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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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magnitude smaller than other simple intercalators such as ethidium (Bresloff & Crothers, 1975), proflavin (Li & Crothers, 1969), and daunomycin (Gabbay et al., 1976) and also established the positive correlation between slowness of DNA dissociation and inhibition of RNA synthesis. While the kinetics of binding to calf-thymus DNA show three slow processes (Muller & Crothers, 1968; Bittman & Blau, 1975), Krugh et al. (1980) recently have shown that ACTD dissociation from poly(dG-dC)·poly(dG-dC) is characterized by a single slow process.

One explanation for the slow kinetics of interaction of actinomycins with DNA, originally put forward by Muller & Crothers (1968), involves conformational changes in the peptide portion of the antibiotic as the rate-determining step. An alternative proposal has been presented by Sobell (1974) that ascribes the slow rate constants to changes in polynucleotide conformation. Relatively little experimental data are available that pertain to this point directly. Several observations, however, indirectly support the first of the above hypotheses. Actinomine, an analogue without the peptide rings, is characterized by a DNA binding curve very similar to that of ACTD but displays the very rapid kinetics of binding found with other intercalators (Muller & Crothers, 1968). Also, the presence of supercoils in the DNA has little effect on the slow kinetics of association (Bitterman & Blau, 1975).

We have been interested in establishing to what extent changes in peptide conformation play a role in determining the slow kinetics of actinomycin–DNA binding. In a previous study (Shafer et al., 1980), we examined the effects on DNA dissociation kinetics resulting from replacement of proline, the 3′-amino acid, in both pentapeptide lactones with proline analogues. The substitutions involved pipecolic acid, the six-membered proline analogue, and azetidine-2-carboxylic acid, the four-membered proline analogue. In that study, referred to as I in what follows, large and opposing changes in the enthalpy and entropy of activation for dissociation were found as a result of those amino acid substitutions. The net effect of these changes in activation parameters was a decrease in the dissociation rate constant by approximately a factor of 2 for each change in ring size of the imino acid from six to five to four atoms. Substitution of alloisoleucine for valine at the 2′-amino acid position had little effect on the kinetics.

The sensitivity of the DNA dissociation kinetics to the structure of the 3′-amino acid implied a possible role for that amino acid in determining the time scale of DNA binding. Furthermore, we found the free energy of activation (20–22 kcal/mol) to be similar to that observed for the cis–trans isomerization of proline and other N-substituted amino acids (Portnova et al., 1970; Maia et al., 1971; Torchia & Bovey, 1971; Love et al., 1972; Brandts et al., 1975; Cheng & Bovey, 1977). This led us in I to propose cis–trans isomerization of the Val–Pro peptide bond as the rate-determing step in the DNA dissociation kinetics for this class of compounds.

In this study we extend our earlier work in several ways. First, we include new actinomycin analogues that are substituted in only one of two pentapeptide 3′ positions. Second, we report results on binding isotherms for all the actinomycin analogues, thereby permitting determination of DNA binding constants in addition to dissociation rate constants. The results of these experiments further establish the importance of the 3′ position in the pentapeptide lactone rings and also provide evidence for one peptide exerting a greater role in the DNA kinetics than the other. Thus, while the geometry of the actinomycin–DNA complex may display a certain degree of symmetry, the dynamics of the binding process may be governed by structural asymmetry.

