Spectroscopic analysis of the equilibrium and kinetic DNA binding properties of several actinomycin analogs

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ABSTRACT

Experiments are described that measure DNA dissociation kinetics and thermal denaturation temperatures for a series of actinomycin analogs containing, in the 3' amino acid position, pipecolic acid, proline or azetidine-2-carboxylic acid. Also included are studies on actinomycin C₃. Analysis of the temperature dependence of the slowest rate constant for DNA dissociation shows that both the enthalpy and entropy of activation increase as the ring size of the 3' amino acid decreases from six to five to four. All compounds increase the DNA melting temperature to the same extent except for the analog containing pipecolic acid, which shows a smaller effect. These results are discussed in terms of a possible role for conformational changes in the actinomycin pentapeptide lactone rings in determining the slow DNA dissociation rates for this class of intercalators. It is suggested that cis-trans isomerization of proline may be important in this regard.

INTRODUCTION

Actinomycin D is one of the most powerful inhibitors of RNA synthesis available and is used both as a chemotherapeutic agent in the treatment of certain malignancies as well as a tool for the study of various cellular processes, such as RNA transcription. The biological activity of actinomycin D is assumed to be related to its interaction with DNA and this interaction has been studied intensely in the past fifteen years (1-5). Its complex with DNA also serves as a model for protein-nucleic acid interactions (5).

The importance of the dissociation kinetics of actinomycins from DNA in conferring biological activity was first established by Muller and Crothers (6), who also showed that the kinetics of DNA binding by these compounds included a slow, multistep process. In particular, they observed that a complete description of the dissociation curve, at high ratios of DNA to drug, required three exponential terms. Muller and Crothers (6) originally ascribed the complicated dissociation and association curves to sequential steps involving conformational changes in the peptide rings necessary for the strong anchoring of the molecule to the DNA duplex. Very recently, however, Krugh

and coworkers have obtained evidence that the three components in the dissociation curve from calf thymus DNA were due to three different binding sites rather than arising from a multistep binding process for one site (7). They also found that dissociation of ACTD from the synthetic duplex poly(dG-dC).poly(dG-dC) was a single exponential decay, further supporting their contention that the multi-exponential decay obtained with calf thymus DNA was due to site heterogeneity. The single dissociation rate constant, however, was observed to depend on the ratio of polynucleotide to drug concentrations.

The time scale for the on and off rates for actinomycin binding to DNA (8,8) is several orders of magnitude slower than that for other simpler intercalators, such as proflavine (9), ethidium (10) and daunomycin (11). Muller and Crothers (6) originally proposed that conformational changes in the pentapeptide lactone rings attached to the chromophore were responsible for the slow interaction kinetics. Alternative explanations have been presented; for example, the possibility of polynucleotide conformational changes leading to the slow kinetics has been suggested by Sobell (5). In a kinetic analysis of the association reaction of several actinomycins with DNA, however, Bittman and Blau found no significant difference in the rate constants for the slow binding steps between linear and supercoiled DNA (8).

The experiments described below have been designed to explore the possible role that conformational properties of the peptide rings may play in determining the slow time scale for actinomycin-DNA interactions. In these experiments, we analyze the DNA binding of several actinomycin analogs which differ in one amino acid in the pentapeptide lactone ring. Actinomycin pip2 (12) and azetomycin II (13) differ from actinomycin D in that the size of the 3' amino acid ring changes by one methylene group, as shown in Figure 1. Actinomycin C₃ differs from actinomycin D in that D-valine is replaced by D-alloisoleucine. In all the above compounds, both pentapeptide rings are identical. The nomenclature of the actinomycins is discussed in the reviews by Meienhoffer and Atherton (3) and Hollstein (4).

MATERIALS AND METHODS

Calf thymus DNA was purchased from Sigma (Type I), as was actinomycin D (C₁, IV, ACTD). Azetomycin II (AZETII), actinomycin C₃ (ACTC), and actinomycin pip 2 (PIP2) were generous gifts of Dr. M. A. Apple and Dr. J. V. Formica. Sodium lauryl (dodecyl) sulfate (SLS) was obtained from Calbiochem. Poly (dG-dC).poly (dG-dC) was purchased from PL Biochemicals. Buffer salts were reagent grade. Concentrations were determined spectrophotometrically using the molar extinction coefficients $\epsilon_{260} = 6800$ per nucleotide for DNA and $\epsilon_{440} = 24,450$ for ACTD (8). The same extinction coefficient was used for all actinomycin analogs.

