Localized chemical reactivity in DNA associated with the sequence-specific bisintercalation of echinomycin

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Four complementary footprinting and probing techniques utilizing DNAase I, methidiumpropyl EDTA (MPE)·Fe^{II}, diethyl pyrocarbonate (DEPC) and KMnO₄ as DNA-cleaving or DNA-modifying agents have been applied to investigate the sequence-specific binding to DNA of the antitumour antibiotic echinomycin. A 265 bp *EcoRI*–*PvuII* DNA restriction fragment excised from plasmid pBS was used as a substrate. Six regions of protection against DNAase I cleavage were located on the 265-mer: three sites encompass the sequences 5'-TCGA or 5'-GCGT and the three others contain 5'-GpG (CpC) dinucleotide sequences where the inhibition of DNAase I cutting by echinomycin is less pronounced. In contrast, MPE·Fe^{II} cleavage allows identification of only three echinomycin-binding sites on the 265-mer: two sites contain the sequence 5'-TCGA and one encompasses the sequence 5'-ACCA. Cleavage of DNA by MPE·Fe^{II} in the presence of echinomycin remains practically unaffected at the sequence 5'-GCGT, despite its identification by

DNAase I as a strong site for binding the antibiotic, as well as at the two other sequences containing GpG steps. With both DNAase I and MPE·Fe^{II}, enhanced DNA cleavage is evident at AT-rich sequences in the presence of echinomycin. Enhanced reactivity towards KMnO₄ and DEPC provides clear evidence for sequence-dependent conformational changes in DNA induced by the antibiotic. The experiments reveal that KMnO₄ reacts most strongly with thymines located around, but not necessarily adjacent to, an echinomycin-binding site, whereas the carbethoxylation reactions caused by DEPC occur primarily at the adenine residues lying immediately 5' or 3' to the dinucleotide that denotes an echinomycin-binding site. The results reported here demonstrate that DEPC and KMnO₄ serve as sensitive probes for different states of the DNA helix. It seems that the reaction with KMnO₄ involves transient unstacking events, whereas the carbethoxylation reaction of DEPC requires larger-scale helix opening.

INTRODUCTION

Bisintercalation of the antitumour antibiotic echinomycin (Figure 1) causes considerable unwinding of the DNA which allows the two quinoxaline chromophores to slip between the base pairs of the double helix and the cyclic peptide portion to fit in the minor groove [1–3]. It is now well established that this quinoxaline depsipeptide opts preferentially for CpG sequences [3,4]. The sequence selectivity is apparently mediated by the formation of three hydrogen bonds between the alanine residues of the ligand and the N-3s and the 2-amino group of the guanines in the recognition site [5–8]. Recent studies from our laboratory have emphasized the determinant role of the exocyclic 2-amino group of guanine exposed in the minor groove for echinomycin–DNA sequence-specific recognition [9,10].

Knowledge of sequence-selective recognition of DNA by drugs usually comes from footprinting studies. Substantial, but rather widely dispersed, literature exists on the footprinting of echinomycin to DNA. Among the DNA-cleaving agents employed to detect echinomycin-binding sites on DNA, there have been enzymes such as DNAase I [11–15], DNAase II [11] and micrococcal nuclease [16]. In other studies, transition-metal complexes, such as methidiumpropyl EDTA (MPE)·Fe^{II} [17,18], and acridine-based photosensitizing reagents [19] have been employed, together with a variety of DNA conformational probes including diethyl pyrocarbonate (DEPC), KMnO₄, potassium tetrachloropalladate and osmium tetroxide [20–23]. Despite the wide range of cleaving agents used to date, there is no

report systematically comparing the information yielded by these different probes. Accordingly, as part of our continued effort to understand the molecular aspects of the interaction of quinoxaline antibiotics with their DNA target, we have performed a direct comparative study employing (i) DNAase I and MPE footprinting procedures to analyse the sequence-preferential binding of echinomycin to DNA and (ii) KMnO₄ and DEPC as probes to detect echinomycin-induced conformational changes in DNA. A restriction fragment 265 bp long obtained from plasmid pBS was used throughout the study. This piece of DNA was deliberately chosen because it contains only a few CpG steps and in particular no 5'-ACGT tetranucleotide sequences to which echinomycin binds avidly [17,18]. The use of a DNA fragment lacking the most favoured echinomycin-binding sites provides opportunities for investigating binding to less favoured (e.g. 5'-TCGA) and secondary (e.g. GpG, TpG) sites, affording a more sensitive means of examining the relative ability of different footprinting probes to detect weak interactions.

