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Bleomycin May Be Activated for DNA Cleavage by NADPH–Cytochrome P-450 Reductase†

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ABSTRACT: In the presence of NADPH and O_2, NADPH–cytochrome P-450 reductase was found to activate Fe(III)–bleomycin A_2 for DNA strand scission. Consistent with observations made previously when cccDNA was incubated in the presence of bleomycin and Fe(II) + O_2 or Fe(III) + C_6H_5IO, degradation of DNA by NADPH–cytochrome P-450 reductase activated Fe(III)–bleomycin A_2 produced both single- and double-strand nicks with concomitant formation of malondialdehyde (precursors). Cu(II)–bleomycin A_2 also produced nicks in SV40 DNA following activation with NADPH–cytochrome P-450 reductase, but these were not accompanied by the formation of malondialdehyde (precursors). These findings confirm the activity of copper bleomycin in DNA strand scission and indicate that it degrades DNA in a fashion that differs mechanistically from that of iron bleomycin. The present findings also establish the most facile pathways for enzymatic activation of Fe(III)–bleomycin and Cu(II)–bleomycin, provide data concerning the nature of the activated metallobleomycins, and extend the analogy between the chemistry of cytochrome P-450 and bleomycin.

The bleomycins are a family of antitumor antibiotics elaborated by *Streptomyces verticillus* as Cu(II) complexes (Umezawa et al., 1966; Carter, 1978; Crooke, 1978; Umezawa, 1979). In the presence of Fe (Sausville et al., 1976, 1978a,b), Cu (Oppenheimer et al., 1981; Murugesan et al., 1982), or Co (Chang & Meares, 1982), the bleomycins have been shown to effect DNA strand scission; their ability to degrade DNA is believed to represent the basis of their therapeutic efficacy (Umezawa, 1979). Although substantial progress has been made in understanding the nature of the process(es) by which bleomycin may be activated for DNA cleavage (Burger et al., 1981, 1982; Giloni et al., 1981; Murugesan et al., 1982), and the explicit mechanism(s) by which cleavage occurs (Hecht, 1979; Giloni et al., 1981; Wu et al., 1983), a number of questions remain unanswered. Among these is the way in which bleomycin is activated in situ.

To date, bleomycin has been activated successfully in vitro in the presence of Fe(II) + O_2 (Sausville et al., 1976, 1978a,b), Fe(III) + H_2O_2 or ethyl hydrogen peroxide (Burger et al., 1981, 1982), Cu(I) + O_2 (Oppenheimer et al., 1981), and Co(III) + $h\nu$ (Chang & Meares, 1982). Murugesan et al. (1982) have recently shown that Fe(III)·BLM[1] and Cu(II)·BLM can be activated with oxygen surrogates such as iodosobenzene, in analogy with observations made previously for cytochrome P-450 and for a number of Fe(III)–porphyrins (Hrycay et al., 1975; Lichtenberger et al., 1976; Gustafasson et al., 1979). Moreover, the bleomycins so activated mediated chemical transformations remarkably similar to those reported for cytochrome P-450 (analogues) (Groves et al., 1980; Tabushi et al., 1980) and were also capable of effecting DNA strand scission in the absence of dioxygen (Aoyagi et al., 1982; Murugesan et al., 1982).

Cytochrome P-450, a component of the microsomal electron transport system, is activated in situ by NADPH–cytochrome P-450 reductase in the presence of NADPH and O_2. The microsomal electron transport system has been shown to activate BLM for DNA cleavage in a reaction that was dependent on NADPH and O_2 (Yamanaka et al., 1978; Trush et al., 1982a,b) and enhanced by Fe(III) (Trush, 1983). In a preliminary report, NADPH–cytochrome P-450 reductase was also shown to support DNA cleavage by Fe(III)·BLM (Scheulen et al., 1981). Presently, we confirm the activation of Fe(III)·BLM by NADPH–cytochrome P-450 reductase and demonstrate that activated Cu(II)·BLM can also be obtained enzymatically and mediates DNA cleavage. Also described are the nature of NADPH–cytochrome P-450 reductase mediated activation of the metallobleomycins, with regard to required components, preferred pathways, and time course of activation, and important characteristics of the enzymatically activated bleomycins and the processes by which they mediate DNA strand scission.

