Diethylpyrocarbonate and permanganate provide evidence for an unusual DNA conformation induced by binding of the antitumour antibiotics bleomycin and phleomycin

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ABSTRACT

DNA structural changes induced by bleomycin have been investigated using diethylpyrocarbonate and permanganate as probes under conditions in which the antibiotic binds to, but does not cut the DNA. Diethyl-pyrocarbonate shows an enhanced reaction with adenines in the presence of the antibiotic in the sequences GTA>GCA>GAA, on the 3' side of the drug cutting site (GPy). Permanganate ions display an enhanced reactivity at the second pyrimidine of the sequence GPyPy. The results are consistent with a model in which bleomycin distorts the structure of the base pair on the 3' side of its binding site.

INTRODUCTION

The bleomycins are a group of glycopeptide antibiotics which are widely used in the treatment of cancer [1,2]. They are believed to act by binding to and degrading DNA in a reaction which requires molecular oxygen and ferrous ions [3-5]. Two types of DNA lesions have been described. Under anaerobic conditions the predominant reaction involves breakage of the N-glycosidic bonds with subsequent release of free nucleic acid bases in decreasing order T>C>A>G, leaving DNA strands which are alkali labile [6-8]. The alternative reaction pathway requires additional oxygen and results in direct strand scission at neutral pH [9]. Cleavage of DNA in vitro occurs most frequently at the dinucleotide steps GpC and GpT, especially when the G is preceded by a pyrimidine residue [10-12]. DNA cleavage is generally a single-stranded event, since the extent of cleavage on one strand is independent of that on the opposing strand [12], although a separate reaction pathway generating double-stranded cuts has also been described [13].

It has been shown that the complex of bleomycin with

cobalt ions is very stable [14]. This complex still binds to DNA but does not result in subsequent strand cleavage [15]. Footprinting experiments have demonstrated that this binding reaction is sequence specific and that cleavage only occurs at those regions to which the antibiotic is already selectively bound [15].

There have been conflicting reports as to the precise nature of the interaction between bleomycin and DNA although it has been proposed that the bulk of the drug is located in the DNA minor groove [16]. Bleomycin has been shown to induce DNA lengthening and unwinding characteristic of intercalators with the bithiazole ring parallel to the base pairs [17,18], although such a mechanism seems unlikely for the phleomycins. In a previous footprinting study bleomycin and phleomycin were shown to induce enhancements in DNAaseI cleavage in runs of A and T residues surrounding some of the drug binding sites [15]. It has also been shown that bleomycin can render DNA sensitive to cleavage by a single strand specific endonuclease [19]. Clearly these drugs do cause some local alterations in DNA structure although it is not clear how these effects are related to their ability to cleave the DNA strands.

In this paper we report the results of footprinting experiments using diethylpyrocarbonate and potassium permanganate as probes for studying drug-induced changes in DNA structure. Diethylpyrocarbonate (DEPC), which reacts with A and G residues, has previously been used as a probe for Z-DNA [20,21], hairpin loops [22,23] and Hoogsteen base pairs [24]. Potassium permanganate reacts with pyrimidine residues in single stranded DNA and can be used as a tool for DNA sequencing [25,26]. The results of these studies suggest that bleomycin can radically alter the conformation of the nucleotide adjacent to its cutting site, and that this occurs before base release or strand cleavage.

MATERIALS AND METHODS

Drugs and enzymes

Bleomycins-B₄ and -CHP and phleomycins-E and -CHP were prepared, metal-free, as previously described [27]. Stock

tyrT DNA

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5'-AATTCGGTTAACCTTTAATCCGTACGGATGAAAATTACGCAACCAAGTTCATTTTTCTCAACGTAACAC
  0      10      20      30      40      50      60
3'-ATGGGCCAATTGGAAATTAGGCAATGCCTACTTTTAATGCGTTGGTCAAGTAAAAAGAGTTGCATTGTG

TTTACAGCGCGCGTCATTTGATATGATGCGCCCCGCTTCCCGATAAGGGAGCAGGCCAGTAAAAAGCATTAC
  70      80      90      100     110     120     130
AATGTGCGCGCGCAGTAACTATACTACGCGGGGCGAAGGGCTATTCCCTCGTCCGGTCATTTTTCGTAATG

CCCGTGGTGGGGGTTCCG-3'
  140     150
GGGCACCAACCCCAAGGGCT-5'
  
