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Specific interactions between DNA left-handed supercoils and actinomycin D

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The interactions between the natural cyclopentapeptide antibiotic actinomycin D (ACT) and circular pBR322 DNA have been studied by freezing the topological state of the DNA in the complex by topoisomerase I reaction. Both supercoiled and relaxed DNAs, in the complexes at low antibiotic/DNA base-pair ratios, showed a dramatic decrease in linking number that cannot be explained by taking into account only the generally accepted unwinding of 28° for each ACT molecule bound. Recent results derived from the crystallographic analysis of the complex between GpC and ACT suggest that ACT could mediate non-covalent cross-links between distant sections of DNA. Bridges between ACT and different sections of the pBR322 double helix could also explain our results. Two-dimensional gel electrophoresis of ACT-relaxed pBR322 DNA complexes reveals that all supercoils induced by ACT are negative. Two models of the complexes which correspond to the stabilization of DNA crossing by one or two molecules of ACT are proposed. In both cases the ability of ACT to stabilize only DNA left-handed supercoils is derived from the chirality of ACT, when it interacts with DNA.

1. Introduction

In recent years, the major role of topology in recognition processes involving DNA has been convincingly demonstrated. The complexes between DNA and numerous biologically active molecules which untwist the double helix (intercalators) have been extensively analyzed (ref. 1 and references cited therein). Base-sequence-specific intercalators, such as actinomycin D (ACT), are of particular interest for obtaining information on specific interactions involved in recognition mechanisms.

However, despite extensive research, the molecular structure of the complex between DNA and ACT is still a proposal [2–4]. Important structural features are provided by model systems, mainly complexes between ACT and oligonucleotides, which indicate that the phenoxazone ring intercalates into the double helix at the GpC sequences, while the two cyclopentapeptide rings are engaged in a couple of hydrogen bonds with guanines [1,5]. Recently, the crystal structure of the ACT-d(GpC) complex, at 1.5 Å resolution [6], revealed some structural features that were rather different with respect to the classic intercalation mode. On the basis of these results, Takusagawa and Berman [7] suggested that the phenoxazone moiety could be intercalated between two guanines belonging to two different sections of DNA double helix, so that ACT could mediate a sort of non-covalent cross-link between distant regions of DNA in the Z conformation. We have analyzed the binding of ACT to both supercoiled and relaxed pBR322 DNA, freezing the topological state of the DNA in the complexes through the reaction

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with topoisomerase I, with the aim of obtaining information on the features of ACT binding to DNA at very low ACT/DNA binding ratios. In fact, it is well known that under these conditions, important biological processes, such as DNA transcription [8], are influenced significantly by ACT.

2. Materials and methods

2.1. Plasmid pBR322 DNA

Plasmid pBR322 DNA was amplified in *Escherichia coli* 600. Supercoiled DNA was isolated by CsCl density gradient centrifugation in the presence of ethidium bromide (EtBr). The percentage of supercoiled vs. nicked form was 90% as judged by densitometric analysis of agarose gel negatives.

2.2. Topoisomerase I

Topoisomerase I was extracted from chicken erythrocyte nuclei as described previously [9] and used in a buffer containing 0.2 M NaCl, 10 mM Tris-HCl (pH 7.8), 1 mM EDTA. The reaction was carried out at an enzyme/DNA ratio equal to 1 U (Keller)/μg for 1 h at 37°C.

2.3. Actinomycin D

ACT was a gift from Merck, Sharp and Dohme. Antibiotic solution was prepared by direct weighing and dissolving in double-distilled water. It was stored at 4°C in the dark. The concentration was checked by recording the absorption spectrum and using an extinction coefficient of $\epsilon_{440} = 24\,450$ [10].

2.4. Complexes between ACT and supercoiled or relaxed pBR322 DNA

Complexes were prepared by adding to DNA solution ($c = 1$ μg/μl) different amounts of the ACT solution in water ($c = 1 \times 10^{-6}$ M) and waiting for at least 1 h. The results are not affected by longer incubation times.

