Daunomycin inhibits the B $\rightarrow$ Z transition in poly d(G-C)

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ABSTRACT

The cancer drug daunomycin is an effective inhibitor of the B $\rightarrow$ Z transition in poly d(G-C) in 4 M NaCl. Both the rate and extent of the B $\rightarrow$ Z transition are decreased by the drug, as judged by equilibrium and kinetic studies. Daunomycin can, under some conditions, convert Z form DNA back to B form. Drug binding to poly d(G-C) in 4 M NaCl is slow and highly cooperative, consistent with a role for daunomycin as an allosteric effector on the B $\rightarrow$ Z equilibrium. Since daunomycin binds preferentially to alternating purine-pyrimidine sequences, which are the very sequences capable of undergoing the B $\rightarrow$ Z transition, these effects may be an important part of the mechanism by which the drug inhibits transcription and replication.

INTRODUCTION

The deoxypolynucleotide poly d(G-C) undergoes a cooperative transition from one double helical form to another at high salt concentrations (1). This transition corresponds to a shift from the right handed B form DNA to the left handed Z form, as shown by a variety of physical techniques (2-8). Other sequences containing an alternating purine-pyrimidine structure can also undergo the B $\rightarrow$ Z transition (9-13). Stretches of Z DNA may co-exist with adjacent sequences in the B conformation (14-and references therein). Antibodies to Z DNA bind specifically to regions in *Drosophila* polytene chromosomes, indicating the natural occurrence of Z DNA, and leading to the suggestion of a role of Z DNA in genetic activity or in the specific organization of the chromosome (15). Recently, a class of proteins that specifically bind to Z DNA has been isolated from *Drosophila* (16).

Daunomycin is an anthracycline antibiotic widely used in the treatment of human cancers (17-19). The drug binds tightly to DNA by intercalation (20,21), inhibiting both DNA replication and RNA transcription (22-24). Daunomycin binds preferentially to alternating purine-pyrimidine sequences (25). Since these are the very sequences which may undergo the B $\rightarrow$ Z transition, the effect of daunomycin on the conversion of B DNA to Z form

DNA is of interest, and may be important for understanding the drug's mode of action.

In the experiments described here, I show that daunomycin inhibits both the rate and the extent of the B $\rightarrow$ Z transition in poly d(G-C) at 4 M NaCl. At high drug/b.p. ratios, daunomycin can convert Z form DNA back to B form. Drug binding to poly d(G-C) at 4 M NaCl is highly cooperative. These observations are consistent with the preferential binding of daunomycin to B form DNA, and with a role for the drug as an allosteric effector on the B $\rightarrow$ Z equilibrium.

MATERIALS AND METHODS

Poly d(G-C)

Poly d(G-C) was obtained from P and L Biochemicals (Milwaukee), treated with S1 nuclease and fractionated as previously described (25). The polymer preparations used had a sedimentation coefficient of 10.9S and showed the ultraviolet absorbance spectrum and thermal denaturation profile expected for double helical poly d(G-C). The concentration of poly d(G-C) was estimated assuming an extinction coefficient of $16,800 \text{ M}^{-1}$ (base pairs) cm^{-1} at 254 nm.

Equilibrium Measurements of the B $\rightarrow$ Z Transition

The conversion of poly d(G-C) from the B to the Z form results in the inversion of the circular dichroism spectrum, and a red shift in the ultraviolet absorbance spectrum, with a decrease in the ratio A_{260}/A_{295} from 8.6 to 3.2 (1). The ratio of A_{260}/A_{295} was used to monitor the B $\rightarrow$ Z transition. Concentrated poly d(G-C) was added to buffered NaCl solutions (containing 6 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 1 mM Na_2EDTA , pH adjusted to 7.0 with 1 M NaOH) to give a final polymer concentration of 20 μM (b.p.). Following a 2 hr equilibration, the ultraviolet absorbance spectrum of each sample was recorded, and the ratio $R = A_{260}/A_{295}$ calculated. Samples containing daunomycin were corrected for the absorbance of the added drug.

Kinetics of the B $\rightarrow$ Z Transition

Kinetic studies on the B $\rightarrow$ Z transition were performed on a Cary 219 spectrophotometer, equipped with a Neslab circulating water bath and thermal programmer. The difference in absorbance at 295 nm relative to a reference solution containing poly d(G-C) in 2 M NaCl was recorded continuously as a function of time following the addition of poly d(G-C) to a solution containing 4 M NaCl. The final concentration of polymer was 40 μM (b.p.), and the addition of polymer and mixing were completed within 20 s. For samples

containing daunomycin, identical amounts of drug were added to the reference solution, in order to correct for the absorbance of the bound drug. Temperature was maintained at 26° C.

