Sequence-selective binding of phleomycin to DNA

Keith R. FOX,*† Geoffrey W. GRIGG† and Michael J. WARING*

*Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD, U.K., and †CSIRO Division of Molecular Biology, P.O. Box 184, North Ryde, Sydney 2113, Australia

The binding of phleomycin and bleomycin to DNA has been investigated by studying their effects on cleavage by DNAase I and micrococcal nuclease. In the presence of cobalt, cleavage of DNA by the antibiotics is suppressed, yet they still provide protection from nuclease attack in regions surrounding the drug cleavage sites. We conclude that cleavage by phleomycin occurs at bonds around which the antibiotic is already selectively bound.

INTRODUCTION

The phleomycins and the structurally related bleomycins are glycosidic antibiotics which are used in cancer chemotherapy [1,2]. They appear to act by causing DNA strand breakage and degradation [3,4] in a reaction that has been shown to require stoichiometric amounts of Fe^{2+} [5,6]. Current evidence suggests that the reaction mechanism involves the oxidation of Fe^{2+} to Fe^{3+} , with the production of reactive species which damage the DNA [7–9].

Cleavage of DNA *in vitro* has been shown to occur best at dinucleotide sequences GpC and GpT, and it is known that bonds are most susceptible when the G is preceded by a pyrimidine residue [10–12]. DNA damage appears to be primarily a single-stranded event, since the extent of cleavage on one strand is independent of that on the opposite strand, presumably because the reactive species do not diffuse far from the site at which they are generated [12]. An outstanding question is whether the cleavage specificity arises direct from selective binding of the antibiotic to DNA or whether only a subset of sites to which the antibiotic is bound are liable to strand scission.

Arguably the best method for assessing the sequence selectivity of a DNA-binding ligand is that of ‘footprinting’ [13–20]. In this technique a DNA fragment of known sequence, carrying a radioactive label at one end, is digested by a chemical or enzymic agent in the presence or absence of the ligand. The products of the reaction are separated on denaturing polyacrylamide gels, and the positions of bound ligand molecules can be determined from the gaps in the otherwise continuous ladder of bands. However, before this technique can be applied to such ligands as phleomycin, conditions must be found which permit the binding to the DNA, yet prevent the subsequent strand cleavage. We have solved this problem for the phleomycin/bleomycin group of antibiotics by working with their cobalt complexes, which have previously been shown to be very stable [21]. We find that these complexes bind to DNA in sequence-selective fashion, yet do not subsequently cleave the DNA. As a result, standard footprinting procedures

become applicable, so that the sites of physical binding of the antibiotic can be compared with the sites at which strand cleavage occurs in the Fe^{2+} -promoted reaction.

MATERIALS AND METHODS

Drugs and enzymes

Phleomycins E and CHP and bleomycins B₄ and CHP were prepared, metal-free, as previously described [22]. Stock solutions (1 mM) were prepared in 10 mM-Tris/HCl/10 mM-NaCl, pH 8.0, and stored at -20°C . DNAase I was purchased from Sigma and stored as a 7200 units/ml solution in 150 mM-NaCl/1 mM-MgCl₂ at -20°C . Micrococcal nuclease was purchased from P-L Biochemicals and stored as a 2500 units/ml solution in 50 mM-Tris/HCl, pH 7.6, containing 2 mM-CaCl₂ at 4°C .

DNA fragments

The 160-bp *tyrT* and the 166-bp *ptyr2* DNA fragments were isolated from plasmids pKMA-98 and pMLB1048 respectively as previously described [18]. Relevant portions of these sequences are shown in Figs. 2 and 3 (below). They were each labelled at the 3'-end of the endonuclease-*EcoRI* sites by using reverse transcriptase and [α -³²P]dATP. The residue numbering scheme used to describe bond numbers in these fragments corresponds to that previously employed [17–20].

Footprinting

DNAase I footprinting was performed as previously described [17–20]. Drug solutions were incubated with 400 μM -CoCl₂ before addition to the DNA, yielding a final cobalt concentration of 200 μM and antibiotic concentration as required. All control digests contained an equivalent amount of cobalt. Those lanes showing cleavage by the antibiotic alone were obtained by mixing the metal-free drug solution with the DNA for periods of 1–2 min before stopping the reaction with EDTA. Although no Fe^{2+} was added to these mixtures, the cleavage probably resulted from an iron complex generated by trace amounts of this metal, since it could

Abbreviation used: bp, base-pair.