Materials and Methods

Calf-thymus DNA (type I) and ACTD were purchased from Sigma Chemical Co. Azetomycin II (AZETII) and actinomycin C_3 (ACTC) were obtained from Dr. M. A. Apple [see Figure 1 in Mirau & Shafer (1982) for structures]. Actinomycin pip 2 (PIP2), actinomycin pip 1β (PIP1β), actinomycin V (ACTV), and azetomycin I (AZETI) were obtained from Dr. J. V. Formica (Formica et al., 1968; Formica & Apple, 1976). All buffer salts were reagent grade. Concentrations were determined spectrophotometrically by using molar extinction coefficients of $\epsilon_{260} = 6600$ (per nucleotide) for DNA and $\epsilon_{440} = 24\,450$ (Bittman & Blau, 1975) for all actinomycin analogues. All solution studies, except thermal denaturation (T_m) experiments, were carried out in BPES buffer, consisting of 0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.18 M NaCl, and 0.01 M Na_2EDTA (disodium ethylenediaminetetraacetate) in doubly distilled water. T_m measurements were done in 0.01 × BPES buffer. Stock solutions of DNA were prepared, clarified by centrifugation, and extensively dialyzed against BPES buffer prior to use. Drug–DNA dissociation kinetics and T_m profiles were determined as described in I. Equilibrium binding isotherms were obtained by titrating small aliquots of concentrated DNA solutions into a solution of the drug and following the change in absorbance at 440 nm on a Beckman Acta CIII double-beam spectrophotometer. Initial drug concentration was typically 4 μM in thermostated, 10-cm cylindrical cuvettes. Care was taken to assure that equilibrium was reached following each addition of DNA. The resultant Scatchard plots were analyzed by using eq 10 of McGhee & Von Hippel (1974) by nonlinear regression: $r/c = K(1 - nr)[(1 - nr)/(1 - (n - 1)r)]^{n-1}$. Here c is the free drug concentration, r is the amount of bound drug per DNA base pair, n is the number of base pairs per binding site, and K is the binding constant. All analogues, except ACTC, differ from ACTD only at the 3′-amino acid position: PIP1β and PIP2 contain one and two residues of pipecolic acid, respectively; AZETI and AZETII contain one and two residues of azetidine-2-carboxylic acid, respectively; ACTV has one residue of 4-ketoproline. For the singly substituted analogues PIP1β, AZETI, and ACTV, the site of the substitution has been determined only in ACTV, which has 4-ketoproline in the β (quinoid) peptide ring [H. Brockmann, personal communication, as quoted in Meienhofer & Atherton (1977)]. ACTC differs from ACTD at the 2′ position, containing D-alloisoleucine instead of D-valine on both peptide rings.

Results

Equilibrium Measurements. The effect of the various actinomycin analogues on the thermal denaturation of calf-thymus DNA is presented in Table I. Since drug-induced alterations in the T_m reflect relative affinities of the drug for double-stranded vs. single-stranded DNA, they do not necessarily provide information on the absolute affinity for double-stranded DNA. In the case of actinomycin, however, it has been shown that complex formation is specific for double-stranded DNA, with little binding to the strand-separated form (Reich & Goldberg, 1964). Assuming this to be true for the analogues under consideration in this study, we may conclude that the higher the T_m, the greater the affinity for double-stranded DNA. The validity of this assumption will be examined below in the analysis of the binding curves.

As can be seen in Table I, the T_m values cover a broad range. Values from I have been included for completeness. Substitution of only one of the prolines with azetidine-2-carboxylic

Table I: Equilibrium DNA Binding Properties of Actinomycin Analogues

	ΔT_m (°C)[a]	$10^6 K$ (M^{-1})	n (base pairs)
ACTD	10.6	3.2	9.8
PIP2	3.3	0.33	10.8
AZETII	10.6	1.9	8.3
PIP1β	9.4	0.62	9.9
AZETI	12.9	3.3	9.1
ACTV	17.4	8.9	6.9
ACTC	10.4	3.1	9.7

[a] Measured in 0.01 × BPES buffer, relative to DNA alone.

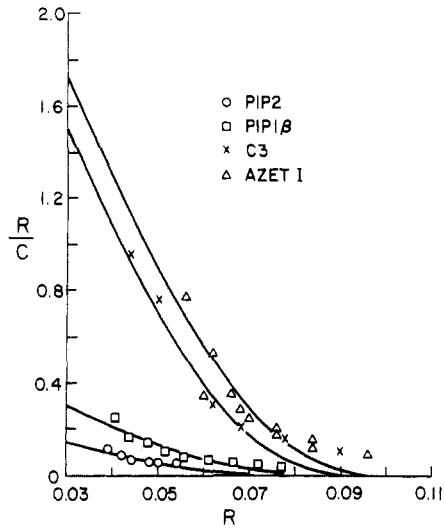

FIGURE 1: Scatchard plots for binding to DNA. Experimental points plus best fit (solid line) to eq 10 (see Materials and Methods) of McGhee & Von Hippel (1974).