MATERIALS AND METHODS

Antibiotic

Echinomycin was obtained from Parke-Davis and dissolved to a concentration of 100 µM in 10 mM Tris/HCl, pH 7.0, containing 10 mM NaCl and 40% (v/v) methanol. The stock solution was diluted to working concentrations with appropriate volumes of this buffer so as to yield a final methanol concentration of 10% (v/v) in the footprinting reactions. Under these conditions

Abbreviations used: MPE, methidiumpropyl EDTA; DEPC, diethyl pyrocarbonate.

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Figure 1 Structure of echinomycin

methanol is known not to affect the nuclease activity [11,22]. An absorption coefficient of $11\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$ was used to determine echinomycin concentration from absorbance measurements at 325 nm [2].

Chemicals and biochemicals

Ammonium persulphate, Tris base, acrylamide, bisacrylamide, ultrapure urea, boric acid, tetramethylethylenediamine and dimethyl sulphate were from BDH. Formic acid, piperidine, hydrazine and formamide were from Aldrich. Photographic requisites were from Kodak. Bromophenol Blue and xylene cyanol were from Sigma Chemical Co. Nucleoside triphosphates labelled with ^{32}P (α -dATP and γ -ATP) were obtained from NEN-Dupont. Unlabelled dATP was Ultra-pure grade from Pharmacia. Restriction endonucleases *Eco*RI and *Pvu*II (Boehringer Mannheim, Germany) were used according to the supplier's recommended protocol in the activity buffer provided. Alkaline phosphatase, T4 polynucleotide kinase and avian myeloblastosis virus reverse transcriptase were from Pharmacia. Bovine pancreatic DNAase I (EC 3.1.21.1; Sigma Chemical Co.) was stored at a concentration of 7200 units/ml in a solution of 20 mM NaCl, 2 mM MgCl_2 and 2 mM MnCl_2 , pH 8.0, at -20°C , and was freshly diluted to the desired concentration immediately before use. MPE was a gift from Professor Peter Dervan (California Institute of Technology, Pasadena, CA, U.S.A.). All other chemicals were analytical-grade reagents, and all solutions were prepared by using doubly deionized Millipore-filtered water.

DNA purification and labelling

The plasmid pBS (Stratagene, La Jolla, CA, U.S.A.) was isolated from *Escherichia coli* by a standard SDS/NaOH lysis procedure and purified by banding twice in CsCl/ethidium bromide gradients. Ethidium was removed by several propan-2-ol extractions followed by exhaustive dialysis against Tris/EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer before digestion by the restriction enzymes. Amplification of plasmid copy numbers was effected by treatment of bacteria with chloramphenicol. The DNA fragment for footprinting was prepared by 3'- ^{32}P -end-labelling of the *Eco*RI-*Pvu*II double digest of pBS plasmid using [α - ^{32}P]dATP (6000 Ci/mmol) and AMV reverse transcriptase or by 5'- ^{32}P -

end-labelling of the *Eco*RI/alkaline phosphatase-treated pBS plasmid using [γ - ^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase followed by treatment with *Pvu*II. The digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE buffer (89 mM Tris/borate, pH 8.3, 1 mM EDTA). After autoradiography, the band corresponding to the 265 bp fragment was excised, crushed and soaked in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) overnight at 37°C . This suspension was filtered through a Millipore 0.22 μm filter, and the DNA was precipitated with ethanol. After washing with 70% ethanol and vacuum drying of the precipitate, the labelled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

DNAase I footprinting

These experiments were performed essentially according to the original protocol of Drew and Travers [24] as described in [11]. Reactions were conducted in a total volume of 10 μl . Samples (3 μl) of the labelled DNA fragment were incubated with 5 μl of the buffer solution containing the desired echinomycin concentration. After 30–60 min incubation at 37°C to ensure equilibration of the binding reaction, the digestion was initiated by the addition of 2 μl of a DNAase I solution, the concentration of which was adjusted to yield a final enzyme concentration of about 0.01 unit/ml in the reaction mixture. The extent of digestion was limited to less than 30% of the starting material so as to minimize the incidence of multiple cuts in any strand ('single-hit' kinetic conditions). Optimal enzyme dilutions were established in preliminary calibration experiments. After 3 min, the digestion was stopped by the addition of 3 μl of an 80% formamide solution containing tracking dyes. Samples were heated at 90°C for 4 min and chilled in ice for 4 min before electrophoresis.

