.

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tions has also been widely studied.[13] Phase diagrams of lipid−DNA complexes also proved to be complex.[14,15]

Cationic lipids are widely used to transfect DNA across the cell membrane for gene delivery applications.[16,17] However, the process is rather poorly understood, and the search for the most efficient transfection agents is mostly empirical. Here we do not study DNA transfection across a membrane, but we investigate the first stage of the transfection process, namely, DNA−lipid binding and DNA condensation.

It has been observed that monovalent and divalent cations, such as sodium and magnesium, do not condense DNA. Usually the smallest charge that the cation has to possess to condense DNA is +3.[18] Cobalt hexammine and spermidine are the most widely used trivalent cations that condense DNA.[19] However, monovalent alkylammonium cations that have sufficiently long aliphatic chains also efficiently condense DNA. This is because, in addition to electrostatic attraction forces of a monovalent cation, cationic lipids also possess aliphatic tails that tend to bind to each other due to hydrophobic interactions. Lipid−DNA and lipid−lipid binding may not be experimentally distinguishable from DNA condensation because the two processes occur highly cooperatively and simultaneously. The energetic parameters reported in this study pertain to the sum of all simultaneously occurring processes, including sodium displacement, lipid binding, and condensation of the DNA−lipid complex. Aggregation and precipitation of the condensed complex occurred in every case where binding was observed.

Most isothermal titration calorimetry experiments of DNA−lipid binding in the previous studies have been carried out with lipids in micellized[20] or liposomal (vesicular)[21,22] forms. The study by Spink[20] attempted to dissect the energetics of lipid micellization and binding to DNA. Calorimetric titration study of nonmicellized dodecyltrimethylammonium binding to DNA[9] showed a cooperative binding peak similar to the ones reported in this study.

A systematic calorimetric study of cationic lipid with varied hydrophobic tail length binding to DNA has been lacking. Furthermore, there is a need to distinguish the energetics of lipid binding from counterion displacement by carrying out experiments at various ionic strengths. There is also a need for a systematic investigation of the dependence of energetics on various lipid structure features such as double-tailed lipids. The energetic approach to understand DNA−lipid interaction could be complementary to structural studies. Unfortunately, there are no atomic resolution DNA−lipid cocrystal structures in the Nucleic Acid Database nor are there NMR structures of the complexes in solution, which might help assign energetic results to particular structural features of the complex. However, calorimetric results and models give considerable insight into the role of atomic-level groups in the interacting partners.

We show that every additional methylene group of the aliphatic lipid chain increases the association constant about 4-fold by increasing the binding cooperativity. The enthalpy remains endothermic, relatively small, and practically independent of aliphatic chain length or salt concentration. Binding is weaker at high salt because of reduced electrostatic entropy but is stronger with increased chain length because of increased hydrophobic entropy. As expected for an aggregation process, lipid−DNA interaction thermodynamics is dependent on DNA concentration. Finally, as expected for most hydrophobic reactions, the binding strength is practically independent of temperature because of compensating entropic and enthalpic contributions to the process.

Materials and Methods

Preparation of DNA. Two kinds of double-stranded DNA were used in this study—plasmid pUC118 DNA and calf thymus DNA. *Escherichia coli* strain DHP5-α with pUC118 plasmid was obtained from Dr. Anath Das, University of Minnesota, and is available from Worthington Biochemical Corporation (Lakewood, NJ). Cells were grown in 6 L of LB medium containing 50 μg/mL ampicillin. Plasmid was isolated and purified using Qiagen (Germany) Endo Free Giga plasmid isolation kit. Plasmid size is 3168 base pairs. Size homogeneity was confirmed to be above 98% by agarose gel electrophoresis. Plasmid DNA stock was prepared by dissolving dried DNA in water (∼0.5 mg/mL) with 10 mM NaCl. DNA concentration was determined spectrophotometrically using the relationship 1 A_{260} = 50 μg/mL = 0.15 mM DNA phosphate (nucleotide).

Calf thymus DNA (catalog no. D-1501) was purchased from Sigma Co., St. Louis, MO. Stock solution was prepared by dissolving dry DNA in water (∼1 mg/mL) with 10 mM NaCl. The solution was sonicated on ice five times for 15 s. Obtained DNA fragment sizes, as confirmed by agarose gel electrophoresis, were about 1000 base pairs. Titration calorimetry experimental results were similar when comparing the two sources of DNA. Most experiments were carried out using plasmid DNA because it is homogeneous in size and data were less noisy.

Chemicals. The following amines were purchased from Aldrich Co., Milwaukee, WI. Propylamine (%), propylammonium hydrochloride, octylamine (99%, cat. no. O-580-2), nonylamine (98%, cat. no. N3,-100-1), decylamine (95%, cat. no. D240-4), undecylamine (98%, cat. no. U140-0), tridecylamine (98%, cat. no. T5,800-9), tetradecylamine (96%, cat. no. T1,020-0), dihexylamine (97%, cat. no. 13,120-2), dioctylamine (98%, cat. no. D20,114-6), didecylamine (98%, cat. no. 26,497-0), trihexylamine (96%, cat. no. 18,399-7), trioctylamine (98%, cat. no. T8,100-0), tetrahexylammonium chloride (98%, cat. no. 26,-383-4), tetraheptylammonium bromide (98%, cat. no. 23,784-1), tetraoctylammonium bromide (98%, 29,413-6), didecyldimethylammonium bromide (98%, cat. no. 38,231-0), didodecyldimethylammonium bromide (98%, 35,902-5). The following amines were purchased from Acros Organics (currently obtainable through Fisher Scientific): octylamine hydrochloride (99%, cat. no. 41643-1000), dodecylamine (98%, cat. no. 11766-1000). *N*-dodecyl trimethylammonium bromide (purity not declared, cat. no. US 18218) was obtained from USB corporation, Cleveland, OH, and Sigma Co. (cat. no. D-8638). Tetradecyltrimethylammonium bromide (cat. no. T-4762) and hexadecyltrimethylammonium bromide (cat. no. H-5882) were also obtained from Sigma Co.

Alkylamine aqueous solutions were prepared by dissolving them in water containing equivalent amount of hydrochloric acid. Liquid amines were stored in tightly sealed containers because they react with atmospheric CO_2. Solid amines were kept in desiccators to prevent accumulation of moisture.

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Isothermal Titration Calorimetry of Ligand Binding. Isothermal titration calorimetry was performed with a Microcal (Northampton, MA) MCS calorimeter at 25.0−58.0 °C temperatures by keeping the cooling circulating bath temperature constant at 20 °C. The cell volume was 1.3438 mL. Two milliliters of pUC118 DNA solution in 10 mM NaCl (unless otherwise specified), pH 6.8−6.9, with no buffer, was added to the calorimeter cell. After careful washing, the cell was prerinsed with the portion of the same DNA solution before each titration experiment. The concentration of the DNA phosphate groups (nucleotides) in the cell was 0.33 mM (unless otherwise specified) at the beginning of titration. Various ligand solutions, pH 7.0 ± 0.4, were injected in 40 portions of 6.25 μL with a 250-μL injection syringe, at 3-min intervals. A control experiment was carried out for each ligand to find out the heats of ligand dilution by omitting DNA from the solution in the cell. In most cases, when the heat of dilution was less than 5% of the heat of binding, it was disregarded. When the heat of dilution was above 5% (and the heat of dilution was nearly constant throughout the control experiment) and the peaks of the same size could be visible at the end of the actual binding experiment, then the heat of dilution was subtracted to obtain the heat of binding. If every one of these conditions could not be fulfilled, then the data were regarded as unreliable and were not included in this study.

Heat measurement of the calorimeter was calibrated electrically and verified by carrying out the Tris base protonation reaction with hydrochloric acid ($\Delta H = -47.6$ kJ/mol).

Analysis of the Titration Calorimetry Data. Raw data curves were integrated using Microcal Origin software as described in the instrument manual. The baseline was not adjusted to improve data fitting with the model to accurately represent the original data. The cooperative binding model was designed to generate lines to best represent the experimental data as described in the Results section. The area under the experimental curve and the model curve were adjusted to match each other.

During a titration calorimetry experiment, the concentration of DNA in the cell decreases slightly. For example, when the starting concentration was 0.333 mM DNA phosphate, then the final concentration after 40 injections was 0.296 mM. A correction to account for this change was found to be not necessary because the integral molar enthalpy was affected by less than 3%, and most heat was evolved during initial injections.

