Hoogsteen base pairs proximal and distal to echinomycin binding sites on DNA

(diethyl pyrocarbonate-mediated DNA-cleavage patterns/DNA polymorphism/allostery transitions/non-Watson-Crick base pairs)

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Contributed by Peter B. Dervan, September 29, 1986

ABSTRACT Forms of the DNA double helix containing non-Watson-Crick base-pairing have been discovered recently based on x-ray diffraction analysis of quinoloxaline antibiotic-oligonucleotide complexes. In an effort to find evidence for Hoogsteen base-pairing at quinoloxaline-binding sites in solution chemical "footprinting" (differential cleavage reactivity) of echinomycin bound to DNA restriction fragments was examined. We report that purines (A>G) in the first and/or fourth base-pair positions of occupied echinomycin-binding sites are hyperreactive to diethyl pyrocarbonate. The correspondence of the solid-state data and the sites of diethyl pyrocarbonate hyperreactivity suggests that diethyl pyrocarbonate may be a sensitive reagent for the detection of Hoogsteen base-pairing in solution. Moreover, a 12-base-pair segment of alternating A-T DNA, which is 6 base pairs away from the nearest strong echinomycin-binding site, is also hyperreactive to diethyl pyrocarbonate in the presence of echinomycin. This hyperreactive segment may be an altered form of right-handed DNA that is entirely Hoogsteen base-paired.

X-ray analyses of triostin A and echinomycin complexed to the hexanucleotide duplex d(CGTACG): (1, 2) and triostin A complexed to the octanucleotide duplex d(GCGTACGG): (3) show that these quinoloxaline bisintercalators bracket a 5'-C-G 3'-dinucleotide and induce Hoogsteen base-pairing (4) of the A-T and G-C base pairs flanking the central Watson-Crick base pairs (5) (Fig. 1). In solution, "footprinting" (differential cleavage reactivity) on DNA restriction fragments reveals that echinomycin has a binding-site size of 4 base pairs (bp) (6). The strong binding sites of echinomycin and triostin A contain the central 2-bp sequence 5'-C-G 3'- with a preference in the first and fourth position for A-T base pairs, though not excluding G-C base pairs (6-8). The sequence 5'-ACGC 3'- contained in one of the solid-state studies (3) is a binding site for echinomycin in solution (ref; 6; Fig. 1). Since the oligonucleotides used in the x-ray crystal studies are small and the bisintercalators are bound at or near the ends of the DNA duplexes, the question arises whether such drug-induced Hoogsteen base-pairing found in the solid state would be observed in solution within a large fragment of DNA at 25°C and pH 7.4.

Diethyl pyrocarbonate, (EtOOC)2O, is a sensitive reagent for the detection of left-handed Z-DNA (9, 10) and cruciform loops (11, 12). Z-DNA hyperreactivity toward (EtOOC)2O relies on the exposure of the N-7 atom of purines adopting the syn conformation, which leads to a chemically modified base (13, 14) and strand scission upon heating (90°C) in the presence of piperidine; thus, syn purines within Z-DNA regions can be identified at nucleotide resolution by sequencing gel techniques (9, 10). Right-handed Hoogsteen base-paired DNA also requires purines to adopt the syn conformation, and this form of DNA would expose the N-1 and N-3 atoms of purines in the major groove (Fig. 1). Therefore, purines rearranging from Watson-Crick to Hoogsteen base-pairing in right-handed DNA might also display differential reactivity to (EtOOC)2O. If this were the case, (EtOOC)2O/piperidine treatment of DNA in the presence of quinoloxaline antibiotics, followed by analysis of the cleavage sites on a high-resolution sequencing gel, would allow mapping of Hoogsteen base pairs at nucleotide resolution. A combination of methidiumpropyl-EDTA-Fe(II) footprinting and (EtOOC)2O hyperreactivity studies would then define the relationship between quinoloxaline antibiotic binding sites and the sites of Hoogsteen base-pairing.