Fig. 1. Structure of actinomycin D and related analogs.

DRUG-DNA DISSOCIATION KINETICS: The kinetics of dissociation of drug-DNA complexes were determined as described by Muller and Crothers (6). Experiments were done in BPES buffer, consisting of 0.08M Na_2HPO_4 , 0.02M NaH_2PO_4 , 0.18M NaCl and 0.01M Na_2EDTA . Complex was formed by adding drug to DNA in a 10 cm cell, mixing well and allowing to stand at constant temperature until the association reaction was complete (30-60 min), as determined by a constant value of the absorbance of the complex at 440 nm. SLS was then added to a final concentration of 3%, the solution was mixed well and the absorbance at 440 nm was monitored as a function of time. The temperature was held constant by circulating water through copper coils surrounding the cell.

Initial experiments showed that identical results were obtained using DNA that had been phenol extracted and sonicated and using DNA that was simply dissolved in BPES buffer. Thus most experiments were performed using DNA that was prepared by simply dissolving in buffer. Furthermore, the dissociation rate constants were independent of the value of P/D (ratio of DNA concentration, in nucleotides, to total drug concentration) when P/D was varied from 20 to 60. Kinetic experiments were run at P/D = 20 in all cases except PIP2, which was run at P/D = 60. This higher ratio was needed to bind up most of the analog, yielding a large signal upon dissociation, similar to the other compounds at P/D = 20. In all cases, the concentration of drug was 3 μM following addition of the SLS to drive the dissociation reaction forward.

THERMAL DENATURATION MEASUREMENTS: Drug-DNA complexes were formed at

a P/D of 20 and the absorbance at 260 nm was followed as a function of increasing temperature on a Beckman Acta CIII spectrophotometer equipped with a multi-sampling accessory. Solutions for thermal denaturation studies were made up in 0.01 x BPES buffer. Temperature control was maintained by circulating water from a heating bath through both the cell holder and through water-jacketed 1 cm cells. The temperature of the bath was raised continuously using a programmed temperature controller and the temperature of the solutions was continuously monitored by placing a thermistor probe (YSI 729) in the reference cell. Solutions were heated at a rate of 0.5°C/min.

ANALYSIS OF DATA: Traces of absorbance as a function of time were digitized into the Prophet computer system for multivariate nonlinear regression analysis. The Prophet system is a national computer resource developed by the Chemical/Biological Information Handling Program of the Division of Research Resources, National Institutes of Health (14). The curves were fit to a sum of two exponentials, as shown in eq. (1), using a Marquardt-Levenberg algorithm (15).

$$A(\infty) - A(t) = \Delta A_1 e^{-k_1 t} + \Delta A_2 e^{-k_2 t}. \quad (1)$$

In this equation, $A(t)$ is the absorbance at 440 nm at time t , $A(\infty)$ is the absorbance at infinite time and ΔA_1 and ΔA_2 are the amplitudes contributed by the different dissociation processes to the total dissociation curve. Results are reported in terms of rate constants, k_i and characteristic dissociation times, τ_i , where $\tau_i = 1/k_i$.

Arrhenius plots of the rate constants were constructed using the equation:

$$\ln \frac{k}{T} = -\frac{\Delta H^\ddagger}{R} \left(\frac{1}{T} \right) + \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R}, \quad (2)$$

from which the enthalpy of activation, $\Delta H^\ddagger$, and the entropy of activation, $\Delta S^\ddagger$, were determined by least squares linear regression. In eq. (2), k_B and h are Boltzmann's constant and Planck's constant, respectively.

Thermal denaturation curves were constructed by digitizing traces of absorbance and temperature as a function of time and then establishing plots of absorbance versus temperature by polynomial interpolation. Correction for volume expansion was included (16) and results plotted in terms of the fraction denatured, F , defined by $F = (A(T) - A(\text{initial})) / (A(\text{final}) - A(\text{initial}))$. Here, $A(T)$ is the absorbance at temperature T , $A(\text{initial})$ and $A(\text{final})$ are the absorbances at the beginning and end, respectively, of the transition; all absorbances refer to 260 nm for thermal denaturation. The thermal denaturation temperature, T_m was determined as the temperature corresponding to $F=0.5$.

