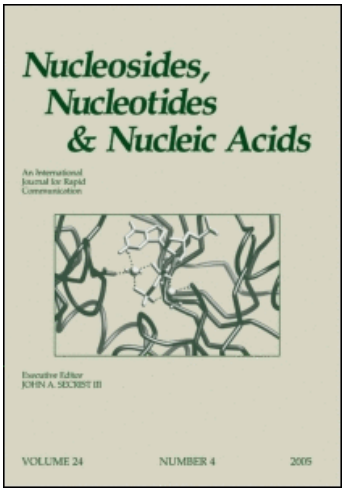

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DAUNOMYCIN BINDING TO DEOXYPOLYNUCLEOTIDES WITH ALTERNATING SEQUENCES: COMPLETE THERMODYNAMIC PROFILES OF HETEROGENEOUS BINDING SITES

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DAUNOMYCIN BINDING TO DEOXYPOLYNUCLEOTIDES WITH ALTERNATING SEQUENCES: COMPLETE THERMODYNAMIC PROFILES OF HETEROGENEOUS BINDING SITES

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ABSTRACT

Complete thermodynamic binding profiles for the interaction of the anticancer drug, daunomycin with natural DNA and synthetic deoxypolynucleotides were described. Fluorescence titration method was used to estimate the equilibrium binding constants. Binding isotherms were found to be surprisingly complex in some cases, presumably because there were heterogeneous sites even in simple deoxypolynucleotides of repeating sequence. Some polynucleotides consisting of alternating sequence contain at least two different binding sites for daunomycin.

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The binding affinity of the primary binding sites of alternating and non-alternating sequences was found to differ by two orders of magnitude. An isothermal microtitration calorimeter was used to directly measure the binding enthalpy at 25°C with a high sensitivity. The binding enthalpy of poly[d(A-T)] was found to be -5.5 Kcal/mol, which was much lower than any other polynucleotides, while the binding constant of the high affinity sites, was similar. In this report, the complete thermodynamic profiles of daunomycin binding to deoxypolynucleotides were reliably shown for the first time.

Key Words: Alternating sequences; Anthracyclines; Daunomycin; Thermodynamic profiles

INTRODUCTION

Daunomycin(daunorubicin) is an anthracycline antibiotic. The anthracyclines are also widely used in cancer chemotherapy and are thought to act by direct interaction with DNA[1] and topoisomerase II.[2] Daunomycin binds to DNA by intercalation of the aromatic rings of the drug molecules between the base pairs. Understanding of the energetics and specificity of drug-DNA interactions with clinically used anthracyclines is important for designing new or improved anticancer drugs. Several reviews have been published for drug-DNA interactions[3,4] and for daunomycin-DNA interactions.[5,6] The structure of poly[d(A-C)]:poly[d(G-T)] and poly[d(A-G)]: poly[d(C-T)] using linear dichroism,[7] the association of poly[d(A-C)]:poly[d(G-T)] in eukaryotic genomes,[8] and the existence of trimer 5′-d(CAG)- or 5′-d(CGG)-repeats in the human genomes related to genetic diseases[9] have also been reported. The binding profiles of daunomycin for a series of polynucleotides excluding any contributions of the heat capacity changes were reported, thus far.[6] The enthalpies were measured by a differential stopped-flow microcalorimetry. The equilibrium binding constants were obtained by assuming that the binding interactions were explained by the neighbor-exclusion model.[10] In this report, however, some polynucleotides were found to contain at least two binding sites for daunomycin. These results imply that even simple polynucleotides of repeating sequence have heterogeneous sites. In order to explain the heterogeneity of binding sites, a different model was required to estimate reliable binding constants. Several experimental techniques were used to obtain the complete thermodynamic profiles for the binding interaction of daunomycin with a natural DNA and a series of synthetic deoxypolynucleotides. Fluorescence titration method was used to estimate the equilibrium binding constants. Isothermal microtitration calorimetry was used for the direct measurements of the binding enthalpy at 25°C. The physico-chemical parameters of daunomycin binding to various DNA templates were described.