Echinomycin binding sites were determined by MPE-Fe(II) footprinting (6, 15) on a 628-bp restriction fragment. The reactivity of purines toward (EtOOC)2O was examined on the same restriction fragment in the presence of identical concentrations of echinomycin. In the absence of echinomycin, (EtOOC)2O does not react with the 628-bp fragment of DNA to a significant extent under the reaction conditions chosen. However, at low binding levels of echinomycin, we find hyperreactivity to (EtOOC)2O at adenine and guanine bases in the first and/or fourth base-pair positions of the echinomycin binding sites assigned from the MPE-Fe(II) footprinting at 37°C and pH 7.4. In addition, we find that the adenines in a 12-bp segment of A-T DNA that is 6 bp distal to the nearest strong echinomycin binding site become increasingly reactive toward (EtOOC)2O as the concentration of echinomycin increases.

MATERIALS AND METHODS

Dithiothreitol, calf alkaline phosphatase, T4 polynucleotide kinase, restriction endonucleases, and the Klenow fragment of Escherichia coli DNA polymerase I were purchased from Boehringer Mannheim; T4 DNA ligase, from New England Biolabs; Et2CO3, from Aldrich; and bovine pancreatic DNase I, from Worthington. Echinomycin was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, and concentrations were determined spectrophotometrically by using ε255 = 11,500 M-1 cm-1 (7). Buffers were of two types: 10 mM Tris, pH 7.4/50 mM NaCl (TN buffer) and 50 mM sodium cacodylate, pH 7.2/10 mM KCl/10 mM MgCl2/5 mM CaCl2 (CKMC buffer).

Plasmid Construction. Two complementary oligonucleotides, 1 (5'-AGCTTATATATATAAAAAGGGGGGG-GGTCGATAG) and 2 (5'-GATCCTATCAGACCCCATCCCATATATATATATA), were synthesized on an automated DNA synthesizer using phosphoramidite chemistry (16, 17). Purification of the oligonucleotides via gel electrophoresis was followed by 5' phosphorylation with ATP and T4 kinase. Hybridization of oligonucleotides 1 and 2 yielded

Abbreviation: MPE-Fe(II), methidiumpropyl-EDTA-Fe(II).

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Footprinting. A 16-µl solution of buffer (32P-end-labeled restriction fragment, echinomycin (dissolved in methanol), and sonicated deproteinized calf thymus DNA was incubated for 15 min at 37°C. Next, 2 µl of 80 µM MPE:Fe(II)/160 µM Fe(NH4)2(SO4)2 solution was added, and the mixture was mixed and allowed to equilibrate another 15 min at 37°C. DNA strand scission was initiated by adding 2 µl of a freshly prepared 40 mM dithiothreitol solution. Cleavage at 37°C was terminated after 20 min by freezing on dry ice, followed by two precipitations with ethanol. The final concentrations in the 20-µl reaction mixture were 400 µM DNA base pairs; 10 mM Tris (pH 7.4); 50 mM NaCl; 8 µM MPE:Fe(II); 16 µM Fe(NH4)2(SO4)2; 10% (vol/vol) methanol; 0, 12.5, 25, 50, or 100 µM echinomycin; and 4 mM dithiothreitol.

