Echinomycin binding to the sequence CG(AT)_nCG alters the structure of the central AT region

Keith R.Fox* and Edwina Kentebe

Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, UK

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ABSTRACT

DNA fragments containing the sequence CG(AT)_nCG have been used in footprinting experiments to assess the effect of echinomycin, which binds to the CG steps, on the structure of the central AT region. DNAase I normally cuts ApT much better than TpA; in the presence of the drug this preference is retained but cleavage at TpA is enhanced. Changes in cleavage by micrococcal nuclease have also been observed. Echinomycin renders alternate adenines hyperreactive to diethylpyrocarbonate. The results suggest that echinomycin induces structural changes in regions surrounding its binding site and that these can be cooperatively propagated over several turns of the DNA helix.

INTRODUCTION

The quinoxaline antibiotics, of which echinomycin is the best studied example, are antitumour agents which act by binding to DNA via the mechanism of bifunctional intercalation [1,2]. Echinomycin has long been known to bind best to GC-rich DNAs [3]; footprinting studies have confirmed this selectivity and identified the preferred site to be the dinucleotide CpG [4,5]. DNAase I footprinting studies have shown that as well producing blockages echinomycin induces enhancements in enzyme activity in regions surrounding its binding site [5]. These are often found in runs of A and T and are probably due to drug-induced changes in DNA structure [5]. Similar changes are observed with actinomycin [6]. Various other enzymic and chemical probes have confirmed that echinomycin and other ligands can induce DNA structural changes in regions remote from their actual binding sites.

An extreme example of DNA structural changes caused by echinomycin is seen in the crystal structures of the ligand bound to short DNA fragments which reveal the presence of Hoogsteen base pairs surrounding the intercalated quinoxaline chromophores [7,8]. The existence of such radical changes has been probed in various solution studies with equivocal results. NMR studies suggest that Hoogsteens form in certain sequences (ACGT) but not others (TCGA) [9]. Footprinting studies with diethylpyrocarbonate [10–13] and osmium tetroxide [12] show that echinomycin does induce structural changes, however, these

are not consistent with the formation of Hoogsteen pairs, and appear to reflect drug-induced unwinding of the DNA helix.

In this study we have probed the ability of echinomycin to alter DNA conformation by constructing DNA fragments which contain regions of alternating AT residues, known to adopt an unusual alternating-B structure which is easy to deform [14,15], adjacent to good echinomycin binding sites. These fragments have been used in footprinting experiments with a variety of cleavage agents known to be sensitive to DNA structure.

MATERIAL AND METHODS

Antibiotics

Echinomycin was obtained from Dr M.J. Waring, University of Cambridge and stored at 4°C as a 1mM solution in dimethylsulphoxide. The drug was diluted to working concentrations immediately before use in Tris-HCl pH 8.0 containing 10mM NaCl.

DNA fragments

Plasmids containing regions of alternating AT adjacent to echinomycin binding sites (CpG) were prepared as follows. Plasmid pUC19 [16] was linearised by digestion with AccI (GT/CGAC) and the ends were filled in with dCTP and dGTP using reverse transcriptase. Poly(dA-dT) was purchased from Pharmacia and dissolved in 10mM Tris-HCl containing 10mM NaCl. 0.1 units of this polynucleotide was digested with DNAase I (15units/ml) for 2 mins to generate short fragments. These were made blunt ended by filling in with dATP and dTTP using reverse transcriptase. The short AT-fragments were ligated into the AccI digested pUC19, transformed into E. coli TG2 and screened for inserts in the usual way by picking white colonies. The resulting fragments were cut with HindIII, labelled with α -³²P dATP using reverse transcriptase and cut again with EcoRI releasing the EcoRI-HindIII polylinker fragments containing the AT insert in the AccI site. The single-end labelled polylinker fragments were isolated from 6% polyacrylamide gels, sequenced by the Maxam-Gilbert method and used as substrates for footprinting experiments. The two fragments used in this work contained the inserts (TA)₅T and (AT)₁₇A within the local sequence AGTCG[CGACCT. In some experiments DNA fragments were

* To whom correspondence should be addressed

Figure 1. DNAase I digestion and DEPC modification of AT inserts in the absence (con) and presence of echinomycin. For the DNAase I digests each pair of lane corresponds to digestion by the enzyme for 1 and 5 mins. The concentration of echinomycin (μ M) is indicated at the top of the gel lanes. Tracks labelled G correspond to dimethylsulphate- piperidine markers specific for guanine. Arrows indicate guanines which are part of the echinomycin binding sites (CpG) flanking the inserts. The alternating AT inserts are indicated by square brackets.

labelled at the EcoRI end by changing the order of addition of EcoRI and HindIII.

