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Equilibrium and Kinetic Measurements of Actinomycin Binding to Deoxyribonucleic Acid in the Presence of Competing Drugs

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SUMMARY

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Competitive binding studies were performed by addition of daunomycin, ethidium bromide, and mithramycin to DNA-bound actinomycin D. Equilibrium and kinetic measurements demonstrated that these drugs displace actinomycin from its strong binding sites on DNA. Actinomine, an analogue of actinomycin that lacks the peptide lactones, did not displace DNA-bound actinomycin under conditions similar to those used for daunomycin, mithramycin, ethidium bromide, and an ethidium analogue in which the phenyl group is replaced by a methyl group. The competing drugs may modify actinomycin-DNA interaction by interfering with binding of the cyclic peptides of actinomycin and by inducing a structural distortion in DNA.

INTRODUCTION

Actinomycin D (C_1) binds specifically to double-helical DNA at GC base pairs and causes a structural distortion in DNA, resulting in specific inhibition of DNA-dependent RNA synthesis in many different cell types (1–6). Several molecular models of the actinomycin-DNA complex, based on the available binding data, have been proposed since 1963 (3, 6–8). The most recent and detailed model (6) describes a phenoxazone chromophore intercalated between GC and CG base pairs, with the peptide lactones situated in the narrow groove and occupying a distance of approximately six pairs; among the important stabilizing forces is hydrogen bonding from the 2-amino group of guanine to the threonyl residue of the cyclic peptide in the minor groove.

Various investigators have studied drug binding to DNA in the presence of competing drug or dye molecules. In such studies the competing drugs chosen had been used extensively in binding studies with nucleic acids, and conclusions were reached about the identity or nonidentity of the binding sites on DNA and about the nature of the binding of the drug under investigation. For example, it was concluded from spectral studies that DNA binding sites for ethidium bromide, proflavine, methylene blue, and quinacrine are identical (9–11), whereas ethidium bromide and actinomycin showed noncompetitive binding (9). Fluorometric and calorimetric studies were reported recently for the simultane-

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ous binding of actinomycin and daunomycin to DNA, and the proposal was made that the two drugs bind to DNA at independent sites (12). The same conclusion was reached in an earlier study in which the selective displacement of actinomycin from a complex of actinomycin, daunomycin, and DNA was observed upon addition of mercuric ion (13).

We have investigated the strong actinomycin binding process in the presence of daunomycin, ethidium bromide, and mithramycin. The competitive equilibrium and kinetic binding studies reported here demonstrate that, in contrast to the reports cited above, these drugs interfere with the binding of actinomycin to DNA. The relation between actinomycin binding to DNA and the binding of other drugs is discussed.

MATERIALS AND METHODS

Actinomycin D (referred to here as actinomycin) was obtained from Merck Sharp & Dohme. Actinomine and daunomycin were generously supplied by Dr. Edward Reich. Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) was obtained from Boots Pure Drug Company. 3,8-Diamino-5-ethyl-6-methylphenanthridinium bromide was a gift from Dr. Michael J. Waring. Concentrations of antibiotics were determined spectrophotometrically using the following extinction coefficients: actinomycin, 24,450 M^{-1} cm^{-1} at 440 nm; actinomine, 22,500 at 445 nm; daunomycin, 9860 at 475 nm; ethidium bromide, 5600 at 480 nm; and 3,8-diamino-5-ethyl-6-methylphenanthridinium bromide, 3600 at 465 nm. Calf-thymus DNA was dissolved, centrifuged, and dialyzed as previously described (14). Concentrations of DNA solutions in terms of nucleotide phosphorus were determined spectrophotometrically using an extinction coefficient ϵ(P) of 6600 M^{-1} cm^{-1} at 260 nm.

All experiments were performed in 0.01 M potassium phosphate or sodium phosphate buffer, pH 7.0, unless otherwise noted.