Results

Linear Alkyl-1-ammonium Cation Binding to DNA. We first considered the structurally simplest lipidlike cation, alkylammonium chloride, binding to DNA. A typical experimental isothermal titration calorimetric curve of undecylammonium binding to DNA is shown in Figure 1. There is little heat emited or absorbed with initial injection of the ligand. Then there is a gradual increase in the size of positive peaks, and eventually they decrease until all peaks remain small and negative. A control experiment, where DNA was omitted from the cell, showed that the small negative peaks are associated with the heat of dilution of the ligand. Both raw data titration curves were integrated. Then the integrated curve obtained without DNA was subtracted from the curve obtained in the presence of DNA, yielding curves as shown in Figures 2−6, 8 (datapoints only).

As in a previous study[9], we found that all tested cationic lipidlike ligands bound to DNA with endothermic enthalpy at 25 °C. The enthalpic contribution opposed the binding reaction. Therefore, the reaction could be driven only by the positive change in entropy. These enthalpies and entropies cannot be directly assigned to the binding reaction because a series of reactions occur simultaneously. First, a sodium cation must be displaced from the counterion atmosphere upon alkylammonium binding to DNA, because double-stranded DNA cannot be completely stripped of counterions. Such counterions are not bound specifically. Instead they are thought to be fully hydrated and held nearby by nonspecific electrostatic forces.[23] Therefore, we actually study a competition reaction between the two ligands:

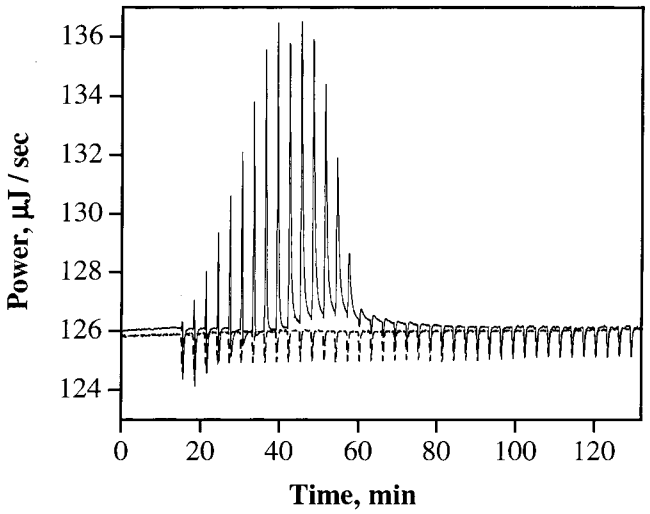

Figure 1. Raw data isothermal titration calorimetric curves of undecylammonium chloride binding to plasmid pUC118 DNA. Solid line shows the dependence of power on time for each injection of undecylammonium onto the DNA solution. The dashed line shows a titration at identical conditions except that DNA was omitted from the solution in the calorimetric cell providing the heats of ligand dilution during titration.

$$DNAP^- \cdots Na^+ + RNH_3^+ + Cl^- \leftrightarrow DNAP^- \cdot RNH_3^+ + Na^+ + Cl^- \quad (1)$$

A second reaction, DNA condensation, occurs simultaneously with the ligand displacement:

$$n DNAP^- \cdot RNH_3^+ \leftrightarrow (DNAP^- \cdot RNH_3^+)_n\downarrow \quad (2)$$

The two reactions cannot be studied independently because they occur at the same time in a highly cooperative manner. Therefore, the energetics that we measure should be assigned to the sum of all these processes.

Alkylammonium chlorides are significantly soluble in water and do not form micelles (at 2 mM concentration, in low salt solutions) up to the length of alkyl chain of 14 carbon atoms (tetradecylammonium chloride). Therefore, titration calorimetry yields the heats associated with processes (1) and (2) without the interfering heat of lipid demicellization.

Application of the Zimm−Bragg DNA Melting Model To Simulate Lipid Binding Curves. Now we apply a Zimm−Bragg-type model [24,25] to quantitatively describe alkylamine binding to DNA. The model was originally applied to DNA melting, expressing the fraction of melted DNA (Θ) as a function of two parameters, s and σ:

$$\Theta = \frac{1}{2} + \frac{1}{2}\left(\frac{s-1}{\sqrt{(s-1)^2 + 4s\sigma}}\right) \quad (3)$$

The parameter s describes the strength of association at a single site (base pairing), and the parameter σ is the cooperativity.

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Cationic lipidlike alkylammonium binding to DNA can be described in a manner mathematically and conceptually similar to DNA melting. The binding process is highly cooperative, that is, an additional ligand molecule prefers to bind next to the previously bound ligand rather than to a portion of DNA with no bound ligand molecules. The binding strength (K) of such interaction is equivalent to the parameter s in the DNA melting model. The cooperativity σ shows how many times smaller is the probability to find ligands bound isolated from each other than adjacent to each other. For convenience, we introduce cooperativity ω ($\omega = 1/\sigma$) which shows how many times greater is the probability to find ligands bound adjacent to each other than away from each other. The fraction of bound ligand per DNA phosphate (Θ) can be expressed as a function of the binding strength (K) and cooperativity (ω):

$$\Theta = \frac{1}{2} + \frac{1}{2}\left(\frac{K-1}{\sqrt{(K-1)^2 + 4\frac{K}{\omega}}}\right) \quad (4)$$

Localization (binding) constants of Na^+ cations at the DNA surface are described by the following relationship where α is equal to 1 at low ionic strength ([NaCl]→0) and $^1/_2$ at high ionic strength ([NaCl] → ~1 M):[26]

$$K_{Na^+} = \left(\frac{[\text{cation}^+]_{surf}}{[Na^+]}\right)^{\alpha} \quad (5)$$

where $[\text{cation}^+]_{surf}$ is the concentration of all cations localized at DNA surface, approximately equal to 1 M, and independent of sodium concentration in bulk solution $[Na^+]$. Similarly, the binding constants of alkylammonium cations are equal to

$$K_{RNH_3^+} = \left(\frac{[\text{cation}^+]_{surf}}{[RNH_3^+]K_{sp}}\right)^{\alpha} \quad (6)$$

where $[RNH_3^+]$ is the concentration of free alkylammonium cation and K_{sp} is the specificity competition constant which shows the extent to which a hypothetical amine without the hydrophobic tail, binds more strongly to DNA than does Na^+. If only nonspecific electrostatic forces played a role here, K_{sp} would be equal to unity because both cations are singly charged. If sodium binds stronger than the hypothetical amine, K_{sp} is smaller than unity. The best fit of all our experimental data yielded K_{sp} equal to 0.028.

Electrostatic alkylammonium binding constant may be expressed as

$$K_{elec} = \frac{K_{Na^+}}{K_{RNH_3^+}} = \left(\frac{[RNH_3^+]}{[Na^+]}K_{sp}\right)^{\alpha} \quad (7)$$

and the overall alkylammonium binding constant K in eq 4 is the product of electrostatic binding constant and cooperativity:

$$K = K_{elec}\omega = \left(\frac{[RNH_3^+]}{[Na^+]}K_{sp}\right)^{\alpha}\omega = \left(y_f\frac{[DNAP]_{tot}}{[Na^+]}K_{sp}\right)^{\alpha}\omega \quad (8)$$

where y_f is the ratio of free ligand concentration $[RNH_3^+]$ to total DNA phosphate (nucleotide) concentration $[DNAP]_{tot}$.

We now express the fraction of bound ligand using eqs 4 and 8:

$$\Theta = \frac{1}{2} + \frac{1}{2} \times \left(\frac{\left(y_f\frac{[DNAP]_{tot}}{[Na^+]}K_{sp}\right)^{\alpha}\omega - 1}{\sqrt{\left(\left(y_f\frac{[DNAP]_{tot}}{[Na^+]}K_{sp}\right)^{\alpha}\omega - 1\right)^2 + 4\left(y_f\frac{[DNAP]_{tot}}{[Na^+]}K_{sp}\right)^{\alpha}}}\right) \quad (9)$$

Here $\alpha = 1$ for reactions which are carried out at low ionic strength up to about 50 mM NaCl. A slight correction for α was necessary only for reactions occurring at higher ionic strength.

Titration calorimetry measures the heat δH evolved upon addition of the ligand. It is assumed that the heat is proportional to the amount of bound ligand, independent of the reaction progress. This heat is equal to the enthalpy because the pressure is constant. A series of enthalpies are obtained during the titration calorimetry experiment as a function of y, added ligand concentration divided by total DNA phosphate concentration in the cell:

$$y = y_f + \Theta = \frac{[RNH_3^+]_{tot}}{[DNAP]_{tot}} \quad (10)$$

Therefore:

$$\delta H(y) = \Delta H\frac{\partial\Theta}{\partial y} \quad (11)$$

where ΔH is the integral molar enthalpy of ligand binding. This integral enthalpy is obtained experimentally by summing all heats evolved during each addition of the ligand.