2.5. Analysis of ACT-DNA complexes on gel electrophoresis

Complexes between ACT and supercoiled or relaxed pBR322 DNA, at different molar ratios, were treated with topoisomerase I (1 U(Keller)/μg DNA) for 1 h at 37°C, after addition of NaCl to obtain a final concentration of 0.2 M. The reaction was stopped and DNA was purified by extraction with phenol (twice) and ether followed by alcohol precipitation. The sample was dissolved in 4×10^{-2} M Tris-acetate (pH 7.9) and 1×10^{-3} M EDTA and analyzed by electrophoresis on agarose gel 1.2%.

According to this procedure, the enzyme transforms uncomplexed DNA into a fully relaxed molecule, whereas in the presence of a complex only those regions that are not blocked by ACT can be relaxed. At the end of the enzyme-catalyzed reaction the antibiotic can be removed from the DNA and the linking number (which represents the $\Delta Tw + \Delta Wr$ in the complex) can be evaluated by measuring the electrophoretic mobility on an agarose gel in comparison with supercoiled and relaxed standard DNA molecules [11].

Two-dimensional gel electrophoresis was carried out to determine the handedness of supercoiling. Electrophoresis in the first dimension was carried out on a vertical apparatus in 1% agarose gel slabs in the above-described buffer. Electrophoresis in the second dimension was performed after soaking the gel in a solution of EtBr in electrophoresis buffer. Different EtBr concentrations were adopted, depending on the ΔLk of samples. ΔLk corresponds to the difference between the linking number (Lk) of DNA alone and that of DNA in the complex with ACT, after treatment with topoisomerase I, and represents the variation in partitioning between twisting (Tw) and writhing (Wr) caused by the antibiotic. The electrophoresis was run in a buffer containing the same concentration of EtBr [12].

3. Results and discussion

Complexes between ACT and supercoiled pBR322 DNA have been formed over the range of

ACT/DNA molar ratios (r) of 10^{-3}–10^{-1}. DNA molarity refers to the number of base-pairs. Taking into account that pBR322 DNA contains 4360 base-pairs, r ranges between 4 and 436 ACT molecules per plasmid molecule. The complexes were relaxed with topoisomerase I, ACT was phenol-extracted and the topological state of DNA was analyzed by agarose gel electrophoresis. This approach [13] has the advantage of freezing the DNA/ACT topological state, which can then be measured in the absence of the antibiotic, on gel electrophoresis.

To rule out the possibility that ACT influences the enzyme activity, the same experiment has been carried out in parallel using pBR322 DNA previously relaxed. Fig. 1a and b shows that in both cases DNA seems to be fully supercoiled as shown by one-dimensional gel electrophoresis when ACT/DNA ratio r is equal to 0.02. Since in one-dimensional gel electrophoresis topoisomers with ΔLk higher than 12–13 are not resolved, one-dimensional gel electrophoresis was carried out in the presence of different amounts of EtBr. The trend of ΔLk vs r in the two cases is reported in fig. 1c.

The ratio r is calculated considering that all the ACT added to the solution is bound to DNA. This assumption is in good agreement with the association constant measured by Muller and Crothers [14]. The values of ΔLk are systematically higher in the case of supercoiled pBR322 DNA than for relaxed DNA, as is to be expected since the binding is energetically favoured in the case of negative supercoiled DNA [15]. We have also plotted the line corresponding to the variation of ΔLk in the case of the normal ACT unwinding angle of 28° per bound molecule [1]. On increasing r the ΔLk curve obtained experimentally moves closer to the theoretical line up to $r = 0.05$, where the values become almost coincident.

