High-Resolution Proton Nuclear Magnetic Resonance Analysis of Conformational Properties of Biosynthetic Actinomycin Analogues†

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ABSTRACT: High-resolution proton nuclear magnetic resonance (^{1}H NMR) has been used to study the conformation and dynamics of biosynthetic analogues of actinomycin D that have been substituted at one or both of the 3′-amino acids with azetidine-2-carboxylic acid, pipecolic acid, or 4-ketoproline for one or both of the prolines. The results of measurements made in organic solvents indicate that the amino acid residues on the α and β pentapeptide lactone rings experience different magnetic environments in all the analogues studied. Analysis of the $J_{\alpha NH}$ coupling constants and temperature coefficients for the valine and threonine amide protons indicates that substitution at the 3′ position has little effect on the conformational and hydrogen-bonding properties of the amino acids at the 1′ or 2′ position. Examination of the low-field position of the H^{α} proton of the 3′-amino acid reveals that the biosynthetic substitutions were made at a unique site on either the α or the β pentapeptide ring and that only one conformation of proline, azetidine-2-carboxylic acid, or pipecolic acid is observed. In D_2O, the dynamics of the prolines appear to differ; one remains in a preferred conformation while the other fluctuates at a rate that is intermediate on the NMR time scale. A possible relationship between the conformational and dynamic properties and the DNA binding and kinetic properties is discussed.

Nuclear magnetic resonance (NMR) has been frequently used to to study the conformation and dynamics of biological molecules in an attempt to relate these properties to their biological activity. Actinomycin D, Figure 1, is an anticancer drug whose biological activity is believed related to its ability to bind DNA (Meienhofer & Atherton, 1977). X-ray crystallography (Jain & Sobell, 1972) and NMR (Krugh & Nuss, 1979) have been used to construct a detailed model for the antinomycin–DNA complex. The complex is stabilized by the intercalation of the phenoxazone ring between the base pairs as well as numerous van der Waals' contacts and hydrogen bonds between the pentapeptide lactone rings and the double helix.

We have been studying the equilibrium and kinetic DNA binding properties of actinomycin analogues that contain altered amino acids in the 2′ and 3′ position in an attempt to determine what role the peptides play in the DNA binding properties (Shafer et al., 1980; Mirau & Shafer, 1982). The analogues (Figure 1) contain two prolines (actinomycin D, ACTD), two azetidines (AZETII), two pipecolic acids (PIP2), or one proline and one azetidine (AZETI), one pipecolic acid (PIP1β), or one 4-ketoproline (actinomycin V) at the 3′ position. In this study we compare the conformational, dynamic, and hydrogen-bonding properties of these analogues with the parent compound. Examination of the model built from the 2:1 complex of deoxyguanosine with actinomycin shows intimate contacts between the peptides and the helix (Sobell & Jain, 1972). Thus, we might expect the DNA binding properties to be related to actinomycin peptide conformation. Also, since the GC sequence binding preference is thought to be related to the ability of actinomycin to hydrogen bond to the intercalation site (Sobell & Jain, 1972), we have studied the hydrogen-bonding ability of the analogues. Finally, we hope to gain some insight into the kinetic properties by monitoring the dynamics of the drug in solution.

Materials and Methods

Actinomycin D and deuterated solvents were obtained from Sigma. Actinomycin analogues, isolated and characterized as previously described (Formica et al., 1968; Formica & Apple, 1976), were generous gifts of Dr. J. V. Formica.

^{1}H NMR experiments were performed at 360 MHz on a modified Bruker HXS360 spectrometer at the Stanford Magnetic Resonance Laboratory. Typically, the concentration of actinomycin was 2 mM. Spectra were gathered in 16K data points by using a sweep width of ±2.5 kHz, a 45° nonselective radio-frequency pulse, and a 2-s recycle time. Spectra in organic solvents were referenced to the residual solvent peak but are reported with respect to tetramethylsilane (Me_4Si). Spectra in D_2O were referenced externally to 4,4-dimethyl-4-silapentane-1-sulfonate at 0 ppm. Peak assignments were made by comparison to previously published actinomycin D spectra (Arison & Hoogsteen, 1970; Lackner, 1975; Victor et al., 1969; Conti & De Santis, 1970) and its analogues (Mauger, 1975), spin-decoupling experiments, and the known chemical shifts of the amino acids (James, 1975).