**DNase I Footprinting.** A 16-µl solution of buffer (32P-end-labeled restriction fragment, echinomycin (dissolved in methanol), and sonicated deproteinized calf thymus DNA was incubated for 15 min at 37°C. Next, 4 µl of dithiothreitol (1 mM)/DNase I (0.33 µg/ml) was added, mixed, and allowed to stand for 2 min at room temperature. DNase I digestion was terminated by addition of 5 µl of 3 M NH4OAc/0.25 M EDTA and 150 µl of cold ethanol. The reaction mixtures were then precipitated with ethanol and washed. The final concentrations in the 20-µl DNase I digestion mixtures (before the stop solution) were 400 µM DNA base pairs, 50 mM 32P-end-labeled DNA restriction fragments. Lanes: 1 and 16, intact DNA; 2 and 17, MPE:Fe(II)-mediated cleavage of DNA in the absence (+) of echinomycin; 3-6 and 18-21, MPE:Fe(II)-mediated cleavage of DNA in the presence (+) of echinomycin at 12.5, 25, 50, and 100 µM, respectively; 7 and 22, (EtOOC)2O/piperidine cleavage of DNA in the absence of echinomycin; 8-11 and 23-26, (EtOOC)2O/piperidine cleavage of DNA in the presence of echinomycin at 12.5, 25, 50, and 100 µM, respectively; 12 and 14, Maxam–Gilbert chemical-sequencing guanine-specific reaction; 13 and 15, Maxam–Gilbert chemical-sequencing purine-specific reaction. The gel was scanned from the bottom to the arrow (at left). Brackets denote the 5' (T-A)_4 3' segment hyperreactive to (EtOOC)2O.

FIG. 1. (Upper) Diagram of the complex between a quinoxaline bisintercalator and a right-handed DNA duplex similar to that shown in ref. 3. The quinoxaline rings are represented by dark bars and the -cyclic depsipeptide backbone is drawn as an oval. The first and fourth base pairs are Hoogsteen-paired and the central C-G is Watson–Crick-paired. (Lower) Schematic drawings show T-A and C-G Hoogsteen base pairs. The C-G Hoogsteen base-pairing requires a protonated cytosine.

FIG. 2. Autoradiogram of 5′ (lanes 1–13) and 3′ (lanes 14–26) 32P-end-labeled DNA restriction fragments. Lanes: 1 and 16, intact DNA; 2 and 17, MPE:Fe(II)-mediated cleavage of DNA in the absence (+) of echinomycin; 3–6 and 18–21, MPE:Fe(II)-mediated cleavage of DNA in the presence (+) of echinomycin at 12.5, 25, 50, and 100 µM, respectively; 7 and 22, (EtOOC)2O/piperidine cleavage of DNA in the absence of echinomycin; 8–11 and 23–26, (EtOOC)2O/piperidine cleavage of DNA in the presence of echinomycin at 12.5, 25, 50, and 100 µM, respectively; 12 and 14, Maxam–Gilbert chemical-sequencing guanine-specific reaction; 13 and 15, Maxam–Gilbert chemical-sequencing purine-specific reaction. The gel was scanned from the bottom to the arrow (at left). Brackets denote the 5′ (T-A)_4 3′ segment hyperreactive to (EtOOC)2O.
sodium cacodylate (pH 7.2), 10 mM KCl, 10 mM MgCl₂, 5 mM CaCl₂, 10% (vol/vol) methanol, 66 ng of DNase I per ml, 200 μM dithiothreitol, and 0 or 100 μM echinomycin.

(EtOOC)₂O Footprinting. A buffered 19-μl solution of labeled restriction fragment, echinomycin (dissolved in methanol), and sonicated deproteinized calf thymus DNA was incubated for 15 min at 37°C. The mixture was cooled to 0°C, and 1 μl of (EtOOC)₂O was added. The mixture was then incubated for 15 min at room temperature with occasional mixing. The concentrations of DNA, buffer, methanol, and echinomycin were identical to those in the MPE-Fe(II) or DNase I protocols above. (EtOOC)₂O modification of the DNA was terminated by two precipitations with ethanol, followed by vacuum-drying. The resulting pellet was resus-