MPE footprinting

This was carried out by the procedure initially developed by Van Dyke and Dervan [25]. Briefly, 2 μl of labelled DNA (~ 500 c.p.s.) was mixed with 5 μl of echinomycin at the desired concentration, and the drug-DNA complex was left to equilibrate for at least 30 min at 37°C . Successively 1 μl of MPE (100 μM ; portions stored at -20°C), 1 μl of a freshly prepared 100 μM solution of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$, then 1 μl of dithiothreitol

(40 mM) were added to initiate the cleavage reaction. After 4 min incubation at room temperature, the reaction was stopped by freeze-drying. Samples were freeze-dried and washed at least twice with 50 μ l of water. The DNA in each tube was resuspended in 5 μ l of formamide/TBE loading buffer, denatured at 90 °C for 4 min then chilled in ice for 4 min before loading on to the sequencing gel.

DEPC reactions

Reactions were performed by protocols described previously [22,26]. Briefly, the DNA samples were diluted with the echinomycin solution and left to equilibrate for a minimum of 30 min at 37 °C. DEPC (1 μ l) was then added and the mixture was left at room temperature for a further 15 min with frequent mixing. The reaction was stopped by adding 200 μ l of 0.3 M sodium acetate, and the DNA was precipitated with ethanol, then washed twice with 70 % ethanol. The DNA was subsequently cleaved at the modified residues by boiling for 20 min in 1 M piperidine. Samples were lyophilized and resuspended in the gel-loading buffer.

KMnO₄ reactions

The labelled DNA fragment was incubated with various concentrations of echinomycin (5–20 μ M) in a total volume of 100 μ l at 37 °C for 30 min. Then 4 μ l of a freshly made up solution of 25 mM KMnO₄ in water was added and the mixture incubated for 4 min at room temperature. The reaction was stopped by addition of 350 μ l of a solution containing 1 % 2-mercaptoethanol in ethanol. The DNA was recovered by precipitation. Samples were then treated with piperidine as described above for the DEPC reaction.

Electrophoresis and autoradiography

DNA-cleavage products were resolved by PAGE under denaturing conditions (0.3 mm thick, 8 % acrylamide containing 8 M urea) capable of resolving DNA fragments differing in length by one nucleotide. Electrophoresis was continued until the Bromophenol Blue marker had run out of the gel (about 2.5 h at 60 W, 1600 V in TBE buffer; BRL sequencers model S2). Gels were soaked in 10 % acetic acid for 15 min, transferred to Whatman 3 MM paper, dried under vacuum at 80 °C, and subjected to autoradiography at –70 °C with an intensifying screen. Exposure times of the X-ray films (Fuji R-X) were adjusted according to the number of counts per lane loaded on each individual gel (usually 24 h).

Densitometry and data reduction

Autoradiographs were scanned using a multi-channel computer-operated gel scanner at the Medical Research Council Laboratory of Molecular Biology, Cambridge, by kind permission of Sir Aaron Klug and Dr. J. M. Smith. Gel profiles were plotted and displayed on a raster graphics screen. Baseline-corrected scans were analysed by integrating all the densities between two selected boundaries using the interactive program GELTRAK [27] developed specifically for quantitative analysis of footprinting gels. Each resolved band on the autoradiograph was assigned to a particular bond within the pBS fragment by comparison of its position relative to the sequencing standards generated by

treatment of the DNA with dimethyl sulphate (G), formic acid (G+A) or hydrazine (T+C) followed by piperidine-induced cleavage at the modified bases in DNA [28].

RESULTS

Location of echinomycin-binding sites

Two complementary footprinting methods utilizing the enzyme DNAase I and the metal complex MPE·Fe^{II} were used to visualize at nucleotide resolution the position and length of individual echinomycin-binding sites in the DNA fragment. DNAase I and MPE·Fe^{II} were allowed to cleave the 265 bp *EcoRI*–*PvuII* restriction fragment, 3'- or 5'-labelled on one or other of the complementary strands, in the absence and presence

Figure 2 DNAase I footprinting of echinomycin on the 265 bp *EcoRI*–*PvuII* restriction fragment excised from plasmid pBS

The duplex DNA was 3'-end-labelled at the *EcoRI* site with [α -³²P]dATP in the presence of AMV reverse transcriptase. The cleavage products of the DNAase I digestion were resolved on an 8 % polyacrylamide gel containing 8 M urea. The concentration (μ M) of the drugs is shown at the top of the appropriate gel lanes. The tracks labelled Ct contained no drug. The tracks labelled G and G+A represent dimethyl sulphate/piperidine and formic acid/piperidine markers specific for guanine and purine residues respectively. The lane marked DNA contains the ³²P-labelled DNA alone, incubated without antibiotic or enzyme. Numbers at the left side of the gels refer to the numbering scheme as used in Figure 4.

