Interaction of bleomycin with a bent DNA fragment

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The interaction of bleomycin with a kinetoplast DNA fragment has been examined using various footprinting techniques. This DNA adopts a bent structure and displays an unusually low gel mobility on account of its phased runs of adenines. The bleomycin–cobalt complex increases the mobility of this DNA fragment, in contrast with other DNAs which show a decreased rate of gel migration, suggesting that the antibiotic removes DNA bending, possibly via an unwinding mechanism. Removal of the bending is confirmed by hydroxy-radical footprinting which produces a more even ladder of bands in the presence of the ligand. Cleavage by bleomycin is at the sequence G-pyrimidine, though not all such sites are affected to the same extent and some cutting is found at GA and GG. DNAase I footprinting confirms the antibiotic-binding sites but reveals that some strong cleavage sites do not yield footprints. Bleomycin renders adenines on the 3' side of its cleavage sites (GT, GC and GA) hyper-reactive to diethyl pyrocarbonate.

INTRODUCTION

The bleomycins are a family of structurally related glycopeptide antibiotics which are currently used in cancer chemotherapy (Blum *et al.*, 1973; Crook & Bradner, 1977). It is proposed that they act by causing DNA strand breakage in a reaction that requires stoichiometric amounts of Fe(II) and molecular oxygen (Takita *et al.*, 1978; Burger *et al.*, 1981). The molecular details of the binding of bleomycin to DNA have not been fully determined but various physicochemical measurements suggest that it binds in the minor groove (Kuwahara & Sugiura, 1988). The antibiotic unwinds DNA by 12° and lengthens it by 0.31 nm (3.1 Å), an observation that has been proposed to be due to partial intercalation of the bithiazole moiety (Povirk *et al.*, 1979, 1981; Henichart *et al.*, 1985; Levy & Hecht, 1988).

Bleomycin cleaves DNA in a sequence-specific fashion with a preference for G-pyrimidine (GC = GT > GA > GG) (D'Andrea & Haseltine, 1978; Murray & Martin, 1985). Bleomycin forms a highly stable complex with Co(II) which binds to, but does not cleave, DNA (Fox *et al.*, 1987). Footprinting studies of the Co(II)–bleomycin complex have revealed that the drug protects from DNAase I cleavage around its cutting sites, suggesting that specificity is the result of binding rather than the cleavage mechanism (Fox *et al.*, 1987). However, recent studies have indicated that bleomycin can also efficiently cleave other repetitive sequences, notably ApT, suggesting that the sequence selectivity may also be due to structural factors, rather than simply the formation of specific hydrogen bond contacts with particular DNA base substituents (Fox, 1990a).

Bleomycin binding enhances DNAase I cleavage in runs of A and T adjacent to some of its binding sites (Fox *et al.*, 1987), and renders DNA sensitive to cleavage by a single-strand-specific endonuclease (Sleigh & Grigg, 1974). The drug also renders specific adenine and thymine residues, adjacent to its binding sites, hyper-reactive to diethyl pyrocarbonate (DEPC) and potassium permanganate (Fox & Grigg, 1988). These studies provide compelling evidence that bleomycin induces changes in local DNA structure.

In the present study we have investigated the effects of DNA local structure on bleomycin cleavage by studying its interaction with a kinetoplast DNA fragment. This DNA, found in the mitochondria of trypanosomes, contains phased runs of adenine residues spaced at approximately 10 bp intervals and has an

anomalously low rate of migration in polyacrylamide gels (Kitchin *et al.*, 1986). It is proposed that the molecule is highly bent. Several models have been proposed to account for the bending; the most widely accepted is that there is a deflection in the helical axis at the junction between the A tracts and the surrounding regions of B-DNA (Burkhoff & Tullius, 1987; Katahira *et al.*, 1990). Poly(dA) tracts are known to adopt an unusual structure with a narrow minor groove, in which the bases are highly propeller-twisted (Nelson *et al.*, 1987). Previous studies have shown that several other DNA-binding drugs can remove the bending of this DNA fragment, increasing its gel mobility (Griffith *et al.*, 1986; Nielsen *et al.*, 1990; Fox, 1990b; Cons & Fox, 1990; Barcelo *et al.*, 1991) and affecting DNAase I digestion patterns (Fox & Kentebe, 1990; Mendoza *et al.*, 1990). In this study we have assessed the effect of bleomycin on the mobility of this DNA fragment and have attempted to correlate this with its sequence-selective binding and cleavage.