MATERIALS AND METHODS

Materials

A variety of synthetic polynucleotides, such as poly[d(A-C)]:poly-[d(G-T)], poly[d(A-G)]: poly[d(C-T)], poly[d(G-C)]:poly[d(G-C)], poly[dG]: poly[dC], poly[dA]:poly[dT], poly[d(A-T)]: poly[d(A-T)] were obtained from Pharmacia LKB Biotechnologies (Piscataway, NJ) and dialyzed overnight against a large volume of an appropriate buffer (BPES). The buffer of BPES consists of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, 0.20 M NaCl, at pH 7.0. Salmon testes DNA was purchased from Sigma Chemical Co. (St. Louis, MO) and was sonicated and purified as described previously.[11] Daunomycin was purchased from Sigma Chemical Co. and was used without purification. The concentration of deoxypolynucleotides, and salmon testes DNA was determined by using the following wavelengths and extinction coefficients ($M^{-1}cm^{-1}$) in terms of DNA base pairs: poly-[d(A-C)]: poly[d(G-T)], $\lambda_{max} = 254$, $\varepsilon = 16,800$; poly[d(A-G)]:poly[d(C-T)], $\lambda_{max} = 256$, $\varepsilon = 11,400$; poly[d(G-C)]:poly[d(G-C)], $\lambda_{max} = 254$, $\varepsilon = 16,800$; poly[dG]:poly[dC], $\lambda_{max} = 253$, $\varepsilon = 14,800$; poly[dA]:poly[dT], $\lambda_{max} = 260$, $\varepsilon = 12,000$; poly[d(A-T)]:poly[d(A-T)], $\lambda_{max} = 262$, $\varepsilon = 13,200$; salmon testes DNA, $\lambda_{max} = 260$, $\varepsilon = 13,100$. Molar coefficients of 11,500 ($M^{-1}cm^{-1}$) at 480 nm was used for free daunomycin and 7000 ($M^{-1}cm^{-1}$) at 480 nm was used for bound daunomycin with DNA. Absorbance spectra were measured using a UVIKON 923 (Kontron Instruments; Milano) or a Cary 3E (Varian; Palo Alto, CA) spectrophotometer.

Binding Studies. Fluorescence titration experiments were conducted on a photon counting fluorometer (Greg 200, I.S.S., Champaign, IL) or using a Perkin-Elmer 560-40 fluorometer with a xenon lamp. An excitation wavelength of 480 nm and an emission wavelength of 555 nm were used. Titrations were performed at a fixed daunomycin concentration by increasing the concentration (bp) of DNA. Alternatively, titrations were done at a fixed DNA concentration by increasing the daunomycin concentration at 25°C.[11] The concentration of free drug (C_f) was calculated,

$$C_f = C_t(I/I_o - P)/(1 - P) \quad (1)$$

where C_t is the known added amounts of total drug, I_o and I, is the fluorescence intensity of drug in the absence and in the presence of DNA, respectively, and P is the ratio of the observed quantum yield of fluorescence of the totally bound drug to that of the free drug. The concentration of bound drug, C_b was then obtained by the difference,

$$C_b = C_t - C_f. \quad (2)$$

Binding data were analyzed by the nonlinear least fitting to the simple neighbor exclusion model,[10]

$$r/C_f = K(1 - nr)\{(1 - nr)/(1 - [n - 1]r)\}(n - 1) \quad (3)$$

where K is the binding constant for an isolated DNA site with the same binding affinity and n is the neighbor exclusion parameter. Another nonlinear least squares fitting mode, which is an uncoupled saturation model for the independent sites with different binding affinity can reliably describe the binding interaction of daunomycin with DNA. An uncoupled saturation model was applied by using the software FitAll (MTR software, Toronto, Canada). As the mathematical derivations for independent sites with different binding affinity were described,[12] this model is used for determining the equilibrium constants for the ligands binding to a mixture of binding site, or substrates to a particular enzyme. The general form of the function is,

$$r = P_1 + \sum [P_{2i}P_{2i+1}C_f/(1 + P_{2i}C_f)] \quad (4)$$

where C_f is the free drug concentration, r is the binding ratio, i is the number of uncoupled (parallel) reactions for the independent binding sites from 1 to 5, P represents the parameters: P_{2i} are the equilibrium constants for each different binding sites, P_1 is the limiting value of r-bound when C_f is zero, and P_{2i+1} are the limiting values of r-bound at high C_f for each binding site.