Results

Chemical Shifts. Figures 2 and 3 show the low-field region of the 360-MHz spectra in benzene-d_6 of actinomycin D and its analogues, which contain azetidine-2-carboxylic acid, pipecolic acid, or 4-ketoproline in place of one or both of the prolines. Spectra in benzene-d_6 were typically of higher resolution than in other solvents such as $CDCl_3$. While the spectra are complex, several conformational features are apparent. In all analogues, for example, separate resonances are observed for the amino acid residues on the α and the β peptides. This is also true for the high-field region of the spectra where separate signals are observed for the valine, methylvaline, and sarcosine methyl groups (data not shown). This difference in magnetic environment has been observed previously in actinomycin D (Conti & De Santis, 1970; Arison & Hoogsteen, 1970; Angermen et al., 1972) and actinomycin II (Mauger, 1975) and indicates that the asymmetry of the phenoxazone chromophore is propagated through the peptide portion.

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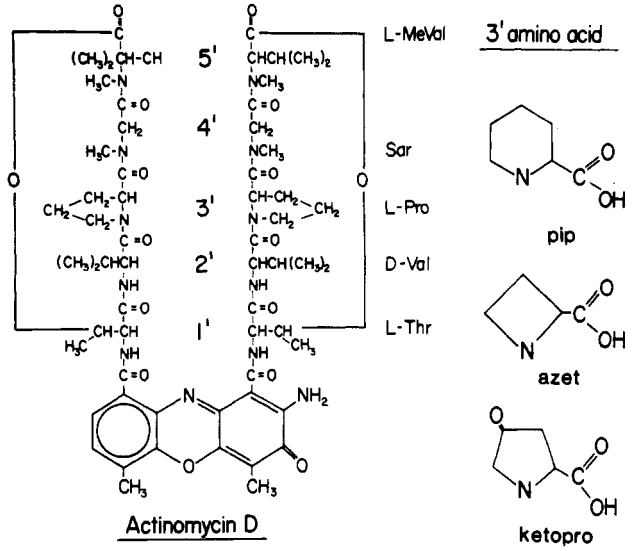

FIGURE 1: Structure of actinomycin D and analogues.

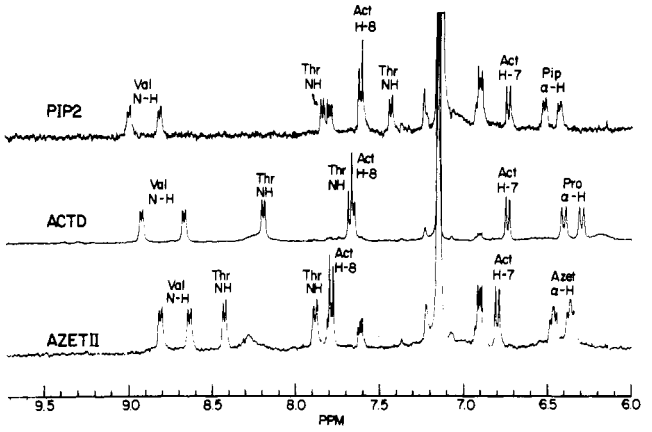

FIGURE 2: Low-field 360-MHz ^1H NMR spectra of actinomycin analogues PIP2, ACTD, and AZETII in benzene-d_6. Concentrations were 2 mM and 200 transients were recorded with a 2-s recycle time.