acid resulted in an increase in T_m somewhat larger than that of ACTD, while the single substitution with pipecolic acid led to a slightly smaller effect. By far, the greatest increase in T_m was found with ACTV in which 4-ketoproline is substituted for one of the prolines. Thus, it appears that the largest deviation from ACTD arises from PIP2, the doubly substituted pipecolic acid analogue, and ACTV, the singly substituted 4-ketoproline analogue. All the other analogues produce results in fair agreement with ACTD.

Binding isotherms for several of the analogues are displayed in Figure 1, along with the best fit to the McGhee–Von Hippel equation. Results for all the compounds are presented in Table I in terms of K and n. The values of K cover a 30-fold range of (0.3–9) × 10^6 M^{-1} and parallel the pattern observed in the T_m results discussed above. PIP2 is the weakest binding compound while ACTV is by far the strongest binding compound. ACTV, also known as actinomycin X_2, was similarly found to be the strongest DNA binding analogue in the series studied by Muller & Crothers (1968). PIP1β has a binding constant considerably smaller than expected from its effect on the T_m, but the other compounds fall in a small range of values, (2–3) × 10^6 M^{-1}, for K, consistent with the T_m results. Overall, the analogues may be classified in three groups: those that bind more strongly than ACTD (ACTV), those with similar affinities (ACTC, AZETI, AZETII), and those that bind less strongly (PIP1β, PIP2).

Table I also lists the values of n, the number of base pairs per binding site, for the series of analogues. These values show relatively little change from one compound to the other. It can be seen that the smallest value of n occurs with ACTV. The next lowest value of n corresponds to the substitution of both prolines with the smaller proline analogue while the largest value of n is observed for the double substitution with the larger proline analogue. Thus there appears to be a small effect of the size of the 3′-amino acid ring on the binding site size. This effect is also apparent in the singly substituted AZETI but not in the PIP1β analogue. The large value of n found here, compared to $n = 4$ found for ACTD binding to poly(dG-dC)·poly(dG-dC) (Winkle & Krugh, 1981), presumably arises from the G-C specificity of ACTD and the heterogeneous lattice of binding sites on calf-thymus DNA. These effects are not accounted for in the McGhee–Von Hippel analysis.

Examination of the fit of the experimental data points to the McGhee–Von Hippel isotherms in Figure 1 reveals a common feature of lack of fit at high r values. Since Krugh et al. (1980) have recently provided evidence for heterogeneity of binding sites for ACTD on calf-thymus DNA, the presence of additional types of weaker binding sites is most likely responsible for this lack of fit. However, it is clear that all analogues bind strongly to duplex DNA ($K > 10^5$ M^{-1}) and the general agreement between T_m effects and binding constants supports the notion that these drugs do not bind to a significant extent to strand-separated DNA. The variation in K and n presumably reflects details of the peptide–DNA interactions, primarily at nucleotides adjacent to the intercalation site.

Kinetic Measurements. We have also analyzed the effect of the proline analogues on the DNA dissociation kinetics. As observed by Muller & Crothers (1968), the dissociation curves, in the presence of excess DNA, show more than one exponential component. As our studies were performed by hand mixing of the drug–DNA solution with detergent solution, only the two slower components were observed. More rapid mixing techniques, such as stopped flow, are required to reveal the fastest process. Thus all our experimental traces have been analyzed in terms of a two-exponential decay curve. While the original explanation of the multiexponential kinetic curves involved peptide conformational changes of the bound ligand at one site (Muller & Crothers, 1968), more recent evidence suggests the presence of several classes of binding sites with different binding affinities and different dissociation rates (Krugh et al., 1980). While some results are presented for the faster dissociating complex below, the emphasis of our discussion is placed on the most slowly dissociating form.