To fit the model curves to our experimental titration calorimetry curves, we expressed the derivative of Θ as:

$$\frac{\partial\Theta}{\partial y} = \frac{\Theta(i\text{th injection}) - \Theta((i-1)\text{ injection})}{y(i\text{th injection}) - y((i-1)\text{ injection})} \quad (12)$$

Figures 2−6 and 8 show experimental ITC datapoints and their curve fits using the model described above. Such a simple model with only two fitting parameters for low salt solutions (K_{sp} and ω) and three fitting parameters for high salt solutions (K_{sp}, ω, and α) should not be expected to reproduce small experimental details. However, the model predicts the positions and general shapes of the experimentally observed peaks as a function of alkyl chain length, salt concentration, and DNA concentration.

Electrostatic and Hydrophobic Contributions to the Gibbs Free Energy. Alkylammonium cations bind to DNA due to the combination of electrostatic and hydrophobic forces. We asume that the Gibbs free energies of the two contributions are additive:

$$\Delta G = -RT\ln K = \Delta_{elec}G + \Delta_{h\Phi}G \quad (13)$$

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The electrostatic component of the Gibbs free energy of binding is equal to

$$\Delta_{elec}G = -RT\alpha \ln\left(\frac{[RNH_3^+]}{[Na^+]}K_{sp}\right) = -RT\alpha \ln\left(y_f\frac{[DNAP]_{tot}}{[Na^+]}K_{sp}\right) \quad (14)$$

where R is the gas constant, T is the temperature in Kelvin, α is the empirical parameter in eq 5, $[RNH_3^+]$ is the concentration of unbound alkylammonium cation, and $[Na^+]$ is the concentration of unbound sodium ions.

The hydrophobic contribution to the Gibbs free energy of binding depends on m, the number of carbon atoms in the alkyl chain:

$$\Delta_{h\Phi}G = RT \ln \omega = \Delta_0 G + \Delta_{CH_2}Gm \quad (15)$$

where $\Delta_0 G$ is the reference free energy of hydrophobic effect, and $\Delta_{CH_2}G$ is the contribution to the free energy from a methylene group. In terms of the cooperativity factor ω, eq 15 can be rewritten as:

$$\omega = \omega_0 \Delta\omega^m \quad (16)$$

Here $\Delta\omega$ is the increment in the cooperativity factor per methylene group. This increment should be equal to about four, that is, alkyl chain elongation by one methylene group increases the tendency of ligand binding adjacent to each other by about 4 times. A factor of 4.24 has been found in many systems where alkyl chain length was varied, including alkane solubilities [27,28] and the aggregation of alkylamines upon deprotonation.[29−31]

The position of the maximum of each peak in Figures 2−6, and 8 can be accurately predicted using the following relationship which is derived from eqs 8 and 10 when $K = 1$:

$$y_{max} = \frac{1}{2} + \left(\frac{[Na^+]}{[DNAP]_{tot}}\right)\left(\frac{1}{K_{sp}\omega^{1/\alpha}}\right) \quad (17)$$

Similarly, the width of a peak (δy) can be found from the reverse derivative of eq 9 when $K = 1$

$$\delta y = 1 + \frac{4[Na^+]}{\alpha[DNAP]_{tot}K_{sp}\omega^{(1/\alpha+1/2)}} \quad (18)$$

Gibbs free energies of the combined processes 1 and 2, including the electrostatic and hydrophobic contributions for various ligands and experimental conditions, are summarized in Tables 1−5.

Electrostatic and Hydrophobic Contributions to Enthalpy. Enthalpies of processes 1 and 2 for various ligands also have contributions arising from electrostatic and hydrophobic interactions. However, the observed enthalpy was nearly independent of the ligand aliphatic tail length making it difficult to dissect contributions to the enthalpy arising from headgroup and aliphatic tail. One way to dissect these contributions is to assume that the enthalpy of ammonium phosphate crystallization (per ion pair) and the enthalpy of alkylammonium headgroup binding to DNA are equal. In other words, we assume that the enthalpies observed in inorganic reactions for specific bond formation carry over to large macromolecules when the same kind of bonds are formed. We believe that this assumption is reasonable, but the uncertainty of such additivity is significant.

The calorimetric enthalpy of ammonium phosphate dissolution in water is equal to −3.0 kJ/mol.[32] There are three ammonium groups per one phosphate anion in the crystal. Therefore, we would expect the enthalpy of one ammonium-phosphate pair formation to be about +1.0 kJ/mol.

Alternatively, we can calculate the electrostatic enthalpy of cation binding to DNA by using the relationship:[19]

$$\Delta H = zRT(\nu - 1) \quad (19)$$

where z is the valence of the cation and $\nu = 1.4$ is the experimentally determined exponent in the equation for the temperature dependence of the dielectric constant of water:

$$\epsilon = \epsilon^*(T^*/T)^\nu \quad (20)$$

where ϵ^* is the dielectric constant of water at $T^* = 298$ K. According to relations 19 and 20, the predicted electrostatic enthalpy of monovalent cation binding to DNA is equal to +0.99 kJ/mol, for divalent is +1.98 kJ/mol, and for trivalent is +2.97 kJ/mol. Thus, we assume both from the ammonium phosphate crystallization and electrostatic calculations that the electrostatic component of binding enthalpy is about +1.0 kJ/mol at 25 °C (Table 1).

The hydrophobic component of the binding enthalpy is obtained by subtraction:

$$\Delta_{h\Phi}H = \Delta H - \Delta_{elec}H \quad (21)$$

Total, hydrophobic, and electrostatic entropies of processes 1 and 2 were calculated and presented in Tables 1−5 using the standard thermodynamics equation

$$T\Delta S = \Delta H - \Delta G \quad (22)$$

Dependence on Alkyl Chain Length. As we see from Figure 2, binding of linear alkylammonium chlorides to DNA is stronger—peaks are shifted to the left—when the alkyl chain of the ligand is longer. Nonylammonium chloride was the shortest alkylamine that bound to DNA at our experimental conditions. Its binding peak is shifted significantly to the right as compared to ligands with longer alkyl chains. In other words, it took more decylammonium than undecylammonium cation to displace sodium and condense DNA. The longer the alkyl chain, the stronger the binding and condensing capability of the ligand.

The curves in Figure 2 were fitted using the above-described model by applying an additional constraint, namely, that each additional methylene group increases the cooperativity ω by a factor of 4.24 which was described above. Consistency of such model with the experimental data indicates that each additional methylene group increases the association constant by the same value and the contribution to the Gibbs free energy is constant (Table 1). It also indicates that the logarithm of the solubility

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Table 1. Thermodynamics of Linear Alkylammonium Binding to pUC118 DNA with Sodium Displacement (eq 1) and Condensation of the Alkylammonium−DNA Complex (eq 2) as a Function of Alkyl Chain Length[a]

ligand	ω	K	ΔG, kJ/mol	$\Delta_{h\phi}G$, kJ/mol	ΔH, kJ/mol	$\Delta_{h\phi}H$, kJ/mol	$T\Delta S$, kJ/mol	$T\Delta_{h\phi}S$, kJ/mol	ΔS, J/mol·K	$\Delta_{h\phi}S$, J/mol·K
					Errors and Uncertainties of the Values					
−	±10%	±10%	±1	±1	±1.0	±2.0	±2	±3	±7	±10
					Formulas Used To Fit Experimental Data or Calculate Values Below					
−	b	c	(13)	(15)	d	(21)	(22)	(22)	(22)	(22)
C_9	47.2	132	-12.1	-9.55	6.0	5.0	18.1	14.6	60.7	48.8
C_{10}	200	560	-15.7	-13.1	6.44	5.44	22.1	18.6	74.3	62.4
C_{11}	848	2370	-19.3	-16.7	6.42	5.42	25.7	22.2	86.3	74.4
C_{12}	3600	10100	-22.9	-20.3	5.40	4.40	28.3	24.8	94.9	83.0
C_{13}	15260	42700	-26.4	-23.9	5.65	4.65	32.1	28.5	107.5	95.6

[a] Ligand structures are shown in Figure 7. Initial DNA concentration in the cell (as phosphate, [DNAP]) was 0.33 ± 0.02 mM, NaCl concentration both in the syringe and the cell was 10 ± 1 mM, temperature was 25 ± 1 °C. Hydrophobic contributions were estimated as explained in the text. Electrostatic contributions did not vary on alkyl chain length according to the model and were equal to: $\Delta_{elec}G = -2.6 \pm 1.0$ kJ/mol, $\Delta_{elec}H = 1.0 \pm 1.5$ kJ/mol (electrostatic contribution to the enthalpy, equal to 1/3 of the enthalpy of ammonium phosphate crystallization, also confirmed by (19)), $T\Delta_{elec}S = 3.6 \pm 2.5$ kJ/mol, and $\Delta_{elec}S = 11.9 \pm 8$ J/mol·K. Gibbs free energies and entropies are relative to the 1 M free ligand reference state. [b] Cooperativity parameter, obtained by fitting experimental integrated ITC curves shown in Figure 2, using formulas 8, 9, and 16. [c] Association strength, similar in meaning to binding constant, obtained by fitting experimental integrated ITC curves according to formulas 8 and 9. [d] Enthalpy of processes 1 and 2, equal to area under experimental integrated ITC curves.