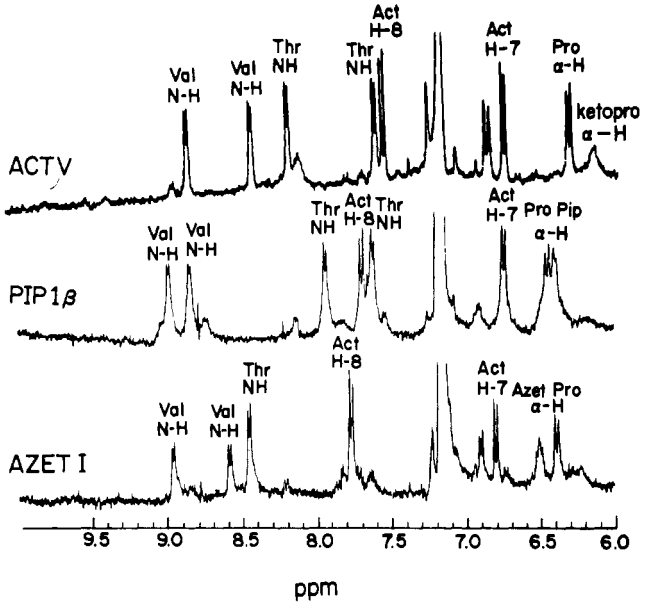

FIGURE 3: Low-field 360-MHz ^1H NMR spectra of actinomycin analogues ACTV, PIP1β, and AZETI in benzene-d_6. Same conditions as in Figure 2.

An anomalous feature that is common to all analogues is the low-field position of the H^α proton of the 3'-amino acid. Figures 2 and 3 show that these peaks appear between 6.0 and 6.5 ppm instead of their normal position of about 4.5–5.5 ppm (James, 1975). Lackner (1975) has proposed that their low-field position is due to the proximity of the H^α proton to the threonine carbonyl on the same pentapeptide lactone ring; evidently this feature is unperturbed by amino acid substitution at the 3' position. We have observed this same low-field position for all the analogues in benzene-d_6, $CDCl_3$, Me_2SO-d_6, and D_2O; thus it is not an artifact of organic solvents but rather a conformational feature resulting from cyclization of the pentapeptide lactone rings. Most sensitive to amino acid substitution are the chemical shifts of the valine and threonine amide protons and the H8 proton on the phenoxazone chromophore.

Table I: Coupling Constants and Temperature Coefficients of Valine in Actinomycins[a]

analogue	$J_{\alpha NH}$[b] valine 1	$\alpha \times 10^3$[c] valine 1	$J_{\alpha NH}$ valine 2	$\alpha \times 10^3$ valine 2
ACTD	5.3	−4.6	5.9	−3.6
AZETII	6.2	−6.5	6.3	−6.0
PIP2	5.4	−5.1	5.6	−4.2
AZETI	5.0	−5.5	4.3	−4.4
PIPIβ	6.1	−4.9	5.4	−4.1
ACTV	5.7	−4.2	5.7	−4.2

[a] All results are for benzene-d_6. [b] Valine 1 refers to the low-field valine; values are in hertz. [c] α is the temperature coefficient of the chemical shift in ppm/°C.

$J_{\alpha NH}$ Coupling Constants and Temperature Coefficients. Measurement of the $J_{\alpha NH}$ coupling constants and temperature coefficients of the amide proton on the valines and threonines may be used to probe the effect of amino acid substitution at the 3' position on the conformation, hydrogen bonding, and solvent exposure of the 1'- and 2'-amino acids. Table I lists the valine $J_{\alpha NH}$ coupling constants for the analogues. The data, as in the case of the threonine resonances, show no systematic deviation with substitution, indicating that substitution has little effect on the conformation about the C^α–N bond in the 1'- and 2'-amino acids.

Table I lists the temperature coefficients of the valine amide protons for all of the analogues. Venkatachalapathi & Balaram (1981) have shown that temperature coefficients are related both to the extent of hydrogen bonding and to the degree of solvent exposure in benzene. They observed that the temperature coefficients for compounds in benzene-d_6 are about 2.5 times larger than those observed in Me_2SO, where temperature coefficients less than 0.003 ppm/°C are thought to be indicative of intramolecular hydrogen bonding (Deslauriers & Smith, 1980). While not all of the threonine resonances were amenable to this analysis due to their overlap with other peaks, the temperature coefficients for the observable ones were close to zero and also showed no systematic or large variation with substitution. These data show that the hydrogen-bonding properties or solvent exposure of the amide protons of the 1'- and 2'-amino acids are not greatly perturbed by substitution at the 3' position.