The results of the sodium dodecyl sulfate induced dissociation of the monosubstituted analogues PIP1β, AZETI, and ACTV are shown in Figure 2. Both PIP1β and AZETI show a bend in the semilog plot at early times (~250 s) while ACTV shows such a break at significantly longer times (~1250 s), indicating the presence of a much slower process than observed for the former two analogues. When dissociation of ACTV from DNA is followed for a considerably longer time, as illustrated in the inset of Figure 2, this slower component is very apparent, the more well-defined bend occurring at approximately the same time as that observed in Figure 2 for this compound.

All experimental traces were fitted very well by a double-exponential decay, and the resulting time constants (inverse rate constants) are summarized in Table II for both the slow and fast components. For ease of comparison, results are also included for the disubstituted analogues. While there is some variation in the fast time constant among the whole set of analogues, this variation is not as large nor does it show the same pattern as that found for the slow time constant.

Regarding the slow time constant, it can be seen that of the three monosubstituted analogues, both ACTV and PIP1β show

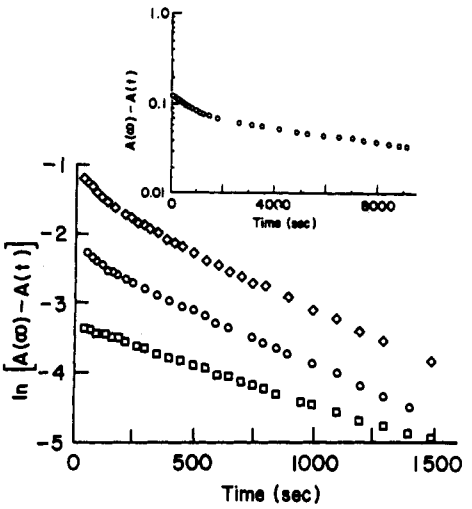

FIGURE 2: DNA dissociation curves for PIP1β (◇), AZET1 (○), and ACTV (□) showing absorbance changes with time, measured at 440 nm. Inset shows data for ACTV. All curves had similar intercepts on the ordinate, and thus the lower two curves have been displaced vertically for visual clarity.

Table II: DNA Dissociation Time Constants at 25 °C

	τ_{fast} (s)	τ_{slow} (s)
ACTV	66	10 910
PIP1β	70	350
AZETI	75	609
ACTD[a]	40	735
PIP2[a]	57	278
AZETII[a]	77	1 360
ACTC[a]	45	592

[a] From Shafer et al. (1980).

a significant deviation from the parent compound, ACTD. This effect is quite large in that ACTV has a time constant almost 15 times greater than ACTD. AZETI has a somewhat smaller time constant than ACTD. In terms of the complete set of analogues under consideration, three groups may be discerned on the basis of the magnitude of the slow time constant: analogues that dissociate more rapidly than ACTD (PIP2 and PIP1β), analogues with time constants similar to ACTD (AZETI, ACTC, ACTD), and those that dissociate more slowly than ACTD (AZETII and ACTV). This classification differs from that based on the binding constants only with respect to AZETII.

It is of interest to note that substitution of both prolines with azetidine-2-carboxylic acid results in almost a doubling of the slow time constant, relative to ACTD, while the singly substituted analogue has a rather similar time constant as ACTD. Substitution of one proline by pipecolic acid results in a time constant that lies between that produced by double substitution and that of ACTD, although closer to the former. Finally, while no analogue is available containing 4-ketoproline substituted for both prolines, it is evident that the single substitution results in the most dramatic change of all.

The effect of temperature on the dissociation kinetics of the monosubstituted analogues has been evaluated as in I, and the resulting Arrhenius plots are presented in Figure 3. These plots have been analyzed to yield activation enthalpies and entropies, which are shown in Table III, along with free energies of activation. One of the most striking aspects of the data in Table III is that the free energy of activation remains relatively constant throughout the series of compounds while the enthalpies and entropies show quite large, opposing changes. Thus most of the differences in energetics are compensated by changes in entropic factors.

