Footprinting studies on the effect of echinomycin on the structure of a bent DNA fragment

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The interaction of echinomycin with a kinetoplast DNA fragment which contains phased runs of adenine residues has been examined by various footprinting techniques. DNAase I footprinting confirms that all drug-binding sites contain the dinucleotide CpG. However, not all such sequences are protected. Three sites, each of which is located between two adenine tracks in the sequence GCGA, are not protected from DNAase I attack. Enhanced cleavage by DNAase I, DNAase II and micrococcal nuclease is observed in regions surrounding drug-binding sites. The results suggest that echinomycin alters the conformation of the AT tracks, making them more like an average DNA structure. Echinomycin renders adenine residues in the sequence CGA hyper-reactive to diethyl pyrocarbonate.

INTRODUCTION

The quinoxaline antibiotics, of which echinomycin is the best-studied example, are antitumour agents which act by binding to DNA [1]. This they do by the mechanism of bifunctional intercalation, in which the two quinoxaline chromophores are simultaneously inserted into the DNA helix, forming a two-base-pair sandwich [2]. Echinomycin has long been known to bind best to GC-rich DNAs [3]. More recent footprinting studies have confirmed this selectivity and identified the binding site to be the dinucleotide CpG [4,5]. DNAase I footprinting studies have revealed that, as well as producing blockages at its binding sites, echinomycin, in common with other sequence-selective ligands, induces enhancements in enzyme activity in surrounding regions [5]. These enhancements are frequently found in runs of A and T and have been interpreted as resulting from drug-induced changes in DNA structure [5,6].

Crystal structures of echinomycin bound to several short DNA fragments have been determined and reveal the unusual feature that the bases surrounding the CpG-binding site are in the Hoogsteen, rather than the normal, Watson–Crick, configuration [7,8]. Various solution studies have attempted to confirm this observation [9–14]. N.m.r. studies with echinomycin complexed to tetranucleotides have shown that Hoogsteen pairs may form with ACGT but not TCGA [9]. Several chemical probes have been used to determine the occurrence of these structures in longer DNA fragments, the most notable being diethyl pyrocarbonate (DEPC) [11–14]. The general conclusion is that echinomycin renders the DNA more reactive to several chemical agents. However, these changes can not be accounted for by invoking the formation of Hoogsteen pairs and are best explained by some unwinding of the DNA duplex.

In the present study we investigated the effects of echinomycin on a kinetoplast DNA fragment. This DNA contains many runs of adenine residues spaced at 10 bp intervals, and as a result is highly bent [15,16]. We have assessed the effect of echinomycin on the unusual structure and how this may limit drug binding.

MATERIALS AND METHODS

Echinomycin was obtained from Dr. M. J. Waring (Department of Pharmacology, University of Cambridge, Cambridge, U.K.) and stored as a 1 mM stock solution in dimethyl sulphoxide

at 4 °C. The antibiotic was diluted to working concentrations in aqueous buffers immediately before use. Because of its very low aqueous solubility, the antibiotic precipitates from solution on dilution, so that concentrations quoted are only approximate.

DNA fragments

Plasmid pPK201/CAT, containing the kinetoplast DNA [15], was a gift from Dr. T. D. Tullius (Department of Chemistry, Johns Hopkins University, Baltimore, MD, U.S.A.), and was transformed into *Escherichia coli* TG2. The 270 bp (*Eco*R1–*Hind*III) kinetoplast DNA fragment was isolated and labelled by digestion with either *Hind*III or *Eco*R1, labelled with [α -³²P]dATP using reverse transcriptase and cleaved again with the other enzyme. The labelled product was eluted from a 5%-(w/v)-polyacrylamide gel. The sequence of this kinetoplast DNA fragment is shown in Fig. 2 (below).

Footprinting

Enzymic footprinting with DNAase I, DNAase II and micrococcal nuclease (MNase) was performed as previously described [5,6,17]. Reaction with DEPC was achieved by mixing 5 μ l of a drug–DNA complex with 5 μ l of DEPC for 10 min at 37 °C. The reaction was stopped by dilution and precipitation before boiling the samples in 10% (v/v) piperidine followed by freeze-drying. The products of footprinting reactions were resolved on 6% polyacrylamide gels containing 8 M-urea. Gels were fixed in 10% (v/v) acetic acid before drying under vacuum at 80 °C and autoradiography at –70 °C using an intensifying screen.