Semilog plots of $\ln(A_{295}^{\circ} - A_{295})$ versus time for B $\rightarrow$ Z transition are nonlinear, due to the length heterogeneity of poly d(G-C) samples (1). I, therefore, have used the mean relaxation time (26)

$$1/\tau^* = (1/\Delta A_{295}^{\circ}) (d\Delta A_{295}/dt)_{t \rightarrow 0}$$

as a measure of the rate of the transition, where ΔA_{295} is the absorbance at time t, and ΔA_{295}° is the overall change in absorbance between the initial and final states. The mean relaxation time is thus the normalized initial rate of the reaction.

Daunomycin Binding

Binding of daunomycin to poly d(G-C) was monitored by absorbance or fluorescence spectroscopy, as previously described in detail (20,25). The binding ratio, r, refers to the amount of daunomycin bound per mole of base pair. Binding data at 2 M NaCl was fit to the neighbor exclusion model

$$r/C_f = K(1-nr) \left\{ \frac{(1-nr)}{(1-(n-1)r)} \right\}^{n-1} \quad (1)$$

where K is the intrinsic binding constant, n the exclusion parameter, and C_f the free drug concentration (31). Fitting was performed using a non-linear least squares fitting routine available in the PROPHET Computer Resource, as previously described (25).

RESULTS

Daunomycin inhibits the extent of the B $\rightarrow$ Z transition, as shown by equilibrium studies illustrated in figure 1. In the absence of daunomycin a midpoint in the transition curve at approximately 2.9 M NaCl is seen, while the ratio A_{260}/A_{295} decreases by a factor of 2.7 at 4 M NaCl. Both of these measures are in tolerable agreement with previous studies (1). In the presence of daunomycin, both the extent of the reaction and the midpoint of the transition are seen to vary with the ratio of added drug. At high ratios of added drug, the B $\rightarrow$ Z transition is effectively inhibited.

Kinetic experiments show that both the rate and the extent of the B $\rightarrow$ Z transition are inhibited by daunomycin, as summarized in figure 2 and Table I. Estimates of the extent of the reaction made from these kinetic experiments

Figure 1. Effect of daunomycin on the B $\rightarrow$ Z equilibrium. The ratio $R = A_{260}/A_{295}$ divided into the A_{260}/A_{295} ratio measured at 1 M NaCl (R^0) is shown as a function of NaCl concentration. Total poly d(G-C) concentration is 20 μ M (b.p.), $T = 25^\circ$ C. Key: $\bullet$ No added daunomycin; $\circ$ 0.08 moles daunomycin/mole b.p. added; $\blacktriangle$ 0.198 moles daunomycin/mole b.p.; $\blacklozenge$ 0.36 moles daunomycin/mole b.p.

Figure 2. Kinetics of the B $\rightarrow$ Z transition. The change in absorbance at 295 nm of a 40 μ M poly d(G-C) solution containing 4 M NaCl relative to a reference solution containing poly d(G-C) in 2 M NaCl is shown. The reaction was initiated as described in Methods. Key: $\circ$ no added daunomycin; $\square$ 0.06 moles daunomycin/mole b.p. added; $\triangle$ 0.1 moles daunomycin 1 mole b.p. added; $\blacklozenge$ 0.2 mole daunomycin/mole b.p. added.

Table I. Rate and Extent of the B $\rightarrow$ Z Transition in the Presence and Absence of Daunomycin

r_{added}	$1/\tau^*, \text{s}^{-1}$	ΔA_{295}°	Extent of Rxn
0	2.3×10^{-3}	0.122	100%
0.06	1.4×10^{-3}	0.120	98
0.1	0.6×10^{-3}	0.108	89
0.2	0.4×10^{-3}	0.088	72

The mean relaxation time ($1/\tau^*$) for the transition of poly d(G-C) to Z form was measured as described in the text. The total optical change (ΔA_{295}°) between the initial and final states is also shown. The extent of the reaction was calculated as follows:

$$\text{Extent of rxn(\%)} = (\Delta A_{295}^\circ) / (\Delta A_{295}^\circ)_{r=0} \times 100$$

are in good agreement with those obtained from the equilibrium studies shown in Figure 1.