† Present address and address for correspondence and reprint requests: Department of Physiology and Pharmacology, School of Biochemical and Physiological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

be prevented by prior incubation with EDTA. No strand cleavage could be detected even after 45 min incubation of the DNA with phleomycin in the presence of 200 μM -CoCl₂.

A similar procedure was used for footprinting with micrococcal nuclease (a final enzyme concentration of 1 unit/ml in 50 mM-Tris/HCl/2 mM-CaCl₂ buffer, pH 7.6, was used). It is worth noting that micrococcal nuclease cleaves the O-5'-P bond, whereas DNAase I and piperidine (used in the G-specific marker lanes) cleave the O-3'-P bond. As a result there is an extra phosphate group present on labelled fragments in the G-track, so that fragments in this lane run between 0.5 and 1.5 bonds faster than corresponding fragments in the micrococcal-nuclease digests.

Autoradiography

After electrophoresis at 1500V for 1½–2 h, gels were transferred to Whatman 3MM paper, dried under vacuum at 80 °C and subjected to autoradiography at –70 °C with an intensifying screen. Autoradiographs were scanned with a Joyce–Loebl scanning microdensitometer. For the DNAase I digestion experiments, results are presented in the form of a differential cleavage plot, as previously described [17–19], representing $\ln(f_a/f_c)$, where f_a and f_c are the fractional extents of cleavage at any bond in the antibiotic-treated and control lanes respectively. For micrococcal nuclease the results are presented as percentage cleavage at each bond in the antibiotic-treated lanes relative to that in the control.

RESULTS

Fig. 1 shows autoradiographs of the products of digestion by both DNAase I and micrococcal nuclease using two different DNA fragments in the presence and absence of phleomycin E. These are presented in a quantitative form in Figs. 2 and 3. Looking first at the cleavage by the antibiotic alone (lanes P), it is readily apparent that cleavage always occurs at sites located one bond to the 3' side of a guanine residue. On closer examination the 'strongest' cleavage sites are found at positions 21, 56 and 61 in *tyrT* DNA and 23, 54 and 87 in *ptyr2* DNA, each of which corresponds to the trinucleotide step PyGPy. Weaker cleavage is found at the PuGPy steps, with occasional cleavage at GpA (positions 44 and 79 in *tyrT* DNA). The dinucleotide GpG is never cleaved. This pattern of phleomycin-specific cleavage is entirely consistent with that previously described [10–12].

Turning next to the DNAase I digestion patterns in Fig. 1, it is clear that, in the presence of cobalt, cleavage by the antibiotic is totally suppressed, even in the longest incubations. The DNAase I cleavage pattern produced in the presence of cobalt–phleomycin is very different from that in the control lane. Clear protected regions are apparent, and these are generally located around each of the antibiotic cleavage sites. On inspecting the differential cleavage plots (Figs. 2 and 3) it can be seen that virtually all the antibiotic cleavage sites correspond to regions of blockage in the DNAase I plot. The only exceptions to this rule occur at positions 44 and 79 in *tyrT* DNA and 66, 69 and 94 in *ptyr2* DNA, most of which correspond to weaker cutting sites at the step GpA. It is not possible to assess rigorously the antibiotic-binding-site size from these data, since many of the phleomycin-binding sites

overlap. However, it is worth noting that in *ptyr2* DNA the isolated cleavage site at position 54 corresponds to a region of protection extending for four to five nucleotides.

A similar pattern of antibiotic cleavage and protection from DNAase I cleavage was observed for the other analogues tested. No significant differences were found. However, with the CHP derivatives (which have a simple side chain [22]), a larger antibiotic concentration was required (10–20 μM) to produce a clear footprinting pattern, possibly reflecting weaker binding to DNA. It seems likely that the side chains do not directly affect the sequence selectivity, but may serve as anchors to increase the strength of binding.