Assignment and Conformation of the 3'-Amino Acid. The unusually low field position of the H^α proton of the 3'-amino acid allows us to study the conformation and dynamics of the amino acid, which appear to play a crucial role in the DNA binding kinetics (Shafer et al., 1980; Mirau & Shafer, 1982). The relationship between the conformation and the $J_{\alpha\beta}$ coupling has been examined in detail for proline and azetidine (Pogliani et al., 1975; Meraldi et al., 1978).

It is first worth noting, however, that separate resonances are observed for the 3'-amino acids on the α and the β pentapeptide lactone rings. Figure 3 shows that in actinomycin

Table II: Magnitude and Multiplicity of $J_{\alpha\beta}$ Coupling Constants of 3′-Amino Acids[a]

analogue	amino acid[b]	$J_{\alpha\beta}$[c]	amino acid[b]	$J_{\alpha\beta}$[c]
ACTD	Pro	9.3 (d)	Pro	9.3 (d)
AZETII	azet	5.8 (t)	azet	5.8 (t)
PIP2	pip	6.4 (d)	pip	6.4 (d)
AZETI	Pro	9.3 (d)	azet	5.8 (t)
PIP1β	Pro	9.3 (d)	pip	6.4 (d)
ACTV	Pro	9.0 (d)	4-ketopro	(s)

[a] All results are for benzene-d_6 at 25 °C. [b] azet, azetidine-2-carboxylic acid; pip, pipecolic acid; 4-ketopro, 4-ketoproline. [c] In hertz; the multiplicity (singlet, doublet, or triplet) is given in parentheses.

V and PIP1β only the higher field proline has been substituted while the opposite is true for AZETI. This demonstrates that the biosynthetic amino acid substitutions have been made at a unique site on either the α or the β pentapeptide in the case of these analogues.

Assignments in the neighborhood of 6.5 ppm for the asymmetrically substituted actinomycins, or anisoactinomycins, may be made in the following manner. The two proline H^{α} proton pseudodoublets in ACTD have been assigned in $CDCl_3$, with deuterium labeling (Lackner, 1971a,b): the higher field doublet is due to the β-peptide proline. Examination of the ACTV spectrum in Figure 3 shows two resonances just below 6.5 ppm, one a clear doublet and the other a broad singlet. On comparison with the spectrum of ACTD in Figure 2, assuming the assignments in benzene-d_6 are the same as in $CDCl_3$, one would conclude that the β peptide has been substituted in ACTV. That this assignment, based solely on spectral comparison, is correct is confirmed by the structure determination of ACTV by chemical means [H. Brockmann, private communication, as quoted in Meienhofer & Atherton (1977)], which established the presence of 4-ketoproline on the β peptide.

The other two anisoactinomycins, PIP1β and AZETI, may be analyzed similarly, except for the assignment of peaks to the α or β peptide chain. For example, in AZETI, examination of the shape of the peaks at 6.5 ppm in Figure 3 and the coupling constants in Table II results in the obvious assignment of the triplet to azetidine and the doublet to proline. However, locating the azetidine substitution on one of the two pentapeptide rings is not possible. In a like manner, the assignments for PIP1β may be made on the basis of coupling constants, and as in the case of AZETI, determination of the particular pentapeptide in which substitution has occurred remains uncertain. Determination of the exact location of the substitution in AZETI and PIP1β will be of interest in that the site of substitution plays an important role in the DNA binding kinetics of these analogues (Mirau & Shafer, 1982). This problem is being pursued by using mass spectrometric techniques.

