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A biphasic nature of the bleomycin-mediated degradation of DNA

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The bleomycin-mediated digestion of DNA in the presence of ferrous ion, molecular oxygen, and dithiothreitol is characterized by a fast initial reaction, which is followed by a much slower process. The fast degradation is due to the fast activation of the bleomycin-Fe(II) complex and the subsequent fast reaction of the activated complex with DNA. The rate determining step for the slow process is reactivation of the bleomycin-Fe(III) complex. The apparent rate constants for both reactions increase with increasing ionic strength. The latter, unusual results are interpreted in terms of inhibition of bleomycin turnover by binding of cationic species with DNA at low ionic strength.

Bleomycin; DNA digestion; Ionic strength

1. INTRODUCTION

Bleomycin is a family of metal-complexing glycopeptide antitumor agents that are used clinically in the treatment of certain cancers [1]. The physiological basis of drug action is apparently due to the degradation of the DNA double helix [2]. Many studies are consistent with the essential requirements for the cofactors ferrous ion and molecular oxygen in BLM-mediated digestion of DNA [2]. The Fe(II)·BLM chelate reversibly forms a ternary complex with oxygen, and the ternary complex is then activated through one-electron reduction. It has been suggested that in the absence of any added reducing agent this electron is obtained by disproportionation of two ferrous complexes to give 'activated bleomycin' and an inactive Fe(III)·BLM complex. The reaction of the active BLM complex with deoxyribose portions of DNA [2], produces DNA strand breaks and the inactive Fe(III)·BLM complex. The latter complex can be reduced back to Fe(II)·BLM. Thus, BLM acts catalytically with each turnover requiring two electrons and one molecule of dioxygen.

The in vitro system consisting of DNA, BLM, molecular oxygen, ferrous ion, and DTT (the iron reducing agent) has been generally accepted as an excellent model that mimics closely the conditions in vivo for the BLM-mediated digestion of DNA. This system is being used extensively in studying BLM chemistry [2–4]. As part of a detailed series of studies on BLM-induced degradation of DNA under the general conditions given above [3,4], we report here that DNA is digested in a biphasic process. Another objective of this paper is to develop a mechanism for explaining the puzzling biphasic effect of BLM on DNA. The phenomenon is investigated using viscometric, electrophoretic, and HPLC methods.

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Abbreviations: BLM, bleomycin; DTT, dithiothreitol; Pipes, 1,4-piperazinebis(ethanesulfonic acid)

2. MATERIALS AND METHODS

2.1. *Bleomycin samples*

Metal-free BLM-A2 and blenoxane (a mixture consisting mainly of BLM-A2 and BLM-B2) were obtained from the Bristol-Myers Co. through the courtesy of Dr William T. Bradner. Stock solutions (3.2×10^{-4} M) were prepared using deionized water and stored at −18°C. Concentrations were

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determined spectrophotometrically at 290 nm with use of an extinction coefficient of 14000 $M^{-1} \cdot cm^{-1}$ [5].

2.2. *DNA*

High molecular mass calf thymus DNA (Worthington Biochemical) was used in all experiments. The commercial sample was purified and extensively dialyzed in a Pipes buffer as previously described [6]. Concentration of DNA-P of the stock solution (3.9×10^{-3} M) was determined spectrophotometrically at 260 nm, using an extinction coefficient of 6600 $M^{-1} \cdot cm^{-1}$.

2.3. *Buffers*

All experiments were conducted in Pipes buffers without EDTA. The buffers contained 0.01 M Pipes and were adjusted to pH 7.00 with NaOH. Solid NaCl was added to give the desired ionic strength.

2.4. *Viscometry*

All viscometric experiments were conducted in Cannon-Ubbelohde series 75 semimicrodilution viscometers, which were maintained at a constant temperature of 37 ± 0.01°C in a Cannon model M-1 water bath. Solutions were added to the viscometer with modified Hamilton syringes that had extensions for inserting the syringe all the way to the bottom sample reservoir of the viscometer. The stock solutions were added in the following order to reach the final volume of 1.480 ml: a Pipes buffer, DNA, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, DTT. The flow time was measured (± 0.05 s), then BLM was added, and the flow time was monitored as a function of time. In all experiments the molecular ratios of DNA to BLM were high. Under these conditions and in the presence of DTT, the DNA viscosity changes in time are described by the following biphasic equation (eqn 1), where η_0 is the initial reduced specific viscosity for DNA before the addition of BLM, η is the reduced specific viscosity for DNA at the digestion time t, k_f is the apparent rate constant for the first (fast) process, k_s is the apparent rate constant for the second (slow) process, and a and b are the amplitudes for the fast and slow phases, respectively.

$$\left(\frac{\eta}{\eta_0}\right)_t = ae^{-k_f t} + be^{-k_s t} \quad (1)$$

2.5. *Electrophoresis*

A standard method was used for separation of DNA oligomers on a 1% agarose gel and then stained with ethidium bromide for visualization [6].