Experimental Procedures

SV40 DNA and agarose were purchased from Bethesda Research Laboratories; 2-thiobarbituric acid and calf thymus

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[1] Abbreviations: BLM, bleomycin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide.

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DNA were from Sigma Chemical Co. Blenoxane was obtained from Bristol Laboratories through the courtesy of Dr. William Bradner and was fractionated (Chien et al., 1977; Oppenheimer et al., 1979) to provide bleomycin A_2. NADPH–cytochrome P-450 reductase (EC 1.6.2.4; NADPH:ferricytochrome oxidoreductase) was isolated from rat liver microsomes as described by Guengerich et al. (1982) and was stored at −40 °C as a 20% glycerol solution. The preparation was found to be reasonably stable over a period of >1 year; the specific activity for cytochrome *c* reduction (Strobel & Dignam, 1978) was found to be ~30 μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$.

DNA Strand Scission Experiments. Reaction mixtures (40 μL total volume) contained 15 μM SV40 DNA (i.e., DNA nucleotide), 5 μM BLM A_2, 5 μM $FeCl_3$ or $CuCl_2$, 10 μM NADPH, and 0.02 μg of NADPH–cytochrome P-450 reductase in 0.15 M potassium phosphate buffer, pH 7.6.[2] Reactions were initiated by the simultaneous addition (within ~30 s) of metal ion, NADPH, BLM A_2, and cytochrome P-450 reductase to the buffered solution of DNA. When metal·BLM complexes were formed prior to reaction, this was done separately in unbuffered solutions; the metallobleomycins were then added to the reaction mixtures containing DNA and NADPH ~1 min before initiation of the reactions by addition of cytochrome P-450 reductase. Reaction mixtures were incubated for 5–60 min at 25 °C and then quenched by addition of NaEDTA to 1 mM final concentration. The reaction mixtures were then treated with a gel loading solution (5 μL); the combined solution contained 0.025% bromphenol blue, 3% glycerol, and 0.3% sodium dodecyl sulfate. Individual samples were loaded immediately onto a 1.2% agarose slab gel (20 × 25 × 0.6 cm) containing 1 μg/mL ethidium bromide. Following electrophoresis at constant voltage (40 V for 16 h) in 40 mM Tris–acetate, pH 7.8, containing 5 mM NaOAc and 1 mM NaEDTA, the gels were visualized (UV light box) and photographed (Kodak no. 9 filter; Polaroid type 55 P/N film). Negatives were analyzed on a scanning densitometer, and peak areas were determined as described (Lloyd et al., 1978).

Malondialdehyde Production. Reaction mixtures (0.2 mL total volume) were prepared in duplicate and contained 300 μM calf thymus DNA, 100 μM bleomycin A_2, 1 mM NADPH, 1 μg of NADPH–cytochrome P-450 reductase, and either 100 μM $FeCl_3$ or 100 μM $CuCl_2$ in 20 mM sodium cacodylate buffer, pH 7.0. Reactions were initiated by the simultaneous addition of metal, NADPH, bleomycin, and NADPH–cytochrome P-450 reductase and were incubated at room temperature for 15 min. The reactions were quenched by the addition of 0.8 mL of a solution, pH 2.0, containing 42 mM 2-thiobarbituric acid and 1 mM EDTA. Each combined solution was incubated at 90 °C for 10 min and then cooled and used for determination of A_{532}. The amount of malondialdehyde was calculated by assuming $\epsilon_{532} = 1.6 \times 10^5$ (Waravdekar & Saslaw, 1959).

Results

As shown in Table I, bleomycin was activated for cleavage of supercoiled SV40 DNA by NADPH–cytochrome P-450 reductase. DNA strand scission required NADPH–cytochrome P-450 reductase, bleomycin, and NADPH; essentially no DNA cleavage occurred when any of these components was omitted. Also required for DNA strand scission was O_2.[3] The effect of metal ions on bleomycin activation by NADPH–cytochrome P-450 reductase is also shown in Table I. Some DNA cleavage was evident in the absence of added metal, due presumably to the presence of adventitious metal(s). The addition of either Fe(III) or Cu(II) to the reaction mixture effected significant enhancement of strand scission.