Isothermal Microtitration Calorimetry. The binding enthalpy of daunomycin with DNA was measured directly using isothermal titration calorimetry (Hart Scientific; Pleasant Grove, Utah). The heat changes caused by drug-DNA interactions were collected by the microtitration calorimeter. The reaction cell and the titrant tubes were contained in a temperature bath. Thus, the temperatures of the titrant and titrate were similar. Titrations were performed by incremental addition with individual injection of titrant being made continually into the titrate in a reaction cell. For a chemical calibration, heat of ionization of water and heat of protonation of Tris were measured and then compared to those published values at different temperature.[13] For measuring the enthalpy of binding reactions, 1 mL of DNA solutions at the concentration of approximately 1 mM (bp) in BPE was put in the reaction cell. Then, samples were equilibrated for the constant temperature at 25°C by mixing with a stirrer at least for 2 h. Daunomycin solutions contained in a 100 μL syringe were put in a buret and set for about 15 min for the equilibration of temperature. The setup conditions of the data-collecting unit are 200 sec for initiation, and 500 sec for the intervals of each injection. By each injection, 2 μM daunomycin was mixed with DNA and the heat changes were

collected by a computer. The above procedures were carried out by putting BPE buffer alone in a reaction cell in order to compensate the heat of dilution by the addition of drug solution into the reaction cell. The normalized binding enthalpies were estimated by compensating the heat of dilution for the observed binding reactions.

RESULTS

Binding of Daunomycin to Deoxypolynucleotides. Binding isotherms for the interaction of daunomycin with a series of synthetic deoxypolynucleotides were obtained using the fluorometric methods. The direct plot of daunomycin binding to poly[d(A-T)] as an example is presented by free drug concentration (C_f) vs. binding ratio (r). Attempts to fit the binding isotherms to the neighbor-exclusion model were unsuccessful as shown in Fig. 1 (A). This model cannot describe most regions of binding isotherms by a huge deviation between the calculated curve and the experimental data. The fitted curve showed a significant deviation from the binding data in Fig. 1 (A). While the binding data were presented in the form of a Scatchard plot shown in Fig. 1 (B), there seemed to be no systematic deviation between the calculated values on the fitted curve and the binding data. The neighbor-exclusion model can, however, account for the shape of the Scatchard plot, but cannot account for the shape of the direct plot (C_f vs. r_b). This is why the binding isotherms of daunomycin to other polynucleotides were presented in the plots of C_f vs. r. Binding data may be reliably analyzed by another binding model. An uncoupled saturation model[12] was applied to explain the experimental binding data. Data from the equilibrium fluorescence measurements were processed using the software Origin (MicroCal Inc., USA) and fitted to the appropriate equation using the nonlinear least-squares fitting subroutine. The results of analysis imply that there is heterogeneity of binding sites even in simple polynucleotides of an alternating sequence like poly[d(A-T)]. Thus, a new model is required to account for the binding interactions of daunomycin to DNA. Binding constants of a series of deoxypolynucleotides were estimated by the nonlinear least-squares fit of binding data to the simple neighbor exclusion model (Eq. 3), and the uncoupled saturation model (Eq. 4). Because of the existence of multiple types of binding sites, several of the binding isotherms are not simple, and not reliably explained by neighbor-exclusion model as an example, shown in Fig.1 (A), even though there is no such resulting effects by the Scatchard equation, shown in Fig. 1 (B). In contrast to this discrepancy demonstrated in Fig.1, the calculated curves of Eq. 4 were fitted much better to the binding data of non-alternating and alternating sequences shown in Fig. 2. It was known that the complete titration curves of drug binding to a class of noninteracting sites should cover a logarithmic interval in free drug