RESULTS

Characteristic times for the two slowest dissociation steps from calf thymus DNA at 25°C for ACTD, ACTC, PIP2 and AZETH are presented in Table 1. Since dissociation experiments were done by hand mixing, only two rate processes were observed. Preliminary stopped-flow experiments indicated the presence of a third process, as originally reported by Muller and Crothers (8). Typical traces of absorbance versus time, along with computer determined fit, are shown in Figure 2. The time constants we have obtained for ACTD and ACTC differ from those reported by Muller and Crothers (8); the most likely explanation lies in the method of determining the fitted rate constants.

Examination of the results presented in Table 1 shows that significant changes in the dissociation kinetics for the slow step are produced by small changes in the ring size of the 3' amino acid of the pentapeptide lactones. As the ring size of this amino acid decreases from 6 to 5 to 4 members, the dissociation times (rate constants) increase (decrease) steadily; a factor of approximately two is observed for each substitution. The magnitude of the changes resulting from these structural perturbations does not become fully apparent, however, until the temperature dependence of the dissociation rate constants is analyzed for the enthalpy and entropy of activation, discussed below. The faster process appears to entail different interactions. The following discussion will concentrate on the slow dissociation step.

Dissociation kinetics at various temperatures were investigated for ACTD, AZETH, ACTC and PIP2. Results from these studies for the slowest dissociation step are shown in Figure 3 and Table 2. There is a definite trend in the activation parameters in going from PIP2 to ACTD to AZETH. The enthalpy of activation steadily increases as does the entropy of activation. This increase in both terms has an opposing effect on the rate constant. For example, the difference in activation enthalpies alone for ACTD and

Table 1. DNA Dissociation Times at 25°C in BPES Buffer.

Analog	$\tau_{\text{slow}}(\text{s})^a$	$\tau_{\text{fast}}(\text{s})^a$
PIP2	278	57
ACTD	735	40
AZETH	1360	77
ACTC	592	45

a. Results on triplicates reproducible to within 15% or better.

Fig. 2. Absorbance changes at 440 nm due to dissociation of actinomycin analogs from calf thymus DNA; a: PIP2, b: ACTD, c: AZETII. Smooth line represents fit to experimental points.

PIP2 would result in a much larger difference in rate constants than observed. The corresponding large changes in activation entropies cancel this effect to a great extent. In all cases, however, the differences in activation enthalpies dominate the resulting difference in rate constants.

An important difference between the substitution of alloisoleucine for valine at the 2' position in ACTC and the substitution of different ring sizes in the 3' amino acid

Fig. 3. Temperature dependence of rate constants for the slow dissociation step from calf thymus DNA; $\square$: PIP2, $\circ$: ACTD, ∇ : AZETII, Δ : ACTC.

Table 2. Activation Parameters for Slow DNA Dissociation Step.

Analog	$\Delta H^\ddagger$ (kcal/mole) ^a	$\Delta S^\ddagger$ (e.u./mole) ^a	$\Delta G^\ddagger$,25°C(kcal/mole)
PIP2	11.5±1.1 ^b	-30.8±3.7 ^b	20.7
ACTD	20.1±1.3	-4.4±4.3	21.4
AZETII	23.0±0.4	+4.4±1.4	21.7
ACTC	19.1±0.6	-7.1±2.7	21.2

a. Calculated by linear least squares regression from eq. (2).

b. Standard error from regression.

in AZETII and PIP2 is revealed in Table 2. In the former, the observed change in dissociation rate constant, compared to ACTD, arises from small changes in the activation enthalpies and entropies, while in the latter, the changes in rate constants are due to large but opposing differences in the enthalpy and entropy of activation. Thus, when proline is conserved, so are the the activation parameters, but when changes are made in the proline structure, large changes are incurred in the activation parameters.

The effect of the various analogs on the thermal denaturation of calf thymus DNA is shown in Figure 4 and Table 3. At equal values of P/D, the ACTD, ACTC and AZETII

Fig. 4. Thermal denaturation curves at 280 nm for: #: DNA alone, x: PIP2, +: ACTD, O: AZETII, *: ACTC.

Table 3. Thermal Denaturation Temperatures, T_m , of Drug-DNA Solutions in 0.01 x BPES Buffer.