Figure 3 MPE·Fe^{II} footprinting of echinomycin on the 3'-end-labelled 265 bp *EcoRI*–*PvuII* restriction fragment

Other details as for Figure 2.

of increasing concentrations of echinomycin. Examples of autoradiograms resulting from each type of footprinting are presented in Figures 2 and 3. Binding of echinomycin prevents both DNAase I and MPE from cleaving DNA. Substantial areas of decreased band intensity in the presence of the antibiotic compared with control lanes can readily be seen at several positions along the DNA sequence. DNAase I footprinting reveals sites of reduced cleavage located around nucleotide positions 35, 70, 90, 100 and 160 on the 3'-labelled strand (Figure 2). Even though the sites of reduced cleavage are easily identifiable, there is no area where the DNAase I cutting is totally abolished so as to produce a 'gap' in the gel as seen in most previous DNAase I studies of echinomycin [11,15]. This reflects the fact that, as mentioned in the Introduction, the chosen fragment lacks strong echinomycin-binding sites such as 5'-ACGT sequences [17,18]. The binding sites for the ligand on DNA are also detectable using MPE·Fe^{II}-generated hydroxyl radicals as footprinting probes, but in this case the footprints are much weaker than with DNAase I, as

other authors have noted in similar circumstances [25]. Regions of attenuated cleavage by MPE·Fe^{II} are sufficiently pronounced to be identifiable by the trained eye around nucleotide positions 60, 80 and 100 on the gel shown in Figure 3 but densitometric analysis of these autoradiograms is required to permit any accurate estimation of the location and approximate size of echinomycin-binding sites.

We have obtained single-band resolution for about 90 positions in the various autoradiograms scanned, corresponding to information on the probability of DNAase I and MPE·Fe^{II} cutting at about 100 different base pairs of the 265-mer. Differential cleavage plots derived from both the DNAase I and MPE·Fe^{II} cleavage patterns are presented in Figure 4. Clearly the cleavage rate by DNAase I and, to a much lesser extent, by MPE is subject to considerable modulation by local binding of echinomycin. With both DNA-cleaving agents, AT-rich sequences are cut with higher efficiency in the presence of the antibiotic. The sequences 5'-AAGCTTTT (49–56) and 5'-TAATT (75–79) in particular are rendered much more susceptible to DNAase I attack. The enhanced cleavages are known to result from a combination of at least two events: redistribution of the cleavage reagent and drug-induced variations in the width of the minor groove of the DNA [29]. With MPE, the sites of enhanced cleavage are less pronounced but can also be observed at these same AT-rich sequences, which are normally cleaved with lower efficiency by MPE·Fe^{II} in the absence of drug.

The gels and differential cleavage plots together reveal six sites (I–VI) of antibiotic-induced inhibition of DNAase I cutting. These sites encompass the sequences (all 5'→3') AGTCGACCT (30–38, site I), TCCCTT (59–64, site II), TGAGGG (68–73, site III), TCGAGCTTGCGTAA (80–94, sites IV and V) and ATGGTC (97–102, site VI). A seventh site can be discerned on the gel around position 160 but it lies beyond the region accessible to densitometric analysis; it probably corresponds to the sequence 5'-CCGGCTCGT which contains two potential echinomycin-binding sites juxtaposed. The DNA cleavage by DNAase I is strongly inhibited at site V corresponding to the sequence CGGT, i.e. a purine–pyrimidine alternation with a canonical CpG step. The extent of protection from nuclease cleavage is equally intense at sites I and IV which both contain the sequence TCGA, whereas it is notably less inhibited at sites II, III and VI which do not contain a CpG step but all present at least two consecutive GC base pairs. In these cases, taking into account the 3'-stagger introduced by the enzyme on DNA cleavage, we can locate the probable sites of antibiotic binding at the sequences TCCC (site II), AGGG (site III) and TGGT (site VI). Within these sites the CC and GG dinucleotide sequences most likely represent the step clamped by echinomycin. However, in each case it is possible to move the tetranucleotide by one or two bases in either the 3'- or 5'-direction so as to include other NpG or CpN sites.