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**FIG. 3.** (A–D) MPE-Fe(II)-mediated DNA cleavage-inhibition patterns in the presence of echinomycin at 12.5, 25, 50, and 100 μM, respectively, bound to 100 bp of the 628-bp fragment, as determined by densitometry from the autoradiogram in Fig. 2 (lanes 3–6 and 18–21). (E–H) (EtOOC)₂O/piperidine cleavage patterns in the presence of echinomycin at 12.5, 25, 50, and 100 μM, respectively, bound to the same 100 bp of DNA, as determined by densitometry from the autoradiogram in Fig. 2 (lanes 8–11 and 23–26). MPE-Fe(II) cleavage-inhibition patterns (A–D) are shown as histograms with the height proportional to the reduction of cleavage at each nucleotide compared with MPE-Fe(II)-mediated cleavage of unprotected DNA. (EtOOC)₂O cleavage patterns (E–H) are shown as arrows, with the height proportional to the enhancement of cleavage at each nucleotide compared with the (EtOOC)₂O/piperidine cleavage of unbound DNA. The scale at the bottom corresponds to the first 5′ thymidine in the EcoRI site of plasmid pDMG10, defined as position 1. Boxes are the assigned echinomycin binding sites based on the model in refs. 6 and 15.
pended in 30 μl of 1 M piperidine, heated for 10 min at 90°C, and lyophilized twice. The (EtOOC)_2O control contained methanol (10%, vol/vol) and was subjected to the identical piperidine workup as were the samples containing echinomycin.

Sequencing Gel Electrophoresis. Base-specific cleavage reactions were performed as described by Maxam and Gilbert (21). Samples from (EtOOC)_2O, MPE-Fe(II), or DNase I cleavage protocols were resuspended in 90% formamide buffer and heat-denatured for 2 min at 90°C. The denatured samples were then loaded onto an 8% 1:19 crosslinked polyacrylamide gel containing 50% (wt/vol) urea. The 40 x 34 cm wedge gel was 0.2 mm thick at the comb and 0.6 mm thick at the base. Electrophoresis was carried out at 1200 V for 4 hr. The gel was dried and then autoradiographed without intensification for 4 days at ~70°C with Kodak X-Omat AR film. Densitometric analysis of the autoradiogram was carried out with an LKB Ultrascan XL 2222 laser densitometer.

RESULTS

Purines Proximal to Echinomycin Binding Sites Are Hyperreactive to (EtOOC)_2O. The patterns of MPE-Fe(II) and (EtOOC)_2O-mediated DNA cleavage of the 5’ and 3’ 32P-end-labeled 628-bp EcoRI-BglI restriction fragment of plasmid DNA were visualized on the autoradiogram of a high-resolution gel (Fig. 2). In the absence of echinomycin, we observe an even DNA cleavage pattern with MPE-Fe(II) (control lanes 2 and 17) and very slight cleavage of purines with (EtOOC)_2O/piperidine (control lanes 7 and 22). In the presence of echinomycin, the cleavage patterns for MPE-Fe(II) and (EtOOC)_2O/piperidine differed significantly from those of controls (Fig. 2, lanes 3–6 and 18–21 vs. 8–11 and 23–26). Echinomycin at 12.5–100 μM protected DNA (400 μM in base pairs) from cleavage mediated by MPE-Fe(II). The echinomycin binding sites on 100 bp of this restriction fragment, assigned from diminished cleavage with MPE-Fe(II), were (5’-3’) TCGA, TCGA, TCCT, ACGCCGGACGC, TCGT, CCGG, TCACCGG, GCCA, AGGT, and GCGG. In the presence of echinomycin, hyperreactivity toward (EtOOC)_2O occurred at purines (A > G) flanking a central dinucleotide, (5’-3’) C-G > C-C or G-G at these same sites (Fig. 3). In data not shown, the quinoxaline antibiotic triostin A showed similar behavior. However, unlike echinomycin and triostin A, other small DNA binding molecules such as the intercalator-groove binder actinomycin D or the groove binder distamycin A did not produce purine hyperreactivity toward (EtOOC)_2O.