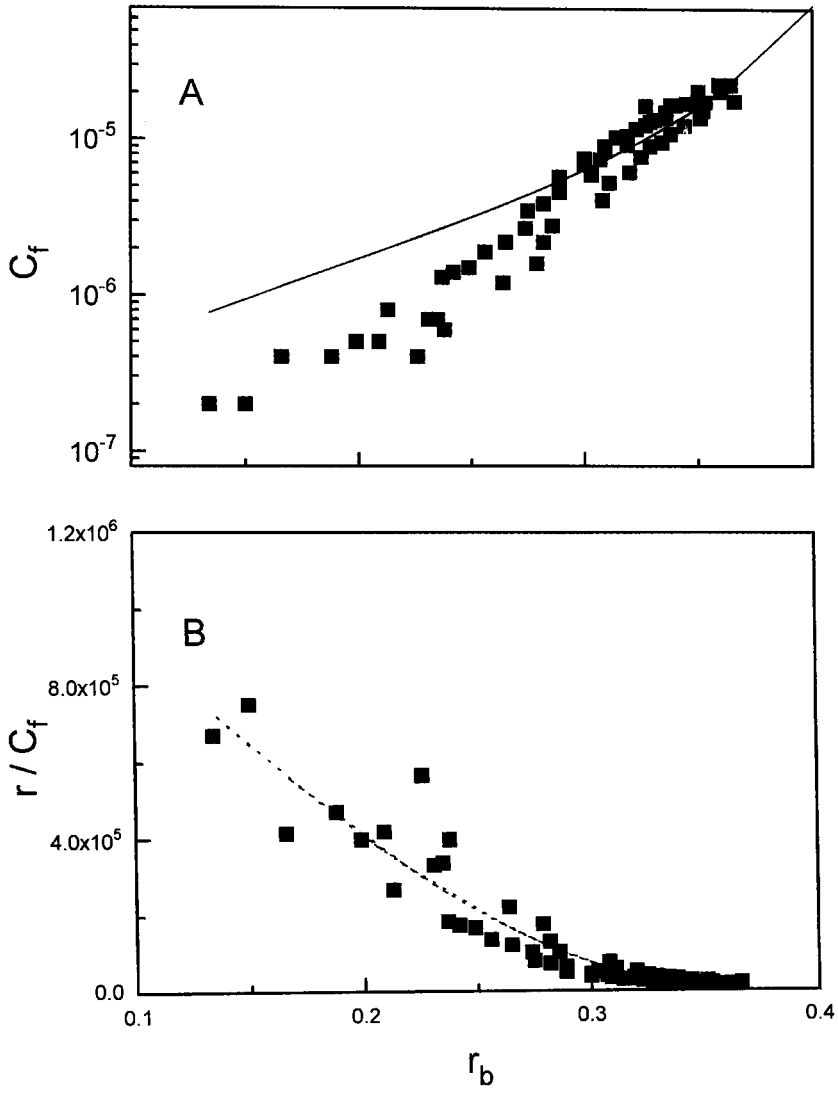

Figure 1. (A) Binding isotherm in the form of direct plot for daunomycin-poly[d(A-T)] interaction. Data are presented as the free drug concentrations vs. binding ratio. (B) Data are shown in the form of a Scatchard plot of r/C_f vs. binding ratio(r). Binding data are presented as symbols and the lines are the calculated curves by the neighbor-exclusion model.

concentration (C_f) of 1.9 units.[14] The binding data of non-alternating poly[dA] and alternating poly[d(A-T)] were represented as evenly dispersed over two logarithmic unit of the concentration of free drug shown in Fig. 2. Interestingly, there are about two orders of magnitude difference of the free drug concentration between the alternating and non-alternating sequence at the same binding ratio. The shape of the binding isotherm of poly[d(A-G)]