By contrast, MPE·Fe^{II} allows identification of only three of the sites detected with DNAase I. Two sites, I and IV, can be detected on each of the complementary strands. They both correspond to the sequence 5'-TCGA. Site IV which is particularly well resolved is 4±1 bp in size, and a 1 bp shift of the maxima of the cleavage inhibition pattern can be easily observed in the differential cleavage plot. Such a 3'-stagger is typical of minor-groove occupation by the antibiotic [17,25]. The third area of MPE·Fe^{II} cleavage inhibition can be clearly seen on the gel in Figure 3 around nucleotide position 100. The differential cleavage plot in Figure 4 reveals that this site (VI) encompasses the sequence 5'-ACCA on the 3'-end-labelled strand of the 265-mer. No MPE footprint could be detected at the corresponding sequence on the complementary strand, although a neat footprint was seen at this position with DNAase I. This is probably related

Figure 4 Differential cleavage plots comparing the susceptibility of the 265-mer DNA fragment to cleavage by DNAase I (—) and MPE·Fe^{II} (----) in the presence of 10 μ M echinomycin

(a) Differential cleavage of the 5'-end-labelled strand; (b) differential cleavage of the complementary 3'-end-labelled strand. The ordinate scales for the two strands are inverted, so that the deviation of points towards the lettered sequence (negative values) corresponds to a ligand-protected site and deviation away (positive values) represents enhanced cleavage. Vertical scales are in units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the drug and f_c is the fractional cleavage of the same bond in the control, given a closely similar extent of digestion in each case. The line drawn represents a three-bond running average of individual data points, calculated by averaging the value of $\ln(f_a) - \ln(f_c)$ at any bond with those of its two nearest neighbours. The results are displayed on a logarithmic scale for the sake of convenience.

to the lower resolution of the gel obtained with the 5'-end-labelled DNA. Owing to the 5'-terminal chemistry produced by MPE·Fe^{II}, radioactive material is present between the oligomers on the sequencing gel which leads to a high background, especially near the top of the gel corresponding to the longer DNA fragments. Similar observations have been reported by Dabrowiak et al. [30]. It is striking that MPE·Fe^{II} allows the detection of site IV but not of sites II and III which also contain a GpG dinucleotide step. We note, however, that the differential cleavage plot contains an undulation at these loci indicating that the DNA cleavage may be slightly reduced at these two positions. But the most surprising feature is that the DNA cutting by MPE·Fe^{II} remains unaffected at the CpG step at positions 90–91 whereas the same sequence is perceived by DNAase I as being a strong binding site for echinomycin (site V).

Echinomycin-Induced structural changes in DNA

Structural changes in DNA were investigated by studying the effect of the antibiotic on the reactivity of KMnO₄ and DEPC towards DNA. These two chemical probes react at specific positions in the nucleotide bases (see the Discussion section), and subsequent treatment with hot piperidine leads to scission of the glycosidic bond and resultant cleavage of the sugar-phosphate backbone. The patterns of KMnO₄- and DEPC-mediated strand cleavage of the 3'-end-labelled 265-mer restriction fragment in the presence and absence of various concentrations of the antibiotic are shown in Figure 5. In both cases, only minor cleavage occurs in the control lanes as these probes interact only weakly with unmodified double-stranded DNA. The 5,6-double bond of thymines and the N-7 position of guanines are only weakly accessible to the permanganate ions and DEPC respectively, when the DNA is in a normal B-form conformation. When echinomycin binds to DNA, the bases adjacent to the intercalation site unstack allowing the chemical probes much

easier access to the major groove. Thus, on binding to DNA, echinomycin strongly potentiates the reaction of KMnO₄ and DEPC at specific sites in the molecule.

KMnO₄ is induced to react at a restricted number of thymine residues whereas many adenine (and fewer guanine) residues throughout the 3'-labelled DNA duplex become hyper-reactive to DEPC in the presence of echinomycin (Figure 5). The intensity of the bands in the echinomycin-containing lanes is by no means uniform, indicating that the distortion of the helix, which is sensed by the two chemical probes, varies locally according to the sequence to which the drug is bound. To gain a better impression of the effect of echinomycin on the rate of reactivity of the probes with the DNA, the autoradiograms were scanned with a micro-densitometer and the extent of reactivity of the chemicals at defined nucleotide positions was quantified. The distribution of KMnO₄- and DEPC-hyper-reactive sites on both strands of the 265-mer is summarized in Figure 6 together with the regions of cleavage inhibition by DNAase I. It appears that KMnO₄ is induced to react predominantly when the reactive thymine is flanked by an adenine residue on either its 3' or its 5' side. Only four thymine residues within the 3'-end-labelled DNA exhibit fairly strong susceptibility towards KMnO₄ attack. These thymines (at positions 28, 45 and the doublet 76–77) all correspond to TpA or ApT steps located in the vicinity of a drug-binding site. Such steps were similarly identified in previous studies using other DNA fragments and the osmium tetroxide–pyridine complex as a thymine-specific probe [21,23]. Thymine nucleotides located within a region of DNAase I-inhibited cleavage are either weakly reactive (e.g. T66 and T70 on the 3'-labelled strand) or not reactive at all (e.g. T35 on the same strand). In a stretch of three or four consecutive thymines, it seems that only the one at the 5' end of the tract is rendered more susceptible to KMnO₄ attack. For example, in the two short runs of thymines at position 53–56 and 78–80, the 5'-terminal thymine residue (i.e. T53 and T78) displays a hyperreactivity in the presence of

Figure 5 Reaction of the 265-mer 3'-end-labelled DNA fragment with DEPC and KMnO_4 .

