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EQUILIBRIUM BINDING OF DAUNOMYCIN AND ADRIAMYCIN TO CALF THYMUS DNA

TEMPERATURE AND IONIC STRENGTH DEPENDENCE OF THERMODYNAMIC PARAMETERS

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Abstract—Absorbance and fluorescence quenching monitoring of the binding of the anthracyclines adriamycin (ADM) and daunomycin (DNM) to calf thymus DNA, provides reproducible binding data only when moderate drug/DNA molar ratios are used in the assays. Under these conditions, the fraction of DNA-bound drug, in equilibrium with free anthracycline, which can be reliably detected, ranged from 40–60% to 80–95% of the total added drug, depending upon ionic strength and temperature. Use of the neighbour exclusion model adequately fits such data and predicts that (i) the affinity of ADM for binding to the DNA is always higher than that corresponding to DNM, under similar experimental conditions, (ii) the binding constant for both drugs exhibits a strong salt and temperature dependence, and (iii) the exclusion parameter, indicative of the size of the anthracycline binding sites on the DNA, equals 3.1 ± 0.4 and 3.3 ± 0.4 base pairs for ADM and DNM, respectively, and is independent of salt concentration.

The salt and temperature dependence of the binding constant is used to estimate the thermodynamic parameters involved in the interaction of the drugs with the DNA. Binding of the drugs is an exothermic process and the binding free energy arises primarily from a large negative enthalpy which, as the entropy, strongly depends upon ionic strength, and is much larger than predicted by polyelectrolyte theory. The enthalpy and entropy changes observed, appear to compensate each other over the entire range of salt concentrations used, and may arise from a complex variety of contributions, including salt-induced changes in secondary structure of the DNA, as indicated by circular dichroism techniques.

Adriamycin (ADM)§ and daunomycin (DNM) are anthracycline antibiotics widely used as antitumor agents [1, 2]. Both drugs bind to DNA by intercalation between base pairs and inhibit DNA replication and RNA transcription [3–7]. Although these drugs are structural analogues and have similar biochemical properties, DNM has been shown effective in the treatment of acute lymphocytic and myelogenous leukemias, while ADM, which is also an active antileukemic drug, is of much greater interest because of its activity against a broad spectrum of solid tumors [2]. These properties have determined that studies on the interaction of these agents with DNA have been performed [8–15] to assess more fully their structure/activity status.

Binding isotherms have been reported as Scatchard plots showing a pronounced curvature at high drug/DNA ratios which, depending on the different authors, has either been neglected [9–13] or interpreted as resulting from neighbour exclusion effects [8, 14] or from heterogeneity of the DNA binding sites [12].

Understanding the molecular mechanism(s) of the drug/DNA binding process requires a thermodynamic analysis of the interactions involved. However, the reports on binding enthalpies and entropies are scarce and thus, the thermodynamics of the interaction has received little attention. Based on the temperature-dependence of the apparent binding constant, Zimmerman [16] has recently reported a thermodynamic study of the interaction of ethidium and acridine dyes to DNA, in which the binding constant was derived from the linear portion of the Scatchard isotherms. Also, Chaires [17] has reported the thermodynamics of the interaction between DNM and DNA, using the neighbour exclusion model [8, 18, 19] to fit the experimental data. Furthermore, the enthalpy and entropy of some intercalators binding to DNA have been found to be strongly dependent on ionic strength [17, 20, 21] and it has been suggested that an enthalpy–entropy com-

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§ Abbreviations used: DNM, daunomycin (daunorubicin); ADM, adriamycin (doxorubicin); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DNA, deoxyribonucleic acid; CD, circular dichroism.

pensation may be a general feature of intercalation reactions, although the physical basis of this phenomenon remains unknown. In an attempt to address more fully some of the above issues, we report here comparative studies on the binding of ADM and DNM to calf thymus DNA.