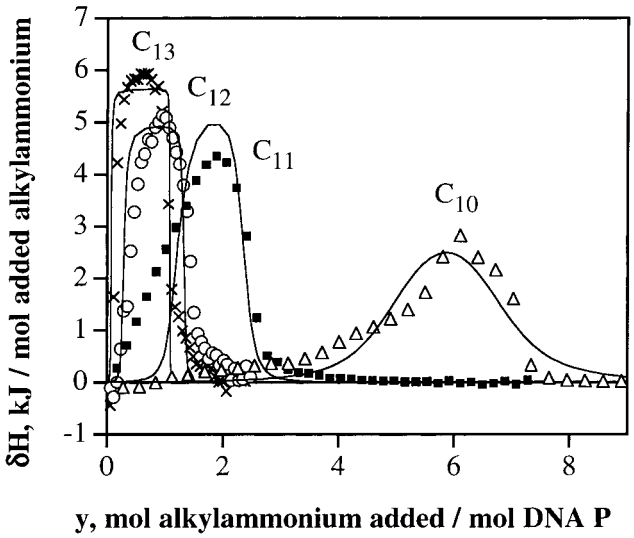

Figure 2. Integrated isothermal titration calorimetry curves (datapoints) of alkylammonium with varying alkyl chain length binding to pUC118 DNA after the subtraction of ligand dilution heats: (×) tridecylammonium (C_{13}), (○) dodecylammonium (C_{12}), (■) undecylammonium (C_{11}), (△) decylammonium (C_{10}). Solid lines represent fitting with a single fitting parameter (ω) according to the model as explained in the text. All binding curves were highly cooperative. Enthalpies of binding were endothermic, opposing the binding. Alkylammonium cations with longer tails bound stronger than the ones with shorter aliphatic tails.

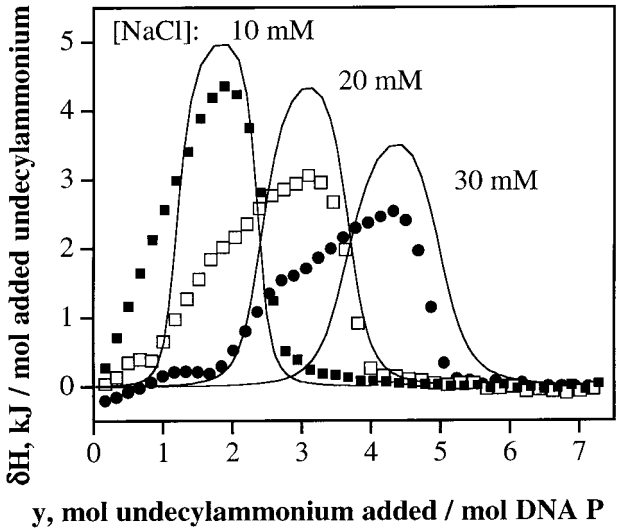

Figure 3. Integrated titration calorimetry curves (datapoints) of undecylammonium binding to pUC118 DNA at various sodium chloride concentration in the cell and syringe: (■) 10 mM, (□) 20 mM, and (●) 30 mM. Solid lines represent fitting with a single fitting parameter (K) according to the model as explained in the text. Increased NaCl concentration competed with undecylammonium and shifted the peak to the right.

of the DNA−lipid complex is inversely proportional to the alkyl chain length.

The enthalpy of binding and condensation was practically independent of the alkyl chain length. The enthalpy varied from +6.4 ± 0.8 kJ/mol for decylammonium to +5.4 ± 0.7 kJ/mol for dodecylammonium interaction with DNA (Table 1). This relatively small and positive enthalpy indicates that alkylamines form a mobile and disordered coating on the surface of DNA. If the lipids formed a solid aggregate, then the enthalpy would be expected to be highly exothermic as was found upon aggregation of alkylamines such as dodecylamine.[29]

The electrostatic component of the Gibbs free energy of lipid binding to DNA is independent of alkyl chain length (Table 1). The actual values depend on the reference state which we assign in a conventional way to a 1 M concentration of free (unbound) lipid. However, each additional methylene group contributes −3.58 kJ/mol of energy to the Gibbs free energy of alkylamine binding to DNA independently of the reference state or experimental concentration. This value exactly matches the series of alkane and alcohol solubilities and alkylamine aggregation.[30]

The hydrophobic component of the entropy is positive and nearly identical in its magnitude to the Gibbs free energy value because the enthalpy contribution is nearly exactly zero. Electrostatic component of the entropy was also positive and constant. The reaction is driven entirely by entropy, and increasingly by hydrophobic entropy with increasing alkyl chain length, just as predicted from the hydrophobic effect.

Dependence on Salt Concentration. Sodium competes with alkylammonium for a cationic binding site on DNA. Therefore, it is expected that doubling salt concentration would shift the calorimetric binding peak toward the twice higher alkylammonium concentration. Figure 3 shows the calorimetric curves of undecylammonium chloride binding to DNA at 10, 20, and 30 mM total sodium chloride concentrations. Relative amount of bound sodium is below 0.33 mM (equal or slightly lower than

Table 2. Thermodynamics of Undecylammonium and Dodecylammonium Binding to pUC118 DNA as a Function of NaCl Concentration in the Cell and the Syringe

[NaCl], mM	α	*K*	Δ*G*, kJ/mol	$\Delta_{elec}G$, kJ/mol	Δ*H*, kJ/mol	$\Delta_{elec}H$, kJ/mol	*T*Δ*S*, kJ/mol	$T\Delta_{elec}S$, kJ/mol	Δ*S*, J/mol·K	$\Delta_{elec}S$, J/mol·K
Errors and Uncertainties of the Values										
±1	±0.01	±10%	±1	±1	±1.0	±1.5	±2	±2.5	±7	±8
Formulas Used To Fit Experimental Data or Calculate Values Below										
−	*b*	*c*	(13)	(14)	*d*	*e*	(22)	(22)	(22)	(22)
Undecylammonium + DNA										
10	1.00	2370	−19.3	−2.6	6.4	1.0	25.7	3.6	86	12
20	1.00	1190	−17.5	−0.8	7.0	1.6	24.5	2.4	82	8
30	1.00	791	−16.5	0.2	5.4	−0.1	21.9	−0.2	73	−1
Dodecylammonium + DNA										
10	1.00	10100	−22.9	−2.6	5.4	1.0	28.3	3.6	95	12
20	1.00	5040	−21.1	−0.8	4.7	0.3	25.8	1.1	87	4
30	1.00	3360	−20.1	0.2	4.2	−0.2	24.3	−0.4	81	−1
60	0.98	1700	−18.5	1.9	4.0	−0.4	22.4	−2.3	75	−8
100	0.95	1100	−17.3	3.0	5.4	1.0	22.7	−2.0	76	−7
200	0.89	630	−16.0	4.3	4.0	−0.4	20.0	−4.7	67	−16

[a] Initial DNA concentration in the cell (as phosphate, [DNAP]) was 0.33 ± 0.02 mM, temperature was 25 ± 1 °C. Electrostatic contributions were estimated as explained in the text. Hydrophobic contributions did not vary on ionic strength according to the model and are as given in Table 1. Gibbs free energies and entropies are relative to the 1 M free ligand reference state. [b] Parameter α, obtained by fitting integrated ITC curves shown in Figures 3 and 4, using formulas 8 and 9; α deviates from unity only at NaCl oncentrations higher than ∼50 mM. [c] Association strength, similar in meaning to binding constant, obtained by fitting experimental integrated ITC curves according to formulas 8 and 9. [d] Enthalpy of processes 1 and 2, equal to area under experimental integrated ITC curves. [e] Electrostatic enthalpy obtained by $\Delta_{elec}H = \Delta H - \Delta_{h\phi}H$ under assumption that $\Delta_{h\phi}H$ does not vary with salt concentration.

