Communications to the editor

DNA STRUCTURES REQUIRED FOR BLEOMYCIN BINDING

Sir:

Bleomycin (Blm) causes strand scission of DNA *in vitro* as well as *in vivo*[1]. We have been determining its activity by a sensitive and quantitative method based on introducing a single breakage into the superhelical SV40 DNA with Blm under restricted conditions[2]. In previous papers[2,3] we reported that SV40 DNA was protected from Blm-induced cleavage by DNA of different origin, *e.g.* poly (dG-dC)*, and poly (dA-dT)*, but not by tRNA of *Escherichia coli*, apurinic acid, poly dA, poly dT and some deoxyribooligonucleotides. The protective effect was designated "protection by competitive binding". As an extension of this study, deoxyribonucleotides of various sizes and sequences have been tested for this effect. These studies have led us to propose that Blm preferentially binds to double stranded DNA at some purine/pyrimidine neighboring sequences.

The experiments were conducted as reported previously[2,3]. In brief, a 200 μl reaction mixture contained ^{3}H-SV40 DNA (approximately 2×10^{-3} A_{260nm} unit, 4,000 dpm), 1 μg of Blm-B_2, 50 mM Tris-HCl, pH 9.0 (adjusted at 0°C), and an indicated amount of a deoxyribonucleotide (a possible protector). The reaction was started by adding Blm to the rest of the reaction mixture, continued at 0°C for 30 minutes and terminated by adding 10 μl of 5 N NaOH. The mixture was then centrifuged through an alkaline sucrose density gradient to separate the reaction products sedimenting at 16S and 18S from the unreacted substrate sedimenting at 53S. In a control run which contained no protecting deoxyribonucleotide, approximately 60% of the original superhelical form of DNA was converted to the nicked open circular form. The percentage of protection was calculated as (1 − rate of strand scission** in a test run/rate of strand scission in a control run) × 100.

From the results of Experiment 1 in Table 1, we see that Blm showed extensive binding with every purine-pyrimidine alternating copolymer (double stranded), some binding with poly dG·poly dC; and slight or no binding with any of the four homopolymers or poly dA·poly dT. These results suggest the following: (1) DNA must be double stranded, (2) some purine-pyrimidine neighboring sequences on each strand are desirable, although a G strand paired with a C strand is somewhat acceptable, (3) thymine is not required for binding, although its selective release is thought to be a cause of strand scission[4]. It should be remembered that there was no extensive strand scission of DNA under our assay conditions because of the low concentration of Blm and the absence of any enhancing factor such as dithiothreitol. The conclusion (1) is not contradictory to our previous observations that heat denatured DNA and alkaline denatured poly (dA-dT) were good protectors[3] because denatured DNA contains some double-stranded regions and denatured poly (dA-dT) anneals rapidly. With respect to conclusion (2), it should be noted that poly (dA-dC)·poly (dG-dT) showed the strongest protection followed by poly (dG-dC) and poly (dA-dT) in that order and that these were even better protectors than calf-thymus DNA. The most preferable structure, therefore, seems to be an area including alternating G and T on one strand and alternating A and C on the other strand[5]. In this structure, a G·C pair(s) may be more important than an A·T pair(s). This assumption rests on the observation that poly dG·poly dC (but not poly dA·poly dT) showed some protection in this experiment and that poly (dC,dG) showed stronger protection than poly (dA,dT) in Experiment 2 (see below). It was also interesting to find that a copolymer of unnatural bases, poly [dI-(5-Br·dC)], was a good protector.

The requirement for double stranded structures and the preference for G·C to A·T were demonstrated again in Experiment 2. Here, copolymers with random sequences of 2 bases were examined. Protection was significant only with poly (dG,dC) and poly (dA,dT) and both can form double stranded regions. Poly (dG, dC) was over 10 times more effective than poly (dA, dT).

* Polymers of this type were expressed as poly dG-dC and poly dA-dT in our previous paper (Ref. 3).

** For details, see the legend to Fig. 2 of Ref. 2.

In the hope of finding the minimum structure to which Blm can bind, we tested a pair of oligonucleotides with complementary sequences, d(pT-pG)$_x$ and d(pC-pA)$_y$, in various combinations in Experiment 3. No protection was observed with either d(pT-pG)$_{6\sim9}$ alone or d(pC-pA)$_{6\sim9}$ alone. Since both oligonucleotides are single stranded, this result is reasonable. In contrast, there was considerable protection if both were present simultaneously, even at very low concentrations. In this experiment, no annealing process was conducted; the complementary oligonucleotides were only mixed in a reaction mixture at 0°C before the reaction was initiated. In other combinations, we found that one oligonucleotide can be as small as a tetranucleotide, provided that the other is somewhat larger. This result seems very reasonable considering the molecular size of Blm. A space-filling model of Blm B1′, the active member of the Blm family with the smallest terminal amine[6], can extend over 4 base-pairs along the grooves of DNA. The lack of protection by dinucleotides, even in combination with larger oligonucleotides, may also be ascribed to instability of the double stranded structure.

Various physical inspections of the complex of Blm and an appropriate oligonucleotide will further clarify Blm action at the molecular level.

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Table 1. Protection of SV40 DNA from bleomycin action by simultaneous addition of various poly- and oligonucleotides

Added nucleic acid	Amount added ($\times10^{-3}A_{260nm}$ unit)	% Protection
Exp. 1. Homo- and copolynucleotides		
calf-thymus DNA	2.5	52.7
poly (dG-dC)	2	77.2
poly (dA-dT)	2	53.2
poly (dA-dC)·poly (dG-dT)	1	82.9
poly [dI-(5-Br·dC)]	1	69.3
poly dA·poly dT	100	0
poly dG·poly dC	10	25.3
	100	97.3
poly dC	100	0.7
poly dG	100	3.8
poly dT	100	0
poly dA	100	0
Exp. 2. Copolymers with random sequences		
poly (dC, dT)	100	0
poly (dI, dT)	100	0
poly (dA, dC)	100	22.8
poly (dC, dG)	1	76.2
poly (dA, dT)	1	6.7
	10	52.0
Exp. 3. Oligonucleotides		
d(pTpG)$_{6\sim9}$	100	0
d(pCpA)$_{6\sim9}$	100	3.3
d(pTpG)$_{6\sim9}$+d(pCpA)$_{6\sim9}$	1	34.1
	10	96.6
d(pTpG)$_4$+d(pCpA)$_4$	10	30.0
	100	100.0
d(pTpG)$_3$+d(pCpA)$_3$	10	17.7
	100	100.0
d(pTpG)$_2$+d(pCpA)$_2$	10	0
	100	13.8
d(pTpG)$_{6\sim9}$+d(pCpA)$_2$	10	8.5
	100	86.7
d(pTpG)$_{6\sim9}$+d(pCpA)	100	4.0
d(pTpG)$_{6\sim9}$+d(CpA)	100	1.1
d(pTpG)$_2$+d(pCpA)$_{6\sim9}$	10	0
	100	60.0

Copolymers with random sequences, purchased from P. L. Biochemicals, Inc., are single stranded polymers prepared from deoxynucleoside triphosphates and terminal addition enzyme. Each polymer contains about equal amounts of two bases which are arranged in a completely random sequence. Repeating sequence oligodeoxynucleotides, purchased from Collaborative Research, Inc., contain 3′,5′-phosphodiester linkages and a terminal 5′-phosphate. They are prepared by chemical polymerization of suitably blocked dinucleotides.

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