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135-base-pair DNA fragment

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3'-AACCGTCCTGTTCCCGTCGAGACGTTTGACATTTTGCCCTGTTTCCGAAAGGGGACCGAATGTGCGTTTT
  10      20      30      40      50      60      70

CCCTTCCCGGAAAGGACTCCTCCACTCGCCGTGGACCTGAGCCCTACCGCGACCTTCA-5'
  80      90      100     110     120     130
  
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70-base-pair DNA fragment

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3'-AAATTACGCCATCAAATAGTGTCAATTTAACGATTGCGTCAGTCCGTGGCAGACATACTTTAGATTGTTACGC-
  40      50      60      70      80      90      100
  
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130-base-pair DNA fragment

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5'-CACATTTCCCGGAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACTATAAAAAATA
  4270      80      90      4300      10      20

GGCGTATCACGAGGCCCTTTTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCCA-3'
  30      40      50      4360 .      10      20
  
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Figure 1. Sequences of the DNA fragments employed. Only the strand bearing the radioactive label is shown (circled).

solutions (1mM) were prepared in 10mM Tris-HCl pH 8.0 containing 10mM NaCl and stored at -20°C. Restriction enzymes were purchased from either New England Biolabs or Northumbria Biologicals Ltd.

DNA fragments

The 160 base pair tyrT DNA fragment was isolated and labelled at either of its 3' ends as previously described [28]. The 135mer was isolated from plasmid pXbs1 [29] by cutting with restriction enzymes HindIII and Sau3A1 as previously

described [30] and labelled at the 3' end of the HindIII site using reverse transcriptase and α -[^{32}P]dATP. The 130 and 70 base pair fragments were isolated from plasmid pBR322 by cutting with HindIII (position 29), labelling with α -[^{32}P]dATP using reverse transcriptase and cutting again with HhaI (positions 103 and 4259). The two radiolabelled fragments were then separated on a 6% non-denaturing polyacrylamide gel.

Chemical reactions

Diethylpyrocarbonate. DNA samples (1 μ l) were mixed with 2 μ l of bleomycin or phleomycin solution (2-30 μ M) containing 200 μ M CoCl_2 and left to equilibrate for 30mins. The cobalt is added to prevent the DNA cleavage by the antibiotic [15]. To this was added 5 μ l of diethylpyrocarboate (DEPC) and this mixture was left at 37°C for 15 mins. with occasional mixing. The reaction was stopped by adding 200 μ l 0.3M sodium acetate and the DNA was precipitated with ethanol. The DNA was subsequently cleaved at the modified residues by boiling for 30mins in 10% piperidine. Samples were then lyophilized and dissolved in the gel loading buffer containing 10mM EDTA, 0.1% bromophenol blue, 1mM NaOH and 90% formamide.

Potassium permanganate. Bleomycin-DNA and phleomycin-DNA complexes were prepared as described above for DEPC. To these 4 μ l of 100mM potassium permanganate was added and the reaction stopped after 1 or 5 mins by adding 2 μ l mercaptoethanol. The DNA was precipitated and cleaved with piperidine as described above. In experiments with permanganate or DEPC little or no cleavage was observed prior to treatment with piperidine, indicating that these reagents have modified but not cleaved the DNA backbone.

Dimethylsulphate and methylene blue. Experiments using dimethylsulphate and methylene blue as footprinting probes were performed as previously described [28].

Gel electrophoresis

Reaction products were fractionated on 8% (w/v) polyacrylamide gels containing 7M urea. Gels were run for about 2 hours, fixed in 10% acetic acid, transferred to Whatman 3MM paper, dried under vacuum and subjected to autoradiography at -70°C with an intensifying screen.

RESULTS

Diethylpyrocarbonate

The patterns of DEPC mediated DNA strand cleavage in the presence and absence of various concentrations of bleomycin-B₄ are presented in Figure 2 for four labelled DNA fragments. For each DNA, cleavage in the control lanes is weak and is restricted to purine residues. The pattern in the presence of bleomycin is significantly different and several bands show a markedly increased sensitivity to modification. The strongest enhancements on each fragment are at position 61 on the 70mer, positions 4336, 4296 and 4273 on the 130mer, positions 19, 32, 67 and 135 on the lower strand of tyrT DNA and positions 127, 119 and 61 on the upper strand of tyrT DNA. All of these correspond to reaction at adenine residues. On each DNA strand all the trinucleotide steps GTA generate adenines which are hyperreactive to DEPC. The sequence GCA is modified less often and is unaffected at position 92 on the tyrT lower strand and 38 on the upper strand. The sequence GAA is also occasionally modified at the second adenine residue. Each of these enhancements is adjacent to a bleomycin cutting site (GPY> GA >>GG). Enhancements in DEPC mediated strand cleavage are always at adenine residues (not guanine) and are never found in the step GGA. Other enhancements, which are not directly adjacent to bleomycin cutting sites can also be seen, especially around position 50 on the tyrT lower strand, and are less readily explained. Presumably these represent longer range changes in DNA structure mediated by bleomycin binding.