DNA phosphate concentration), therefore it can be neglected. As expected, increase in the salt concentration shifted the peak to the right. However, at 20 and 30 mM NaCl, the shape of the binding peaks changed too, becoming broader, so that the model curves fit the experimental data points rather poorly. We should keep in mind though, that the discrepancy is not very large: it is <1 kJ/mol which is approximately the uncertainty of our measurements. Because of such uncertainty it is difficult to elaborate further on the details of the titration peaks.

The overall enthalpy of dodecylammonium binding was practically independent of salt concentration in the range of 10−200 mM NaCl. Therefore, the decrease in association strength upon increase in salt is fully due to the change in entropy of binding (Table 2). The height of the peak decreased significantly upon increasing the salt concentration, but the peak broadened so the area under the peak remained practically unchanged at higher salt.

Under the assumption that only the electrostatic contributions are affected by variation of the salt concentration we see that the electrostatic enthalpy of undecylammonium and dodecylammonium binding to DNA is scattered around zero and varied from −0.4 to +1.6 kJ/mol. This scatter is quite random and is approximately equal to the scatter and repeatability of the data. The hydrophobic component of the enthalpy is slightly more positive, consistent with small positive enthalpies for hydrophobic interactions. The difference between C_{11} and C_{12} (+5.42 and +4.40) is not significant.

At high salt concentration, as predicted by the model, the parameter α needs to be smaller than unity in order to fit the observed data. For example, at 10 and 20 mM NaCl the model nicely fits the experimentally measured heats of titration with dodecylammonium (Figure 4) when α = 1.00. At 60 mM NaCl, α = 0.98 best predicts the position of the peak, but not the broadened shape of the peak. At 200 mM sodium chloride α ≈ 0.89 best predicted the position of the peak. Without this adjustment, the peak would have occurred at about twice higher ligand concentration than was experimentally observed. In other words, at high NaCl (above 50 mM), additional sodium ions did not compete with alkylammonium cations as strongly as at low concentration of NaCl. Furthermore, the model predicts that α should approach 0.5 when the concentration of NaCl approaches ∼1 M.

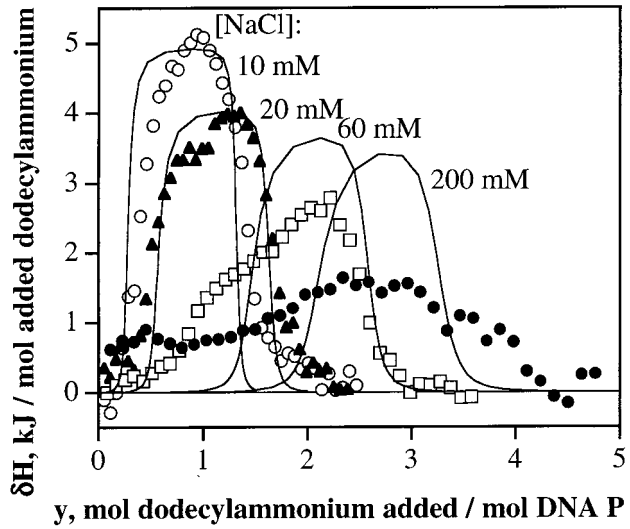

Figure 4. Integrated titration calorimetry curves (datapoints) of dodecylammonium binding to pUC118 DNA at various NaCl concentration in the cell and syringe: (○) 10 mM, (▲) 20 mM, (□) 60 mM, (●) 200 mM. Solid lines represent fitting with a two fitting parameters (*K* and α) according to the model as explained in the text. At high NaCl concentration (above about 50 mM) there is a need to take into account that α is not equal to unity; it's values are listed in Table 2.

There could be several possible explanations why the titration peak is broadening at higher salt concentration in a different fashion than predicted by the model. First, high salt may affect the hydrophobic interactions and not only the electrostatic interactions as shown by the model. Second, high salt reduces solubility and may cause partial micellization of alkylammonium cations even before their binding to DNA. Third, the scatter of data increases at higher salt concentrations, increasing the uncertainty of the experimental measurements.

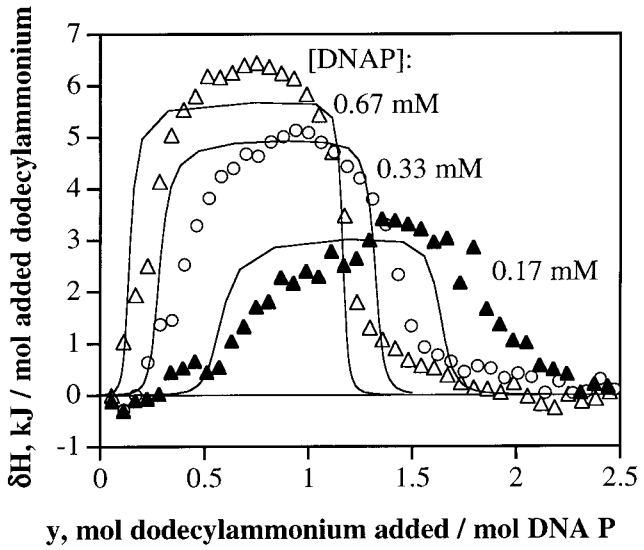

Figure 5. Integrated titration calorimetry curves (datapoints) of dodecylammonium binding to pUC118 DNA at various DNA concentrations in the cell by keeping DNA−ligand concentration ratio unaltered: (△) 0.67 mM, (○) 0.33 mM, and (▲) 0.17 mM expressed as DNA phosphate. Solid lines represent fitting with a single fitting parameter (K) according to the model as explained in the text. At higher DNA concentration the peak is shifted to the left as predicted by the model.

Table 3. Thermodynamics of Dodecylammonium Binding to pUC118 DNA as a Function of Initial DNA Concentration in the Cell (as Phosphate, [DNAP]) (ITC Curves shown in Figure 5)[a]

[DNAP], mM	K	ΔG, kJ/mol	$\Delta_{elec}G$, kJ/mol	ΔH, kJ/mol	$\Delta_{elec}H$ kJ/mol	$T\Delta S$, kJ/mol	$T\Delta_{elec}S$, kJ/mol	ΔS, J/mol·K	$\Delta_{elec}S$, J/mol·K
0.167	5040	−21.1	−0.8	2.4	−2.0	23.6	−1.1	79	−4
0.33	10100	−22.9	−2.6	5.4	1.0	28.3	3.6	95	12
0.67	20100	−24.6	−4.3	5.7	1.3	30.3	5.6	102	19

[a] [NaCl] was 10 ± 1 mM both in the cell and the syringe, temperature was 25 ± 1 °C. Electrostatic contributions were estimated as explained in the text. Hydrophobic contributions did not vary on DNA concentration according to the model and are as given in Table 1. Gibbs free energies and entropies are relative to the 1 M free ligand reference state. Errors and uncertainties except for [DNAP] of ±0.015 mM and the formulas used to estimate values are the same as in Tables 1 and 2.

Dependence on DNA Concentration. The model predicts that the position of the binding peak would be shifted upon changing DNA concentration (eq 9). Therefore, the titration experiments were carried out at several DNA concentrations (0.167, 0.333, and 0.667 mM phosphate), while keeping the ratio of ligand: DNA concentrations unaltered. Concentrations of dodecylammonium chloride were respectively 2, 4, and 8 mM in the syringe.

As expected, the results strongly depended on DNA concentration. At the lowest DNA concentration (0.167 mM DNA phosphate), the binding peak was significantly smaller than at higher concentrations (Figure 5). Experimental enthalpies were respectively 2.4 ± 1.2, 5.4 ± 0.8, and 5.7 ± 0.7 kJ/mol (Table 3). These results demonstrate how important it is to repeat experiments at several DNA concentrations. Decreasing DNA concentration below 0.33 mM yields enthalpy that is not fully evolved. However, increasing the concentration above 0.33 mM does not significantly change the enthalpy. Therefore, other experiments were carried out at 0.33 mM concentration.

When binding energetics depends on reactant concentration, one should look for an aggregation reaction.[31] DNA−lipid interaction is exactly such case where DNA−lipid complex condenses at the end of titration. Therefore, the binding Gibbs free energy depends on DNA concentration. However, only the electrostatic component of the Gibbs free energy and entropy depends on concentration. The hydrophobic component is concentration-independent.