Absorption measurements. Absorption spectra were recorded on a Cary 14 spectrophotometer. Spectral titrations were carried out at 25° using an expanded (0–0.1) slide wire and a cell of 1-cm path length and 5.5-ml capacity as described previously (14).

Kinetic measurements. Stopped-flow studies were conducted with a Durrum-Gibson stopped-flow spectrophotometer equipped with a Tektronix 564 oscilloscope and Polaroid camera. The temperature was maintained constant at 25.0° with a Lauda K2-R temperature circulator. The competing drugs were mixed with DNA prior to the stopped-flow experiment. The procedure by which the interaction of actinomycin with DNA was deconvoluted into five separate rate processes was described earlier (14). In the stopped-flow studies, DNA that had previously been subjected to shearing by passage through the stopped-flow apparatus five times was used. The molecular weight of the five-times sheared DNA (4×10^6) was not diminished appreciably when passed through the stopped-flow apparatus again.

CD measurements. CD spectra were recorded at approximately 23° on a Cary 60 spectropolarimeter equipped with a 6001 CD attachment. Cells of 1- and 2-cm path lengths were used. Molecular ellipticity [θ] is reported in units of (deg cm^2) $dmole^{-1}$

RESULTS

Results of experiments involving competition between actinomycin and ethidium bromide for binding sites on DNA are shown in Figs. 1 and 2. Addition of ethidium bromide to the actinomycin-DNA complex at a constant ratio of DNA to actinomycin causes a shift in the absorption maximum of actinomycin to shorter wavelengths and an increase in the extinction coefficient of actinomycin, with no alteration of the wavelength of the isosbestic point.

CD spectra (Fig. 2) also show that displacement of DNA-bound actinomycin occurs on addition of ethidium bromide or daunomycin. Analyses of the changes occurring in the CD spectrum of actinomycin bound to the strong binding sites of DNA on addition of these drugs were performed at approximately 370 and 420 nm in order to minimize contributions to the CD from

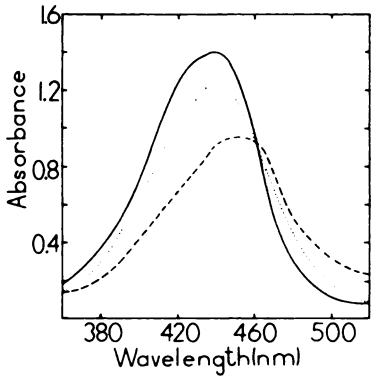

FIG. 1. *Visible absorption spectra of actinomycin, actinomycin-DNA complex, and actinomycin in the presence of DNA and ethidium bromide*

——, actinomycin (57 μM); – – –, actinomycin (57 μM) and DNA (0.83 mM); ····, actinomycin (57 μM), DNA (0.83 mM), and ethidium bromide (0.39 mM). The solution in the reference beam was buffer when the spectra of free actinomycin and DNA-bound actinomycin were recorded. When ethidium bromide was present, a solution of ethidium bromide (0.39 mM) and DNA (0.83 mM) was placed in the reference beam.

ethidium-DNA and daunomycin-DNA complexes. (At these wavelengths the molecular ellipticities of free ethidium and free daunomycin were similar to those of DNA-bound ethidium and DNA-bound daunomycin, respectively.) Addition of 3,8-diamino-5-ethyl-6-methylphenanthridinium bromide, an analogue of ethidium bromide in which the phenyl group is replaced by a methyl group, to DNA-bound actinomycin caused changes similar to those observed on addition of ethidium bromide at comparable concentrations (data not shown). This suggests that under these conditions binding of the phenanthridinium chromophore to DNA produces a local distortion that results in displacement of actinomycin from DNA. Actinomine, an analogue of actinomycin in which each peptide lactone is replaced by an *N,N*-diethylaminoethyl group, had almost no effect on the CD spectrum of DNA-bound actinomycin when added at a molar concentration 2.8 times that of actinomycin. Free actinomine has no CD spectrum in the 350–500-nm region. To examine the effects of actinomine on the molecular ellipticity of DNA-bound actinomycin, difference CD spectra were recorded at wavelengths where the molecular ellipticity of bound actinomine is small relative to that of actinomycin (350–365-nm region).