Several products have been shown to be formed from DNA concomitant with Fe·BLM-mediated DNA strand scission. According to the experimental conditions employed, this in-

Table I: DNA Cleavage by NADPH–Cytochrome P-450 Reductase Activated Fe(III)·BLM

reaction mixture	supercoiled SV40 DNA (mole fraction ± SD)[a]	malondialdehyde (nmol)[b,c]
complete	0.46 ● 0.15 (n = 9)	1.53
−Fe(III)	0.70 ± 0.13 (n = 6)	0
−Fe(III), +Cu(II) (5 μM)	0.42 ± 0.15 (n = 9)	0
−NADPH	0.93 ± 0.01 (n = 2)	0
−NADPH–cytochrome P-450 reductase	0.87 ● 0.03 (n = 3)	0
−BLM A_2	0.96 ± 0.01 (n = 2)	0

[a] Reaction mixtures containing 15 μM SV40 DNA, 5 μM $FeCl_3$, 5 μM BLM A_2, 10 μM NADPH, and 0.02 μg of NADPH–cytochrome P-450 reductase in 0.15 M potassium phosphate buffer, pH 7.6, were incubated for 15 min at 25 °C. The mole fraction of cccDNA remaining was determined by densitometry following agarose gel electrophoresis. [b] ±0.1 nmol. [c] Reaction mixtures containing 300 μM calf thymus DNA, 100 μM $FeCl_3$, 100 μM BLM A_2, 1 mM NADPH, and 1 μg of NADPH–cytochrome P450 reductase in 20 μM sodium cacodylate buffer, pH 7.0, were incubated for 15 min at 25 °C.

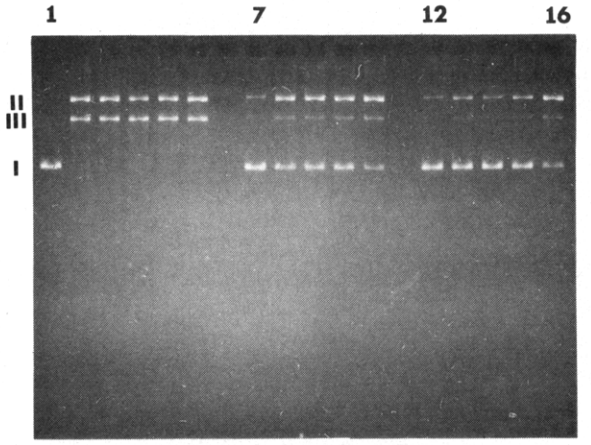

FIGURE 1: Time course of DNA strand scission by bleomycin. Reaction mixtures contained 15 μM SV40 DNA in 0.15 M potassium phosphate buffer, pH 7.6, and 5 μM $Fe(NH_4)_2(SO_4)_2$ + 5 μM BLM A_2 (lanes 2–6), 5 μM $FeCl_3$ + 5 μM BLM A_2 + 10 μM NADPH + 0.02 μg of NADPH–cytochrome P-450 reductase (lanes 7–11) or 5 μM $CuCl_2$ + 5 μM BLM A_2 + 10 μM NADPH + 0.02 μg of NADPH–cytochrome P-450 reductase (lanes 12–16). Incubations were carried out at 25 °C for 1, 5, 10, 15, or 30 min (lanes 2–6, 7–11, and 12–16, respectively). Untreated SV40 DNA is shown in lane 1.

[2] Addition of increasing amounts of $Fe(NH_4)_2(SO_4)_2$ to a solution of 50 μM BLM A_2 in 0.15 M K_2HPO_4 buffer, pH 7.6, produced a continuous change in the UV spectrum of BLM with isosbestic points at 300 and 270 nm, presumably due to binding of metal by BLM. An end point in the spectral change occurred when 100 μM $Fe(NH_4)_2(SO_4)_2$ was added to the BLM solution, apparently reflecting some coordination of metal by the phosphate buffer.