MATERIALS AND METHODS

Drugs and enzymes

Bleomycin was purchased from Sigma, and stored at –20 °C as a 1 mM stock solution in 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-NaCl. This was diluted to working concentrations immediately before use. DNAase I was purchased from Sigma and stored as a 7200 units/ml solution at –20 °C as previously described (Fox *et al.*, 1987).

DNA fragments

Plasmid pPK201/CAT, containing the kinetoplast fragment (Kitchin *et al.*, 1986), 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) fragment containing the kinetoplast DNA was isolated, and labelled at the 3' end of either strand as previously described (Fox & Kentebe, 1990). The sequence of this DNA fragment is shown in Fig. 2. The *tyr*T and T₆₉ DNA fragments were prepared and labelled as previously described (Cons & Fox, 1990).

Gel mobility

DNA mobility changes in the presence of bleomycin were determined as previously described (Cons & Fox, 1990) using

Abbreviation used: DEPC, diethyl pyrocarbonate.

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0.3 mm thick 20 cm × 40 cm 4.5% polyacrylamide gels run in 90 mM-Tris/borate pH 8.3 containing 2 mM-EDTA. Each gel was divided into two by placing an additional spacer (coated in petroleum jelly) in the centre of the glass plates. Meanwhile, 50 ml of 4.5% acrylamide-bisacrylamide (19:1, w/w) was prepared and divided into two equal samples. The first 25 ml was used to pour one-half of the gel. Once this had set, the remaining 25 ml was adjusted to 40 μ M-bleomycin and 200 μ M-CoCl₂ and used to pour the second half of the gel. In this way we ensure that both sides of the gel, in the presence and absence of antibiotic, are an identical thickness and gel strength, and that each DNA fragment experiences exactly the same drug concentration. These polyacrylamide gels were run at 600 V for about 2 h after which they were fixed in 10% acetic acid, transferred to Whatman 3 MM paper, dried and subjected to autoradiography.

Footprinting

Footprinting reactions with DNAase I and DEPC were performed as previously described (Fox *et al.*, 1987; Fox & Grigg, 1988). Hydroxy-radical footprinting was performed as previously described (Burkhoff & Tullius, 1987; Cons & Fox, 1989). All bleomycin footprints contained 200 μ M-CoCl₂ to prevent cleavage by the antibiotic. Bleomycin cleavage was obtained by incubating the DNA (approx. 1 μ M-base pairs) with 2 nM-bleomycin for 3 min in the presence of 10 μ M-ferrous ammonium sulphate and 100 μ M-dithiothreitol. 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 being dried at 80 °C under vacuum. The gels were exposed to autoradiography at -70 °C using an intensifying screen. Autoradiographs of bleomycin and hydroxy-radical cleavage were quantified by scanning with a Joyce-Loebl Chromoscan III microdensitometer.

RESULTS

Gel mobility

The kinetoplast DNA fragment displays an anomalously low gel mobility on account of its phased runs of adenines which

Fig. 1. Mobility of DNA fragments in the presence of bleomycin

The three fragments were run on a 4.5% polyacrylamide gel in the presence (D) and absence (F) of 40 μ M-bleomycin. a, T₈₉-containing fragment; b, *tyrT* DNA; c, kinetoplast DNA fragment.