Footprinting

DNAase I and micrococcal nuclease (MNase) footprinting and diethylpyrocarbonate (DEPC) modification were performed as

previously described [5,6,11,12,17]. Products of digestion were resolved on 10–15% polyacrylamide gels containing 8M urea, these were run at 1500V for 2hr before fixing in 10% acetic acid and drying under vacuum at 80°C. The dried gels were exposed to autoradiography at -70°C with an intensifying screen. Autoradiograms were scanned using a Joyce-Loebl chomscan 3.

Figure 2. Densitometer traces of DNAase I digests of the AT inserts in the presence and absence of echinomycin.

Figure 3. Micrococcal nuclease digestion of the AT inserts in the absence (CON) and presence of echinomycin. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 mins. Drug concentrations (μM) are shown at the top of the gel lanes. The alternating AT inserts are indicated by square brackets.

RESULTS

DNAase I footprinting

Figure 1 presents DNAase I digests of the $(\text{TA})_5\text{T}$ and $(\text{AT})_{17}\text{A}$

containing DNA fragments in the presence and absence of echinomycin. In the absence of the antibiotic both fragments show cleavage of ApT but not TpA bonds, as reported by other workers

B

S S w S w S w S w S w S w S S

5'-. .GAGTCGATATATATATATATATATATATATATATATATACGACCT. .-3'

3'-. .CTCAGCTATATATATATATATATATATATATATATATATATATGCTGGA. .-5'

S S S w S w S w S w S w S w

Figure 4. a) Densitometer traces of MNase digestion of the AT inserts in the absence (control) and presence (echy) of 100 μ M echinomycin. The crosses indicate products corresponding to cleavage of the TpA bonds. b) Echinomycin induced susceptibility to DEPC modification in the (AT)₁₇A insert. S and w indicate bonds which are strongly and weakly enhanced respectively.

[14,15]. In the presence of echinomycin clear protections are visible around each of the CpG steps; especially noteworthy are those on either side of the insert. More bonds are protected towards the upper (5') side of the inserts, since DNAase I footprints are staggered towards the 3'-side of drug binding sites. As well as these footprints other changes can be seen within the regions of alternating AT. For the (TA)₅T insert drug concentrations of 5 μ M and above show near normal cleavage of the central three ApT bonds, retaining the clear preference for ApT over TpA. However, there is an increased cutting at the intermediate TpA steps so that products resulting from their cleavage are now visible. Densitometer traces for the cleavage of this insert are presented in Figure 2, and confirm the increased cleavage at TpA. For the (AT)₁₇A insert, low echinomycin concentrations (5 μ M) cause increased cleavage at TpA while cutting at ApT is unaffected. Higher concentrations show a reduced cleavage at all bonds in the insert but retain increased cleavage at TpA. The centre of this region is 17 bases (one and a half helical turns) away from an echinomycin binding site. It appears that drug-induced changes in DNA structure in runs of alternating AT are highly cooperative. Similar results are obtained with DNA fragments labelled at the opposite end (EcoRI site). These effects can be seen more clearly in the densitometer traces presented in Figure 2, which confirm the increased cleavage at the TpA steps. It is worth noting that the alterations in nuclease digestion of the AT inserts appear at slightly lower concentrations (5 μ M) than the corresponding footprints at the CpG sites. This differential effect has been noted previously [18] and may suggest that levels of binding which are not sufficient to protect from enzyme attack may yet cause DNA structural changes.

Micrococcal nuclease footprinting

Figure 3. presents the results of micrococcal nuclease (MNase) digestion of the two DNA fragments in the presence and absence of echinomycin. In each case cleavage in the controls is almost exclusively within the insert since the enzyme cleaves only pA and pT bonds and is especially active in runs of alternating A and T. In the presence of echinomycin the outermost cleavage products are absent, an effect which probably results from steric occlusion by the adjacent drug molecule. Cleavage in the rest of the AT insert is modified by the drug. In the drug-free lanes the enzyme has a small (about 50%) preference for cutting TpA over ApT; this preference is altered by the antibiotic generating a more even ladder of cleavage products. Densitometer traces of these data are presented in Figure 4a and confirm the more even cleavage pattern, although there is still a weak preference for cleavage of TpA. The bands at the very bottom (3') are more intense than those closer to the top since MNase possesses a 5'-3' exonuclease activity in addition to its endonuclease properties.

Modification by diethylpyrocarbonate (DEPC)

The pattern of DEPC mediated DNA strand cleavage of the (AT)₁₇A containing fragment in the presence and absence of echinomycin is presented in Figure 1. No cleavage products are visible in the control lanes since this reagent has only weak interaction with double stranded DNA. However, in the presence of echinomycin (5 μ M and above) adenines throughout the insert become hyperreactive to DEPC. The extreme bands, below each of the arrows, correspond to adenines which are distal to echinomycin sites in the sequence CGA. In contrast the only proximal adenine (ACG) at the lower (3')-end of the insert remains unreactive. On inspection an alternating pattern of

cleavage products is evident in which every other adenine is more reactive to the probe. This unusual effect is confirmed by similar experiments on the fragment labelled at the opposite (EcoRI) end (not shown). When data across the two strands are compared (Figure 4b) bands which become hyperreactive to DEPC are found to occur at adjacent adenines which are staggered in the 5' direction.