[3] In three separate experiments, complete reaction mixtures [5 μM Fe(III) + 5 μM BLM] incubated in the absence of O_2 (under argon) contained 0.46 ± 0.047 mole fraction of supercoiled DNA. Control reaction mixtures prepared at the same time in the presence of O_2 produced 0.24 ● 0.098 mole fraction of supercoiled DNA. When Cu(II) was substituted for Fe(III), the reaction mixtures under argon produced 0.52 ± 0.15 mole fraction of supercoiled DNA, while those prepared in the presence of O_2 contained 0.095 ± 0.077 mole fraction of supercoiled DNA.

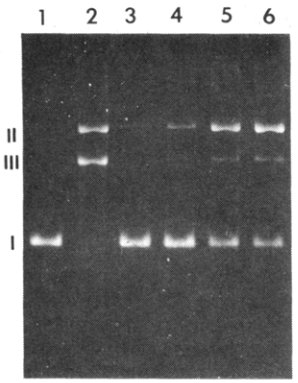

FIGURE 2: Metal dependence of bleomycin-mediated DNA strand scission in the presence of NADPH–cytochrome P-450 reductase and NADPH. Supercoiled SV40 DNA (15 μM) was incubated with 5 μM BLM A_2, 10 μM NADPH, and 0.02 μg of NADPH–cytochrome P-450 reductase in 0.15 M potassium phosphate buffer, pH 7.6, at 25 °C for 15 min. Individual incubations contained no added metal (lane 4), 5 μM $FeCl_3$ (lane 5), or 5 μM $CuCl_2$ (lane 6). Control reactions contained SV40 DNA (lane 1), DNA + 5 μM $Fe(NH_4)_2(SO_4)_2$ + 5 μM BLM A_2 (lane 2), or DNA + 5 μM BLM A_2 + 0.02 μg of NADPH–cytochrome P-450 reductase (lane 3).

cluded malondialdehyde or its presumed precursor, base propenal (Burger et al., 1980; Giloni et al., 1981; Burger et al., 1982; Wu et al., 1983). DNA strand scission by enzymatically activated Fe·BLM was also shown to result in the formation of malondialdehyde (Table I); the amount of malondialdehyde produced by enzymatically activated Fe·BLM was in the same general range as that produced by chemically activated Fe(II)·BLM control samples. Interestingly, no malondialdehyde was formed as a result of DNA cleavage by enzymatically activated copper BLM.

The time course of DNA strand scission by 5 μM Fe(III)·BLM and 5 μM Cu(II)·BLM in the presence of 0.02 μg of NADPH–cytochrome P-450 reductase is shown in Figure 1. As illustrated in the figure, for both metallobleomycins there was an increased extent of conversion of SV40 form I DNA to forms II and III as the period of incubation was increased successively from 1 to 30 min. In contrast, DNA strand scission by the same concentration of Fe(II)·BLM ($+O_2$) was complete within 1 min. Thus, the enzymatic activation of Fe(III)·BLM and Cu(II)·BLM appeared to be rate limiting under the experimental conditions employed. This conclusion was examined further by varying the amount of NADPH–cytochrome P-450 reductase employed for BLM activation. As the amount of enzyme was increased from 0.01 to 0.1 μg, the extent of DNA cleavage obtained over a period of 15 min increased both for Fe·BLM and for Cu·BLM (data not shown).

Also investigated in more detail was the metal dependence of DNA cleavage by BLM in the presence of NADPH–cytochrome P-450 reductase. As shown in Figure 2, both form II and form III DNA were observed when SV40 form I DNA was incubated with 5 μM Fe(III)·BLM (lane 5) or Cu(II)·BLM (lane 6) in the presence of 0.02 μg of NADPH–cytochrome P-450 reductase. In contrast, much less cleavage was obtained, relative to an untreated DNA control (lane 1), when either NADPH or metal were omitted (lanes 3 and 4, respectively). For both Fe(III) and Cu(II), the extent of DNA cleavage was also shown to increase steadily as a function of metal concentration (Figure 3).