MATERIALS AND METHODS

Chemicals. DNM and ADM hydrochlorides were purchased from Sigma Chemical Co. (St Louis, MO). Stock solutions of the anthracyclines were prepared in water (about 1 mg/ml) and stored in the dark at −10°. Dilutions of drug stocks into the appropriate buffers were prepared immediately before use and their concentration determined spectrophotometrically. Extinction coefficients used for DNM at 480 nm, a wavelength of optimal spectral change upon binding to DNA, and at the isosbestic point of 540 nm, were those used in Ref. 8. Similarly, ADM was found to have absorbance parameters independent of ionic strength with extinction coefficients at 480 nm of 11,200 M^{-1} cm^{-1} and 7000 M^{-1} cm^{-1} for the free and DNA-bound forms of the drug, respectively, and an isosbestic point at 540 nm with a molar extinction coefficient of 5000 M^{-1} cm^{-1}.

Stock solutions of calf thymus DNA (Sigma) were prepared at 3–4 mg/ml in 10 mM Tris buffer, pH 7.5, 1 mM EDTA, saturated with argon and submitted to ultrasound. Sonication conditions were chosen so that (i) no denaturation of the DNA was produced, as indicated by full preservation of thermally induced hyperchroism at 260 nm as compared to the unsonicated DNA solution, and (ii) the average length of double stranded DNA pieces produced by sonication was approximately of 6000 base pairs, as estimated by agarose electrophoresis using ECO-R1 restriction fragments of lambda phage DNA as standards. Sonicated DNA preparations were dialysed and stored at −30°. The concentration of DNA in dilute solutions was determined spectrophotometrically by using a molar extinction coefficient at 260 nm of 12,824 M^{-1} (base pairs) cm^{-1}.

Binding studies. Absorbance measurements were taken in a Zeiss DMR-11 spectrophotometer, thermostated by a Heto Denmark circulating water bath. Quartz cuvettes of 5 cm path length were used throughout. Spectrophotometric titrations were performed either by adding aliquots (5 μl) from a concentrated drug stock solution directly into a cuvette containing a fixed amount of a DNA solution (10–40 μM in base pairs, depending upon the experiments) or by reversing this procedure and titrating a drug solution (3–7 μM) with aliquots from a concentrated DNA stock. In both methods, each addition was followed by a 5 min equilibration period after which the absorbance at 480 nm and at 540 nm were recorded. The concentration of bound and free drug was determined from the observed absorbances using the extinction coefficients indicated above.

Fluorescence titration experiments were performed using a Fica 55 MK spectrofluorimeter. The fluorescence intensity exhibited by both drugs in the absence and in the presence of added DNA, were determined by exciting at 480 nm and recording the emission at 555 nm. The emission values were used to calculate the concentration of bound and free drug as described [8]. In our studies, the ratio between the observed fluorescence intensity of the totally bound drug to that of the free drug was 0.04 ± 0.01 for either of the drugs used, and was invariant with temperature and ionic strength.

Binding data were cast into the form of a Scatchard plot [22] of r/Cf vs r, where r is the number of moles of drug bound per mole of DNA base pairs and Cf is the concentration of free drug, and computer fitted to the neighbour exclusion model [18, 19]:

$$r/Cf = K_i(1 - nr)\,[(1 - nr)/(1 - (n - 1)r)]^{n-1} \quad (1)$$

where K_i is the apparent binding constant to an isolated DNA binding site and n is the exclusion parameter [8]. The computer program calculates non-linear, least-squares, best-fit values for K_i and n and displays the results graphically.

Circular dichroism (CD). CD spectra were measured in a Jovin Yvon Mark III dichrograph at 0.2 nm/sec scanning speed, using 5 cm-path quartz cuvettes. CD results are the mean values of at least three determinations and are expressed as molar ellipticities (θ) in units of degrees × cm^2 × $dmol^{-1}$.