An analogous series of kinetic experiments were performed using ethidium bromide (data not shown). The effect of ethidium and daunomycin on the mean relaxation time of the B $\rightarrow$ Z transition is compared in figure 3. The two intercalators are equally efficient as inhibitors of the transition.

Daunomycin can, under certain conditions, convert Z form DNA back to B form, as shown in figure 4A. In this experiment, the formation of Z DNA was interrupted by the addition of daunomycin. At low ratios of drug/b.p.,

Figure 3. Comparison of the effect of ethidium and daunomycin on the rate of the B $\rightarrow$ Z transition. The rates of the B $\rightarrow$ Z transition in the presence and absence of added ethidium $\circ$ or daunomycin $\bullet$ was measured as described as a function of added drug. Within experimental error, no difference between the two compounds can be distinguished.

Figure 4. Reversal of the B → Z Transition by Daunomycin and Ethidium. (A) The B → Z transition was initiated as described in the text, but interrupted at the point indicated by the arrow by the addition of daunomycin at the following molar ratios (moles DM/mole b.p.): ○ 0.0; □ 0.225; △ 0.56; ◇ 0.9. Identical amounts of drug were added to the reference solution, and the absorbance change at 295 nm monitored continuously following the addition. (B) The B → Z transition was interrupted at the point indicated by the arrow by the addition of a 0.67 molar ratio of either daunomycin □ or ethidium △.

the transition is slowed, but as the ratio increases the B → Z transition is reversed. The rate of the Z → B conversion is dependent on the molar ratio of added daunomycin. Ethidium bromide appears more efficient in reversing the B → Z transition, as seen in figure 4B. At identical molar ratios of added drug/b.p., the rate of the Z → B conversion is more rapid for ethidium than for daunomycin (figure 4B).

The binding of daunomycin to poly d(G-C) at 4 M NaCl shows cooperative behavior that deviates markedly from the binding isotherm expected from the neighbor exclusion model under these ionic conditions (figure 5). The binding of the drug to poly d(G-C) under these conditions is slow, in contrast to the rapid binding kinetics observed at lower salt concentrations (Chaires, J.B., unpublished data). The binding isotherm seen in figure 5 for 4 M NaCl is consistent with the preferential binding of daunomycin to B form DNA, and the allosteric conversion of Z to B DNA by the drug. The slow binding kinetics reflect the kinetics of the Z → B conversion. The allosteric model proposed by Dattagupta *et al.* (32) may be used to interpret the 4 M binding data. This model proposes two DNA conformations in equilibrium, with drug binding to each form described by the intrinsic binding constants K_1 and K_2 , and exclusion parameter n_1 and n_2 . In this case, form 1 may be equated with Z DNA and form 2 with B DNA. From the 4 M binding

Figure 5. Binding of Daunomycin to Poly d(G-C). Amounts of free and bound daunomycin were determined spectroscopically. The symbol $\square$ refers to drug binding in 2 M NaCl, and the solid line is the least-squares fit of that data to the neighbor exclusion model with $K_1 = 3.3 \times 10^5 \text{ M}^{-1}$ and $n = 3.0$ b.p. The dashed line is the expected binding isotherm in 4 M NaCl, based on the neighbor exclusion model, and the known salt dependence of daunomycin binding to DNA (20). The symbols (O, +) are the observed binding data at 4 M NaCl. The symbol (+) is data obtained by rapidly titrating a poly d(G-C) sample in 4 M NaCl. The symbol (O) refers to binding data obtained at 4 M NaCl, but allowing for a 90 min equilibration after each addition of drug. The pairs of points connected by an arrow show the values obtained immediately after the addition of drug, and following the 90 min incubation. These results show that daunomycin binding to poly d(G-C) in 4 M NaCl is slow and cooperative, consistent with preferential binding of the drug to B-form DNA, and with a role for the drug as an allosteric effector on the B $\rightarrow$ Z equilibria.

data shown in figure 5, the ratio K_2/K_1 is estimated to be greater than 50, indicating that drug binding to B form is strongly favored, and is the driving force for the allosteric conversion of Z to B DNA under these conditions.

DISCUSSION

The results presented here show that the potent cancer drug daunomycin can effectively inhibit both the rate and the extent of the B $\rightarrow$ Z transition in poly d(G-C) at 4 M NaCl. The drug can, under certain conditions, reverse the B $\rightarrow$ Z transition, and stabilize the B form in ionic conditions that would otherwise favor the Z conformation. Binding of daunomycin to poly d(G-C) at 4 M NaCl is strongly cooperative, consistent with the role of the drug as an allosteric effector of the B $\rightarrow$ Z equilibrium.