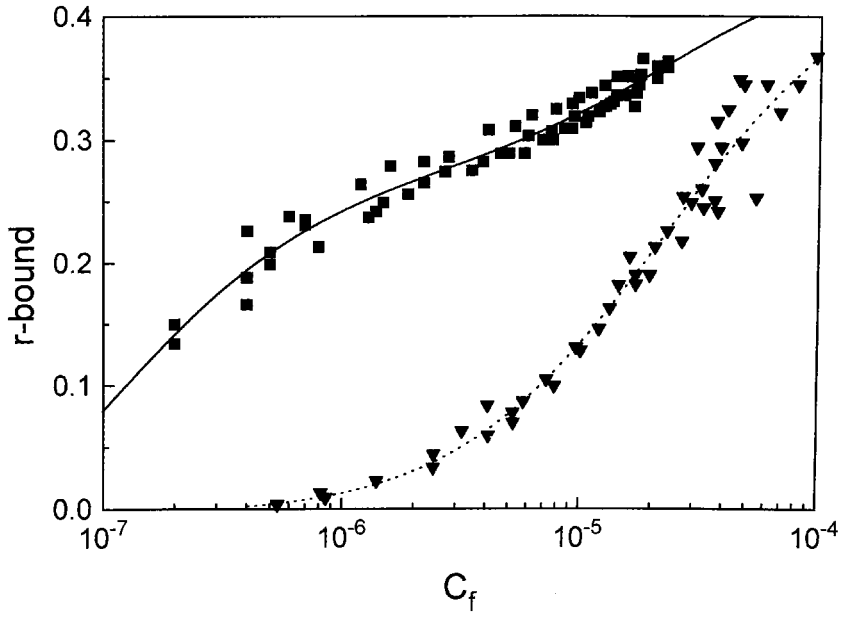

Figure 2. The binding isotherms of daunomycin with deoxypolynucleotides are presented in the form of direct plots of the binding ratio (r_b) vs. log C_f where C_f is the free drug concentration. The binding data of daunomycin with poly[d(A-T)]:poly[d(A-T)] (squares) and poly[dA]:[dA] (triangles). The lines are the calculated curves using an uncoupled saturation model by the least-squares fitting routines.

seems to be parallel to that of poly[d(A-C)] even though it shifts to the right (plots not shown). Partial fluorescence quenching (ca. 60%) was observed in the binding isotherms of non-guanine containing sequences, poly[d(A-T)]. Complete fluorescence quenching was observed for the guanine containing sequences, poly[d(G-T)], poly[d(A-G)], poly[d(G-C)]. Nonlinear least-square fittings of the binding isotherms yield the binding parameters, binding constants, and number of base pairs for binding. The binding affinity of these sequences was found to be different. The binding parameters are summarized in Table 1. Several features can be observed from the binding parameters. First, the uncoupled saturation model described the binding isotherms well. Second, the binding constants seemed to be dependent on the DNA sequence. Third, two different intrinsic binding sites exist describing the binding data of alternating sequence DNA with the uncoupled saturation model, one for high binding affinity and the other for low binding affinity. Fourth, the binding affinity of a primary high binding site K_i is two orders of magnitude different than the binding constants of alternating and non-alternating sequence. Finally, the size of the binding sites of the highest affinity obtained by the uncoupled saturation model was about 3 bp for the alternating sequence.

Table 1. Summary of Daunomycin Binding to a Series of Synthetic Deoxypolynucleotides