The reported results cannot be explained on the basis of a simple intercalation of ACT into DNA, since the ΔLk values for the lower binding ratios should correspond to an untwisting of DNA of about 100° for each antibiotic molecule bound. A possible explanation could be the B-Z transition of a segment of DNA due to ACT interaction, since in this case one ACT molecule should cause at least untwisting of 66°. However, it has been shown that the intercalation of ACT in DNA is

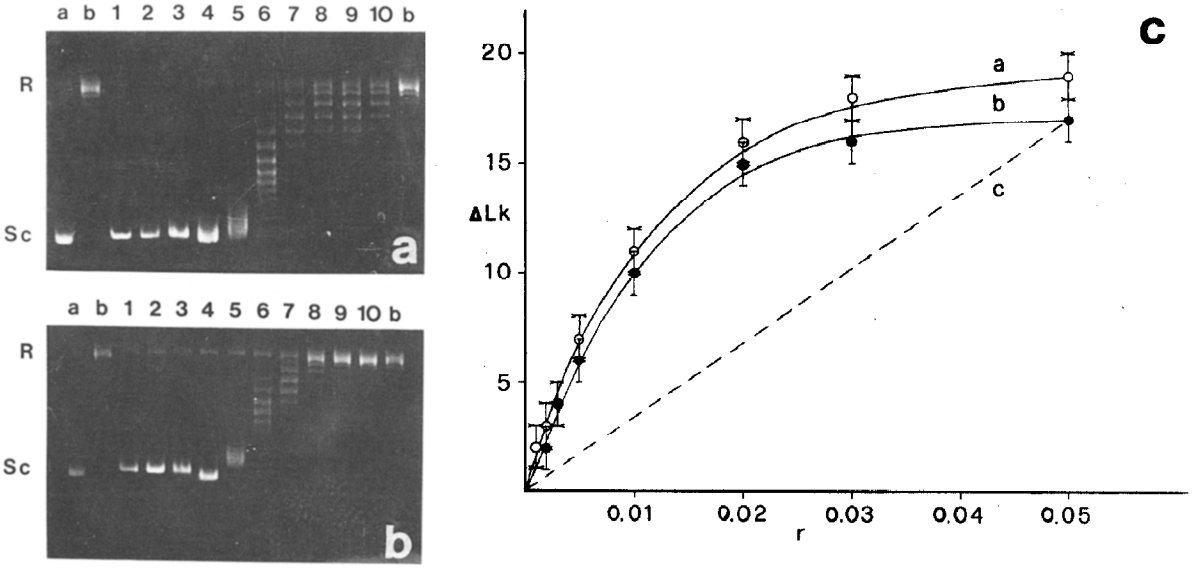

Fig. 1. Agarose gel electrophoresis (1.2%) of (a) supercoiled and (b) relaxed pBR322 DNA-ACT complexes, treated with topoisomerase I (for experimental details see text). (a) Supercoiled DNA; (b) relaxed DNA. Drug/DNA ratios (r): (1) 0.1, (2) 0.05, (3) 0.03, (4) 0.02, (5) 0.01, (6) 0.005, (7) 0.003, (8,9) 0.002, (10) 0.001. (c) Variation of linking number of complexes (ΔLk) as a function of r. (Trace a) Supercoiled DNA; (b) relaxed DNA, (c) theoretical line calculated for an unwinding of 28°.

more favorable in the case of B-DNA than Z-DNA and that ACT effectively inhibits the B-Z transition [16]. Moreover, in Z-DNA the binding can occur only in the major groove with too large van der Waals distances, since the pentapeptide rings are not encapsulated in the DNA groups as in the minor groove [17]. Finally, it is worth noting that whereas it is possible to justify a B-Z transition under the coupled effect of ACT intercalation and the torsional stress induced by supercoiling, it is not possible to apply these considerations to the case of relaxed DNA, where similar experimental results were still obtained.

These last considerations appear to rule out the models based on the B-Z transition [7] and suggest that, at very low ACT/DNA binding ratios, specific interactions different from intercalation underlie the effect of ACT on the equilibrium between twisting and writhing of DNA. The difficulty of explaining the reported data by variation in DNA twisting leads one to consider the possible influence of ACT on DNA writhing. According to this hypothesis, the topological effect of ACT at low r could derive from the stabilization of the crossing of supercoiled as well as relaxed DNA; in fact, relaxed DNA is an equilibrium of positive and negative supercoils due to thermal fluctuations [18].