The results of similar experiments using DEPC as a footprinting probe on the 135 base pair DNA fragment are presented in Figure 3. Clear enhancements are present at positions 25 (GCA), 52 (GAA), 62 (GTA) and 84 (GAA) and are in similar sequences to the changes presents in Figure 2.

Potassium permanganate

The results of experiments using potassium permanganate as a footprinting probe are presented in Figure 3 for the 135-mer and Figure 4 for both strands of the tyrT DNA fragment, both in the presence and absence of bleomycin-B₄. The pattern in

Figure 2. Patterns of diethyl pyrocarbonate mediated DNA strand cleavage in the presence of bleomycin- B_2 and 200 μ M CoCl_2 . Lanes labelled A, B, C, D are in the presence of 0, 2, 10 and 50 μ M bleomycin- B_2 respectively. Tracks labelled G indicate dimethylsulphate-piperidine markers specific for guanine. Tracks labelled 'cut' indicate cleavage of the DNA by 5 μ M bleomycin- B_2 alone. The numbering refers to the DNA sequences shown in Figure 1.

Figure 3. Autoradiograph of DEPC and permanganate mediated DNA strand cleavage on the 135mer in the presence of bleomycin-B₄ and 200μM CoCl₂. Tracks labelled A, B, C are in the presence of 0, 5 and 25μM bleomycin-B₄ respectively. The track labelled 'cut' corresponds to cleavage by 5μM bleomycin-B₄ alone. 'G' indicates a dimethylsulphate-piperidine marker specific for guanine.

Figure 4. Patterns of DNA cleavage mediated by methylene blue (MB), dimethylsulphate (DMS) and potassium permanganate (KMnO₄). Tracks labelled con and blm correspond to control and 25µM bleomycin-B₂ treated samples respectively. For the reaction with potassium permanganate each pair of lanes corresponds to reaction with the probe for 15sec and 5 mins. The track labelled 'cut' corresponds to cutting by bleomycin alone.

the control lanes is complex, with cutting occurring mainly at pyrimidines, although cleavage at some purine residues is also evident. In the presence of bleomycin certain bonds are markedly enhanced. Many of these enhancements are adjacent to bleomycin binding sites the most notable being positions 41, 58, 98 and 110 on the 135mer, position 57 on the tyrI lower strand and positions 122, 45, and 20 on the upper strand. These are in the third base of the sequences GTC, GCC, GCT, GTC, GTT, GCC, GTT and GTT respectively. All of these are in the sequence GPyPy. However, not all such sequences are affected, for example GTT at positions 39 and 62 on the tyrI lower strand and GCC at positions 75 and 121 display an unaltered reactivity. It appears that if the base 3' to a bleomycin cutting site is a pyrimidine then it may be rendered more sensitive to modification by permanganate. It should however be noted that these are not the only bases which are rendered more sensitive to permanganate modification. Other bonds show an increased intensity relative to that in the antibiotic-free control lanes, such as those visible in the vicinity of positions 34-36, 72-78, 90-95 on the 135mer. Each of these corresponds to a run of pyrimidine residues, and may reflect some longer-range structural changes induced by the binding of bleomycin.

Dimethylsulphate and methylene blue

Both dimethylsulphate and methylene blue modify DNA so that subsequent treatment with piperidine results in cleavage at guanine residues and they have each been used as footprinting probes [28]. The results of experiments using these agents as footprinting tools in the presence and absence of bleomycin-B₄ with the tyrI DNA fragment labelled on its lower strand are presented in Figure 4. No drug-induced changes can be detected, suggesting that either bleomycin does not alter the conformation of adjacent G residues or that such alterations if they occur are not detected by these probes.