RESULTS

DNAase I footprinting

Fig. 1 shows the results of DNAase I digestion of the kinetoplast DNA fragment in the presence or absence of various concentrations of echinomycin. In the absence of antibiotic, cleavage of the DNA is uneven, so that gaps in the ladder of bands are visible at positions 58–62, 67–72, 77–83, 92–95, 110–115, 121–125, 130–135, 149–156, 161–166 and 180–186 on the top strand and positions 173–178, 182–189, 162–166, 133–137, 123–127 and 111–118 on the bottom strand. Each of these corresponds to a run of A or T residues. In the presence of

Abbreviations used: DEPC, diethyl pyrocarbonate; MNase, micrococcal nuclease.

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Fig. 1. DNAase I footprinting of the kinetoplast DNA fragment labelled at the 3' end of either strand in the absence (con) or presence of various concentrations (5, 25 or 100 μ M) of echinomycin

The drug concentration (in μ M) is shown at the top of the gel lanes. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The position numbers and strand designation correspond to the sequence shown in Fig. 2. The track labelled 'G' is a Maxam-Gilbert dimethyl sulphate/piperidine marker specific for guanine.

echinomycin, the pattern of cleavage is very different: several regions of protection can be discerned, as well as some regions of enhanced cleavage; these are summarized in Fig. 2. Potential echinomycin-binding sites (CpG) are present at positions 34, 57, 73, 85, 106, 136, 147, 198, 219, 221 and 228. Inspection of Figs. 1 and 2 reveals that protection from cleavage is not identical at all these sites, so that, for the labelled top strand, positions 34, 136 and 198 produce clear blockages, positions 57 and 73 are weakly protected, and positions 85, 106 and 147 are unaffected by the presence of the antibiotic. A similar pattern emerges with the label on the bottom strand; positions 198, 73, and 57 are well protected, position 136 is weakly protected and 147, 106 and 85 are hardly affected. It appears that the CpG dinucleotides at positions 85, 106 and 147 do not present good echinomycin-binding sites as detected by DNAase I. The reasons for these differences will be considered further in the Discussion section. Regions of enhanced DNAase I cleavage are also evident.

These are clearest on the labelled top strand and can be seen at positions 44–47, 78–81, 88–91, 99–101, 110–119 and around positions 160, 180 and 205. Most of these enhancements are found in runs of A and T residues as anticipated, though many are not close to good echinomycin-binding sites. For example no CpG sites occur between positions 147 and 198, yet this region contains at least two areas of enhanced cleavage. Although the CG at position 85 does not yield a DNAase I footprint, there are distinct enhancements on either side of this region. Other CG sites, such as those at positions 57 and 73, do not result in enhancements in the surrounding runs of A or T.

Micrococcal nuclease and DNAase II

Digestion patterns for these two enzymes in the presence and absence of echinomycin are presented in Fig. 3 for the kinetoplast DNA bearing a label on the top strand. As expected, MNase does not produce footprints, since this enzyme cuts only pA and

pT bonds. The only exceptions where protection from cutting is evident are at positions 97 and 84. The former does not represent a canonical echinomycin-binding site (though see below), whereas the latter was not protected from DNAase I attack. The pattern of MNase digestion products is, however, very different in the presence of echinomycin. In general more bands are visible in the drug-treated lanes. Although this DNA fragment is relatively AT-rich, several such regions are absent or poorly cut in the control lanes. It has previously been noted that regions of alternating A and T are cut much better by this enzyme than homopolymeric runs of A and T [17]. In the presence of echinomycin, several such runs are cleaved much more readily. This is especially noteworthy around positions 60, 70, 80, 95 and 100 (see Fig. 2).

close to canonical echinomycin-binding sites (53 and 67), whereas others are 10 (or more) bp from the closest CpG (162, 128, 43).