Analog	T_m (°C)	ΔT_m (°C)
DNA alone	57.6	0.0
PIP2	60.9	3.3
ACTD	68.2	10.6
AZETH	68.2	10.6
ACTC	68.0	10.4

compounds increase the T_m to a similar extent, while PIP2 shows much less of an effect. This suggests that the former three compounds bind to native DNA with similar affinities, while the latter one binds less strongly. Since a change in the T_m really reflects a change in the relative affinities of the drug for single- and double- stranded DNA, the thermal denaturation experiments cannot be unambiguously interpreted in terms of relative binding strengths of the drug-double-stranded DNA system. Results presented in Figure 4, however, do show that ACTC, which has a binding affinity for native DNA very close to that of ACTD (6), increases the T_m to the same extent as the ACTD compound. Furthermore, we observed that values of P/D of 60, in the case of PIP2, were required to bind essentially all of the drug, while P/D values less than 20 sufficed for the other three compounds. This is consistent with the small change in T_m produced by PIP2 arising from reduced binding affinity. It is also possible that the difference in the PIP2 analog arises from fewer binding sites available on DNA. Preliminary results using spectroscopic titration support the former interpretation; lack of sufficient amount of analogs prevented a complete determination of binding curves.

The effect of poly(dG-dC).poly(dG-dC) on the visible spectra of PIP2, ACTD and AZETH was determined at a P/D value of 80. A qualitative difference was observed for PIP2 compared to ACTD and AZETH. The spectra of ACTD and AZETH in the presence of the synthetic polynucleotide were shifted 12-15 nm to longer wavelength, relative to free drug, while that of PIP2 was shifted only 3-4 nm. The hypochromism upon addition of poly(dG-dC).poly(dG-dC) was approximately the same, however, in all cases.

DISCUSSION

The experiments described above show that the dissociation kinetics, in particu-

lar the activation enthalpies and entropies, of drug-DNA complexes formed by the three analogs, ACTD, AZETII and PIP2, are quite sensitive to the size of the ring of the 3' amino acid. In the case of ACTD and AZETII, the equilibrium properties of their interaction with DNA appear to be very similar, while the PIP2 analog seems to bind with lower affinity or fewer binding sites to DNA. The sensitivity of the dissociation kinetics to the details of the 3' amino acid suggests a possible role for this residue in determining the slowness of dissociation from DNA for the actinomycins.

The results for ACTC demonstrate that changing the structure of a residue adjacent to proline has relatively little influence on the activation enthalpy and entropy. The small decrease in $\Delta S^\ddagger$ for ACTC relative to ACTD may reflect additional solvent interactions. It is interesting to note that the both ACTC and PIP2 differ from ACTD by one extra methylene group. Obviously, the exact location of this extra group plays a crucial role in determining its effect on the resultant DNA binding characteristics. In addition, we have noted that PIP2 has a much lower aqueous solubility than does ACTC, suggesting the importance of the nature of the methylene group added in affecting the physical characteristics of the drug alone.

The enthalpy and entropy of activation of AZETII differ from those of ACTD by about 4 kcal/mole and 10 e.u./mole, respectively. It is possible that this increase in activation enthalpy arises from conformational constraint of the pentapeptide by the smaller ring of the 3' amino acid. NMR evidence for a reduction in conformational flexibility of azetidine-2-carboxylic acid relative to proline has been obtained by Meraldi et al. (23). The increase in the entropy term, which is negative for ACTD and positive for AZETII, may reflect the difference of one CH_2 group on the hydrophobic interactions occurring in the bound state and the activated complex. The negative $\Delta S^\ddagger$ for ACTD certainly suggests increased exposure to solvent in the activated complex, relative to the bound state. While this type of interaction is probably important in AZETII, it is not strong enough to overcome the increase in entropy due to increased relative motions of the ligand and DNA as the complex dissociates. The importance of hydrophobic interactions in the equilibrium DNA binding properties of ACTD has been well established. Formation of the ACTD-DNA complex is accompanied by an increase in entropy of approximately 39 e.u./mole (24).