2.6. *HPLC*

The products of DNA strand scission by activated BLM include oligonucleotides, four base propenals, and four bases with ratios dependent on the concentration of molecular oxygen [1,2]. In this work, in order to overcome the effect of oxygen concentration on the product distribution, relatively low concentrations of DNA and BLM and a buffer solution equilibrated with air were used. Under such conditions the HPLC analysis of cytosine and thymine, the DNA degradation products, is a convenient way to monitor the BLM-mediated degradation of DNA [3].

Two identical mixtures, as used in the viscometric analyses, were incubated at 37°C and then analyzed by HPLC, one solution after 5 min of incubation and the second solution after 20 min of incubation. An ISCO HPLC system, consisting of a C18 column (5 μm, 4.6 × 250 mm), two model 2350 pumps, a V4 variable wavelength absorbance detector set at 260 nm, and a ChemResearch data management/system controller, was used. Elution was at a rate of 1 ml/min with 0.01 M CF_3COONH_4 in water (A) for 10 min, then with linear gradient of A and 0.01 M CF_3COONH_4 in CH_3CN/H_2O, 4:1 (B) to reach 100% of B after 40 min. The retention times for cytosine and thymine were 4.0 and 12.7 min, respectively.

3. RESULTS

The effects of various concentrations of BLM on the rate of viscosity decreases of DNA solutions, under identical conditions of the initial concentration of ferrous ion and DTT, are illustrated in fig.1. Negligible changes in viscosity are seen in the control experiment (a) in the absence of BLM. Biphasic changes in the viscosity appear upon ad-

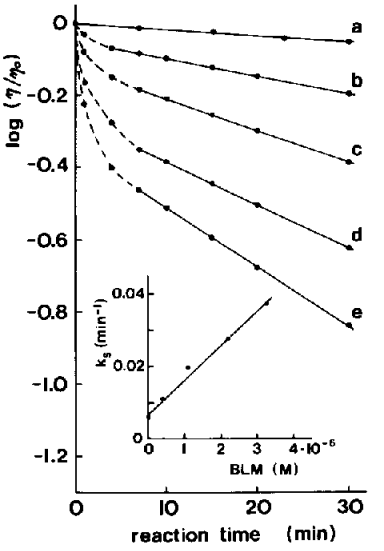

Fig.1. Changes in the reduced specific viscosity of DNA (2.16×10^{-4} M) induced by BLM-A2 in the presence of Fe(II) (7.4×10^{-6} M) and DTT (1.8×10^{-4} M) in a Pipes buffer ($[Na^+]$ = 0.015 M). Experiment, BLM concentration, rate constants k_f and k_s (eqn 1) are given in the order: (a) no BLM, 0.006 min^{-1} (one k only); (b) 4.3×10^{-7} M, 0.90 min^{-1}, 0.0110 min^{-1}; (c) 1.1×10^{-6} M, 1.01 min^{-1}, 0.0205 min^{-1}; (d) 2.15×10^{-6} M, 1.10 min^{-1}, 0.0273 min^{-1}; (e) 3.27×10^{-6} M, 1.22 min^{-1}, 0.0382 min^{-1}. The inset shows changes in k_s as a function of concentration of BLM-A2: k_s = 0.007 + 9708 [BLM], correlation coefficient r = 0.99, confidence level CL > 99%. The corresponding values of k_f and k_s for blenoxane are smaller by ~5% in all experiments b–e.

dition of BLM to the mixtures (b–e). The apparent rate constants, k_f and k_s, increase with increasing concentrations of BLM. The inset of fig.1 shows that the changes in k_s are proportional to the BLM concentration.

Both rate constants, k_f and k_s, increase also with increasing ionic strength of the reactant solutions (fig.2).