The nature of the reactive species responsible for DNA strand scission was also investigated. Figure 4 (left panel) illustrates the lack of effect of the ·OH scavenger dimethyl sulfoxide on the course of DNA strand scission by Fe·BLM

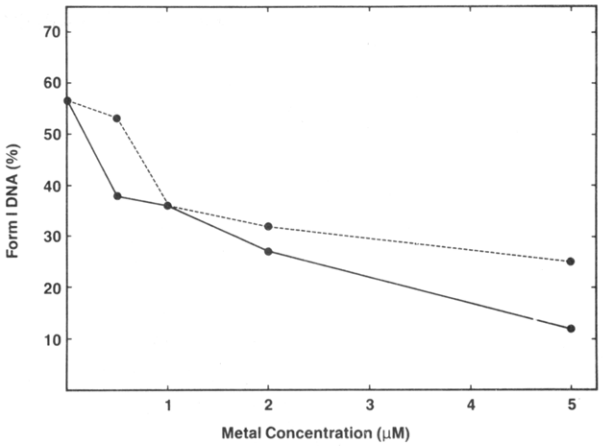

FIGURE 3: Metal concentration dependence of cccDNA strand scission by bleomycin in the presence of NADPH–cytochrome P-450 reductase. SV40 form I DNA (15 μM) was incubated in 0.15 M potassium phosphate buffer, pH 7.6, containing 5 μM BLM A_2, 10 μM NADPH, and 0.02 μg of NADPH–cytochrome P-450 reductase at 25 °C for 60 min. Individual incubation mixtures contained 0–5 μM $FeCl_3$ (—) or 0–5 μM $CuCl_2$ (---). The reaction mixtures were analyzed by agarose gel electrophoresis, as described under Experimental Procedures; conversion of form I DNA to forms II and III was quantitated by densitometric analysis of the gels.

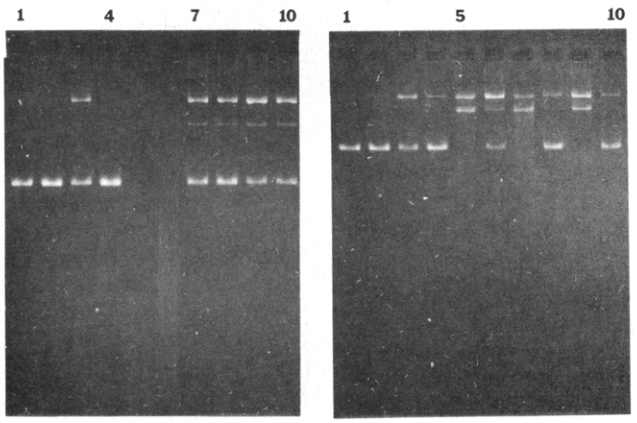

FIGURE 4: Effect of Me_2SO (left panel) and Tiron (right panel) on bleomycin-mediated DNA strand scission. Reaction mixtures contained 15 μM SV40 DNA in 0.15 M potassium phosphate buffer, pH 7.6, and were incubated at 25 °C in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 2.5% Me_2SO (left panel) or 100 μM Tiron (right panel). Individual incubation mixtures also contained 5 μM $Fe(NH_4)_2(SO_4)_2$ (lanes 3 and 4), 5 μM $Fe(NH_4)_2(SO_4)_2$ + 5 μM BLM A_2 (lanes 5 and 6), 5 μM $FeCl_3$ + 5 μM BLM A_2 + 10 μM NADPH + 0.02 μg of NADPH–cytochrome P-450 reductase (lanes 7 and 8), or 5 μM $CuCl_2$ + 5 μM BLM A_2 + 10 μM NADPH + 0.02 μg of NADPH–cytochrome P-450 reductase (lanes 9 and 10). Lanes 1 and 2 were control reactions containing only SV40 DNA. Incubations were conducted for 15 (left panel) or 60 min (right panel).

or Cu·BLM in the presence of NADPH–cytochrome P-450 reductase, an effect that had also been noted previously (Rodriguez & Hecht, 1982) for Fe(II)·BLM in the presence of O_2. However, as shown in Figure 4B, the extent of DNA strand scission by enzymatically activated Fe·BLM and Cu·BLM was inhibited by Tiron, an agent known to destroy O_2^{-}· (Miller, 1970; Strobel & Coon, 1971; Greenstock & Miller, 1975).