The conformation of the 3′-amino acid may be evaluated by the magnitude and multiplicity of the $J_{\alpha\beta}$ coupling constants, which are listed in Table II. For the pyrrolidine ring, two conformers have been observed by NMR (Pogliani et al., 1975) and X-ray crystallography (De Tar & Luthra, 1977) and predicted by molecular mechanics calculations (Ramachandran et al., 1970; Madison, 1977). The "A" and "B" conformers may be distinguished by the dihedral angle χ_1 made by the atoms $N\text{–}C^{\alpha}\text{–}C^{\beta}\text{–}C^{\gamma}$. The A conformer has $\chi_1 > 0$ while the opposite is true for the B conformer (Ramachandran et al., 1970); the value of χ_1 is usually close to $\pm 30°$ (De Tar & Luthra, 1977). The former should appear as a pseudotriplet ($J_{\alpha\beta} \simeq J_{\alpha\beta'}$) while the B conformer should appear as a pseudodoublet ($J_{\alpha\beta'} \simeq 0$). The coupling constants listed in Table II demonstrate that the B conformer is observed in all proline-containing analogues while the azetidines appear to be in an A-like conformation. While no in depth analysis of pipecolic acid has been attempted, Figures 2 and 3 and Table II strongly suggest that only one of the possible conformers is observed. These data indicate that the 3′-amino acid cyclic ring in actinomycins is relatively rigid in organic solutions.

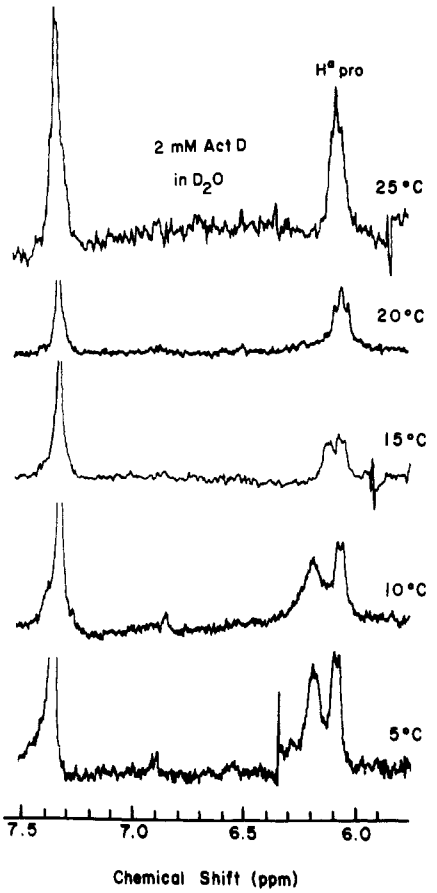

FIGURE 4: Low-field region of the 360-MHz ^1H NMR spectrum of ACTD in D_2O as a function of temperature.

The proton spectrum of actinomycin D has also been studied in D_2O to determine the effect of aqueous solution on the conformation and dynamics of the 3′ prolines. Figure 4 shows the low-field region of the spectra over the temperature range of 5–25 °C. The H^{α} proton of the higher field proline appears as a pseudodoublet with a conformation similar to that observed in organic solution. However, the lower field proline proton is not clearly resolved at 5 °C. Increasing the temperature leads to greater line broadening, followed by a sharpening and, finally, a superpositioning with the higher field resonance. A likely interpretation of these data is that one of the prolines remains in a preferred conformation over this temperature range while the other fluctuates between conformers at a rate that is intermediate on the NMR time scale.

Discussion

Determination of the high-field NMR spectrum allows us to make a detailed study of the conformation and dynamics of actinomycin and the effects of substitution at the 3′ position on these properties. Certain features of actinomycin conformation, such as the proximity of the 3′-H^{α} proton to the threonine carbonyl and the conformation about the 1′ (threonine) and 2′ (valine) amide bonds, are only slightly

perturbed upon substitution. Also, all analogues show small temperature coefficients for the position of the valine amide protons, consistent with the presence of two interpeptide hydrogen bonds between the Val-NH and Val-CO groups. Thus, we may conclude that the salient features of the three-dimensional peptide conformation remain similar in this series of compounds. NMR studies of other actinomycin analogues have revealed similar conformational properties (Lackner, 1975). Chemical shifts do occur, however, in the Pro-H^{α} and Thr-NH resonances of one peptide lactone when the other peptide lactone has been substituted at the 3′ position, indicating the presence of peptide–peptide interactions.