	K[a]	n (b.p.)	K_i[b]	$1/r_i$ (b.p.)
Poly[d(A-G)]: Poly[d(C-T)]	$5.02(+/- 0.12) \times 10^5$	2.33(+/− 0.03)	$6.66(+/- 1.38) \times 10^6$	3.33
Poly[d(A-C)]: Poly[d(G-T)]	$3.58(+/- 0.15) \times 10^6$	2.59(+/− 0.05)	$1.49(+/- 0.86) \times 10^6$	3.25
Poly[dG]: Poly[dC]	$1.58(+/- 0.18) \times 10^4$	2.90(+/− 0.18)	$6.65(+/- 1.22) \times 10^4$	5.56
Poly[d(G-C)]: Poly[d(G-C)]	$8.11(+/- 0.24) \times 10^5$	2.29(+/− 0.04)	$3.45(+/- 0.30) \times 10^6$ $5.64(+/- 1.30) \times 10^2$	3.13
Poly[dA]: Poly[dT]	$1.64(+/- 0.08) \times 10^4$	1.48(+/− 0.09)	$4.36(+/- 0.64) \times 10^4$	2.17
Poly[d(A-T)]: Poly[d(A-T)]	$1.55(+/- 0.09) \times 10^6$	2.65(+/− 0.04)	$8.65(+/- 0.75) \times 10^6$ $4.12(+/- 3.31) \times 10^4$	2.78

[a]Values are the result from the nonlinear least-squares fits of binding data to noncooperative neighbor exclusion model. K is the intrinsic binding constant for the binding of a drug to an isolated site, and n is the exclusion parameter in base pairs.
[b]Equilibrium binding constants K_i are obtained from the nonlinear least-squares fitting of binding data to an uncoupled saturation model which is described for the binding of ligands to a mixture of independent binding sites (i). The r_i denotes the binding ratio at the infinate binding of the independent binding site. The first values of binding constants represent the highest binding affinity between the two potential binding sites.

Determination of Enthalpy for Daunomycin Binding to DNA. The binding enthalpies were measured using an isothermal microtitration calorimeter. All the calorimetric titrations were measured after the instrument was carefully calibrated by standard chemical reaction. Figure 3 represents an example of the response curve (i.e., a calorimetric thermogram) of daunomycin with poly[d(G-C)]. The total reaction heat was calculated by estimating the area of peaks. Baseline is stable during the titrations. In this study, the binding ratio of each increment was maintained at 0.025 by assuming that the concentration of free drug was negligible per each titration. Thus, the added drug molecules assumed to be fully bound to DNA. In brief, the binding enthalpies of these DNA sequences were obtained as follow.[15] The uncorrected binding enthalpies of drug-DNA interaction were obtained by using the equation, $Q = \Delta HV \sum C_b$, where Q is the accumulated heat obtained by integrating each peak of thermograms (shown as an example in Fig. 3) per each injection, V is the reaction volume, and $\sum C_b$ is the accumulated concentration of bound drug per each injection, respectively. By putting the accumulated heat (Q) as y-axis and the accumulated concentration of bound drug ($\sum C_b$) as x-axis, correspondingly, the uncorrected binding enthalpies can be obtained from the slopes of these plots. In addition, the heat of dilution was determined by the separate titrations of daunomycin solution into the buffer. The heat of dilution of daunomycin into buffer at