Preactivation of Fe·BLM prior to addition of SV40 form I DNA was also studied. The results are given in Table II, which illustrates that preincubation periods up to 60 min in the absence of DNA had no effect on the ultimate extent of SV40 DNA strand scission. An interesting effect was also observed when the metallobleomycins were preformed prior

Table II: DNA Cleavage by Bleomycin as a Function of Activation Time[a]

activation time (min)	SV40 DNA (%) form I	form II	form III
1	0	66	34
30	0	50	50
60	0	68	32
untreated SV40 DNA	96	4	0

[a] Reaction mixtures containing 5 μM $FeCl_3$, 5 μM BLM A_2, 10 μM NADPH, and 0.02 μg of NADPH–cytochrome P-450 reductase in 0.15 M KH_2PO_4 buffer, pH 7.6, were incubated for 1, 30, or 60 min and then treated with 15 μM SV40 DNA and incubated for an additional 60 min. Control reactions contained untreated SV40 DNA and 15 μM SV40 DNA + 5 μM Fe(II)·BLM A_2. The percent of form I, II, and III DNA was determined by densitometry following agarose gel electrophoresis.

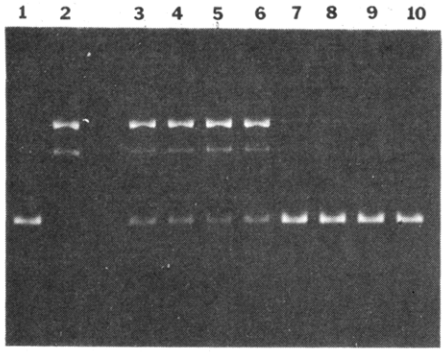

FIGURE 5: Activation of preformed metal–BLM complexes for DNA strand scission by NADPH–cytochrome P-450 reductase. Complexes of Fe(III)·BLM A_2 (lanes 3–6) or Cu(II)·BLM A_2 (lanes 7–10) were preformed over a period of 1 min before addition to reaction mixtures containing 15 μM SV40 form I DNA and 10 μM NADPH in 0.15 M potassium phosphate buffer, pH 7.6. After an additional 1 min, the reactions were initiated by the addition of 0.02 μg of NADPH–cytochrome P-450 reductase and incubated at 25 °C for 5 (lanes 3 and 7), 15 (lanes 4 and 8), 30 (lanes 5 and 9), or 60 min (lanes 6 and 10) and then analyzed by agarose gel electrophoresis. Control reactions containing SV40 DNA (lane 1) and 15 μM SV40 DNA + 5 μM $Fe(NH_4)_2(SO_4)_2$ + 5 μM BLM A_2 (lane 2) were incubated at 25 °C for 60 min.

to addition of NADPH–cytochrome P-450 reductase. As shown in Figure 5, when Fe(III)·BLM was preformed for 1 min prior to enzymatic activation, subsequent incubation with NADPH–cytochrome P-450 reductase produced a species that mediated strand scission of SV40 DNA. The reaction with DNA was complete within 5 min and proceeded to essentially the same extent as the comparable assay in which Fe(III) and bleomycin A_2 were combined at the same time as NADPH–cytochrome P-450 reductase and DNA (cf. Figure 1). Slightly greater DNA strand scission was obtained with Fe(III)·BLM when the preincubation period was extended to 10 min (data not shown). In contrast, when Cu(II) + BLM was preincubated for 1 min prior to incubation with NADPH–cytochrome P-450 reductase, no significant DNA cleavage resulted (Figure 5, lanes 7–10). The same lack of DNA degradation was noted following a 10-min preincubation of Cu(II) and BLM (data not shown).