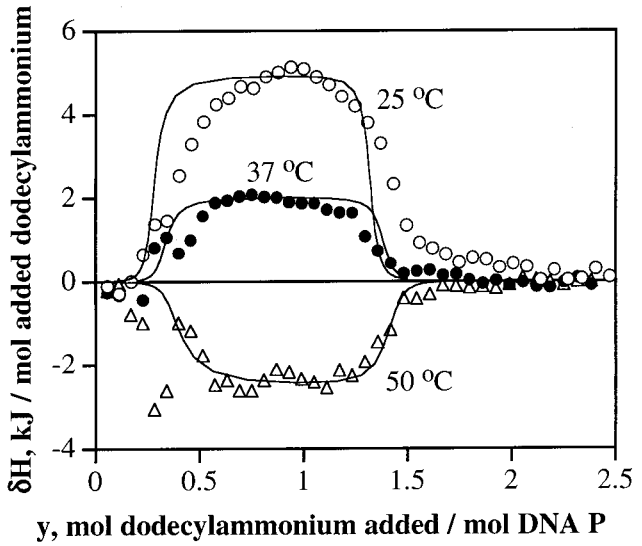

Figure 6. Integrated titration calorimetry curves (datapoints) of dodecylammonium binding to pUC118 DNA at various temperatures: (○) 25 °C, (●) 37 °C, and (△) 50 °C. Solid lines represent fitting with a single fitting parameter (ω) according to the model as explained in the text. At higher temperatures the enthalpy is decreasing yielding the heat capacity that is consistent with the hydrophobic interactions.

Dependence on Temperature: The Heat Capacity. Figure 6 shows calorimetric titration curves of dodecylammonium binding to DNA obtained at several temperatures. An integration of the area under such peaks yields the overall integral molar enthalpy of processes 1 and 2 at various temperatures. As expected from hydrophobic interactions, the enthalpy of binding decreases with increasing temperature and changes sign at around 43 °C, becoming exothermic. Experimental values of the enthalpy at four temperatures are listed in Table 4, spanning the range of 25 to 58 °C. Linear fit of the enthalpies show essentially linear dependence of the enthalpy on temperature with a discrepancy of less than 0.5 kJ/mol. From the slope of the enthalpy linear fit on temperature we obtain the constant pressure heat capacity ΔC_p as −302 ± 50 J/(mol·K).

Gibbs free energy dependence on temperature was negligible (Table 4), varying from −22.9 to −22.1 kJ/mol at 25 and 50 °C, respectively. This difference is so small that an attempt to derive enthalpy, entropy, and heat capacity as temperature derivatives of Gibbs free energy would yield nearly meaningless results. This uncertainty of van't Hoff method is similar for many hydrophobic interactions,[30] because Gibbs free energy is often nearly temperature-independent. Large enthalpy variation with temperature is compensated by the hydrophobic component of entropy (Table 4).

However, it is possible to estimate electrostatic and hydrophobic contributions to the enthalpy at various temperatures by applying additivity. The heat capacity of ammonium phosphate crystallization is about +476 ± 80 J/mol·K (our estimate from [32] and [33]). Under the assumption that this heat capacity is independent of temperature we calculate the electrostatic

(33) Yaws, C. L. *Chemical Properties*; McGraw-Hill: New York, 1999.

Table 4. Thermodynamics of Dodecylammonium Binding to pUC118 DNA as a Function of Temperature (ITC curves shown in Figure 6)[a]

temp, °C	ω	K	ΔG, kJ/mol	$\Delta_{elec}G$, kJ/mol	$\Delta_{h\phi}G$, kJ/mol	ΔH, kJ/mol	$\Delta_{elec}H$, kJ/mol	$\Delta_{h\phi}H$, kJ/mol	ΔS, J/mol·K	$\Delta_{elec}S$, J/mol·K	$\Delta_{h\phi}S$, J/mol·K
25	3600	10100	−22.9	−2.6	−20.3	5.4	1.0	4.4	95	12	83
37	3000	8400	−22.4	3.2	−25.6	2.1	6.7	−4.6	79	12	68
50	2700	7600	−22.1	9.4	−31.5	−2.5	12.9	−15.4	61	11	50
58	∼2500	∼6900	∼−21.9	∼13.2	∼−35.1	−4.3	∼16.7	∼−21.0	∼53	∼11	∼42

[a] Sodium chloride concentration [NaCl] was 10 ± 1 mM both in the cell and the syringe, DNA concentration (as phosphate [DNAP]) was 0.33 ± 0.015 mM. Electrostatic and hydrophobic contributions were estimated as explained in the text. Gibbs free energies and entropies are relative to the 1 M free ligand reference state. Errors and uncertainties and the formulas used to estimate values are as in Tables 1 and 2.

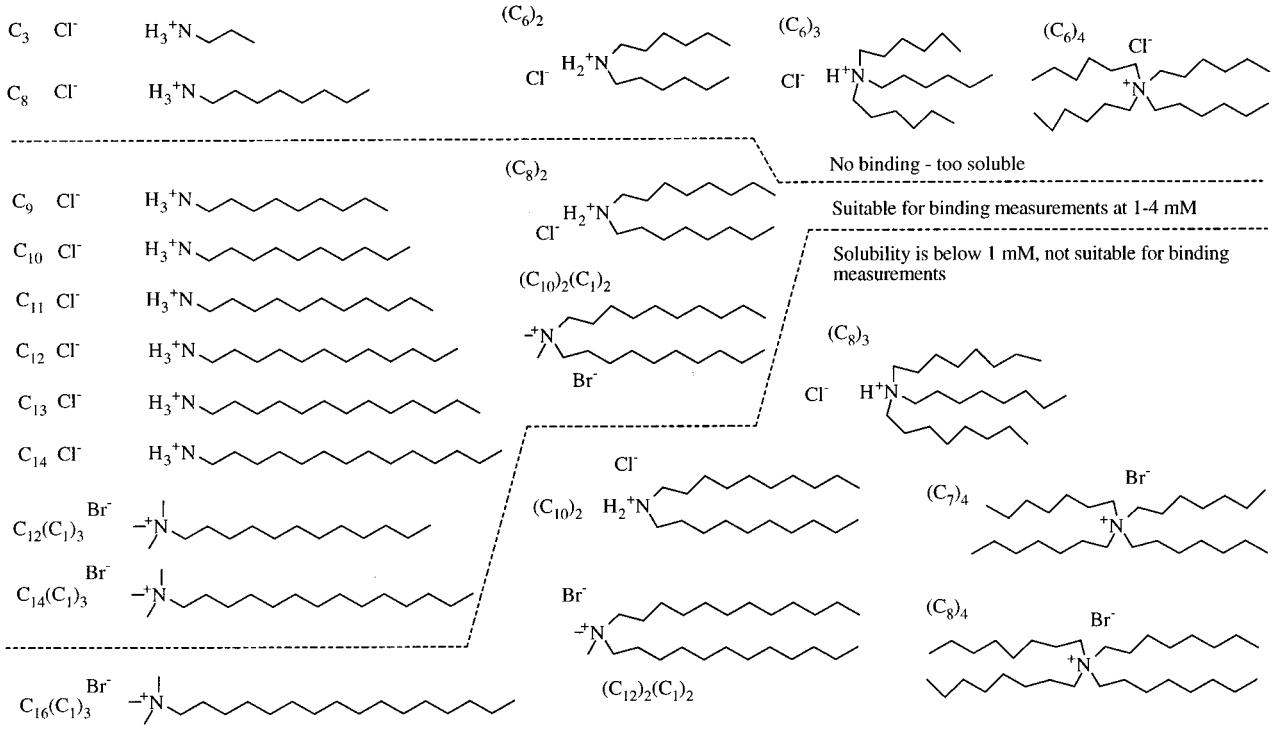

Figure 7. Chemical structures of ligands used in this study. First column shows single-tailed ligands, second column shows double-tailed ligands, and triple- and quadruple-tailed ligands on the right. Ligands that are above the upper dashed line did not produce any heat upon mixing with DNA, indicating that no binding occurred to a significant extent. Ligands that are shown below the lower dashed line were too insoluble or formed aggregates in water preventing accurate determination of their energetics of binding to DNA.

contribution of the enthalpy to increase from 1.0 kJ/mol at 25 °C to about 16.7 kJ/mol at 58 °C. By subtraction, the hydrophobic enthalpy must be decreasing to an even greater extent yielding the heat capacity of $-302 - 476 = -778 \pm 130$. This value is very close to the predicted value of the heat capacity of dodecane aggregation which is equal to -795 ± 30 J/mol·K.[30] Therefore, the overall energetics appears to be self-consistent. However, application of the ammonium phosphate heat capacity of crystallization to the process of alkylammonium−DNA binding is only approximately correct. The numbers obtained by such additivity should be treated with caution.