Estimation of thermodynamic parameters. The Gibbs' free energy is determined from the apparent binding constant, according to the relation, $\Delta G^\circ = RT \ln K_i$. The binding enthalpy ΔH° is determined from plots of the temperature dependence of the apparent binding constant according to the Van't Hoff relationship $[\partial \ln K_i/\delta(1/T)] = -\Delta H^\circ/R$. The entropy is estimated from the Gibbs' free energy and the enthalpy as $\Delta S^\circ = -(\Delta G^\circ - \Delta H^\circ)/T$.

RESULTS

Absorbance monitoring of the drug–DNA interaction, at drug/DNA ratios ranging from 1:6 to 1:10 or lower, depending upon ionic strength and temperature, indicates that all the added drug is bound to the DNA. Only above those stoichiometric ratios, free anthracycline, in equilibrium with DNA-bound drug, begins to be reliably detected at molar fractions ranging from 0.05 to 0.15 of the total added drug. Also, since DNM or ADM form aggregates in aqueous solutions above 8–10 μM [23], we adopted these as the upper limit in our titration experiments to study the interaction between monomeric anthracyclines and DNA. For these reasons and again, depending upon ionic strength and temperature, our equilibrium binding data are limited to drug/DNA ratios corresponding to percentages of bound drug ranging from 40–60% to 80–95%, as the lower and upper limits, respectively. Imposing these limits results in excellent agreement between absorbance and fluorescence monitoring of the binding process. Figure 1 shows representative Scatchard plots for the adriamycin–DNA (panel A) and daunomycin–DNA (panel B) interactions, over a wide range of temperature and ionic strength. The differences in the slope of the plots indicate that the affinity of ADM for binding to the DNA is always higher than that corresponding to DNM under comparable experimental conditions. An increase in either temperature or salt concentration results in a decreased binding affinity for both drugs.

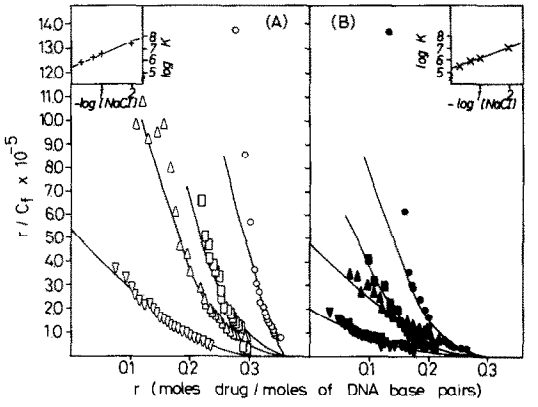

Fig. 1. Representative Scatchard plots for adriamycin (A) and daunomycin (B) binding to calf thymus DNA at 15° (○, ●, △, ▲) and 35° (□, ■, ▽, ▼). Experiments were conducted in 8 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.05 M (○, ●, □, ■) or 0.50 M (△, ▲, ▽, ▼) NaCl, as described under Materials and Methods. Points in the figure are experimental and solid curves represent the non-linear, least-squares fits to equation (1) in the text. The inserts show the double log plot illustrating the influence of ionic strength on the neighbour exclusion model's apparent binding constant. The slope was 0.87 for ADM and 0.84 for DNM.

In an attempt to assess whether the anthracyclines used in this work exhibit positive cooperativity for binding to the DNA, we have used the criteria reported by Kowalczykowsky *et al.* [24]. Figure 2A shows representative "*r*" (moles of bound drug/mole of DNA base pairs) vs free ligand concentration plots, which are expected to be sigmoidal for cooperative binding processes. As shown, no signs of sigmoidal behaviour were observed although the initial parts of the curves cannot be fully defined due to the methodology limitations indicated previously. Also, Fig. 2B shows representative results from titrations of a fixed concentration of drug with increasing amounts of DNA, as plots of the fractional saturation of the ligand vs total DNA concentrations. The curves reached a plateau corresponding to almost 100% of ligand saturation, which is independent of the initial concentration of drug within the 3–7 μM range. Again, this is consistent with noncooperative or moderately cooperative binding processes [24].