Also tested was the effect of "excess" metal ions on the extent of DNA cleavage by Fe(III)·BLM. As shown in Figure 6, the addition of 5 μM Fe(III) to a reaction mixture containing 5 μM Fe(III)·BLM increased the extent of strand scission, especially at longer incubation times. Similarly, when 5 μM Cu(II) was added to preformed Fe(III)·BLM, slight enhancement of DNA degradation was observed, but only at longer reaction times (data not shown). To investigate the possibility that the addition of Cu(II) nontheless may have

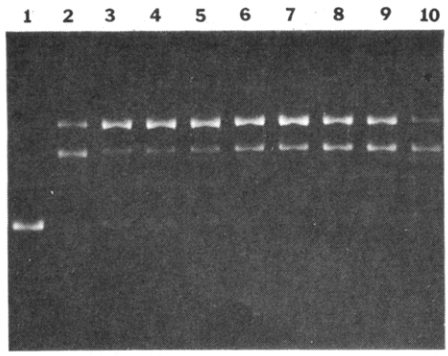

FIGURE 6: Effect of excess Fe(III) on Fe(III)·BLM A_2 mediated DNA strand scission in the presence of NADPH–cytochrome P-450 reductase. Solutions containing 5 μM BLM A_2 were preincubated for 10 min with 5 μM $FeCl_3$ (lanes 3–6) or 10 μM $FeCl_3$ (lanes 7–10) and then added to reaction mixtures containing 15 μM SV40 DNA and 10 μM NADPH in 0.15 M potassium phosphate buffer, pH 7.6. The reactions were initiated by the addition of 0.02 μg of NADPH–cytochrome P-450 reductase and incubated at 25 °C for 5 (lanes 3 and 7), 15 (lanes 4 and 8), 30 (lanes 5 and 9), or 60 min (lanes 6 and 10). Control reactions containing SV40 DNA (lane 1) and 15 μM SV40 DNA + 5 μM $Fe(NH_4)_2(SO_4)_2$ + 5 μM BLM A_2 (lane 2) were incubated at 25 °C for 60 min.

Table III: Effect of Cu(II) Preincubation on DNA Degradation by Fe(III)·BLM[a]

preincubation time (min)	supercoiled[b] SV40 DNA (mole fraction)	malondialdehyde[c] (nmol)
1	0.07	0.26
2	0.12	0.31
5	0.34	0.26
10	0.55	0.38
–Cu(II)	0	3.15

[a] Fe(III)·BLM complexes (preformed for 10 min) were incubated in the presence of DNA, $CuCl_2$, and NADPH for the indicated time, prior to addition of NADPH–cytochrome P-450 reductase. [b] Reaction mixtures contained 15 μM SV40 DNA, 5 μM Fe(III)·BLM, 5 μM $CuCl_2$, 10 μM NADPH, and 0.02 μg of NADPH–cytochrome P-450 reductase in 0.15 M potassium phosphate, pH 7.6. [c] Reaction mixtures contained 300 μM calf thymus DNA, 100 μM Fe(III)·BLM, 100 μM $CuCl_2$, 1 mM NADPH, and 1 μg of NADPH–cytochrome P-450 reductase in 20 mM sodium cacodylate buffer, pH 7.0.

altered the process by which DNA strand scission occurred, the extent of malondialdehyde produced was followed in parallel with DNA strand scission. As indicated in Table III, preincubation of one molar equivalent of Cu(II) with preformed Fe(III)·BLM for 1 min prior to addition of NADPH–cytochrome P-450 reductase had little effect on the extent of DNA strand scission but effected a 10-fold reduction in the amount of malondialdehyde produced. Qualitatively similar results were obtained when the cleavage of SV40 DNA was monitored under conditions identical with those employed for malondialdehyde production (data not shown). As malondialdehyde is produced concomitant with DNA cleavage by Fe·BLM, but not Cu·BLM (cf Table I), one possible interpretation of these results is that DNA cleavage was mediated by activated Cu·BLM following replacement of Fe in the preformed Fe·BLM by Cu(I).