Patterns of DEPC-mediated DNA strand cleavage in the presence or absence of echinomycin are presented in Fig. 4 for kinetoplast DNA labelled on either DNA strand. Reaction in the control lanes is weak and occurs mostly at purine residues. In the presence of echinomycin, several bands show a marked increase in reactivity (summarized in Fig. 2). At the lowest echinomycin concentrations, clear enhancements can be seen at positions 71, 83, 104, 134, 145, 196 and 226 on the labelled top strand. Fewer enhancements are visible on the bottom strand, but can be seen at positions 223, 206, 183, and 131. All the enhancements on the top strand are at adenine residues in the sequence CGA. The lower strand contains only one such sequence (222), which is also rendered hyper-reactive to DEPC. It appears that adenine residues immediately distal to echinomycin sites are rendered hyper-reactive to DEPC. This DNA fragment only possesses one adenine residue that is proximal to an echinomycin site (i.e. ACG), namely that at position 57, and the reactivity of this base is not altered by the presence of the antibiotic. At elevated echinomycin concentrations, additional sites of DEPC hyper-reactivity are visible on the labelled top strand, notably at positions 36 (TGA), 44 (TCA), 50 (TGA), 62 (CCA), 94 (TGA), 114 (CA4) and 156 (CCA). These additional bands could arise from either longer range perturbations in DNA structure or from

Fig. 3. MNase and DNAase II footprinting of the kinetoplast DNA fragment labelled at the 3'-end of the *Hind*III site (top strand in Fig. 1) in the absence (con) or presence of various echinomycin concentrations

Details are as for Fig. 1.

secondary drug-binding sites (not CpG). We favour the second possibility.

DISCUSSION

Sequence selectivity

DNAase I footprinting of echinomycin on this DNA fragment confirms the previous observations that protections from cleavage are located around the dinucleotide CpG. However, not all such sequences are protected; those at positions 85, 106 and 147 do not present good binding sites. Each of these is within the sequence GCGA (TCGC). The only other such sequence on this DNA fragment is found at position 222, where it is not possible to assess the extent of protection, since it overlaps with an adjacent CG. This tetranucleotide is not present on the tyrT DNA which was used in the previous DNAase I footprinting

Fig. 4. DEPC modification of the kinetoplast DNA fragment, labelled at each 3'-end in the presence of various concentrations of echinomycin

The drug concentration (in μM) is shown at the top of each gel lane. 'G' is explained in Fig. 1.

study [5]. Despite the lack of DNAase I footprints at these positions, the adenine residues become hyper-reactive to DEPC, suggesting that drug binding is not prohibited, but is weakened, so that DNAase I is no longer a useful footprinting tool. This is confirmed by the results with MNase, which reveal reduced enzyme attack around position 85. It has previously been suggested that MNase is better at detecting weakly bound ligands than is DNAase I [17].

Why then does echinomycin bind less well to this CG site than all the others? It is possible that this is not merely a function of this sequence *per se*, but of the regions surrounding it. All three of these sites have blocks of A (or T) residues very close on both sides, whereas the other CG sites do not. Bending of the DNA fragment is presumed to occur at junctions between the runs of A and surrounding sequences [16], so that, when a CG is sandwiched between two such structural motifs, the ability to bind echinomycin is reduced. Such an effect may not be peculiar

to echinomycin, since actinomycin D (binding to GC) fails to reveal a footprint around position 65 (K. R. Fox & K. Waterloh, unpublished work).

At low echinomycin concentrations, all the adenine residues which become hyper-reactive to DEPC are immediately adjacent to the dinucleotide CpG. However, on raising the drug concentration, more enhanced bands appear. The simplest explanation for this is that the drug is binding to other (weaker) sites and inducing similar conformational changes in the surrounding bases. These additional sites are often found at NpG or CpN, so that only one of the two base-pairs is 'incorrect'. The existence of secondary binding sites has been suggested by footprinting studies using methidiumpropyl-EDTA [4], as well as by equilibrium-binding isotherms for a variety of natural and synthetic DNAs [3]. These secondary binding sites are never detected by DNAase I and serve to emphasize the importance of employing more than one footprinting probe for rigorously defining preferred drug-binding sites.

DNA structural changes

The kinetoplast DNA fragment is known to possess an unusual structure on account of its phased runs of adenine residues. This results in the DNA possessing an anomalously low mobility on non-denaturing polyacrylamide gels. Drugs such as distamycin, which bind to AT-rich regions, have been shown to remove the bending and so increase DNA mobility. Can echinomycin, binding to regions outside the runs of A and T, alter DNA bending? Echinomycin itself has been shown to have no effect on the mobility of DNA in polyacrylamide gels, presumably because its binding is not strong enough and the drug dissociates during electrophoresis.