We attempted to obtain further information about the molecular nature of the actinomycin-DNA interaction from stopped-flow kinetic studies in which actinomycin was mixed with DNA bound to the intercalative drugs ethidium bromide and daunomycin. The order of affinity of these drugs for DNA, as measured by spectrophotometric titrations, is daunomycin > ethidium ≅ actinomycin.[1] We previously reported that the association of actinomycin with synthetic polydeoxyribonucleotides having defined and homogeneous actinomycin binding sites is characterized by a multiplicity of rate processes and concluded that structural heterogeneity in DNAs was not responsible for the extreme complexity of the kinetics of association (14). The two rapid DNA concentration-dependent reaction steps, labeled k_1 and k_2 in Table 1, are believed to involve binding of the phenoxazone chromophore with DNA in such a manner that the reactants have little freedom of motion in the transition states of these steps. The slower rate processes appear to involve structural perturbations in the polynucleotide backbone induced by interaction with the peptide rings of actinomycin. Under the conditions presented in Table 1, the rates observed in the presence of these drugs, as measured by changes in A_{425}, are believed still to represent rate processes for actinomycin binding to DNA. This conclusion is based on our observations that the rates of association of ethidium bromide with DNA are considerably faster than those of actinomycin; visible absorption spectra indicate that ethidium remains bound to DNA when actinomycin is added to give ethidium to actinomycin molar ratios approximating those used in Table 1; and there is only a small difference in A_{425} between solutions of free daunomycin and of daunomycin in the presence of DNA [Calendi *et al.* (15) observed the maximum difference in absorbance at

[1] L. Blau and R. Bittman, unpublished observations.

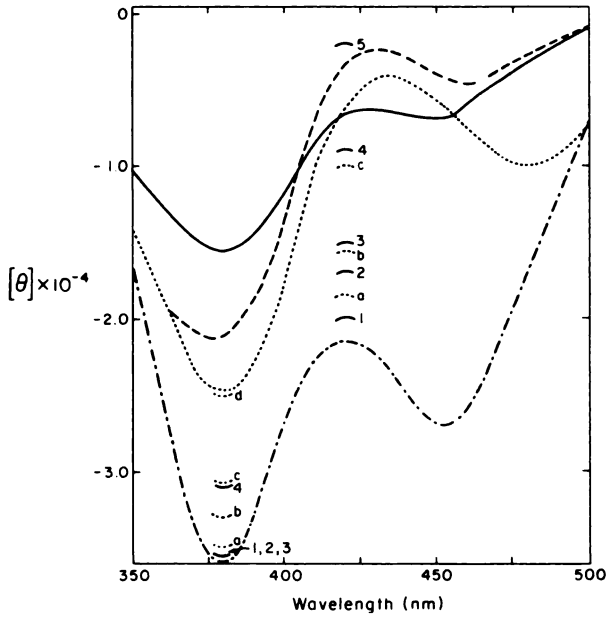

FIG. 2. *CD spectra of free actinomycin and actinomycin in the presence of DNA, ethidium bromide, and daunomycin*