Purines Distal to Strong Echinomycin Sites Are Hyperreactive to (EtOOC)_2O. A 12-bp site with the sequence 5’ TATATATATATA 3’, which was not protected from MPE-Fe(II)-mediated cleavage in the presence of echinomycin and is 6 bp away from the nearest strong echinomycin binding site, 5’ TCGA 3’, became increasingly reactive toward (EtOOC)_2O as the echinomycin concentration was increased. The pattern of adenine-(EtOOC)_2O hyperreactivity within this region was asymmetric to the 3’ side and extended over 10 bp on both strands (bracketed region in Figs. 2 and 4). Densitometric analysis of both MPE-Fe(II)- and (EtOOC)_2O-modified cleavage patterns in the presence of echinomycin are presented in Fig. 3.

DNase I and MPE-Fe(II) Cleave the 5’ (T-A)_k 3’ Site Distal to a Strongly Bound Echinomycin. In the presence of 100 μM echinomycin, the enzyme DNase I showed broad regions of cleavage inhibition on the 628-bp DNA restriction fragment at locations where MPE-Fe(II)-mediated cleavage inhibition patterns revealed several discrete echinomycin binding sites (Fig. 4). However, as with MPE-Fe(II), DNase I cleaved the 5’ (T-A)_k 3’ site in the presence of 100 μM echinomycin with an efficiency comparable to that found in the absence of echinomycin (Fig. 4, compare lanes 15 and 16 with 17 and 18). Moreover, the 5’ (T-A)_k 3’ site is not sensitive to cleavage by nuclease S1 (data not shown), which is specific for single-stranded DNA (22).

DISCUSSION

Hoogsteen Base-Pairing Proximal to Echinomycin Sites. (EtOOC)_2O hyperreactivity of purines in the first and/or fourth positions of the echinomycin binding sites, independently determined by MPE-Fe(II) footprinting, suggests that the Watson–Crick to Hoogsteen base pair structural reorganization of DNA that was discovered by Rich and coworkers in the x-ray crystal structures of quinoxaline antibiotic oligonucleotide complexes may also exist in solution (25°C, pH 7.4). With (EtOOC)_2O, cleavage enhancement at purines in the first and/or fourth positions of echinomycin sites is observed on a sequencing gel, which corresponds to cleavage inhibition at sites 4 bp in size observed with MPE-Fe(II) (Fig.

Fig. 4. Autoradiogram of 5' (lanes 1, 3, 5, 7, 9, 11, 12, 15, 17, 19, and 21) and 3' (lanes 2, 4, 6, 8, 10, 13, 14, 16, 18, 20, and 22) 32P-end-labeled DNA restriction fragments. Lanes: 1 and 2, intact buffered DNA; 3 and 4, MPE-Fe(II)-mediated cleavage of DNA in the absence (−) of echinomycin; 5 and 6, MPE-Fe(II)-mediated cleavage of DNA in the presence (+) of 100 μM echinomycin; 7 and 8, (EtOOC)_2O/piperidine cleavage of DNA in the presence of 100 μM echinomycin; 9 and 10, (EtOOC)_2O/piperidine cleavage of DNA in the presence of echinomycin; 11–14, Maxam–Gilbert chemical sequencing G- and G+A-specific reactions; 15 and 16, DNase I cleavage of DNA in the absence of echinomycin; 17 and 18, DNase I cleavage of DNA in the presence of 100 μM echinomycin; 19 and 20, (EtOOC)_2O/piperidine cleavage of DNA in the presence of 100 μM echinomycin; 21 and 22, (EtOOC)_2O/piperidine cleavage of DNA in the absence of echinomycin. Reactions in lanes 1–10 were performed in TN buffer, and in lanes 15–22 in CKMC buffer. Brackets denote the 5’ (T-A)_k 3’ site hyperreactive to (EtOOC)_2O.
3. Because (EtOOC)$_2$O detects bound quinoxaline antibiotics at nucleotide resolution, (EtOOC)$_2$O can be considered the preferred reagent for footprinting DNA-bound quinoxaline antibiotics, especially in cases where several binding sites are clustered or where they overlap. The finer precision of (EtOOC)$_2$O relative to MPE-Fe(II) is accompanied by greater sensitivity of the former reagent. This is probably because MPE-Fe(II) competes with echinomycin for binding sites whereas (EtOOC)$_2$O does not. The relative reactivity of adenine versus guanine may be due to the ease with which adenine adopts the syn conformation, to the intrinsic reactivities of adenine and guanine toward (EtOOC)$_2$O in Hoogsteen base-paired DNA, or to some combination of both. One explanation for purine hyperreactivity is that right-handed Hoogsteen DNA exposes nucleophilic purine N-1 atoms, making them available for reaction with (EtOOC)$_2$O.