Since we found no evidence for the occurrence of cooperativity in our system, we adopted the neighbour exclusion model [18, 19] to fit our experimental data. We find that such a model adequately fits the data within regions of the Scatchard plots corresponding to a range of 40–50% to 80–85% of DNA-bound ligand. However, it should be noted that significant deviations from the neighbour exclusion predictions are observed when either low ionic strength (below 100 mM of added NaCl) or low temperature conditions are used (see Fig. 1). It has been proposed that these deviations could be due to an incomplete neighbour exclusion model, weaker contributions from ionic binding or a conformational change of either the drug or the DNA as a consequence of salt concentration [25]. The latter possibility has been tested by performing CD studies of both the drug and the DNA. The CD spectrum of DNA shows a main positive band at 275 nm and a negative band at 246 nm [26]. Upon increasing the concentration of NaCl in the buffer (Fig. 3), the main spectral changes occur at the 275 nm peak. The molar ellipticity at 275 nm maintains a constant value at salt concentration below 100 mM and then decreases linearly as the salt concentration increases from 100 to 900 mM (insert to Fig. 3). Conversely, the CD spectrum of drug alone does not show any dependence on salt concentration (data not shown).

Table 1 shows the estimated values for the binding constant (K_i) and the exclusion parameter (n) obtained from fits to the neighbour exclusion model. Imposing the restrictions indicated previously to accept experimental data, results in values of n which appear to be invariant with ionic strength, and equal to 3.1 ± 0.4 and 3.3 ± 0.4 DNA base pairs, for ADM and DNM respectively. Also, we find that the apparent binding constant for either drug decreases as the sodium salt concentration increases, and approaches a limiting value of 6–7 × 10^5 M^{-1} for ADM and 2 × 10^5 M^{-1} for DNM at high salt (0.7–0.9 M).

The ionic strength (50–900 mM NaCl) and temperature (15–40°) dependence of the interaction between the drugs and DNA, has been analysed to determine the thermodynamic parameters involved, all subject to about 10–15% uncertainty. As indicated in Table 1 the estimated thermodynamic parameters are strongly dependent upon ionic strength,

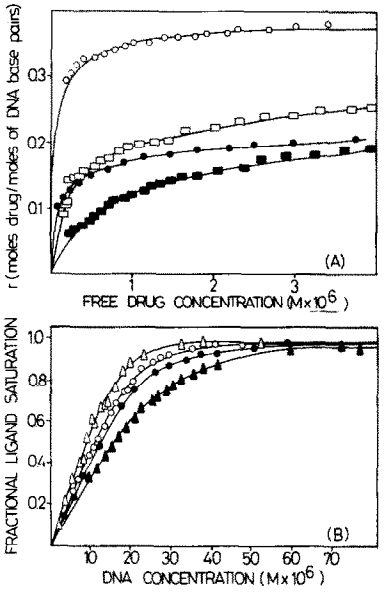

Fig. 2. (A) Binding density of adriamycin (○, □) and daunomycin (●, ■) on the macromolecule (in moles of bound drug per mole of DNA base pairs) vs free ligand concentration. DNA concentration was 20 μM in base pairs. (B) Fractional ligand saturation of adriamycin (△, ○) and daunomycin (▲, ●) vs DNA concentration. Drug concentrations were (△) 4.4 and (○) 5.5 μM for adriamycin and (●) 4.6 and (▲) 7 μM for daunomycin. These titrations were carried out in 8 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.05 M (○, ●), 0.50 M (□, ■) and 185 M (○, △, ●, ▲) NaCl at 25°.