——, free actinomycin (35.8 μM); -·-·, actinomycin (35.8 μM) in the presence of DNA (0.53 mM); – – –, difference CD spectrum of [actinomycin (35.8 μM), DNA (0.53 mM), and ethidium bromide (0.268 mM)] minus [DNA (0.53 mM) and ethidium bromide (0.268 mM)]; -----, difference CD spectrum of [actinomycin (35.8 μM), DNA (0.53 mM), and daunomycin (25.9 μM)] minus [DNA (0.53 mM) and daunomycin (25.9 μM)]. Difference CD measurements were also made at various concentrations of ethidium bromide and daunomycin. The concentrations of actinomycin and DNA were maintained at 35.8 μM and 0.53 mM, respectively, and the molecular ellipticities of solutions were determined at the following molar ratios of DNA to ethidium bromide: *1*, 40; *2*, 16; *3*, 9; *4*, 6; *5*, 2. Similarly, the following molar ratios of DNA to daunomycin were used: *a*, 12.5; *b*, 5.3; *c*, 3.3; *d*, 2.4. The measured ellipticities of the DNA–ethidium bromide and the DNA-daunomycin solutions, which were small at the wavelengths at which analyses were performed, were subtracted from the measured ellipticities of the solutions containing actinomycin, DNA, and ethidium bromide or daunomycin. No evidence of interaction between actinomycin and ethidium bromide or between actinomycin and daunomycin was found. These experiments were carried out in 0.01 M potassium phosphate buffer containing 0.25 M potassium nitrate, pH 6.8.

TABLE 1

Effect of ethidium bromide and daunomycin on kinetics of association of actinomycin D with DNA

Actinomycin was mixed with drug and DNA in the stopped-flow apparatus. The final concentration of actinomycin was 7.5 μM. The ratio of DNA phosphorus to actinomycin was 31.

	DNA P/drug	k_1	ΔT_1	k_2	ΔT_2	k_3	ΔT_3	$10^2 \times k_4$	ΔT_4	$10^3 \times k_5$	ΔT_5
		sec^{-1}	%	sec^{-1}	%	sec^{-1}	%	sec^{-1}	%	sec^{-1}	%
None		111 ± 10	10	7.0 ± 0.3	28	0.45 ± 0.05	30	10.9 ± 1.0	20	7.3 ± 0.5	12
Ethidium	9.1	200	7	5.0 ± 1.0	18	0.34 ± 0.03	38	7.7 ± 0.6	24	5.7 ± 0.7	13
Ethidium	6.9	160 ± 50	3	3.6 ± 0.3	21	0.29 ± 0.05	34	7.4 ± 0.3	25	6.3 ± 0.5	17
Daunomycin	8.0			1.9	14	0.14 ± 0	28	3.3 ± 0.5	31	4.2 ± 0.6	27

475 nm]. Table 1 shows that in the presence of a 4-fold molar excess of daunomycin relative to actinomycin, the first step of actinomycin association with DNA was not detected and the rate constants of the other steps were decreased. Ethidium bromide, at 3.4 and 4.5 times the molar concentration of actinomycin, caused a de-

crease in k_2; at a higher molar excess of ethidium bromide, e.g., a DNA to ethidium ratio of 2, and in buffer containing 0.24 M K^+, the first reaction step was not detected. Thus, at comparable concentrations of competing drug, daunomycin modified the rate constants of actinomycin binding to DNA more than ethidium bromide did. The bulky sugar residues of daunomycin may offer greater steric hindrance to binding of actinomycin than the smaller phenyl and ethyl groups of ethidium bromide.

Equilibrium binding studies were also performed in the presence of mithramycin. Table 2 shows that the binding parameters are modified in the presence of mithramycin. For complex formation, this antibiotic requires the 2-amino group of guanine and a divalent cation (16–18). We performed spectral titrations of mithramycin with DNA. Analysis of the strong binding process of the binding isotherm according to the Scatchard equation (19) gave an apparent equilibrium constant for association approximately 30-fold lower than that for actinomycin binding to DNA in the same buffer (0.01 M potassium phosphate–0.05 M $MgCl_2$, pH 7.0). At the higher DNA to mithramycin ratio used (7.8) binding is competitive, since the strong binding association constant of actinomycin is decreased but the number of binding sites is unaffected. For the binding of mithramycin molecules at actinomycin binding sites, the Scatchard equation for competitive binding, $r_1/c_1 = K_1(1 + K_2c_2) \times (n - r_1) = K^* (n - r_1)$, applies. This equation, which is a modification of an equation by Klotz *et al.* (20), predicts that a Scatchard plot for competitive binding should have the same intercept on the r axis as the simple binding case, but the slope should be smaller in the competitive plot. Competitive binding is consistent with the suggestion that the mithramycin chromophore binds to guanine in helical DNA (18), since actinomycin binding has a general, although not an absolute, requirement for the presence of a purine 2-amino group in double-helical DNA (4). It should be noted, however, that the deoxyguanosine involved in binding mithramycin need not be identical with that in an actinomycin binding site; even in the absence of competing dye molecules, simultaneous binding of actinomycin molecules at sites separated by fewer than approximately six base pairs is precluded by steric interference. In the presence of high concentrations of mithramycin, both the equilibrium constant and number of binding sites are decreased (Table 2). When a large number of mithramycin molecules occupy the actinomycin binding regions, complex formation between actinomycin and DNA is altered, presumably because of steric interference between the peptide lactones of actinomycin and the sugar side chains of mithramycin.