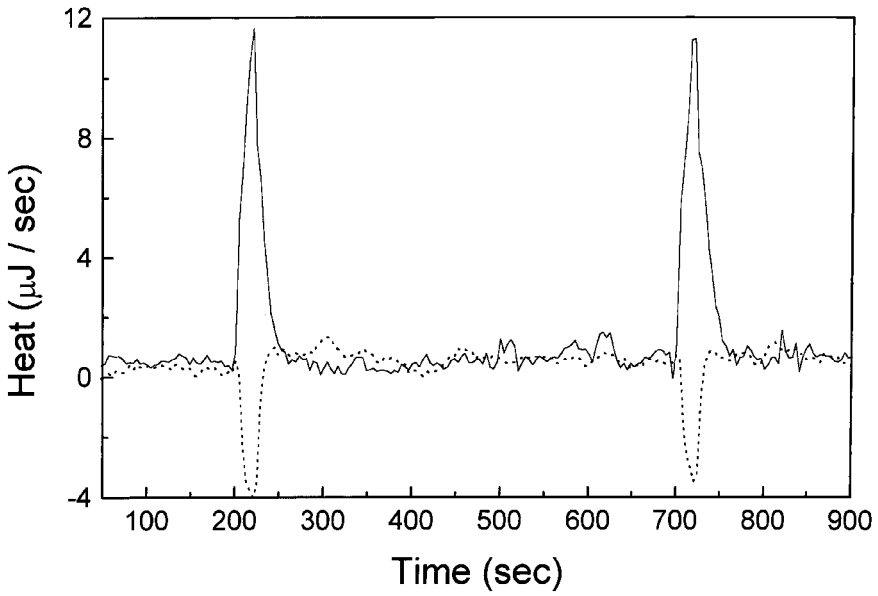

Figure 3. The profile of heat absorbed or released from the addition of daunomycin into buffer solution in the absence (. . . . .) or in the presence (——) of poly[d(G-C)] at 25°C, respectively. The areas of each positive peak represent the heat released by binding daunomycin molecules to the excess amount of DNA per each injection. The first peak is 80 μcal and the second peak is 76 μcal in terms of area by converting the unit of y-axis(J) into calories(cal).

25°C was found to be +2.22 Kcal/mol. Heat of dilution was then subtracted from the uncorrected enthalpies obtained during the titration of daunomycin into DNA solution and estimated by the above method. The resulting negative values of binding enthalpy for the exothermic DNA-drug interactions were summarized with other thermodynamic parameters shown in Fig. 4.

Dissection of the Free Energy. The complete thermodynamic profiles were given for daunomycin binding to salmon testes DNA, poly[d(G-C)], poly[d(A-T)], and poly[d(A-C)] in Fig. 4. The enthalpy values were directly obtained from the calorimetric determinations:

−8.0 Kcal/mol for salmon testes DNA, −8.4 Kcal/mol for poly[d(G-C)], −5.5 Kcal/mol for poly[d(A-T)], and −8.1 Kcal/mol for poly[d(A-C)]. The values of observed free energy (ΔG) were estimated from the binding constants of high affinity sites in Table 1: −8.2 Kcal/mol for salmon testes DNA, −8.9 Kcal/mol for poly[d(G-C)], −9.5 Kcal/mol for poly[d(A-T)], and −8.4 Kcal/mol for poly[d(A-C)], using the standard relationship, $\Delta G = -RT \ln K$.

These binding free energies in conjunction with the binding enthalpies were used for calculating the binding entropies (ΔS): $T\Delta S = \Delta H - \Delta G$. The

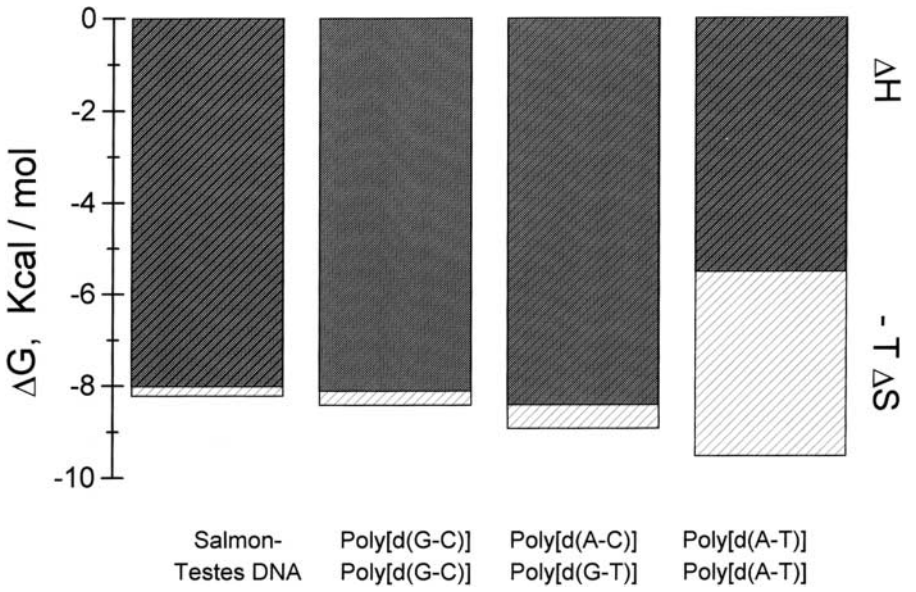

Figure 4. The diagrams of the thermodynamic parameters are represented for daunomycin-DNA interactions at 25°C. The y-axis (Kcal/mol) represents the observed free energy estimated by the binding constants. The black-shadowed regions, ΔH^{o}_{cal} represent the exothermic enthapies of drug-DNA interactions directly obtained by the microtitration calorimeter.