Table 1. Thermodynamic parameters for the interaction of adriamycin and daunomycin with calf thymus DNA*

	NaCl (M)	K_i (25°) (M^{-1})	n§	$-\Delta G°$ (25°) (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (25°) (cal/grad mol)
ADM	—†	2.3×10^7	3.0	10.0	—	—
	0.050‡	7.1×10^6	2.8	9.3	17.2	26.3
	0.100†	3.7×10^6	3.0	8.9	—	—
	‡	4.6×10^6	2.9	9.0	13.1	13.7
	0.185†	1.9×10^6	3.2	8.5	—	—
	‡	1.8×10^6	3.6	8.4	11.7	11.0
	0.500†	7.8×10^5	3.3	8.0	—	—
	‡	1.1×10^6	3.0	8.2	10.3	7.1
	0.700‡	6.2×10^5	3.5	7.8	10.4	8.0
	0.900‡	7.4×10^5	2.8	7.9	11.0	10.4
DNM	—†	8.4×10^6	3.2	9.4	—	—
	0.050‡	2.5×10^6	3.9	8.7	16.0	24.5
	0.100†	9.0×10^5	3.2	8.1	—	—
	‡	1.4×10^6	3.3	8.4	13.3	16.5
	0.185†	7.5×10^5	3.5	8.0	—	—
	‡	5.0×10^5	3.7	7.7	10.6	9.7
	0.500†	3.4×10^5	4.0	7.5	—	—
	‡	3.0×10^5	3.1	7.4	9.8	8.0
	0.700‡	2.2×10^5	3.0	7.3	10.0	9.0
	0.900‡	2.0×10^5	3.0	7.2	10.1	9.7

* Thermodynamic parameters are calculated from the salt and temperature dependence of the binding constant (K_i) predicted by fitting of experimental data to the neighbour exclusion model.

† Binding data are obtained from simultaneous absorbance and fluorescence monitoring, using different DNA preparations.

‡ Absorbance titrations are conducted in 8 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and NaCl at the indicated concentrations.

§ n, exclusion parameter estimated from the neighbour exclusion model (moles of DNA base pairs/mole of drug).

particularly, at concentrations of added NaCl below 200–300 mM. Regardless of salt concentration, binding of either of the anthracyclines used in this work to calf thymus DNA is an exothermic process in which the negative values for the estimated Gibbs' free energy seem to arise from large enthalpic contributions.

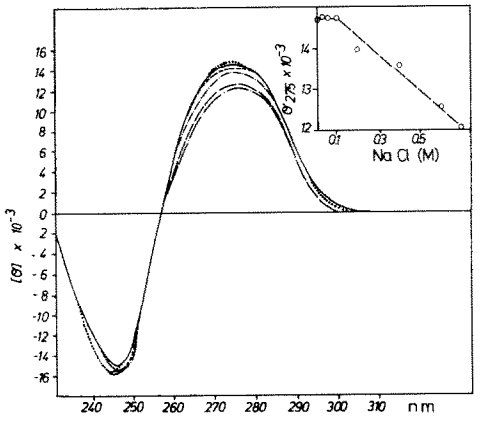

Fig. 3. Circular dichroism spectra of DNA (31 μM in base pairs) in 8 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA (solid line) and the same solution containing (· · · · ·) 50 and 100 mM, (——) 200 mM, (—·—·—) 400 mM, (— —) 600 mM and (—·—) 700 mM of NaCl. Spectra were taken at 25°. The insert shows the changes in the molar ellipticity at 275 nm in the presence of increasing salt concentrations.