To obtain information on the molecular aspects of interactions, we have addressed the question as to whether, in relaxed DNA, ACT is capable of discriminating between positive and negative DNA supercoils. To this end, the handedness of relaxed DNA complexed with ACT, after reaction with topoisomerase I, was determined by two-dimensional electrophoretic analysis, running the second dimension in the presence of EtBr.

As fig. 2 illustrates, DNA supercoils are always left-handed, showing that ACT interaction strongly favours negative supercoils. This result, which is expected for supercoiled DNA, is surprising in the case of relaxed DNA and indicates that the chiral-

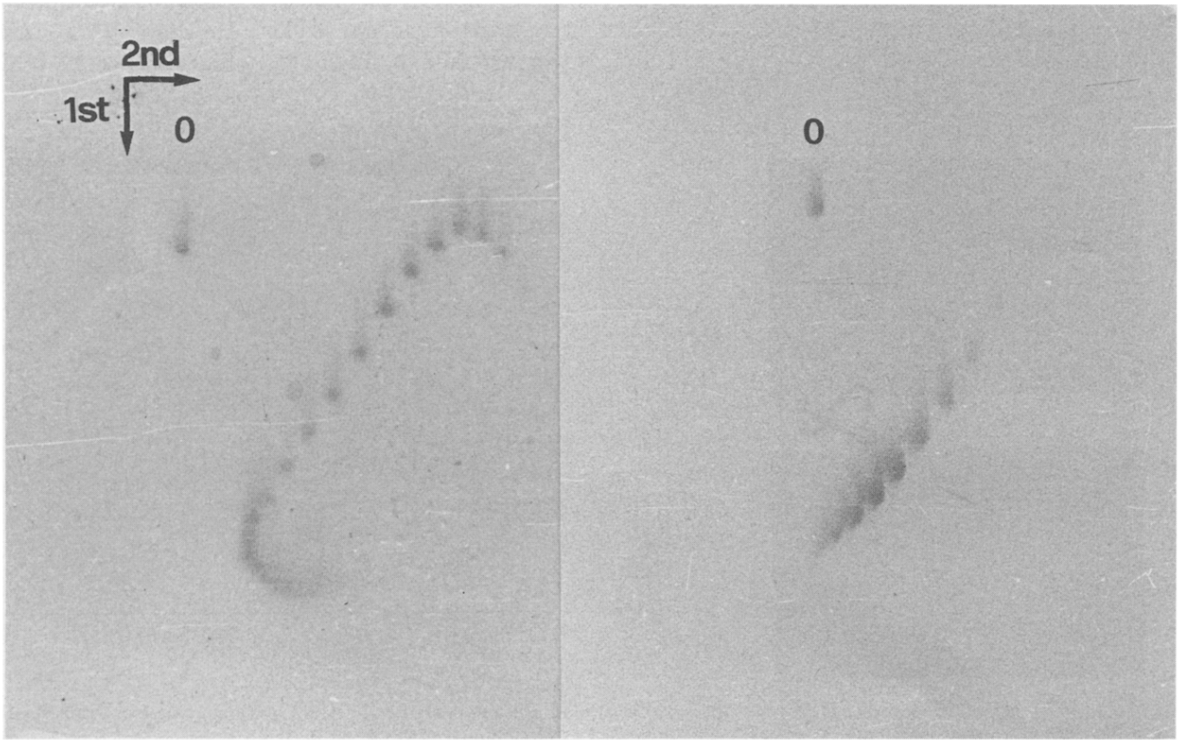

Fig. 2. Analysis of ACT-relaxed DNA complex at $r = 0.004$ (left) and $r = 0.006$ (right) by two-dimensional gel electrophoresis (for experimental details see section 2).