Fig. 2. Sequence of the kinetoplast DNA fragment and its cleavage by bleomycin

The lines refer to the relative cleavage at each site by the antibiotic. The positions for each strand which bear the radioactive label are underlined. Data were taken between positions 30 and 210 for the upper (*HindIII* labelled) strand and 55 and 245 for the lower (*EcoRI* labelled) strand. Also included are summaries of the major DNAase I footprints (boxes) and sites of DEPC-mediated cleavage (filled boxes). DNAase I and DEPC footprinting data were taken between positions 140 and 220 (top strand) and 30 and 130 (bottom strand).

cause the entire sequence to bend through about 360°. We have studied the effect of bleomycin on the mobility of this fragment, under conditions in which the antibiotic does not cut the DNA. We have included two other DNA fragments for comparison: the 160 bp *tyrT* DNA fragment, which has been well studied as a footprinting substrate, and a 130 bp fragment derived from the pUC19 polylinker containing the sequence (T)₈₉·(A)₈₉ ligated into the *SmaI* site (Cons & Fox, 1990, 1991). *TyrT* DNA is useful for showing the effects of the drug on a DNA of average base composition and 'random' sequence, whereas T₈₉ provides a control showing that any effects are due to the bending of the kinetoplast fragment and not merely from its high AT content. The effects of 40 μ M-bleomycin on the mobility of these three DNA fragments in a 4.5% polyacrylamide gel are presented in Fig. 1. This bleomycin concentration was chosen since it is within the range for which sequence-selective binding has previously been demonstrated in footprinting experiments.

It is clear from Fig. 1 that bleomycin causes changes in the mobility of the three fragments. The *tyrT* and T₈₉ fragments show a slight decrease in mobility, typical of that observed in 'band-shift' experiments, and which can probably be attributed to the increased bulk of the complex, or to an increase in the

Fig. 3. DNAase I digestions of kinetoplast DNA in the presence of bleomycin

(a) Kinetoplast DNA labelled at the 3' end of the *Hind*III site. Each sample contained $200 \mu\text{M}$ - CoCl_2 to prevent cleavage by the antibiotic and the following bleomycin concentrations: (1) control, no drug; (2) $1 \mu\text{M}$; (3) $5 \mu\text{M}$; (4) $25 \mu\text{M}$; (5) $100 \mu\text{M}$; (6) $200 \mu\text{M}$. Each lane represents digestion by the enzyme for 2 min. The track labelled G is a dimethyl sulphate/piperidine marker specific for guanine. The residue numbers refer to the numbering scheme in Fig. 2. (b) Kinetoplast DNA labelled at the 3' end of the *Eco*RI site. Each sample contained $200 \mu\text{M}$ - CoCl_2 to prevent cleavage by the antibiotic and the following bleomycin concentrations: (1) control, no drug; (2) $5 \mu\text{M}$; (3) $25 \mu\text{M}$. Each pair of lanes represents digestion by the enzyme for 1 and 5 min. The track labelled G is a dimethyl sulphate/piperidine marker specific for guanine; that labelled Cl shows the pattern of cleavage by the antibiotic alone in the absence of cobalt. The residues refer to the numbering scheme in Fig. 2.

DNA rigidity. The effect on the T_{69} fragment is less than that for *tyrT* DNA, presumably because it contains fewer bleomycin-binding sites [bleomycin shows little or no interaction with poly(dA)·poly(dT)]. This mobility change is unlikely to result from electrostatic effects since the bleomycin- Co^{3+} complex will be either neutral or bear a small positive charge. The change in the molecular charge will be small compared with the overall charge of the DNA fragments.

In contrast, bleomycin causes a pronounced increase in the mobility of the kinetoplast fragment. This by itself may not be impressive, but when compared with the opposite effect on the other DNA fragments it is clear that bleomycin has had some other effect on kinetoplast DNA. The most likely explanation is that the antibiotic has affected the DNA conformation, possibly removing the bending, thereby restoring its mobility closer to that of a normal piece of DNA of equivalent length. Two possible mechanisms can be envisaged. Either bleomycin binds adjacent to the bending loci and straightens the DNA, or the drug lengthens the DNA so that, although some local bending still occurs, the bends are no longer in phase with each other or the helical pitch. The increase in gel mobility then arises from some combination of its binding characteristics including sequence selectivity, DNA unwinding and helix extension.