DISCUSSION

Binding of anthracycline intercalators to DNA from natural sources, remains as a complex issue whose characteristics need to be defined further. A molecular mechanism for the binding process has been proposed only for DNM [27] and comprises a rapid bimolecular association step, followed by two sequential isomerizations involving a variety of interactions between the drug and the DNA lattice. The possible occurrence of cooperativity in the binding process is also a controversial subject [15, 17] and its detection appears to depend upon factors including the ionic strength, the use of natural or synthetic polynucleotides, the drug/DNA ratio and the technique used to monitor the binding process. In this paper, we have analysed the binding of ADM and DNM to calf thymus DNA, by using absorbance and fluorescence quenching methods and found that, in our hands, reliable monitoring of the binding process is limited to moderate drug/DNA ratios corresponding to percentages of bound drug ranging from 40–60% to 80–95% of the total amount of drug present. Within these limits, we have not been able to detect the occurrence of cooperative binding according to criteria proposed in Ref. 24. Furthermore, imposing such limits for the acquisition of binding data results in that (i) there is concordance between absorbance and fluorescence monitoring, (ii) the binding isotherms fit reasonably well to the neighbour exclusion model used by others [8, 14, 19], (iii) the exclusion parameter (n) describing the size

of anthracycline binding sites on the DNA, is constant for each drug, with independence of factors such as ionic strength or temperature, and (iv) the binding constant predicted by the neighbour exclusion model, always higher for ADM than for DNM under similar conditions, exhibits a strong dependence on both ionic strength and temperature.

The observed dependence of K_i on ionic strength suggests a competitive effect between the cationic drugs and Na^+ for binding to the DNA, by which the bound drug(s) can be partly displaced from the polymer by Na^+. Such a dependence could be anticipated from the polyelectrolyte theory [28], based upon the linkage between drug and phosphate ion binding. As indicated in the inserts to Fig. 1, it is apparent that the salt dependence of K_i follows Record's expression

$$\partial \ln K_i / \partial \ln [Na^+] = -Z\psi = \Delta r \qquad (2)$$

where Z is the charge on the drug molecule, and ψ is the fraction of counterions associated with each DNA phosphate group (for double-stranded DNA $\psi = 0.88$). The quantity Δr is the number of counterions released upon binding of a ligand with charge Z. We find $\Delta r = 0.84$ and 0.87 for DNM and ADM, respectively, and thus $Z = 0.95$ and 0.99 for DNM and ADM, respectively, which appear to correspond to the single positive charge carried by both drugs at neutral pH [4].

Equation (2) can also be used to predict what changes in the Gibbs' free energy would be expected on the basis of polyelectrolyte theory. Solving equation (2) for the different concentrations of salt shows that such predictions are in excellent agreement with the free energy changes calculated from the salt dependence of the neighbour exclusion model's binding constants. In contrast to this, the experimental values for the entropy and, particularly, the enthalpy changes with ionic strength are much larger (by 1–2 orders of magnitude) than those predicted from polyelectrolyte theory, with the most dramatic differences being observed below 200–300 mM, where the approximations of polyelectrolyte theory should be most valid. This suggests that polyelectrolyte theory predictions of free energy changes may be fortuitous [8, 17]. Moreover, the enthalpy and entropy changes estimated from the salt dependence of the binding constant, seemingly compensate each other according to a linear relationship (correlation coefficients of 0.993 and 0.996 and slopes ("compensation temperature") of 369 and 387 K for ADM and DNM, respectively), to produce relatively small changes in free energy. A similar situation was reported for ethidium [21] and for DNM binding to calf thymus DNA [17]. In the latter paper, however, the magnitude of the enthalpy and entropy changes reported were even higher than those observed here. This could be due to differences in the preparation of DNA samples or, most likely, to the criteria imposed for acquisition and processing of binding data.

In our interpretation, the above discordance with polyelectrolyte theory indicates that phenomena other than ionic interactions, may also be important in anthracycline binding to DNA and must, therefore, be invoked to explain the salt dependence of enthalpy and entropy changes. In this regard, our data on CD spectral changes of the DNA induced by salt, introduces an additional factor to be taken into account in discussing the salt dependence of the anthracycline/DNA interaction. Changes in the intensity of the 275 nm CD band of DNA has been related to the change in the average rotation of the helix [26]. Thus, changes in DNA secondary structure induced by salt, along with additional structural changes in the DNA induced by binding of the intercalators [29–31], might contribute enthalpic and entropic terms to produce the apparent compensation observed.

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