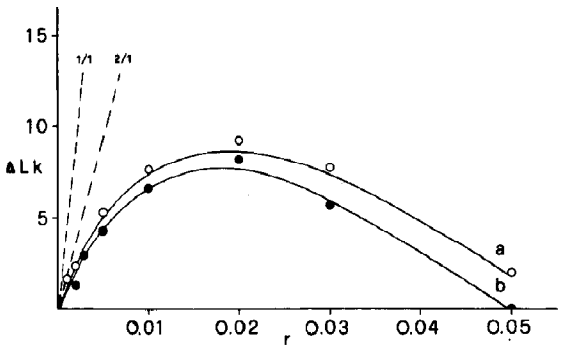

Fig. 3. Theoretical ΔLk curve as a function of r derived as the difference between the experimental ΔLk and theoretical intercalation line; (a) supercoiled DNA; (b) relaxed DNA. Dashed lines, 1/1 and 2/1, represent the theoretical trends in the case of 1/1 and 2/1 ACT-DNA crossing complexes, respectively.

ity of the ACT molecule is relevant in the complex with DNA, in agreement with the previous proposal [3,4] that ACT in DNA complex adopts only one of its two possible chiralities.

Two molecular models of the association complex can be proposed, taking into account the chirality of the ACT molecule, the handedness of DNA supercoils, and the stoichiometry of the complex, namely, whether one DNA supercoil is stabilized by one or two ACT molecules. The trend in the binding was analyzed to obtain evidence on the stoichiometry.

Fig. 3 shows the curves derived by subtracting the theoretical curve for intercalation from the experimental ΔLk curve as a function of r in order to demonstrate the relative trends of unwinding due to intercalation and cross-linking between two different tracts of B DNA. In both supercoiled and relaxed DNA the curves show a maximum corresponding to about 9 supercoils at $r = 0.02$. At higher r, intercalation, increasing the DNA length and stiffness [19], becomes prevalent on cross-linking. One can readily calculate that only a fraction of bound ACT molecules is productive for the stabilization of crossings.

The two dashed lines shown in fig. 3 represent the calculated trends for ΔLk in the case where all ACT molecules stabilize the crossings of DNA. The 1/1 line refers to a model of interaction which involves only one ACT molecule for one crossing, the 2/1 line referring to the case of two ACT molecules. It is evident that the 2/1 line is nearer to the slope of the experimental curves at infinite dilution of ACT; however, taking into account the approximation in the experimental data (we can determine the error in ΔLk measurement to be ± 1), it is difficult to evaluate the stoichiometry only on the basis of these results.

Two possible molecular models of interactions are presented, in the case of 1/1 and 2/1 ACT/DNA stoichiometry, on the basis of the chirality of the ACT molecule and the handedness of DNA crossing.

The two different proposals are shown schematically in figs. 4 and 5. In the first, two guanines, belonging to two different sections of the same DNA molecule, have rotated out of the B-helix and are stacked on the phenoxazone. This model requires the breaking of hydrogen bonds between guanines and cytosines. In fig. 4, a lateral view of the model is schematized in order to indicate the chiral discrimination between negative and positive supercoils. The chirality of ACT fits better with the left-handed crossings of negative supercoils and, additionally, can shift the equilibrium between positive and negative supercoils towards the latter.

An alternative proposal, involving two molecules of ACT for one DNA crossing, is schematically shown in fig. 5. The two ACT molecules are intercalated into two different sections of the DNA molecule, as illustrated in fig. 5d and e. They face each other on the side of the cyclopeptide rings. Beginning from a position in which the phenoxazones of the two molecules lie on the same plane, one molecule must rotate to optimize the van der Waals interactions between the cyclopep-

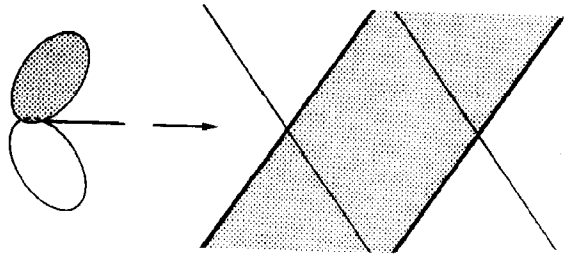

Fig. 4. Schematic illustration of the specific interactions between left-handed crossing of negative DNA supercoils and one ACT molecule.