The DNA was complexed with echinomycin for at least 30 min before reaction with DEPC or KMnO_4 , and subsequent treatment with hot piperidine. Cleavage products were resolved by electrophoresis on an 8% denaturing polyacrylamide gel. The lane marked control (Ct) corresponds to the DNA subjected to the same chemical treatment in the absence of drug. The concentration (μM) of echinomycin is indicated at the top of the appropriate gel lanes. Guanine- and purine-specific sequence markers obtained by treatment of the DNA with dimethyl sulphate and formic acid followed by piperidine were run in lanes marked G and G+A respectively. Numbers between the two panels of the gel refer to the nucleotide sequence of the DNA fragment. Portions of the sequence, with the nucleotides at which the chemical probe has reacted (indicated by arrows), appear at the sides of the gels.

echinomycin whereas the 3'-terminal thymine remains unaffected by KMnO_4 . The same observation apparently holds true as regards reactivity towards DEPC. The DEPC hyper-reactivity occurs at the two 5'-terminal adenine residues situated in the run of four adenines at position 53–56 on the 3'-labelled strand of the DNA. DEPC attack at the 3'-terminal adenines of the adenine tract seems to be blocked. These observations are in perfect agreement with the previously reported specific chemical reactivity patterns of DEPC and KMnO_4 found for short adenine tracts which reflect the significant alteration of the helical axis at the junction between the adenine-tract-specific (sometimes bent)

conformation and the flanking B-DNA [31]. Indirectly, echinomycin further distorts these structures by increasing the extent of base unstacking at the junction.

In contrast with KMnO_4 , DEPC reacts much more strongly with adenines situated at an echinomycin-binding site than with adenines distant from the binding site. This is particularly apparent at the binding sequence 5'-TCGA at positions 32–35 and 80–83 where, consistently on both strands of the duplex, the (underlined) adenine residue is subjected to strong electrophilic substitution. DEPC also strongly alkylates the adenine residues flanking the GG-containing secondary echinomycin-binding

Figure 6 Diagrammatic representation of the DNAase I footprints and DNA strand cleavages produced by DEPC and KMnO_4 on both strands of the 265-mer DNA fragment

Only the region of the restriction fragment analysed by densitometry is shown. The sequences enclosed by grey boxes indicate the positions of inhibition of DNAase I cutting, representing echinomycin-binding sites. The 3'-offset of the boxes on the two strands of the duplex DNA is in accord with the model for asymmetric cleavage by DNAase I across the minor groove of the B-form helix. Arrows indicate sites of echinomycin-mediated reactivity towards DEPC ($\rightarrow$) and KMnO_4 ($\dashrightarrow$). The lengths of the arrows are proportional to the intensity of cleavage.

sites. By contrast, it reacts only weakly with adenines well removed from a known binding sequence [e.g. the adenine residue in the sequence 5'-TGCA (46–49) which is scarcely modified]. These observations agree with the findings of previous workers that DEPC hypersensitivity is strongly dependent on neighbouring sequences [20–22].

In conclusion, the two complementary probes reveal the location of AT base pairs the secondary structure of which has been perturbed by the antibiotic both at a distance from and essentially within the sites of intercalation. DEPC senses principally the variations in DNA structure within the binding sequence caused by antibiotic-induced unstacking of the bases and to a lesser degree the structural modifications that occur some distance from the drug-binding sites. In contrast, KMnO_4 seems much more sensitive to the alterations of DNA structure propagated away from the drug-binding sites than to the disruption of base stacking immediately adjacent to or within the site of intercalation. The amplitude of the structural variations may vary significantly depending on the DNA sequence. But the KMnO_4 - and DEPC-hyper-reactive sites are distributed all along the DNA sequence, indicating that it is the entire secondary structure of the DNA fragment that, to varying degrees, is affected by the binding of echinomycin.