The effects of phleomycin on the cleavage by micrococcal nuclease are also shown in Figs. 1 and 2. This enzyme does not cleave every internucleotide bond, but cleaves best at pT and pA, especially in regions of alternating A and T residues [23–25; K. R. Fox & M. J. Waring, unpublished work], presumably because of their greater 'breathing' motions. As a result of the very uneven pattern in the control digestion, this enzyme is not useful for assessing exact drug-binding sites, but again we notice that regions where micrococcal-nuclease cleavage is visibly inhibited correspond to antibiotic cleavage sites. For example, the enzyme is strongly inhibited between positions 55 and 70, which contain five phleomycin cleavage sites, yet is unaffected between positions 80 and 90, a region in which no antibiotic cleavage is apparent.

It is worth noting that, as well as those antibiotic-induced blockages, there are other regions in which the enzymic cleavage appears markedly increased by the presence of the drug. These enhanced cleavage effects are most easily seen with DNAase I on *tyrT* DNA around positions 30 and 50, corresponding to runs of A and T residues. Enhanced cleavage at these positions has previously been noted in the presence of other antibiotics such as actinomycin, echinomycin and mithramycin [17,19,20] and has been interpreted as resulting from drug-induced changes in the local DNA structure propagated into regions surrounding the ligand-binding sites, rendering them more susceptible to nuclease attack. Clearly by this criterion phleomycin is also able to influence the local helix conformation at certain regions, in agreement with a report that it can sensitize native DNA to attack by *Neurospora crassa* endonuclease *in vitro* [26]. This may yield some clues concerning its mode of binding, and this is considered below.

DISCUSSION

The results presented here demonstrate that, in the presence of cobalt, phleomycin binds to DNA in a sequence-specific fashion, yet does not cause strand breakage. The regions that the cobalt complex protects from nuclease digestion correspond to those surrounding bonds which can be cleaved by the antibiotic. It appears, then, that the specificity of the phleomycin cleavage reaction results from the selective binding of the ligand; cleavage only occurs at these bonds around which the antibiotic is already selectively bound. The binding specificity is little affected by the nature of the side chain, at least for the few derivatives we have examined, although this portion may affect the strength of the interaction. The binding-site size appears to be 4–5 bp.

Fig. 1. Autoradiographs of nuclease digestion patterns in the presence of phleomycin E

The first two panels represent digestion of *tyrT* DNA and *ptyr2* DNAase I in the absence (CON) and presence (PHLEO) of 10 μ M-phleomycin, both in the presence of 200 μ M- CoCl_2 . Each set of lanes shows digestion by the enzyme for 1.5 and 30 min. The right-hand panel represents the digestion pattern of *tyrT* DNA produced by micrococcal nuclease (MNase) in the absence (CON) and presence (PHLEO) of 10 μ M-phleomycin (1 and 5 min digestion). The tracks labelled 'G' represent dimethyl sulphate/piperidine markers specific for guanine, and those labelled 'P' show the pattern of cleavage by phleomycin alone in the absence of cobalt. The residue numbers refer to the numbering scheme previously employed to describe these fragments [17–20] and correspond to those used in Figs. 2 and 3.

It is not possible, from the data presented here, to assess the exact positioning of the antibiotic. It would be interesting to determine which portions of the molecule are directed to the 5' or 3' sides of the GpPy bond being cleaved, but the size of the nuclease probe and the problem of overlapping binding sites do not permit such an analysis. Further experiments using DNA fragments containing isolated phleomycin-binding sites with simple chemical probes may shed further light on this problem.

The phleomycin-induced enhancement of cleavage by DNAase I at certain regions is consistent with previous work suggesting that the antibiotic lengthens the DNA helix by an amount similar to that caused by actinomycin D [27]. This does not necessarily argue for or against an intercalative mode of binding, but confirms that local distortion of the DNA helix can be propagated into neighbouring regions, which, if they are normally resistant to DNAase I cleavage, may thereby be rendered

Fig. 2. Cleavage plots for *tyrT* DNA in the presence of 10 μ M-phleomycin E

The upper panel represents relative cleavage at each site by the antibiotic alone. The centre panel corresponds to a differential cleavage plot showing differences in the relative probability of cutting by DNAase I, with 200 μ M- CoCl_2 present. Positive values indicate enhancements; negative values indicate blockage. The lower panel indicates percentage cleavage by micrococcal nuclease in the presence of the antibiotic and CoCl_2 with respect to that in the control.