DISCUSSION

The results presented in this paper demonstrate that bleomycin and phleomycin can provoke significant changes in the structure of the nucleotide on the 3' side of some of their

cleavage sites. This modification is independent of strand cleavage. Enhanced reactivity towards diethylpyrocarbonate is observed at adenines on the 3' side of the GPy drug binding site; no changes in modification of G residues are apparent. The sequences GTA always gives rise to an enhanced reaction with DEPC, GCA is modified less frequently and modification at GAA is occasionally observed. This is consistent with the known cleavage specificity of the antibiotic for which these sites are cut in order GTA> GCA> GAA>> GGA [10-12]. Increased reactivity towards permanganate is generally observed for the second pyrimidine in the sequence GPyPy, again on the 3' side of the drug recognition site. Before considering the likely structural changes that can give rise to these modification it is worth making a few general observations.

The intensity of the modification does not always correlate with the relative cleavage specificity of bleomycin. For example on the 70mer the major enhancement to DEPC is at position 61 (GCA), yet this corresponds to a relatively weak cleavage site; the strongest drug cutting occurs at position 66 (GTT). Again on the 135mer the best bleomycin cutting sites are at position 63 and 65 (GTA and GTG respectively), yet only the former shows any change in reactivity to DEPC. It is not clear whether there is any relationship between these structural distortions and the ability of the ligand to subsequently cleave the DNA. The drug induced changes in reactivity to DEPC and permanganate are all single stranded events, as too is the cleavage reaction. For example in the sequence GTA the adenine shows increased reaction with DEPC, yet there is no corresponding change on the opposing strand TAC.

The observation that these structural changes are identical for all four bleomycins and phleomycins investigated suggests that the side chain is not responsible. It has previously been reported that the base on the 5' side of the drug cleavage site has an effect on the cutting activity, being greatest for T and least for A residues [12]. Taken together these two different sets of observations demonstrate that bleomycin must interact with sequences on both sides of the GPy recognition site,

although it is not clear how the molecule is oriented about this sequence.

Although the major sites of enhanced sensitivity to DEPC and permanganate are adjacent to bleomycin cutting sites, other positions show a lesser increase in sensitivity towards these two probes. These are presumably the result of bleomycin-induced changes in long-range DNA structure. Where a direct comparison with other footprinting data is possible, for the lower strand of the tyrT DNA fragment [15], these changes correspond to regions of increased sensitivity to DNAaseI.

Structural changes

What then are the changes in DNA structure that cause these alterations in reactivity towards the two chemical probes. DEPC has previously been used to detect Z-DNA [20,21], hairpin loops [22,23] and Hoogsteen base pairs [24]. In each of these cases the probe is presumed to react with purine bases in the syn (rather than the normal anti) configuration. With Z-DNA reaction occurs at the purine N7 (and the exocyclic N6 of adenine) [20,21], whereas in Hoogsteen pairs these groups form the central, unexposed, portion of the base pair and reaction is presumed to occur at purine N1 or N3 [24]. It has previously been shown that double stranded RNA is less reactive towards DEPC than double stranded DNA [31], so that we can conclude that an A-type DNA helix can not be responsible for the observed changes with bleomycin. Permanganate ions are presumed to react with the C5-C6 bond in pyrimidines and have been shown to modify single stranded DNA in the order T>C>G>>A [25]. Single stranded nucleic acids are much more sensitive to reaction than double stranded ones, consistent with reaction at the exposed C5-C6 bond. Although an increase in DEPC reactivity has been previously demonstrated with echinomycin [24, Fox, Portugal & Waring unpublished observations] actinomycin D [24] or other simple intercalators such as ethidium (unpublished observations) do not induce such changes, so that the intercalation event itself can not be responsible for the alterations in DNA structure.

The data are consistent with a model in which

bleomycin and phleomycin can stabilize an unusual base-pair structure on the 3' side of some of their cutting sites. Of the structures considered above we can discount the formation of a Z-DNA like conformation since this is unlikely to be restricted to a single base pair step and would be expected to be generated along a longer length of DNA. The formation of Hoogsteen-like base pairs would explain why guanine residues are not modified since they form Hoogsteen pairs less readily [32]. However, such an interpretation relies on the assumption that DEPC can modify purine N1 and N3 atoms [24], whereas a more likely reaction site is at N7. The most likely structural change responsible for these increases in reactivity towards DEPC and permanganate is the formation of a single-stranded region, involving the looping out of a single base on one DNA strand.

It must be emphasized that the experiments described in this paper can not reveal the exact nature of such a transition, but indicate that an unusual and unexpected structural change has occurred. Further details of the exact nature of the structural alterations must await the determination of a crystal structure of bleomycin bound to an oligonucleotide.

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