Binding of Multi-tailed Ligands. Chemical structures of various ligands used in this study are shown in Figure 7. In addition to single-tailed ligands, we also investigated binding of two-, three-, and four-tailed amines to DNA. Some data could be obtained using such ligands. However, many ligands were too insoluble in water to obtain titration calorimetry data. Furthermore, titration of DNA with ligands that have short aliphatic tails often did not produce any heat because there was no significant binding. The upper line in Figure 7 separates ligands for which the observed heat of binding was negligible (less than 1 kJ/mol) or there was no binding at all. The lower line approximately separates ligands that could not be brought into true aqueous solution to reach 2−4 mM concentration without forming micelles or aggregates. As we see, there were no three- or four-tailed ligands for which binding data could be obtained. However, we obtained reliable and reproducible data for several single-tailed methylated ligands and several double-tailed methylated and nonmethylated ligands.

Figure 8 shows experimental titration calorimetry data and lines generated according to the above-described model with the single fitting parameter ω. Parameters that were used to generate lines are shown in Table 5. The tails of dodecyltrimethylammonium ($C_{12}(C_1)_3$) and dodecylammonium (C_{12}) ligands are the same length; therefore, we use the same cooperativity parameter ω (3600) to fit data for methylated ligand as obtained above for nonmethylated ligand). The parameter that is adjusted here is the specificity constant K_{sp} described in eq 11, because it is expected that the methylated headgroup would have a different competition parameter than the nonmethylated ammonium headgroup. For dodecylammonium cation, K_{sp} was found to be about 0.028. For trimethyl dodecylammonium cation, K_{sp} was significantly smaller, about

Table 5. Thermodynamics of Various Methylated and Doubly Tailed Ligand (Structures Shown in Figure 7, ITC Curves Shown in Figure 8) Binding to pUC118 DNA[a]

ligand	ω	K	ΔG, kJ/mol	$\Delta_{elec}G$, kJ/mol	$\Delta_{h\phi}G$, kJ/mol	ΔH, kJ/mol	$\Delta_{elec}H$, kJ/mol	$\Delta_{h\phi}H$, kJ/mol	ΔS, J/mol·K	$\Delta_{elec}S$, J/mol·K	$\Delta_{h\phi}S$, J/mol·K
$C_{12}(C_1)_3$	3600	1200	−17.6	2.7	−20.3	5	0.6	4.4	76	−7	83
$(C_8)_2$	65	11400	−23.2	−12.8	−10.3	19	∼5	∼14	142	60	82
$(C_{10})_2(C_1)_2$	5000	26000	−25.2	−4.1	−21.1	9.5	∼1	∼8	116	19	97
$(C_{12})_2(C_1)_2$	-	-	-	-	-	4	∼1	∼3	-	-	-

[a] Sodium chloride concentration was 10 ± 1 mM for $C_{12}(C_1)_3$, 0.16 mM for $(C_8)_2$, 0.66 mM for $(C_{10})_2(C_1)_2$, and 0.16 mM for $(C_{12})_2(C_1)_2$ both in the cell and the syringe because the ligand started to aggregate in the syringe at higher NaCl concentrations. DNA concentration (as phosphate [DNAP]) was 0.33 ± 0.015 mM except for $C_{12}(C_1)_3$ where it was 0.50 mM. Electrostatic and hydrophobic contributions were estimated as explained in the text. Gibbs free energies and entropies are relative to the 1 M free ligand reference state. Errors and uncertainties and the formulas used to estimate values are the same as in Tables 1 and 2.

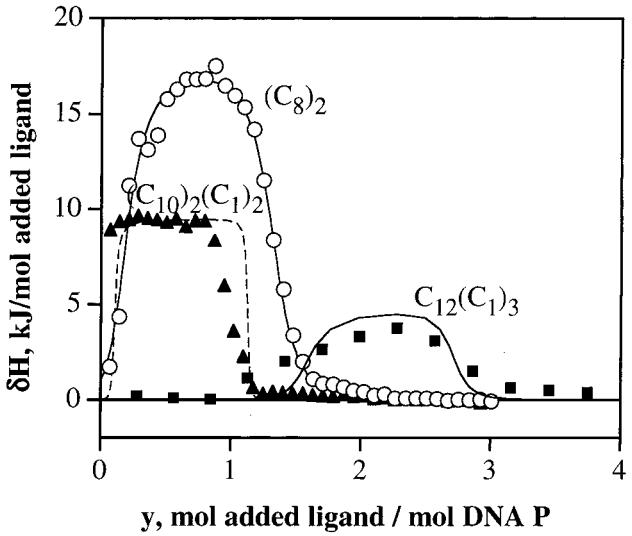

Figure 8. Integrated titration calorimetry curves (datapoints) of various ligand binding to pUC118 DNA: (○) dioctylammonium ($(C_8)_2$), (▲) didecyldimethylammonium ($(C_{10})_2(C_1)_2$), and (■) dodecyltrimethylammonium ($C_{12}(C_1)_3$). Solid lines represent fitting with a single fitting parameter (K_{sp} or ω) according to the model as explained in the text. Double-tailed ligands also bound to DNA in a highly cooperative fashion but significantly more positive enthalpy than single-tailed ligands. Didecyldimethylammonium was partially aggregated in the syringe before injection.

0.0034. This indicates that tetramethylammonium is a significantly poorer DNA binder than ammonium cation. Comparing energetic contributions of $C_{12}(C_1)_3$ and C_{12} we see that hydrophobic components of the energetics are essentially the same. However, the electrostatic component of methylated ligand binding is less favorable, consistent with experimental results.

Comparing double-tailed with single-tailed ligand binding energetics (Table 5), we see that the additional tail only slightly increases the cooperativity parameter. This increase is equal to the contribution of only about two to three methylene groups. The remaining part of the adjacent aliphatic chain is essentially bound to the first aliphatic chain, and little additional energy is gained upon binding to DNA.

Discussion

There are two steps or reactions that can be envisioned to occur during cationic lipid binding to DNA. First, a single lipid molecule binds to DNA. Second, a stoichiometric number of alkylammonium cations (equal to the number of DNA phosphates) bind to DNA, and electrically neutral DNA−lipid complexes condense (aggregate and precipitate). This distinction between two types of reactions is arbitrary because all reactions occur highly cooperatively. It is hard to get the first alkylammonium cation to displace sodium cation because their specificity competition constant is equal to about 0.028. After binding the first cation, it is relatively easy to complete the formation of the complex. To a reasonable approximation, these two reactions can be considered as making additive electrostatic and hydrophobic contributions to the overall Gibbs free energies, entropies, enthalpies, and heat capacities.

Structural Considerations. We would like to interpret our results in terms of a structural model of the DNA−alkylammonium complex. There are at least three distinct modes by which lipid cations may bind to DNA. In the first model, cationic headgroups would be localized within several Å from DNA phosphates, while hydrophobic tails would lay down on the DNA surface. Another model would suggest that ligand cationic headgroups are near DNA phosphates while the aliphatic tails stand perpendicular to DNA surface. The third model [34] suggests that cationic lipids form large micelles, consisting of about 100 lipid molecules, that are bound to DNA with significant portions of DNA not covered by lipid. In analyzing our results we favor the first and disfavor the third model. However, energetic means alone may not be sufficient to unambiguously determine the mode of binding.

Because the heat capacity decrease during binding and condensation is so similar to the predicted heat capacity decrease of aggregating dodecane, one might suggest that the entire aliphatic chain of ligands such as dodecylammonium has to be completely removed from contact with water. To achieve this, aliphatic chains may have to lay down on the DNA surface, displacing all or most water molecules from the DNA hydration layer. The exposed side of the bound lipid molecules then binds to lipid molecules similarly layered on the adjacent DNA helix.

Geometric considerations are consistent with this picture. The DNA diameter is equal to about 20 Å and phosphate charges are distributed every 1.7 Å along the DNA length. Therefore, there is about 107 Å^2 surface area of DNA per one negative charge. The dodecylammonium cation has a length of about 19.5 Å and a width of about 4.7 Å including van der Waals radii. Thus, a bound dodecylammonium cation laying on its side may cover an area of about 90 Å^2. Therefore, a neutralizing amount of ligands could cover nearly the entire DNA surface area if laying on their sides. An advantage of this model is that it explains how an entire lipid chain could be removed from contact with water. A disadvantage of this model is that aliphatic hydrophobic lipid tails have to bind to a relatively hydrophilic DNA surface.