binding interactions of daunomycin with salmon testes DNA seemed to be enthalpy driven. The reactions of daunomycin binding to poly[d(G-C)] and poly[d(A-C)] showed similar enthalpies, -8 Kcal/mol, while both showed relatively low entropy. The contribution of entropy was significant for the reaction of daunomycin with poly[d(A-T)] relatively by representing the low enthalpy and high entropy. The results demonstrate the complete thermodynamic profiles for the binding of daunomycin to a variety of DNA molecules.

DISCUSSION

It can be emphasized that there are differences of shapes and midpoints of titration curves between alternating and non-alternating sequences. For example, the upper strand of poly[d(A-T)] has different possible binding sites, 5′-d(ATA), or 5′-d(TAT) compared to 5′-d(AAA), the only binding site of poly[dA]. The other alternating and non-alternating sequence also show similar characteristics; poly[d(G-C)] has at least two different binding sites, 5′-d(GCG), or 5′-d(CGC), but poly[dG] has a single binding site, 5′-d(GGG). The fluorescence signals were only partially quenched in the absence of guanine at the binding site.[16] For example, the binding of daunomycin to poly[d(A-T)] showed only 60% fluorescence quenching. The details of binding and quenching behaviors of daunomycin with poly[d(A)] were also

reported before.[5] Such results are correlated with a partial fluorescence quenching by intercalating drugs into AT, AU, or IC base pairs.[17] The binding interaction of daunomycin with poly[d(A-T)] was not described well by the neighbor exclusion model shown in Fig. 1 (A). Two distinct curvatures represented in the binding isotherms indicate that there may at least be two possible classes of binding sites. The uncoupled saturation model described the binding data well by assuming that there are at least two independent different binding sites. The calculated curves of this model for the binding data were shown in Fig. 2. The binding isotherms of polynucleotides with alternating sequence were not simple but indicated multiple modes of binding. There were heterogeneous binding sites even in simple polynucleotides, while the dinucleotide steps were different (from 5′ to 3′ for only upper strand): AG, GA for poly[d(A-G)]; AC, CA for poly[d(A-C)]; AT, TA for poly[d(A-T)]; GC, CG for poly[d(G-C)]. The overall structure of the minor groove between AT and TA sequence looked similar. The position of a cleft of AT base pair formed by the thymidine O-2 and adenine H-2, however, displaced to the backbone of DNA from that of TA base pair. The specific recognition of AT and TA dinucleotide by certain protein was also reported.[18] The multiple modes of binding of daunomycin with poly[d(A-T)] seemed to be related to the structural difference, helical twist, tilt, etc., between AT and TA dinucleotide steps. The contribution of the enthalpy and entropy is different in a way of compensation to the free energy depending on the sequence of deoxypolynucleotides shown in Fig. 4. The contribution of $T\Delta S$ is much bigger for poly[d(A-T)] than for poly[d(G-C)], poly[d(A-C)] with daunomycin. The presence of thymidine in the sequence seems to decrease the contribution of enthalpy to the free energy. The characteristics and binding interactions of daunomycin as a topoisomerase II inhibitor with deoxypolynucleotides, however, are enthalpy driven.[19] Thermodynamic parameters for the binding of protoberberines as topoisomerase I inhibitors to DNA, however, indicate that less than half of the energetic force for drug-DNA complexation was found to be enthalpy driven reactions.[20] Recently, the simple way of determining the mode of binding of poly[d(G-C)] and poly[d(A-T)] with DNA sequence specific agents was reported.[21]

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