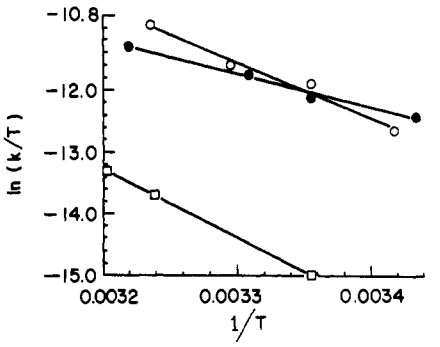

FIGURE 3: Temperature dependence of rate constants for the slow dissociation step from calf-thymus DNA: PIP1β (○); AZETI (●); ACTV (□).

Table III: Activation Parameters for the Slow DNA Dissociation Process

	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu/mol)	$\Delta G^{\ddagger}$, 25 °C (kcal/mol)
ACTV	21.9 ± 0.1	−3.4 ± 0.2	22.9
PIP1β	17.5 ± 1.7	−12.3 ± 5	20.9
AZETI	11.0 ± 0.9	−36.2 ± 3	21.2
ACTD[a]	20.1 ± 1.3	−4.4 ± 4.3	21.4
PIP2[a]	11.5 ± 1.1	−30.8 ± 3.7	20.7
AZETII[a]	23.0 ± 0.4	4.4 ± 1.4	21.7

[a] From Shafer et al. (1980).

On the basis of our previous work described in I, a definite trend was apparent in the dependence of the enthalpy of activation $\Delta H^{\ddagger}$ on the 3′-amino acid ring size in the doubly substituted analogues (see Table III). As the ring size of this residue decreased from six to five to four atoms, the value of $\Delta H^{\ddagger}$ increased from 11.5 to 20.1 to 23.0 kcal/mol. This trend is carried over to the singly substituted analogue PIP1β but not AZETI. In fact, the effect of amino acid ring size is opposite in this latter case: while AZETII is characterized by a larger $\Delta H^{\ddagger}$ than ACTD, AZETI shows a significantly smaller $\Delta H^{\ddagger}$. Similarly opposing changes appear in the values of $\Delta S^{\ddagger}$.

It is very evident from Table III that entropy plays a major role in the DNA dissociation kinetics. This most likely arises from the presence of nonpolar amino acids in the peptide portion of the actinomycins, leading to large hydrophobic effects. Differences in $\Delta S^{\ddagger}$ may also be due to variations in the degrees of freedom in the transition state. The opposite effect on $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the singly substituted analogue AZETI relative to the doubly substituted AZETII may indicate a significant peptide–peptide interaction in the bound drug and/or the transition-state complex.

Discussion

The results presented above extend our initial studies of actinomycins containing substitutions at both proline positions to those containing only one substitution, with one proline remaining. In the case of ACTV, it has been established that the substitution of 4-ketoproline for proline occurs uniquely on the β (quinoid) pentapeptide lactone ring. In the preceding paper (Mirau & Shafer, 1982), we present high-resolution proton nuclear magnetic resonance (^{1}H NMR) evidence that the analogues AZETI and PIP1β also have proline substituted only on one of the two pentapeptide rings. Which of the two rings is substituted remains to be determined. It is important, however, to know that these compounds are not mixtures of isomers containing substitutions on each of the peptide rings.

In that study we also showed that the overall peptide conformation is similar in this series of analogues. Nevertheless, differences were observed in the imino acid ring pucker.

The substitutions at the 3′-amino acid position have considerable effects on both the DNA binding constants and the DNA dissociation times. The binding constants span a 30-fold range among all the analogues while the dissociation rates cover a 40-fold range. The size of the binding site varies only slightly, except for ACTV, which shows a considerably smaller value of n than all the others. As the alterations in structure are very specific and restricted to one or both of the peptide rings (in all cases but two just a difference in ring size of the imino acid), the ensuing changes in DNA binding characteristics suggest an important role for the 3′-amino acid in determining the details of the interaction.