DISCUSSION

The footprinting results with DNAase I and $\text{MPE} \cdot \text{Fe}^{\text{II}}$ reinforce the view that echinomycin binds selectively to CpG-containing sequences but can also tolerate other (secondary) sites such as GpG (CpC)-containing sequences. This important conclusion is in accord with previous footprinting studies in which either enzymic [11–16] or chemical [17–19] cleaving agents were employed. $\text{MPE} \cdot \text{Fe}^{\text{II}}$ appears less efficient than DNAase I in locating the sites to which the antibiotic is less tightly bound (or resides for shorter times). This, too, is as expected: MPE interacts with duplex DNA so as to cleave the backbone at

positions corresponding to ethidium intercalation sites [25]. The hydroxyl radicals generated by the $\text{MPE} \cdot \text{Fe}^{\text{II}}$ complex provoke the abstraction of a hydrogen atom from a deoxyribose residue in the DNA backbone, and the resulting sugar radical then decomposes to give a single-strand gap at the point of attack. In this instance, unlike the situation with DNAase I, there is no direct recognition of nucleotide bases in DNA by the cleaving diffusible species. If we can further assume that the methidium chromophore competes directly with echinomycin in order to intercalate into DNA, this will explain the lower sensitivity achieved with the $\text{MPE} \cdot \text{Fe}^{\text{II}}$ complex than with DNAase I. Our MPE data corroborate those reported by Dervan and co-workers [17,18] as regards the binding of echinomycin to various types of XCGY sequences, but provide the first definitive evidence that MPE can also be used to detect interaction with much weaker binding sites. However, the detection of secondary echinomycin-binding sites by $\text{MPE} \cdot \text{Fe}^{\text{II}}$ footprinting absolutely requires accurate densitometric analysis as visual inspection of a gel is insufficient to reveal weak sites of attenuated DNA cleavage. We note also that $\text{MPE} \cdot \text{Fe}^{\text{II}}$ serves to detect only a subset of echinomycin-binding sites. DNAase I is thus clearly more versatile than MPE as a tool with which to investigate sequence-selective binding of echinomycin (and probably other ligands) to DNA.

Our second set of observations, that echinomycin strongly potentiates the reactivity of both DEPC and KMnO_4 towards specific sequences in DNA, indicate that the antibiotic significantly perturbs the conformation of the helix. KMnO_4 and DEPC are base-specific chemical probes which discriminate respectively in favour of pyrimidine ($\text{T} \gg \text{C}$) and purine ($\text{A} > \text{G}$) residues in single-stranded and disordered regions of double-stranded DNA relative to more ordered structures [32–35]. In spite of their different chemical reactivity, KMnO_4 and DEPC share two essential properties: (i) they both attack the DNA via the major groove, and (ii) they both react with the side of the bases that is not involved in Watson–Crick hydrogen-bonding.

Figure 7 Sites of reactivity towards DEPC and KMnO_4 in an AT base pair

Broken lines represent the hydrogen bonds between the paired bases. Base adducts formed by reaction of DEPC with adenine and of KMnO_4 with thymine are boxed. R represents deoxyribose. The major groove of the helix is indicated by *M* and the minor groove by *m*.

Indeed, the KMnO_4 -reactive C-5-C-6 double bond of thymine lies on the opposite side of the pyrimidine ring from that which forms hydrogen bonds with adenine. Likewise, the N-7 position of guanine which is carboxylated by DEPC lies on the opposite side of the purine ring from the side that hydrogen bonds to cytosine (Figure 7). Therefore KMnO_4 and DEPC reactions do not represent measures of base-pair disruption as they can proceed even if the base-pairing is maintained. However, adduct formation does require out-of-plane attack by the electrophile and thus will be sterically hindered by stacking of neighbouring base pairs (explaining the poor reactivity towards B-DNA). KMnO_4 and DEPC are essentially probes of base stacking.

Given this explanation of the chemical reactivity of the two probes, we can address the following question as to what changes in DNA structure induced by echinomycin cause the observed alterations in reactivity towards DEPC and KMnO_4 . Echinomycin-induced hypersensitivity of purines towards attack by DEPC was originally proposed to reflect the formation of Hoogsteen base pairs in DNA [18]. But several footprinting studies [20,21,23] including a recent quantitative analysis of the binding of echinomycin to DNA incapable of forming Hoogsteen base pairs [15] have clearly argued against any *anti-syn* conformational transition of purine nucleosides occurring on echinomycin binding. It is worth pointing out that Hoogsteen base-pairing has been detected only for the AT base pairs within the sequence 5'-ACGT [6,36], i.e. at a sequence that is not present in the DNA fragment used here. So a transition to Hoogsteen base-pairing cannot be responsible.