In these experiments the bleomycin was incorporated into the gel matrix; as a result it is not possible to determine the effect of drug concentration on the changes in mobility. Such experiments would be prohibitively expensive and would require rigorous control of the gel conditions between separate experiments. We attempted to examine changes in mobility by mixing bleomycin and DNA before running on normal non-denaturing polyacrylamide and agarose gels. Similar experiments with other DNA-binding drugs have demonstrated that the ligands remain bound to the DNA during electrophoresis (Barcelo *et al.*, 1991). Under these conditions we found no changes in the mobility of kinetoplast DNA, suggesting that the drug dissociated from the DNA during electrophoresis.

Bleomycin cleavage

Fig. 2 presents the bleomycin-cleavage pattern of both strands of kinetoplast DNA (an autoradiograph of the cleavage products is also shown in Fig. 3b). All the strong cleavage sites are found at sequences GC and GT. However, although all such sites show some cleavage, the extent of digestion at each site varies considerably along the fragment. On the top strand (*Hind*III labelled) the best cleavage is found around position 160 in the sequence TGCC, flanked on both sides by runs of adenines. Interestingly a very similar sequence, containing an additional G before the bleomycin-cleavage site is located at 170, yet this is cut at only 20 % of the rate. Bleomycin cleavage at several GC and GT dinucleotides is poor, especially on the top strand. Cutting is more even on the bottom (T-containing) strand.

As well as this canonical cleavage at G-pyrimidine, several GA steps are cut with moderate efficiency. In general, the GA step is cut best if the base on the 5' side is a G (183 and 203) and poorly if it is preceded by a run of Cs (71, 135, 197). From the available data, less can be determined about the base on the 3' side since in every case on this DNA fragment this is either A or T. Cleavage is also observed at some GG steps (188, 178, 159, 128, 87, 77). A few other unusual cleavage sites are also apparent. Notable amongst these are the products at 192 and 170, each of which is one base removed from a canonical bleomycin-cleavage site. Around position 170 the symmetrical sequence TGGCCA shows this anomalous cleavage only on the lower T-containing strand.

DNAase I footprinting

Bleomycin-cleavage efficiency is a function of both binding and catalytic rate constants; it is therefore not possible to determine whether the different rates of cleavage at each site are the result of variations in bleomycin-cleavage efficiency *per se*, or arise from alterations in binding of the ligand to DNA. We have therefore examined the binding of bleomycin by DNAase I footprinting. Previous studies with *tyrT* DNA demonstrated that bleomycin cutting correlates with the footprinting pattern,

Fig. 4. Densitometer traces of hydroxy-radical cleavage of kinetoplast DNA in the presence of 100 μ M-bleomycin

Kinetoplast DNA was labelled at the 3' end of the *Hind*III site (a) and the *Eco*R1 site (b). CON indicates the control, BLM corresponds to the trace in the presence of 100 μ M-bleomycin. The numbers correspond to the numbering scheme presented in Fig. 2.

indicating that the cleavage preference arises from the binding specificity of the ligand (Fox *et al.*, 1987). It is also known that the ability of a ligand to generate DNAase I footprints depends on its persistence time on the DNA lattice; those compounds which dissociate the fastest generate poor footprints.

Fig. 3 presents DNAase I-digestion patterns of the kinetoplast DNA, labelled on each strand, in the presence and absence of various concentrations of bleomycin. It is worth remembering that the concentration of bleomycin used in these footprinting studies needs to be over a thousandfold higher than in the simple cleavage reactions, since in the latter case the reaction is catalytic, whereas in the former the stoichiometry of binding is dictated by the equilibrium binding parameters. In the absence of the antibiotic, cleavage of the DNA is uneven and regular gaps in the ladder of bands are apparent, corresponding to the blocks of A and T residues (Fox & Kentebe, 1990). In the presence of bleomycin, the cleavage pattern is altered and a footprinting pattern is generated, though this is less clear than the footprints seen with other DNA fragments. Sites of protection can be seen around positions 30–33, 60–65, 84–87 (top strand, *Hind*III labelled) and 219–222, 189–192 and 155–159 (bottom strand, *Eco*R1 labelled). These footprints are summarized in Fig. 2. Each of these footprints is situated around a good bleomycin-cleavage site. However, not all cutting sites generate clear footprints. For example, the best cleavage on the top strand is found at position 55 yet this region shows very little decrease in DNAase I-cleavage efficiency. A similar effect is seen around position 149 on the bottom strand. Fewer sites of enhanced cleavage are generated than seen in previous studies with *tyrT* DNA though