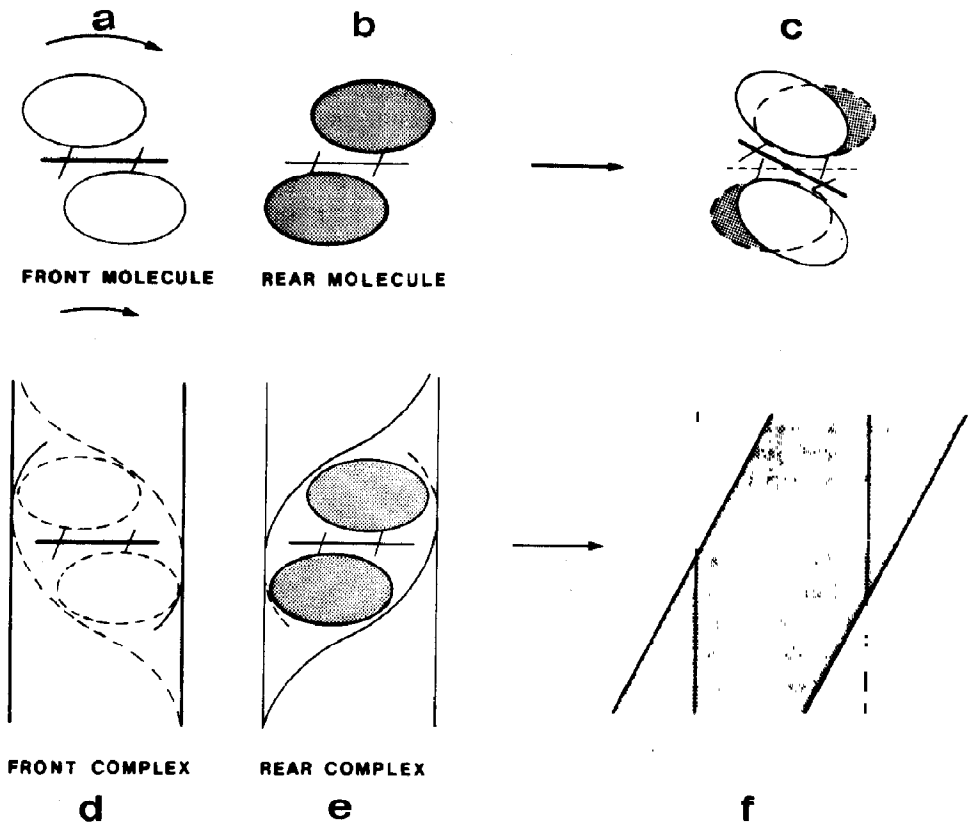

Fig. 5. Schematic depiction of the specific interactions between left-handed crossing of negative DNA supercoils and two ACT molecules. The terms front and rear are relative to the reader.

tide rings, as depicted in fig. 5a–c, where the DNA molecule has been omitted for clarity. Also, in this case the chirality of ACT molecules gives rise to a preference for left-handed crossing of negative supercoils, as shown in fig. 5f, where ACT molecules have been omitted. Also, if our experimental data do not allow one to choose between them due to experimental error, the second model shows better van der Waals contacts and hydrogen bonds between the ACT molecules themselves and with the DNA and does not require the breaking of hydrogen bonds between the DNA bases to intercalate the phenoxazone moiety of the ACT molecule.

In conclusion, it is worth remarking that a relatively small molecule such as ACT, via chiral discrimination, is able to choose between left-handed and right-handed DNA supercoils, a recognition process that, generally, requires specific interactions with proteins.

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