The effects of BLM on DNA viscosity in solutions of the same ionic strength but with increasing concentrations of ferrous ion and in the absence of DTT are given in fig.3 (a–c). The curve d, obtained under the conditions of b but in the presence of DTT, is also included for comparison. In the absence of DTT the first fast decrease in DNA viscosity is clearly defined, but the second slow process virtually disappears in all three experiments, a–c. The initial changes in DNA viscosity are faster as more ferrous ion is added. Again, these changes are faster with increasing ionic strength of the solution (not shown). As can be seen from curve d, the initial changes in the viscosity with time in the presence of DTT are

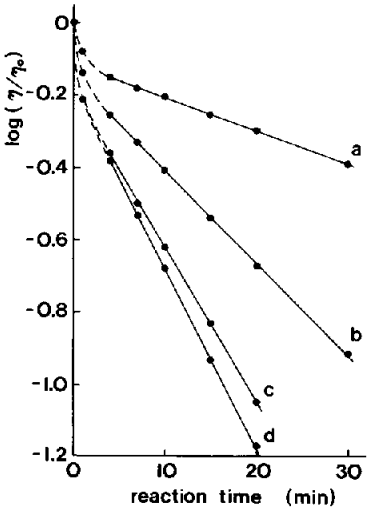

Fig.2. Salt-dependent changes in the reduced specific viscosity of DNA (2.16×10^{-4} M) obtained in the presence of BLM-A2 (1.1×10^{-6} M), Fe(II) (7.4×10^{-6} M) and DTT (1.8×10^{-4} M). Experiment, $[Na^+]$, rate constants k_f and k_s (eqn 1) are given in the order: (a) 0.015 M, 1.01 min^{-1}, 0.0205 min^{-1}; (b) 0.065 M, 1.57 min^{-1}, 0.0595 min^{-1}; (c) 0.115 M, 2.18 min^{-1}, 0.0984 min^{-1}; (d) 0.215 M, 2.20 min^{-1}, 0.1178 min^{-1}. The function $k_s = 0.007 + 1.010\ [Na^+]\gamma$, $r = 1.00$, CL > 99.9%, is obtained for the range $0.015\ M \leq [Na^+] \leq 0.115\ M$; standard activity coefficients γ for NaCl solutions are used.

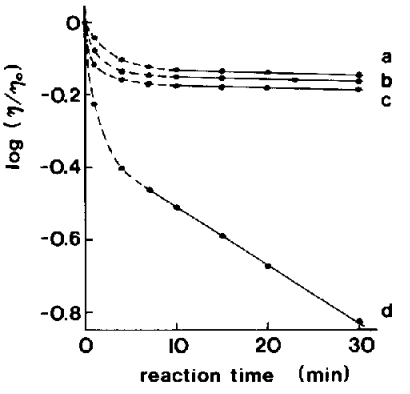

Fig.3. Changes in the reduced specific viscosity of DNA (2.16×10^{-4} M) induced by BLM-A2 (3.27×10^{-6} M) in the absence of DTT and in the presence of various concentrations of Fe(II): (a) 3.3×10^{-6} M; (b) 7.4×10^{-6} M; (c) 1.1×10^{-5} M. Curve d corresponds to experiment b conducted in the presence of DTT (1.8×10^{-4} M) and is incluced for comparison. $[Na^+]$ = 0.015 M for all experiments a–d.

greater than those in the corresponding experiment b conducted in the absence of the iron reducing agent. This result indicates, as expected, that a higher concentration of activated BLM is available in the presence of DTT, under otherwise identical conditions. It should be noted that the addition of DTT to the mixture of the experiment b of fig.3 after 10 min of incubation resulted in the appearance of the second process comparable to that of the experiment d (not shown).

The dual effect of BLM on DNA degradation can also be observed using electrophoretic analysis of the degraded DNA (fig.4). A sharp decrease in the average molecular mass of DNA is observed after the first 5 min of incubation, which is followed by a much slower change. The salt effect, as observed in the viscometric tests, is also clearly visible in these experiments. Thus, both the reduction in the DNA molecular mass after 5 min and subsequent changes are larger than those in the corresponding experiments with lower salt concentration (not shown).

The HPLC analysis of cytosine and thymine released from the digested DNA is shown in table 1. Concentrations of the two bases rise rapidly within the first 5 min of incubation, and much slower subsequent changes are observed. This result is consistent with the biphasic nature of the DNA degradation, as observed in the viscometric