Fig. 5. Pattern of DEPC modification of kinetoplast DNA in the presence of bleomycin

Kinetoplast DNA was labelled at the 3' end of the *Hind*III site (a) and the *Eco*R1 site (b). The DNA was modified by DEPC as described in the Materials and methods section, in the presence of 200 μ M- CoCl_2 and the following bleomycin concentrations: (1) control, no drug; (2) 0.1 μ M; (3) 0.5 μ M; (4) 2.5 μ M; (5) 10 μ M; (6) 20 μ M. The track labelled G is a dimethyl sulphate/piperidine marker specific for guanine. The residue numbers refer to the numbering scheme shown in Fig. 2.

some increased cleavage is evident around positions 38–48, and 91–94 (lower strand) and 208–216 (upper strand). Each of these contain A tracts which are close to bleomycin-cleavage sites. For example, the enhancement at positions 38–48 most likely arises from drug binding at position 33.

Hydroxy-radical cleavage

Although hydroxy-radical cleavage of most DNA fragments produces a fairly even ladder of bands, on account of the small size of the probe, digestion of kinetoplast DNA yields a phased cleavage pattern, corresponding to bending of the DNA helix (Burkhoff & Tullius, 1987). Hydroxy-radical cleavage decreases towards the 3' end of the poly(A) tracts. Densitometer traces derived from hydroxy-radical cleavage of kinetoplast DNA in

the presence and absence of bleomycin (with 100 μM -CoCl₂ present to prevent cleavage) are presented in Fig. 4 for both labelled DNA strands. Both strands show the characteristic phasic cleavage pattern previously reported for this fragment, with decreases in cleavage evident around positions 68–72, 78–83, 88–97, 102–106, 112–117 and 121–124 (upper strand) and positions 165–170, 175–180, 186–190 and 196–200 (lower strand). In the presence of bleomycin no clear footprints are produced though the phasic cleavage pattern is abolished. It is not clear why no hydroxy-radical footprints are obtained, but this is typical of other sequence-selective DNA-binding drugs, including actinomycin (Churchill *et al.*, 1990). The removal of the phased pattern strongly suggests that bleomycin has removed the DNA bending.

DEPC-mediated cleavage

The patterns of DEPC-mediated cleavage in the presence and absence of bleomycin are presented in Fig. 5 for the kinetoplast fragment. The reaction in the control lanes occurs at purine residues. In the presence of bleomycin, several bands show a marked increase in reactivity notably at positions 214 (bottom strand) and 35, 49, 54, 70, 82 and 93 (top strand). These are summarized in Fig. 2. Each of these is located at an adenine two bases to the 3' side of guanine, in the sequences GA, GC or GT. Fewer enhancements are present for the bottom strand since this is T-rich and contains only one such sequence GPyA. For both strands all such sequences are affected (both GPyA and GAA), irrespective of whether they are good bleomycin-cleavage sites.

DISCUSSION

Sequence selectivity

The results presented in this paper confirm that bleomycin cleaves DNA most efficiently in the sequence G-pyrimidine, though significant cleavage is often observed at GA. The simple base selectivity does not appear to have been altered by the bending of the kinetoplast DNA fragment. Previous studies have shown that bleomycin cleavage corresponds to the footprints produced by the cobalt-bleomycin complex (Fox *et al.*, 1987). It is worth noting that the footprints obtained in this work are generally not as clear as those in similar studies with other DNA fragments under identical conditions (Fox *et al.*, 1987). The binding of bleomycin to this bent DNA fragment is weaker than to other natural DNAs. A possible explanation for this is that part of the binding energy is taken up by distorting (unbending) this DNA. It may be significant that the clearest protection is seen around position 35 (top strand) which lies outside the bent region, belonging instead to the polylinker.