The results of equilibrium measurements displayed in Table I show a strong parallel between the effects of various structural alterations on the drug-induced thermal stabilization of duplex DNA and the binding constant for complex formation with duplex DNA. This is expected for ligands that do not bind significantly to single-stranded DNA. Due to experimental difficulties in obtaining precise binding data at low values of r and the use of a necessarily limited model for interpretation of those data, the T_m results are probably more accurate, though perhaps less informative, than the values obtained for K and n. At this point we also mention that recent studies by Winkle & Krugh (1981) have shown evidence for positive cooperativity in the binding of ACTD to calf-thymus DNA at very low r values (<0.02) using tritiated ACTD. As this effect may well exist in the binding of the analogues studied here, the absolute values K and n may be somewhat uncertain. The relative affinities for DNA among the series of analogues, however, should still be reliable.

In several cases, i.e., ACTV, ACTD, PIP2, and PIP1β, the dissociation rates correlate with the binding constants in that the slower the off rate, the stronger the binding constant. AZETI deviates only slightly from this trend, AZETII more so. Quantitative agreement is not necessarily expected, since the amino acid substitution may affect the association as well as dissociation rates, and the binding process is multistep in nature. Thus the kinetics of only one step may not reflect the overall thermodynamic effect of the substitutions. This does appear to occur, however, for the majority of analogues.

The following observation may be made regarding the correlation between biological activity and physical parameters characterizing the actinomycin–DNA interaction. PIP2 is both the least tightly binding analogue and the most rapidly DNA dissociating analogue. Consistent with these properties, it is the least potent in terms of antibacterial activity (Meienhofer & Atherton, 1977). Similarly, ACTV, the most tightly binding and slowest DNA dissociating analogue, is the most potent inhibitor of bacterial growth. The other compounds in this series fall within these extremes, but an exact quantitative correlation of binding or kinetic properties with antibacterial potency cannot be made. This may be due to other factors such as permeability of the drug with respect to the bacterial cell, combining results from different research groups, etc. Finally, it is of interest to note that antibacterial potency does not correlate with therapeutic antitumor efficiency (Meienhofer & Atherton, 1977). Thus much more work is required to elucidate the molecular description of actinomycin antitumor activity.

The experimental results presented above demonstrate that substitution of only one proline on one of the pentapeptide rings can have a large effect on both the equilibrium and the kinetic properties of actinomycin–DNA binding. In the case of AZETI, the replacement of one proline has an opposite effect on the enthalpy and entropy of activation from that due to replacement of both prolines. This may be indicative of the presence of specific interpeptide interactions in the DNA complex that are highly sensitive to the detailed peptide structure and conformation. Such interactions may involve the hydrogen bonds between the N–H of one valine and the C=O of the other valine. These hydrogen bonds have been observed in the ACTD–DNA crystalline complex (Jain & Sobell, 1972) as well as in solution studies of ACTD alone (Victor et al., 1969; Conti & De Santis, 1970; Arison & Hoogsteen, 1970) and complexed with d-pG (Patel, 1974a) and d-pGpC (Patel, 1974b).

There are several pieces of evidence that suggest a certain asymmetry in the role played by the two pentapeptide rings in determining the details of the actinomycin–DNA complex. As mentioned above, the opposite effect of the singly and doubly substituted AZET analogues on $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for dissociation is observed. Second, the largest effect on the off rate as well as on the binding constant is observed for ACTV, a singly substituted analogue. Third, we have presented NMR evidence (Mirau & Shafer, 1982) suggesting different conformational flexibility in the two prolines of ACTD in aqueous solution. Fourth, the X-ray diffraction analysis of the ACTD complex with deoxyguanosine reveals a different pucker for the two prolines. Finally, in this regard we note that the revised model of the ACTD–DNA complex developed by Sobell (1980) involves loss of exact symmetry due to the chromophore asymmetry and, more importantly, due to the fact that only one pentapeptide ring can interact with a guanine residue at the intercalation site.