All three nucleases employed in the present work reveal enhanced cleavage in regions surrounding echinomycin-binding sites. The most dramatic effect is seen with MNase, for which many regions which are refractory to cleavage in the drug-free control become sensitive to enzyme attack. Runs of A or T are resistant to MNase attack because of their increased stability relative to regions of alternating A and T; witness the fact that poly(dA)·poly(dT) 'melts' at a higher temperature than does poly(dA-dT) [19]. This increased stability results from the high propeller twist, which increases the base stacking and results in the formation of an extra bifurcated hydrogen bond [20]. It appears that, when echinomycin binds next to these regions, their structure is modified so that it more closely resembles that of B-DNA. The enhanced rates of cleavage for all three enzymes occur in similar positions. From these data we suggest that echinomycin has removed much of the DNA-bending associated with the runs of A residues. This has been achieved by binding to adjacent regions.

The regions which become hypersensitive to DEPC modification are similar to those reported for other DNA

fragments. At low echinomycin concentrations only adenine residues in the sequence CGA are affected; the one ACG site is unaffected. We are uncertain as to the origin of this difference, but it probably originates from subtle changes in the local unwinding geometries when the quinoxaline chromophores are inserted between purine-purine or purine-pyrimidine steps. N.m.r. studies on tetranucleotides have shown that echinomycin can induce the formation of Hoogsteen base-pairs in the sequence ACGT, but not in TGCA, the opposite of the changes seen with DEPC, so that it seems unlikely that the changes in reactivity observed here are due to Hoogsteen-pair formation.

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REFERENCES

1. Waring, M. J. & Fox, K. R. (1983) in *Molecular Aspects of Anti-Cancer Drug Action* (Neidle, S. & Waring, M. J., eds.), pp. 127-156, Macmillan, London
2. Waring, M. J. & Wakelin, L. P. G. (1974) *Nature (London)* **252**, 653-657
3. Wakelin, L. P. G. & Waring, M. J. (1976) *Biochem. J.* **157**, 721-740
4. Van Dyke, M. W. & Dervan, P. B. (1984) *Science* **225**, 1122-1127
5. Low, C. M. L., Drew, H. R. & Waring, M. J. (1984) *Nucleic Acids Res.* **12**, 4865-4879
6. Fox, K. R. & Waring, M. J. (1984) *Nucleic Acids Res.* **12**, 9271-9285
7. Ughetto, G., Wang, A. H. J., Quigley, G. J., van der Marel, G., van Boom, J. H. & Rich, A. (1985) *Nucleic Acids Res.* **13**, 2305-2323
8. Wang, A. H.-J., Ughetto, G., Quigley, G. J. & Rich, A. (1986) *J. Biomol. Struct. Dyn.* **4**, 319-342
9. Gao, X. & Patel, D. J. (1988) *Biochemistry* **27**, 1744-1751
10. Gilbert, D. A., van der Marel, G. A., van Boom, J. H. & Feigon, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3006-3010
11. Portugal, J., Fox, K. R., Mclean, M. J., Richenberg, J. L. & Waring, M. J. (1988) *Nucleic Acids Res.* **16**, 3655-3670
12. Mendel, D. & Dervan, P. B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 910-914
13. Jeppesen, C. & Nielsen, P. E. (1988) *FEBS Lett.* **231**, 172-176
14. McLean, M. J. & Waring, M. J. (1988) *J. Mol. Recognition* **1**, 138-151
15. Kitchin, P. A., Klein, V. A., Ryan, K. A., Gann, K. L., Rauch, C. A., Kang, D. S., Wells, R. D. & Englund, P. T. (1986) *J. Biol. Chem.* **261**, 11302-11309
16. Burkhoff, A. M. & Tullius, T. D. (1987) *Cell (Cambridge, Mass.)* **48**, 935-943
17. Fox, K. R. & Waring, M. J. (1987) *Biochim. Biophys. Acta* **909**, 145-155
18. Drew, H. R. & Travers, A. A. (1984) *Cell (Cambridge, Mass.)* **37**, 491-502
19. Riley, M., Maling, B. & Chamberlain, M. J. (1966) *J. Mol. Biol.* **20**, 359-389
20. Nelson, H. C. M., Finch, J. T., Bonaventura, F. L. & Klug, A. (1987) *Nature (London)* **330**, 221-226

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