Hoogsteen Base-Pairing Distal to Echinomycin Binding Sites. As noted above, adenines within the (T-A)$_6$ segment show enhanced reactivity with (EtOOC)$_2$O in the presence of echinomycin. This suggests that this segment can adopt a conformation that is non-B-form, since B-DNA is largely unreactive to (EtOOC)$_2$O. The fact that cleavage of the 5′ (T-A)$_6$ 3′ region with MPE-Fe(II) and DNase I is efficient in the absence or presence of echinomycin (100 μM) shows that this sequence is not bound strongly by echinomycin. Because the 5′ (T-A)$_6$ 3′ site is resistant to cleavage by nuclease S1 in the presence of 100 μM echinomycin, this segment is most likely not denatured. If the structural change that is responsible for purine hyperreactivity to (EtOOC)$_2$O is identical for purines distal as well as proximal to echinomycin binding sites, then the data suggest that echinomycin competes with Watson–Crick DNA. The high salt or multivalent cations. This result appears to be in disagreement with molecular mechanical calculations by Kollman and coworkers, who concluded that a Hoogsteen base-paired structure is unlikely to be found in uncomplexed DNA with neutral bases, unless specific ion effects can overcome the significant preference for Watson–Crick pairing due to sugar–phosphate and phosphate–base interactions.

Actinomycin D Bound to DNA Does Not Produce (Et$_2$OOC)$_2$O Hyperreactivity. Echinomycin binds DNA via bisintercalation and helix unwinding accompanying binding (30). The question arises as to whether the observed purine hyperreactivity to (Et$_2$OOC)$_2$O could be due to unwinding of the DNA helix by the intercalator and not a result of a structural reorganization such as Hoogsteen base-pairing. Therefore, another intercalator was screened for its ability to produce purine hyperreactivity to (Et$_2$OOC)$_2$O when bound to DNA. Actinomycin D, a groove binder-intercalator with G-C specificity, failed to produce purine hyperreactivity to (Et$_2$OOC)$_2$O either proximal or distal to its binding sites even at high-binding densities. Because actinomycin D is known to unwind the DNA helix (31), its failure to induce hyperreactivity to (Et$_2$OOC)$_2$O suggests that (Et$_2$OOC)$_2$O responds to a reorganization of purine functional groups rather than to altered exposure of purine nucleophilic sites because of unwinding of the B-DNA helix. Furthermore, we find that triostin A produces a pattern of purine hyperreactivity to (Et$_2$OOC)$_2$O nearly identical to that observed for echinomycin. Both triostin A and echinomycin induce Hoogsteen base-pairing at their binding sites in solid-state crystal structures.

CONCLUSION

In solution under physiologically relevant conditions, purines at the first and/or fourth base-pair positions of echinomycin binding sites become hyperreactive toward (Et$_2$OOC)$_2$O in the presence of echinomycin. From x-ray analysis of quinoxaline–oligonucleotide co-crystals, these same purines were found to be Hoogsteen base-paired. If this correspondence is not fortuitous, then (Et$_2$OOC)$_2$O detects Hoogsteen base-pairing in solution.

We are grateful to the American Cancer Society (NP428) and to the Burroughs Wellcome Company for support.