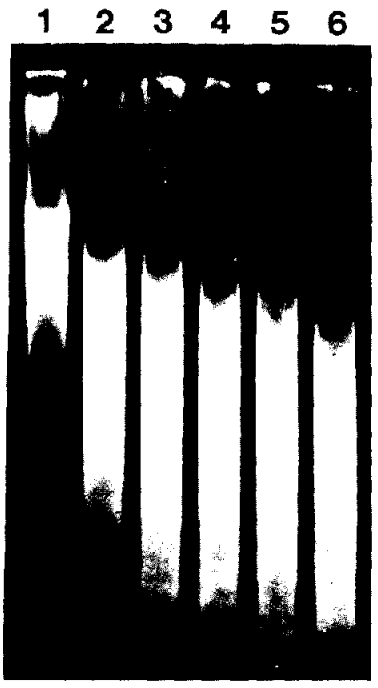

Fig.4. Electrophoretic analysis of DNA cleavage by blenoxane. Six 1.48-ml solutions, each 0.015 M in Na^+, 2.16×10^{-4} M in DNA, 7.4×10^{-6} M in Fe(II), and 1.8×10^{-4} M in DTT, were prepared. Stock solution of BLM was added to samples nos 2–6 to reach the final concentration of 1.1×10^{-6} M, and all six samples were incubated at 37°C. The mixtures were quenched with EDTA after the following periods of time (sample/time): 2/5 min, 3/10 min, 4/15 min, 5/20 min, 6/25 min.

Table 1

Ionic strength- and time-dependent release of cytosine and thymine from BLM-digested DNA

$[Na^+]$, M	Concentration, μM ($\pm$ 0.1 μM)[a]			
	Cytosine		Thymine	
	$t = 5$ min	$t = 20$ min	$t = 5$ min	$t = 20$ min
0.015	0.1	0.2	0.5	0.8
0.065	0.2	0.4	0.8	1.3
0.115	0.5	0.9	1.2	2.2
0.215	1.0	1.7[b]	1.7	2.9[b]

[a] Mean data of three independent measurements

[b] These concentrations correspond to 3.2% and 5.3% of the total amount of cytosine and thymine, respectively, in the DNA

The experimental conditions are identical to those of fig.2

and electrophoretic tests. The salt effect is also seen in these experiments; that is, the release of cytosine and thymine from digested DNA is faster with increasing concentrations of the sodium cation.

4. DISCUSSION

The results of three independent sets of experiments, conducted in the presence of ferrous ion and DTT, demonstrate unambiguously the biphasic nature of the BLM-mediated degradation of DNA. We have shown that the degradation process becomes monophasic in the absence of the iron reducing agent. For the first time we have also demonstrated that the rate for the BLM-induced degradation of DNA increases with increasing ionic strength. This is an unexpected result [7] because the increase in the ionic strength lowers the equilibrium binding constant of the cationic BLM molecule with DNA. It has been demonstrated that this binding is a necessary condition for the DNA degradation [8].

The biphasic effect of BLM on DNA degradation is consistent with a relatively fast formation of the active form of BLM from Fe(II)·BLM and the subsequent fast reaction with DNA (the first fast process) followed by slow reduction of the resultant Fe(III)·BLM complex to Fe(II)·BLM (the rate determining step of the second slow process). The monophasic degradation observed in the absence of the iron reducing agent (fig.3) strongly supports this analysis. It has been suggested previously [2] that under the conditions of experiment a of fig.3 the active BLM complex is obtained by disproportionation of two Fe(II)·BLM complexes in the presence of oxygen, and Fe(III)·BLM is formed as a byproduct. It has been shown [9] that the Fe(III)·BLM complex is stable and does not undergo an exchange reaction with ferrous ion to any significant extent in the time scale of fig.3. This finding together with the observed increasing digestion rates of DNA in the presence of the same amount of BLM and increasing concentrations of the excess ferrous ion (curves b and c in fig.3) are not consistent with the disproportionation of Fe(II)·BLM in the presence of O_2 as the only process leading to the formation of the activated species [2]. The results of fig.3 show that the active form of BLM is also obtained in a bimolecular col-

lision of Fe(II)·BLM·O_2 with free ferrous ion. To be effective, the two disproportionation processes, of BLM complexes and the BLM complex with Fe(II), must occur with at least one species not bound with DNA. This requirement agrees well with the observed salt effect on the DNA digestion in the absence of DTT. Thus, under the conditions of low ionic strength, most of these cationic species are complexed with anionic DNA backbone. They are increasingly released to solution as the ionic strength increases, thus facilitating the activation of BLM in intermolecular reactions. The subsequent reaction with DNA is not a rate determining step for the overall process, under the range of ionic strength studied, as already discussed.

The intriguing salt effect, observed in the presence of DTT, can be explained in a similar fashion. Under these conditions the bulk of Fe(II)·BLM·O_2 is activated and the bulk of Fe(III)·BLM is reduced by direct electron transfer from the iron reducing agent. Again, the data of fig.2 and table 1 show that the two reactions must be more efficient for the species free in solution than bound with DNA.

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