Structural changes

Bleomycin increases the gel mobility of kinetoplast DNA, in contrast with other DNA fragments which show the usual decrease in mobility. It therefore seems that the drug has removed some of the bending associated with this DNA fragment. What is the cause of this effect? Bleomycin has been shown to unwind DNA, an effect which some have interpreted as suggesting the intercalation of the bithiazole portion. The removal of the bending cannot be due to the unwinding alone, since other intercalators such as ethidium and actinomycin do not cause a similar effect, though echinomycin and nogalamycin do (Cons & Fox, 1990). Similarly, the AT-selective minor-groove-binding ligand distamycin increases the mobility of kinetoplast DNA, though to a lesser extent than seen with bleomycin in this paper. It seems that the effect on DNA bending arises from some

combination of the structural changes caused by the ligand and its sequence selectivity.

The ability of bleomycin to unwind DNA is confirmed by the sensitivity of adjacent adenines to modification by DEPC. This has been previously noted for the sequences GTA and GCA (Fox & Grigg 1988). The most likely explanation for this effect is that bleomycin causes a local unwinding of the DNA, exposing the N-7 position of adjacent adenines, rendering them hyper-reactive to DEPC. This unwinding seems to be concentrated in the position 2 bases to the 3' side of guanines and must have implications for the binding mode of this antibiotic. It is not obvious why this increase in reactivity should be restricted to adenines.

Binding versus cleavage

In a previous study we demonstrated that the footprints produced by bleomycin correspond to regions which are cleaved efficiently by the antibiotic (Fox *et al.*, 1987). This suggests that the cleavage specificity is largely determined by the binding of the ligand; the drug cuts best at those regions to which it is already tightly bound. However, we have also shown that bleomycin can efficiently cleave certain non-canonical sequences, especially regions of alternating A and T, but that these do not yield DNAase I footprints (Fox, 1990a). In this case it appears that the ligand dissociates from its cleavage sites so rapidly that it cannot be detected by the probe, yet during its short residence time it efficiently cleaves the DNA. Do the present results shed any light on this paradox? We suggest that these results confirm that tight ligand binding does not necessarily correspond to good drug cleavage sites and vice versa. Some sites are efficiently cleaved, yet do not produce footprints (position 54 top strand); other sites (for example position 86) yield clear footprints but are only weakly cleaved. In general, we note that most of the footprints are found in regions where the G-pyrimidine step is preceded by G or C, whereas the most efficient cleavage sites are in the sequence pyrimidine-G-pyrimidine. Enhanced reactivity towards DEPC is constant at all GNA sites (N = C, T or A). We therefore suggest that bleomycin binding is strongest in GC-rich regions, but that this tight binding does not necessarily lead to efficient catalytic cleavage. Bleomycin degrades DNA in a catalytic reaction; before a second cleavage event can occur it must first dissociate from its first binding site. The sequences cut the most efficiently will then be those from which the ligand can easily dissociate (and which will therefore not yield good footprints). Sites to which bleomycin binds too tightly will trap the ligand and not lead to efficient catalytic cleavage. In this respect bleomycin behaves like a typical enzyme. It should be noted that this analysis compares cleavage by the iron(II) complex with binding of the cobalt(II) complex. However, the metal plays a role in the binding reaction since both the zinc and metal free bleomycin (i.e. in the presence of an excess of EDTA) complexes bind so weakly to natural DNA fragments that they fail to yield footprints (unpublished observations). It is also worth noting that hyper-reactivity towards DEPC occurs at a lower drug concentration (2–5 μM) than the DNAase I footprints (25–50 μM). This may be explained by suggesting that the DNA structural changes persist for longer than the residence time of the ligand on the DNA helix.

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