TABLE 2

Effect of mithramycin on association constant and number of binding sites for actinomycin-DNA interaction

Binding parameters were determined in 0.01 M potassium phosphate buffer containing 0.05 M $MgCl_2$, pH 7.0, in the absence and presence of 6.5 and 18.6 μM mithramycin.

DNA/mithramycin	K_{app}	Nucleotides/site
	μM^{-1}	
	2.76 ± 0.15	16.7
7.8	1.83 ± 0.15	15.6
2.7	1.60 ± 0.26	33.3

Further evidence that mithramycin was effective in causing displacement of actinomycin from DNA was obtained by monitoring the ellipticity at 380 nm. At this wavelength the ellipticity of free mithramycin and the mithramycin-DNA complex is zero. At a 3-fold molar excess of mithramycin to actinomycin, 60% of the DNA-bound actinomycin was displaced, as measured by the decrease in $[\theta]_{380}$ relative to the difference in $[\theta]_{380}$ between free and bound antinomycin. All the DNA-bound actinomycin was displaced by mithramycin when the molar ratio of mithramycin to actinomycin was 5. Thus mithramycin, which is believed to bind to DNA by a mechanism other than intercalation, since it does not produce the removal and reversal of the supercoils of closed circular DNA characteristic of intercalative agents (5), was effective in causing displacement of bound actinomycin. Since the affinity of

mithramycin for DNA is lower than that of actinomycin, the bulky substituents of mithramycin are implicated in the displacement process.

DISCUSSION

CD studies of the displacement of DNA-bound actinomycin support the conclusion drawn from kinetic studies that the peptide lactones play an important role in the actinomycin-DNA interaction. Ethidium bromide and daunomycin are believed to interact with DNA largely via formation of intercalative complexes (21). Displacement of DNA-bound actinomycin by addition of ethidium bromide or daunomycin under conditions of strong site binding is expected, since daunomycin binds to DNA with slightly higher affinity than actinomycin, and ethidium bromide binds with approximately equal affinity;[1] moreover, the stoichiometry of the strong binding process indicates that more ethidium bromide or daunomycin molecules can be bound than the number of actinomycin molecules that are bound to DNA. Thus it appears unjustified to reach conclusions about binding mechanisms (12) based on results showing that daunomycin and ethidium bromide did not cause displacement of DNA-bound actinomycin when added at DNA to drug molar ratios similar to those used in the present study (9, 12, 13). In contrast, actinomine, at a molar concentration approximately 3 times greater than that of actinomycin, did not cause dissociation of the actinomycin-DNA complex. The bulky substituents of the competing drugs, especially mithramycin and daunomycin, may give these agents the ability to interfere with the binding of the cyclic peptides of actinomycin. The guanine specificity of mithramycin and the intercalative binding (with the consequential helix unwinding) of ethidium bromide and daunomycin may cause modification of actinomycin-DNA interaction through distortion of the helix and perturbation of the conformation of the polynucleotide backbone.

[1] L. Blau and R. Bittman, unpublished observations.

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