DISCUSSION

The results presented in this paper demonstrate that echinomycin, binding to the sequence CpG, can alter the conformation of adjacent regions of alternating AT. Cleavage by DNAase I and MNase as well as modification by DEPC are all affected by the antibiotic.

It has previously been shown that alternating AT tracts in random sequence DNA possess an unusual structure which is relatively easy to deform [13,14]. In this structure the poor TpA base stacking is sacrificed to improve the better ApT stacking interaction [13,19]. As a result relatively large changes in structure will only have a slight effect on the energetics, especially at the TpA steps. Since this structure can be easily deformed it is not surprising that ligand binding to adjacent regions can have significant effects. We postulate that in the presence of echinomycin these regions more closely resemble B-DNA, and that stacking at the TpA step is improved. This is confirmed by changes in MNase specificity; in the control TpA is cut best, consistent with its poor base stacking, whereas in the presence of echinomycin cleavage of TpA is more nearly equal to ApT.

These results contrast with previous studies on the effects of actinomycin on the structure of flanking regions of AT [20,21]. In these studies short oligonucleotides containing central actinomycin sites (GC) were investigated by footprinting and NMR and the authors concluded that drug binding stabilised the unusual alternating-B structure. These differences could arise from the nature of the DNA binding ligand; echinomycin is a bifunctional intercalator whereas actinomycin interacts in a monofunctional fashion. Differences could also be due to the length of the DNA fragments under investigation. In this study we have been careful to incorporate the alternating AT regions within longer DNA fragments thereby avoiding end effects and generating a model which more closely resembles the binding of drug to long DNA fragments. It is worth emphasising that these results can not be explained by suggesting the redistribution of DNAase I in the presence of the antibiotic. In an elegant series of quantitative footprinting experiments Ward et al [22] demonstrated that the DNAase I enhancements induced by actinomycin on a 139 base pair DNA fragment were not consistent with DNA structural changes but could be explained by changes in the relative concentration of free DNA to enzyme in the presence of the antibiotic. Such a mechanism can not account for the repetitive changes that we observe in the regions of alternating AT.

The observation that DNAase I cleavage of the longer AT insert is reduced by elevated echinomycin concentrations is interesting. Two explanations can be postulated; either the drug induces a significant proportion of DNA molecules to adopt a conformation which is resistant to DNAase I, or echinomycin itself can bind to such sequences if they are part of longer regions. The lack of such an effect on the shorter AT insert suggests the latter possibility. Echinomycin does not protect AT steps from DNAase I attack in natural DNA sequences [4,5]. However, equilibrium

binding data have shown that the drug binds strongly to poly(dA-dT) ($K=3 \times 10^5 \text{M}^{-1}$) and that this is a highly cooperative process [3]. The cooperative nature of the structural changes is evident since cleavage at TpA is enhanced throughout the insert.

Previous studies have shown that echinomycin renders adenines proximal and distal to its CG binding site hyperreactive to DEPC [10–13]. This was originally interpreted as providing evidence for the formation of Hoogsteen base pairs. However, the major site of reaction of DEPC is at purine N7; in Hoogsteen base pairs this position is involved in base pair hydrogen bonding and should be less accessible to chemical modification. Various NMR studies have shown that the formation of Hoogsteen pairs is not a necessary consequence of echinomycin binding and may occur at some sequences (such as ACGT) and not others (TCGA) [9]. When echinomycin binds to the octanucleotide ACGTACGT the central AT base pairs only form stable Hoogsteens at low temperatures, in contrast to the unconstrained terminal AT pairs which remain in the Hoogsteen conformation at temperatures up to 45°C [23]. It therefore seems likely that the enhancements in reactivity towards DEPC reflect more subtle changes in DNA structure, such as drug-induced unwinding of the DNA helix.

The alternating pattern of adenines hyperreactive to DEPC is puzzling but again could be the result of echinomycin binding within the insert. If this does occur and if the drug binds exclusively to one of TA or AT then alternate adenines would be inside and outside the sandwiched dinucleotide. These would only be staggered in the 5' direction across the two strands if binding occurs at ApT (TpA would give the opposite stagger). Further studies will be necessary to clarify this issue.

We have demonstrated that echinomycin can alter the structure of adjacent AT sequences. It remains to be seen whether this is a general phenomenon, or if it is limited to sequences which are easy to deform.

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