The DNA binding of actinomycins is expected to be highly dependent upon the conformation of the peptides, as the complex is stabilized by the intimate contact between the peptides and the nucleotides adjacent to the intercalation site (Sobell & Jain, 1972). Thus, from the NMR data presented here, we might predict that all analogues will bind DNA, as they may attain similar conformations. This speculation is confirmed in the following paper (Mirau & Shafer, 1982).

The interpretation of the data relating to the threonine amide protons is somewhat variable in the literature. Conti & De Santis (1970) noted that the downfield threonine resonance exchanged rapidly while the upfield one exchanged slowly. They interpreted this as a possible indication of a weak hydrogen bond involving the upfield threonine amide proton. Arison & Hoogsteen (1970) noted that the upfield Thr-NH resonance was consistently deshielded to a greater extent by proton-accepting solvents. Their proposed explanation involved a hydrogen bond between the downfield Thr-NH proton and the C9 phenoxazone ketone, thereby assigning this downfield resonance to the β-peptide threonine. While this assignment was confirmed by the work of Lackner (1971a,b), the agreement is fortuitous in that the upfield resonance was the slowly exchanging one in the report by Conti & De Santis (1970). Thus the proposed H-bond scheme cannot hold.

The apparent confusion regarding the threonine amide resonances has been eliminated in a study by von Dreele & Stenhouse (1974). By simultaneous ^1H NMR and IR studies on the effects of D_2O added to a solution of ACTD in $CDCl_3$, they ascertained that neither Thr-NH was involved in hydrogen bonding. One may thus conclude that the Thr-NH protons in ACTD and AZETI, which possess small temperature coefficients (data not shown), are shielded from solvent. In both of these actinomycins, the upfield Thr-H resonance showed almost no temperature dependence at all, consistent with deuterium exchange studies showing this peak to require significantly longer times for exchange than the downfield peak (Conti & De Santis, 1970).

The reason for this apparent difference in solvent accessibility remains to be clarified. While it appears that the threonine amide protons are not involved in hydrogen bonding in the drug alone, the structural model of the ACTD–DNA complex based on ACTD–deoxyguanosine X-ray crystallographic studies (Jain & Sobell, 1972; Sobell & Jain, 1972) includes a weak hydrogen bond between Thr-NH and the N3 of guanine at the intercalation site. This hydrogen bond, along with the stronger hydrogen bond between the Thr-CO and the NH_2 group of guanine, is postulated to be responsible for the G-C specificity in ACTD binding to DNA. It is intriguing to consider the correlation of solvent inaccessibility in the free drug with hydrogen-bond formation in the ACTD–DNA complex in aqueous solution.

Recent efforts in our laboratory have been directed toward the structural and dynamic aspects of drug–nucleic acid interactions (Shafer et al., 1980; Mirau & Shafer, 1982; Bolton et al., 1981; Mirau et al., 1982). The rigidity of the imino ring conformation in the 3′ position provides a point of interest in terms of dynamics. Analysis of the $J_{\alpha\beta}$ coupling constants of the 3′-amino acid indicates that one conformation is highly preferred for the imino ring in all of the analogues studied. Theoretical calculations (Madison, 1977) and analysis of the ^{13}C NMR relaxation behavior of proline (London, 1978; Deslauriers et al., 1974) predict that the pyrrolidine ring should fluctuate between conformers on a subnanosecond time scale. However, it has been observed that in peptides (particularly cyclic peptides) proline exhibits reduced rates of motion (Deslauriers & Smith, 1980). Actinomycins seem to be an example of this reduced conformational mobility arising from cyclization of the pentapeptide lactone.

Analysis of the temperature dependence of the proline H^{α} proton resonances in D_2O suggests a difference in conformational dynamics in the prolines of the α and β pentapeptide rings. One pyrrolidine ring appears to conserve its rigid structure typically observed in organic solvents while the other shows evidence of enhanced flexibility. In the following paper (Mirau & Shafer, 1982), we provide further evidence of differences between the two pentapeptide rings in terms of DNA binding properties. We also show that the actual site of substitution at the 3′ position, i.e., α or β peptide, plays an important role in determining the DNA dissociation kinetics. Muller & Crothers (1968) first proposed that the extremely slow DNA binding kinetics of actinomycins are due to a conformational change in the peptide moiety, and subsequently we suggested cis–trans isomerization about one or several amide bonds as the particular conformational change in question (Shafer et al., 1980; Mirau & Shafer, 1982). It will be of great interest to determine if such a change occurs and if it occurs in one or both peptide rings. Experiments are under way with ^{15}N NMR to elucidate this problem.