Any intercalating drug may, in principle, favor B form DNA as a binding site, and consequently show behavior similar to that observed in this study for daunomycin. However, a surprising variety has been seen in the limited number of studies that have examined the effect of intercalators on the B $\rightarrow$ Z transition. Only ethidium (27) and now daunomycin have thus far shown the cooperative binding effects illustrated in figure 5. Proflavine and quinacrine do not bind cooperatively to poly d(G-C) under similar conditions (27). In a study examining the effects of intercalators on the initial rate of the B $\rightarrow$ Z transition, proflavine was found a less efficient inhibitor of the transition relative to ethidium, while bismethidium spermine and actinomycin were dramatically more efficient (28). Contrasting behavior for the non-intercalating compounds netropsin and distamycin has been reported. Netropsin will reverse Z DNA back to B form, while distamycin will not (29). Thus, DNA binding drugs do not necessarily effect the B $\rightarrow$ Z equilibrium in the same way. Among those compounds that do inhibit the B $\rightarrow$ Z transition, it is by no means clear if they do so by the same mechanism.

Mirau and Kearns (28) have proposed that the efficiency of inhibition of the B $\rightarrow$ Z transition by intercalators is correlated primarily with the dissociation kinetics of the ligand. The present work vitiates that view. The binding of daunomycin to DNA shows complex kinetics involving at least three steps, with the slowest dissociation rate constant equal to 1.2 s^{-1} , some two orders of magnitude slower than seen for ethidium (Chaires, in preparation). The Mirau-Kearns model suggests that the inhibition of the B $\rightarrow$ Z transition by daunomycin would be more efficient than ethidium, but less efficient than actinomycin, if the inhibitory power of an intercalator correlates with its dissociation kinetics. However, figure 3 shows that the relative efficiency of ethidium and daunomycin in inhibiting the B $\rightarrow$ Z transition is identical. Further, ethidium reverses the B $\rightarrow$ Z transition more readily than does daunomycin (figure 4B). All of this points to the fact that the mechanism by which intercalators inhibit the B $\rightarrow$ Z transition remains poorly understood, and while the kinetics of the intercalator-DNA interaction may play an important role in the inhibition, other factors, such as the exclusion parameter, may also require consideration. These points are currently under active investigation in my laboratory.

Daunomycin strongly prefers B DNA over Z DNA as a binding site. The ratio of the daunomycin binding constant to B DNA to that of Z DNA is over 50, as estimated from the data of figure 5. A possible structural basis for this strong preference for B DNA may be inferred from the studies of

Quigley *et al.* (33), in which the structure of a daunomycin-deoxyoligo-nucleotide complex was determined to atomic resolution. Daunomycin, in that structure, is intercalated with the long axis of its anthraquinone ring system at right angles to the long axis of adjacent DNA base pairs. The acetyl group on the daunomycin A ring and the amino sugar extend in opposite directions to occupy the minor groove of the B DNA helix, providing a natural fit for the drug into the right-handed double helix. Such a favorable geometric fit would not be possible in the left-handed Z DNA helix. Ethidium also prefers B DNA over Z DNA as a binding site. The structural basis of its preference is, however, less clear. Ethidium intercalates with its long axis parallel to the long axis of the DNA base pairs (34), oriented such that its phenyl and ethyl substituents would also lie in the minor groove of B DNA. While these groups are not as bulky as the constituents on the daunomycin molecule that occupy the minor groove, they perhaps contribute to the preference of ethidium for the B DNA conformation.

The role of the B $\rightarrow$ Z transition in gene expression is as yet unknown. Antibodies to Z DNA have, however, demonstrated the occurrence of Z DNA in *Drosophila* polytene chromosomes (15) and within the macronucleus of the hypotrichous ciliate *Stylonychia mytilus* (30), leading to the suggestion that Z DNA may play a role in genetic activity or in the specific organization of the chromosome. Recently, several proteins that bind selectively to Z DNA were isolated from *Drosophila*, and may regulate the B $\rightarrow$ Z conversion in the cell (16). Daunomycin is an effective inhibitor of the B $\rightarrow$ Z transition, and will preferentially bind to the alternating purine-pyrimidine sequences that can undergo the transition. If Z DNA is in fact involved in gene expression, the inhibition of the B $\rightarrow$ Z transition by daunomycin may be an important aspect of the mechanism by which the drug inhibits transcription and replication.

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