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In the second structural model, lipid tails are oriented perpendicularly to DNA and intercalated with tails from lipids bound to adjacent DNA helices. Two lipid molecules standing perpendicularly to DNA can occupy an area of only about 35 Å^2 ($4.7/2)^2 \times \pi \times 2 = 34.7$ Å^2). This area is only about one-third of the area needed to be filled with lipid. Therefore, lipids would have to cluster on the surface on DNA and must leave about two-thirds of the space filled with water. This scenario introduces a lot of water next to lipid tails, which seems unlikely. The advantage of this model, however, is that lipid tails would be bound to each other and not to the hydrophilic DNA surface.

In the third model, suggested by ultrasonic velocity and density measurements,[34] ligands such as dodecyltrimethylammonium bind to DNA by forming large micellelike clusters on the DNA surface. Such large micelles would have much greater positive charge than needed to neutralize DNA, leaving large spans of DNA completely uncovered by lipid. Therefore, the third model is difficult to understand from the electrostatic interaction point of view, which suggests that lipid headgroups should come as closely as possible to negatively charged phosphates.

It was also observed by other researchers (personal communication with C. H. Spink) that ligands such as tetradecyltrimethylammonium prefer binding to AT base pairs over GC base pairs about 2.5-fold. This observation suggested that the methylated headgroup of the ligand is bound to the methyl group of the thymine base in the major grove of DNA helix by hydrophobic interaction. This preference, however, should be valid only for methylated ammonium ligands, a point that remains to be experimentally tested. In our study we did not study the effect of DNA base composition on alkylammonium binding.

It may not be possible to distinguish between all these structural models by energetic means alone. Structural studies on DNA−dodecylammonium aggregate are necessary. For example, neutron diffraction studies could provide the distance between the DNA double helices in the complex and the distribution of lipid relative to DNA constituents.

Energetic Considerations. Despite the uncertainties about the structure of the DNA−lipid complex, the energetics and the mathematical model of interactions appear to be self-consistent. Experimental ITC curves are nicely accounted for by the simulated curves. The discrepancy between the data and the model can partly be explained by errors of reproducibility. However, many titration curves showed systematic deviations from the model. For example, in Figure 3, we clearly see a shoulder of the peaks on the left side. It indicates that the binding reaction started earlier than predicted by the model, to an extent dependent on salt. When NaCl concentration exceeded about 50 mM, the model gave much higher peaks than observed experimentally suggesting that the cooperativity of interaction was lower than predicted by the model at high salt concentration.

Results shown in Tables 1−5 are not of equal reliability. For example, values of integral enthalpy ΔH are directly observed and independent of any assumptions in the model. Precision of the enthalpy depends only on the accuracy of experimental measurement. However, electrostatic and hydrophobic contributions to the enthalpy are obtained by applying additivity principles [35] with all their underlying assumptions. The dependencies of electrostatic and hydrophobic enthalpies on temperature, which depend on the value of the heat capacity of ammonium phosphate crystallization, may be subject to some uncertainty. Extension of enthalpy values obtained for inorganic compounds to macromolecules is promising to help understand energetic contributions of specific parts of interactions. However, it is necessary to clearly state which values are obtained by such procedure and to use such results with caution.

Little reliable data was obtained to characterize double-tailed ligand binding to DNA. Most of the commercially available multi-tailed ligands shown in Figure 7 were either too weak DNA binders or too insoluble in water. Dioctylammonium and didecyldimethylammonium cations were the only ones that were sufficiently soluble and produced titration curves as expected. Interestingly, the enthalpy of their binding to DNA was significantly more endothermic than for single-tailed ligands. This increase was assumed to be due to the increase in hydrophobic contribution to the enthalpy. However, without a sufficiently large variety of ligands, such dissection is rather arbitrary. Didodecyldimethylammonium ligand formed a stable suspension in water. We assume that this lipidlike cation may form a membranelike structure at our experimental concentration. The heat of dilution of this ligand was large and could be assigned to a partial breakup of membranelike structure. However, the parameters for this ligand have significant uncertainty and only enthalpy values are shown in Table 5. The data are likely to represent the process of membranelike complex binding to DNA instead of the binding of single lipid molecules.

Gibbs free energies and cooperativity parameters are quite accurately determined by the fit of experimental data. In most cases, alteration of the value by 10% yielded significantly poorer fits. We estimate that the precision is about ±4%. However, there is an additional parameter, K_{sp}, a single value of which is obtained from fitting all data of linear alkylammonium cations. This value was found to be equal to 0.028. If we assume that the value of K_{sp} should be that of the exchange of the ammonium headgroup with sodium, then this value is inconsistent with the literature value of 2 for ammonium−sodium exchange.[36] It is much closer to the value of ∼0.05 for tetrabutylammonium−sodium exchange. A better model for the exchange reaction might be methylammonium−sodium, but data for this process is not available. Major upward adjustment of K_{sp} resulted in significantly poorer representation of experimental data.

Entropies are obtained by subtraction of Gibbs free energies from enthalpies. Therefore, errors and uncertainties of ΔG and ΔH add to the uncertainties of ΔS, making them the least accurately determined thermodynamic parameters. Furthermore, Gibbs free energy and entropy values depend on the progression of reaction and are meaningless without a reference point. We chose the reference concentration of free ligand to be 1 M. Such concentration is never reached in our experiments, but it is conventional and convenient. Because of the arbitrary reference point, it provides more insight to use Gibbs free energy and entropy increments for various chemical groups (e.g., CH_2) which are independent of concentration chosen, rather than actual ΔG and $T\Delta S$ values shown in Tables 1−5 which depend on the reference chosen.

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For example, $\Delta_{elec}G$ was independent of alkyl chain length, and $\Delta_{h\phi}G$ was the only contributor to ΔG. $\Delta_{elec}G/CH_2$ was equal to zero and $\Delta_{h\phi}G/CH_2$ was equal to $\Delta G/CH_2$, −3.58 kJ/mol (= $RT \ln\Delta\omega$, see eq 16). This value appears to be the signature of the methylene group in various hydrophobic reactions, including but not limited to linear aliphatic compound solubility in water,[28] aggregation from aqueous solutions,[30] lipid binding to DNA (this article), and surfactant binding to proteins.[37] This value, however, appears to be inconsistent with surface tension measurements.[38] There is also a constant increment in entropy upon increasing aliphatic chain length, equal to ∼12 J/mol·K.

The enthalpy was found to oppose lipid−DNA binding, which occurred only due to even greater positive entropy. This entropy is primarily due to the entropy increase accompanying hydrophobic interactions. However, sodium counterion release may have also contributed to the positive change in entropy as discussed by other authors.[23] We believe that the lipid layer around DNA remains in a liquidlike phase and that van der Waals contact energies are weak. If the layer were in a solid phase, we would expect to observe a large exothermic enthalpy of binding as was observed when the same lipids aggregated from aqueous solution into a pure lipid of the solid phase.[29]

Relatively small endothermic enthalpy was observed when studying binding of various cationic ligands to DNA, including cobalt hexammine,[19] cationic alkylamines,[9] (this article), and proteins.[39] All of these cationic ligands have one structural feature in common—positively charged ammonium groups. This may indicate that all such ligands bind in a fashion similar to that of DNA. We know from cystallographic studies that hydrogen bonds are formed between DNA phosphates and ammonium groups of arginine and lysine side chains of proteins in protein−DNA complexes. Similar energetics of binding supports a notion that alkylammonium cations also bind to DNA by forming weak hydrogen bonds.

Conclusions

1. Cationic alkylammonium ligands displace Na^+ cations, bind to DNA, and condense the lipid−DNA complex.

2. Ligands with longer aliphatic chains bind to DNA stronger than short ones, mostly due to highly positive hydrophobic entropy. Relatively small endothermic enthalpy opposes the binding reaction.

3. At high salt concentration the binding is weaker than at low salt due to less favorable electrostatic entropy. Enthalpic contribution is not affected by high salt.

4. At higher temperature the binding is slightly weaker, due to less favorable electrostatic and hydrophobic entropies and electrostatic enthalpy, despite favorable hydrophobic enthalpy.

5. Trimethylalkylammonium ligands bind weaker to DNA than alkylammonium ligands. Double-tailed ligands are only slightly stronger DNA binders than single-tailed ligands.

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