In summary, we have used high-resolution ^1H NMR to study the conformation and dynamics of actinomycins and how these properties are affected by amino acid substitution at the 3′ position. Our results on both singly and doubly substituted analogues suggest that all analogues exhibit a similar overall conformation, but the dynamics of the α and β peptides may differ in aqueous solution. This difference may be significant in terms of DNA binding kinetics.

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Role of Actinomycin Pentapeptides in Actinomycin–Deoxyribonucleic Acid Binding and Kinetics†

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ABSTRACT: Results are reported on equilibrium and kinetic experiments probing the DNA binding properties of a series of actinomycin analogues differing at the 3′-amino acid position. While the parent compound, actinomycin D, contains proline at this position on both pentapeptide lactone rings, the analogues under consideration here contain either azetidine-2-carboxylic acid, pipecolic acid, or 4-ketoproline on one or both pentapeptide rings. This study extends our earlier results on doubly substituted analogues [Shafer, R. H., Burnett, R. R., & Mirau, P. A. (1980) *Nucleic Acids Res. 8*, 1121]. DNA binding constants were determined from Scatchard plots constructed from visible absorption data and covered the range of (0.3–9) × 10^6 M^{-1} for the whole series of analogues. The thermal denaturation temperature of calf-thymus DNA was increased by 3–17 °C. DNA dissociation kinetics, along with enthalpies and entropies of activation, were also determined. The time constant for the slowest dissociation process ranged from 278 to 10900 s. The strongest DNA binding analogue, in terms of the largest binding constant, the largest increase in DNA thermal denaturation temperature, and the slowest DNA dissociation rate, was actinomycin V, which has 4-ketoproline in the β peptide ring, while the weakest DNA binding analogue has pipecolic acid on both peptide rings. Evidence is presented for one peptide ring exerting a greater influence than the other in the interaction with DNA. Also, the possible role of cis–trans isomerization about one or two peptide bonds in determining the slow DNA binding kinetics is discussed.

Actinomycin D (ACTD) is a potent antitumor antibiotic with a strong affinity for double-stranded DNA. It is of practical value as a powerful inhibitor of RNA synthesis as well as a clinical cancer chemotherapeutic agent. In addition, because of the presence of the two pentapeptide lactone rings attached to the phenoxazone chromophore [see Figure 1 in Mirau & Shafer (1982)], interest has arisen in the complex formed between ACTD and DNA in terms of a model for protein–nucleic acid interactions (Sobell, 1974). Because of these properties, ACTD is one of the most extensively studied DNA binding drugs and has been the subject of several recent reviews (Mauger, 1980; Remers, 1978, Meienhofer & Atherton, 1977; Lackner, 1975; Hollstein, 1974).

The experimental studies of Muller & Crothers (1968) and Waring (1970) established intercalation of the phenoxazone chromophore between base pairs as the mode of binding of ACTD to DNA. A more detailed model of the ACTD–DNA complex was developed by Sobell and co-workers (Jain & Sobell, 1972; Sobell & Jain, 1972; Sobell, 1980) based on X-ray diffraction studies of the 2:1 complex formed by deoxyguanosine and ACTD. This model specifies several important drug–DNA interactions, such as particular hydrogen bonds, and predicts that dG-dC is the strongest binding site, in agreement with experimental results (Wells & Larson, 1970).

Thus a high-resolution picture exists of the structure of the ACTD–DNA complex. However, the kinetics of this interaction, which play a major role in determining biological activity, are far less well understood. Muller & Crothers (1968) showed that actinomycins have association and dissociation rate constants for binding to DNA that are several orders of

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