Recently, on the basis of their n.m.r. data on echinomycin-oligonucleotide complexes and the available footprinting data, Gilbert and Feigon [7,8] suggested that the alteration in reactivity of DNA towards chemical probes could result from a combination of two factors: base-pair destabilization and local

unwinding of the helix associated with the bisintercalation of echinomycin. The drug-induced unwinding of the helix engenders a significant decrease in the base twist angle and consequently an expansion of the major groove of the helix, which should favour the access of the probes. But it is perfectly true that DNA unwinding is insufficient in itself to explain the drug-induced hyper-reactivity towards chemical probes. There are at least two lines of evidence that bear out this idea: (i) the structurally simple intercalating agent ethidium bromide decisively unwinds the DNA but has practically no effect on DEPC reactivity [22]; (ii) intercalating drugs that unwind the DNA helix to the same extent and exhibit similar sequence selectivity can produce very different patterns of reactivity towards chemical probes (e.g. the antischistosomal drugs hycanthone and lucanthone and their indazole analogues [37]). There is undoubtedly some other type of DNA structural change that, together with the unwinding effect, allows the chemical probes an easier access to the major groove at AT base pairs. As regards the DEPC reaction, this associated event may well be the destabilization of the AT base pairs adjacent to the CpG step clamped by the antibiotic [38]. For the KMnO_4 reaction, the associated event is more probably related to the stretching and elongation of the helix that results from the intercalative binding process [39]. The increase in the base-pair separation, the flattening of the propeller twist and the modification of the width of the grooves all conspire to expose the 5,6-double bond of thymine and the N-7 position of the purine.

As echinomycin can sometimes interact with ApT steps [13,14], the possibility must be considered that the observed reaction with KMnO_4 might reflect some direct, albeit weak, interaction of echinomycin with ApT sites. Although plausible, we believe that this explanation is unlikely because direct interaction between echinomycin and ApT sites has been detected only using long $(\text{ApT})_n$ blocks, i.e. within sequences not present in the DNA fragment used here. As far as we are aware, there is no report showing that echinomycin interacts with ApT sites within a natural DNA fragment of mixed composition.

Another parameter that has to be carefully considered when using KMnO_4 is the sensitivity of this anionic reagent to local variations in the electrostatic properties of the DNA. Although steric effects cannot be discounted, it is clear that electrostatic effects are the major determinant in the selectivity of permanganate attack [40]. On binding to specific sequences in DNA, echinomycin provokes large-scale distortion of the double-helix structure at sequences flanking the clamped dinucleotide steps. Such major structural changes are clearly DEPC-sensitive but they are apparently not sensed by KMnO_4 . Why? Echinomycin is an uncharged antibiotic and therefore, unlike positively charged DNA ligands, it does not favour the interaction between two anionic species such as KMnO_4 and DNA. Propagated structural changes in DNA are equally likely to result in changed electronic properties of the helix at some distance from the antibiotic-binding sites [41]. Such localized variations of the electronic properties of the DNA at particular sequences could easily promote reactivity towards permanganate anions but not towards DEPC which is a neutral probe.

The present study highlights the importance of the sequence context and the local conformation in the reaction of DEPC and KMnO_4 . Indeed, the most striking observation is that KMnO_4 reacts most strongly with thymines some distance from an echinomycin-binding site whereas the carboxylations induced by DEPC affect principally the adenine residues immediately adjacent to the dinucleotide at the core of an echinomycin-binding site. These experiments emphasize that DEPC and KMnO_4 are sensitive to different states of the DNA helix. It

seems likely that the reaction of KMnO_4 involves transient unstacking events, whereas the reaction of DEPC requires larger-scale helix opening. These observations make interesting comparison with those reported for the related chemical probes osmium tetroxide (which has exactly the same chemical reactivity as KMnO_4) and bromoacetaldehyde (which modifies adenines) [42].

The best-known investigations on the detailed structure of echinomycin–DNA bisintercalation complexes were conducted with short oligonucleotides (for comprehensive reviews see [4,43]). It is clearly important to relate the structural information obtained from these studies on oligonucleotides (mostly using n.m.r.) to the structure of longer natural DNA fragments. In this regard, chemical probes such as those employed here appear to be particularly useful as they can detect large DNA conformational transitions (e.g. B–Z transition, cruciform structure, triple-strand formation) as well as subtle alterations in the DNA structure which result from ligand binding. More detailed proposals as to the exact nature of the geometrical perturbations of the DNA helix induced by echinomycin at a distance from its binding sites must await the results of further experiments using diverse chemical probes as well as DNA fragments containing different types of receptor sequences for echinomycin.

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