A similar comparison of PIP2 and ACTD shows that addition of one CH_2 group decreases that enthalpy of activation by about 9 kcal/mole and decreases the entropy of activation by about 26 e.u./mole. These changes are of considerably greater magnitude than those discussed above for ACTD and AZETII. While they may reflect the influence of the same type of interactions discussed above, their magnitude suggests the possibility of an alternative explanation. One such explanation lies in the observation that the activation enthalpies and entropies for the slowest dissociation step of

PIP2 from DNA were within experimental error of those for the next to slowest step of ACTD from DNA (data not shown). Thus, it is possible that the binding site characterized by tight binding and slow dissociation is not available to PIP2. That is, the change in the 3' amino acid structure leads to a change in the equilibrium properties as well as the kinetic properties of the complex formed with DNA. The data presented above suggesting that PIP2 binds less strongly or with fewer binding sites to DNA than the other analogs support this argument. Also, the qualitative difference between the absorption spectrum of PIP2 bound to poly (dG-dC)·poly(dG-dC) and the absorption spectrum of ACTD and AZETII bound to this synthetic duplex is consistent with a difference in binding site geometry and interactions.

Finally, we can speculate on the nature of the interactions leading to the observed differences in $\Delta H^\ddagger$ for the dissociation of ACTD from DNA compared to the other analogs. Little change was produced in the activation parameters by substituting a methylene group on the valine residue but large changes occurred when an increase or a decrease of one methylene group on the proline residue. These observations lead us to focus on the 3' amino acid residue. One characteristic of such residues is their cis-trans isomerization, which is often observable because the equilibrium energy differences in the two isomers is relatively small. It is possible that dissociation of the tight binding analogs involves isomerization about one of the 3' amino acid peptide bonds. Such a step has been proposed as rate-limiting for the denaturation of proline-containing proteins (25) and in the formation of the triple helix in collagen (26). It is possible that it also plays a role in the dissociation of actinomycins from DNA. While the sensitivity of the rate constants to the structure of the 3' amino acid points to the importance of this residue, we note that the pentapeptide lactone ring contains two other N-substituted amino acids which also may exhibit similar cis-trans isomerization properties. Furthermore, in virtually all of the biosynthetic actinomycin analogs, the 3', 4' and 5' positions always contain N-substituted residues; in particular, the sarcosine residues are conserved (2,3).

NMR studies on the structure of ACTD in various solvents (21) were initially interpreted as being indicative of all peptide linkages in the trans conformation. Further studies (21,22) led to the conclusion that the two peptide bonds involving proline were in the cis conformation. X-ray diffraction analysis of the 1:2 complex of ACTD and deoxyguanosine (27,28) also showed the proline peptide linkages to be in the cis conformation. Thus, assuming the revised interpretation of the NMR data on ACTD in solution to be correct, there appears to be no direct evidence for cis-trans isomerization upon binding. We would like to point out, however, that the ACTD complex with deoxyguanosine lacks a large part of the DNA helix expected to interact with the pentapeptide lactone rings and thus may not be a valid model for the conformational state of the bound

peptide moiety.

In support of the possible significance of cis-trans isomerization in the DNA dissociation process, we note that this isomerization is characterized by an activation free energy of around 20 kcal/mole for various N-substituted amino acids (25,29,30). This is very close to our result for the $\Delta G^\ddagger$ for dissociation of various actinomycins from DNA shown in Table 2. Also, we have carried out some preliminary high resolution NMR studies which show large changes in the chemical shifts of both chromophore and peptide protons in going from PIP2 to ACTD to AZETII (P.A. Mirau and R.H. Shafer, unpublished results). This also indicates an important role for the 3' amino acid in determining the solution conformation of the peptide lactone rings.

It is apparent, however, that the overall kinetics are strongly influenced by the entropy of activation term. A difference of 10 e.u./mole will result in more than two orders of magnitude difference in rate constants. A feature peculiar to actinomycins is the hydrophobic character of the peptide portion. This aspect plays an important role in their aqueous solubility and their equilibrium DNA binding properties. It is natural that it also affects their kinetic DNA binding properties.

The results presented above demonstrate the sensitivity of the DNA dissociation kinetics to the size of the ring in the 3' amino acid for a series of actinomycin analogs. We suggest a possible role for cis-trans isomerization for that amino acid in determining the slow time scale for the interaction of these compounds with DNA. Clearly additional work is needed to further elucidate the details of actinomycin-DNA binding and kinetics and to confirm or refute this hypothesis. We are continuing both kinetic and high resolution NMR studies on model compounds and these as well as other analogs containing substitutions in the 3' amino acid on only one of the pentapeptide lactone rings towards this goal.

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