The sensitivity of the DNA dissociation rates to the structure of the 3′-amino acid and the observation that the various analogues all were characterized by $\Delta G^{\ddagger}$ values of 20–21 kcal/mol led us in I to propose cis–trans isomerization of the Val–Pro peptide bond as the rate-limiting step in DNA binding for these compounds. Obviously, this would imply a different conformation about this bond in the free and bound drug. However, NMR studies (Mauger, 1980; Lackner, 1975) of the uncomplexed drug and the X-ray diffraction studies (Jain & Sobell, 1972; Sobell & Jain, 1972) of the drug complexed with deoxyguanosine both show the same conformation about each peptide bond, i.e., Val–Pro and Pro–Sar in the cis form, the rest in the trans form. There are several reasons to believe that the ACTD–deoxyguanosine complex may not mirror the ACTD–DNA complex in certain aspects. The 1:1 solution complex of ACTD with deoxyguanosine has a large negative ΔH° (−9.1 kcal/mol) and ΔS° (−15.1 eu/mol) as determined by Gellert et al. (1965). This is in sharp contrast to the small but positive ΔH° (2 kcal/mol) and large positive ΔS° (+39 eu/mol) measured for the ACTD–DNA complex (Quadrifoglio & Crescenzi, 1974). Temperature-jump chemical relaxation studies of the interaction of ACTC with deoxyguanosine (Muller & Spatz, 1965) and of ACTD with dinucleotides (Davanloo & Crothers, 1976) showed binding to occur on the millisecond time scale or faster, rather than the extremely slow kinetics with DNA. Thus it appears that a minimum number of base pairs is required to give rise to the thermodynamic and kinetic properties peculiar to the ACTD–DNA complex. This is consistent with the interpretation of the large, positive ΔS° of complex formation with DNA resulting from a reduction in the extent of exposure of the peptide portion to water. Mono- or dinucleotides would not be expected to provide such protection from the solvent.

Experiments are currently under way to determine the DNA length dependence of the binding kinetics with sized DNA oligonucleotides.

We have focused on actinomycins with substitutions mainly at the 3′-amino acid as this position is most subject to incorporation of exogenous amino acids. As noted in I, the 4′ and 5′ positions also consist of N-methylated amino acids, resulting in additional sites capable of undergoing cis–trans isomerization. Recent molecular mechanics calculations of Weiner & Kollman (1981) on the effects of changing one amide bond conformation indicate that the trans isomer of the Val–Pro amide bond is very much higher in energy than the cis isomer, while in the Pro–Sar bond, the two isomers differ in energy by only several kilocalories per mole. This would suggest that the Pro–Sar bond may be more likely to be involved in the DNA dissociation process. Those calculations, however, were done on the free drug, omitting any effect due to the presence of DNA. No study of the solution conformation of ACTD has revealed the presence of more than one conformational isomer, which means that if cis–trans isomerization is responsible for the slow association and dissociation kinetics, it is most likely facilitated by interaction with DNA. This view is consistent with results of DNA binding kinetics that suggest that the slow step occurs when the drug is bound to DNA (Muller & Crothers, 1968).

It is interesting to note that NMR experiments on free pentapeptide lactones, i.e., a single pentapeptide lactone connected to a substituted benzene ring, revealed the presence of two solvent-dependent conformations (Lackner, 1975). Detailed spectral analysis demonstrated that these two conformations differ in that one had both Val–Pro and Pro–Sar bonds in the trans configuration while the other had both of these bonds in the cis configuration. This represents the only experimental evidence for cis–trans isomerization in an actinomycin-like pentapeptide ring and suggests the possibility that ACTD–DNA complex formation may involve both the Val–Pro and Pro–Sar bonds. Such a mechanism would still be sensitive to proline substitution. The presence of only one conformation of the peptide rings in ACTD alone may be due to the interannular Val–NH to Val–CO hydrogen bonds, which serve to lock the pentapeptide in a single structure. Interaction with DNA may then result in loss of those hydrogen bonds, permitting the pentapeptide to take on an alternate conformation.

While the kinetic experiments described here and in I provide suggestive evidence for the role of peptide conformation in ACTD–DNA binding, proof of this hypothesis requires some direct indication of the peptide conformation of ACTD in the presence of DNA. We have initiated such an investigation using ^{15}N NMR on ^{15}N-labeled ACTD that we have recently obtained (R. H. Shafer and J. V. Formica, unpublished results).

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