Fig. 3. Cleavage plots for *pyr2* DNA in the presence or absence of 10 μ M-phleomycin E

The details are as for the upper two panels of Fig. 2.

more susceptible. Similarly the observed inhibition of micrococcal-nuclease activity at regions surrounding the binding site indicates that the antibiotic can locally stabilize the double helix, making 'breathing' of the base pairs less likely.

We thank Dr. Horace Drew and Dr. Maxine McCall for advice and discussion, and Dr. Antonio Aliano for assistance in purifying the phleomycin derivatives. This work was supported by grants from the Cancer Research Campaign, The Royal Society and the Medical Research Council.

REFERENCE

- Blum, R. H., Carter, S. K. & Agre, K. (1973) *Cancer* **31**, 903-914
- Crook, S. T. & Bradner, W. T. (1977) *J. Med.* **7**, 333-428
- Sleigh, M. J. (1976) *Nucleic Acids Res.* **3**, 891-901
- Sleigh, M. J. & Grigg, G. W. (1976) *Biochem. J.* **155**, 87-99
- Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Iitaka, Y. & Umezawa, H. (1978) *J. Antibiot.* **31**, 1073-1077
- Burger, R. M., Peisach, J. & Horwitz, S. B. (1981) *J. Biol. Chem.* **256**, 11636-11644
- Lown, J. W. & Sim, S.-K. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1150-1157
- Burger, R. M., Horwitz, S. B., Peisach, J. & Wittenberg, J. B. (1979) *J. Biol. Chem.* **254**, 12299-12302
- Burger, R. M., Berkowitz, A. R., Peisach, J. & Horwitz, S. B. (1980) *J. Biol. Chem.* **255**, 11832-11838
- D'Andrea, A. D. & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3608-3612
- Takeshita, M., Grollman, A. P., Ohtsubo, E. & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5983-5987
- Murray, V. & Martin, R. F. (1985) *Nucleic Acids Res.* **13**, 1467-1481
- Lane, M. J., Dabrowiak, J. C. & Vournakis, J. N. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3260-3264
- Van Dyke, M. W., Hertzberg, R. P. & Dervan, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5470-5474
- Goodisman, J. & Dabrowiak, J. C. (1985) *J. Biomol. Struct. Dyn.* **2**, 967-979
- Van Dyke, M. W. & Dervan, P. B. (1983) *Nucleic Acids Res.* **11**, 5555-5567
- Fox, K. R. & Waring, M. J. (1984) *Nucleic Acids Res.* **12**, 9271-9285
- Fox, K. R. & Waring, M. J. (1986) *Biochemistry* **25**, 4349-4356
- Low, C. M. L., Drew, H. R. & Waring, M. J. (1984) *Nucleic Acids Res.* **12**, 4865-4879

20. Fox, K. R. & Howarth, N. R. (1985) *Nucleic Acids Res.* **13**, 8695–8714
21. Nunn, A. D. (1977) *Eur. J. Nucl. Med.* **2**, 53–57
22. Grigg, G. W., Hall, R. M., Hart, N. K., Kavulak, D. R., Lamberton, J. A. & Lane, A. J. (1985) *J. Antibiot.* **38**, 99–110
23. Horz, W. & Altenburger, W. (1981) *Nucleic Acids Res.* **9**, 2643–2658
24. Dingwall, G., Lomonosoff, G. P. & Laskey, R. A. (1981) *Nucleic Acids Res.* **9**, 2659–2673
25. Flick, J. T., Eisenberg, J. C. & Elgin, S. C. R. (1986) *J. Mol. Biol.* **190**, 619–633
26. Sleight, M. J. & Grigg, G. W. (1974) *FEBS Lett.* **39**, 35–38
27. Povirk, L. F., Hogan, M., Dattagupta, N. & Buechner, M. (1981) *Biochemistry* **20**, 665–671

Received 15 December 1986/13 February 1987; accepted 20 February 1987