Discussion

NADPH–cytochrome P-450 reductase is a flavin-containing protein that catalyzes the transfer of electrons from NADPH to ferric cytochrome P-450 and to ferrous oxycytochrome P-450 (White & Coon, 1980). The purified NADPH–cytochrome P-450 reductase from rat liver microsomes has been shown to be capable of reducing various electron acceptors such as cytochrome *c* (Vermillion & Coon, 1974; Dignam &

Strobel, 1975) as well as supporting cytochrome P-450 dependent oxidation of organic substrates. In previous studies, we have shown that cytochrome P-450 and bleomycin share common features with regard to activation (e.g., with oxygen surrogates such as C_6H_5IO) and oxygen transfer to small substrates (Aoyagi et al., 1982; Murugesan et al., 1982). The present study extends these analogies to the enzymatic activation of both species; characterized herein are the processes by which Fe·BLM and Cu·BLM are activated enzymatically and certain properties of these activated metallobleomycins.

As illustrated in Table I, in addition to its known role in the activation of cytochrome P-450, NADPH–cytochrome P-450 reductase also activates Fe(III)·BLM A_2. In common with known requirements for cytochrome P-450 activation, the activation of bleomycin occurred only in the presence of NADPH (Table I) and O_2[3] and showed the expected dependence on enzyme concentration and incubation time (Figure 1). Fe(III)·BLM was apparently activated directly by NADPH–cytochrome P-450 reductase; prereduction of Fe(III) prior to BLM binding appeared not to be required, as judged by the lack of effect of preincubation of Fe(III) + BLM on the ultimate extent of enzymatic activation and DNA cleavage (Figure 5). Likewise, addition of excess metal ions to the preformed Fe(III)·BLM complex had little effect on the extent of BLM activation and DNA strand scission (Figure 6), consistent with the interpretation that the enzymatic activation of Fe(III)·BLM by NADPH–cytochrome P-450 reductase need not involve electron transfer from reduced metal ions to the (preformed) Fe(III)·BLM complex. In contrast to results obtained with bleomycin in the presence of microsomes (Trush, 1983), preincubation of Fe(III)·BLM in the presence of NADPH and NADPH–cytochrome P-450 reductase in the absence of DNA did not result in BLM inactivation (Table II).

The efficiency of DNA strand scission by enzymatically activated Fe(III)·BLM was also considered. Under our assay conditions when limiting amounts of NADPH–cytochrome P-450 reductase were intentionally employed, Fe(III)·BLM A_2 produced as many as $\sim 10^3$ DNA strand breaks/enzyme molecule in 15 min. Although carried out under rather different experimental conditions, this compared favorably with the rate of catalysis of cytochrome P-450 mediated oxygenation of cyclohexane (to provide cyclohexanol) by the same protein (Macdonald et al., 1982); moreover, each molecule of NADPH–cytochrome P-450 reductase produced at least 10^2 molecules of malondialdehyde (precursor) from calf thymus DNA. Thus, the activation of Fe(III)·BLM A_2 by cytochrome P-450 reductase proceeded with facility and produced an activated species whose chemistry was similar to that observed when bleomycin A_2 was combined with Fe(II) + O_2 in the presence of DNA (Sausville et al., 1978a,b).

In contrast with the report of Scheulen et al. (1981), we found that BLM could also be activated for DNA cleavage by NADPH–cytochrome P-450 reductase in the presence of NADPH and Cu(II) (Table I; Figures 1–3). Interestingly, while the efficiency of DNA strand scission observed for enzymatically activated Cu·BLM did not differ significantly from that obtained with Fe·BLM, for the Cu complex there was no associated production of thiobarbituric acid reactive material (malondialdehyde) (Tables I and III), indicating that the mechanism of DNA cleavage by activated Cu·BLM must differ fundamentally from that of activated Fe·BLM.

In addition to the chemistry mediated by these activated metallobleomycins, important differences were noted for the process of enzymatic activation per se. Unlike the observations made for the Fe complex, preformed Cu(II)·BLM did not undergo efficient enzymatic activation by NADPH–cytochrome P-450 reductase (Figure 5), suggesting that activated Cu·BLM may be formed by enzyme-mediated reduction of free Cu(II) prior to binding of the metal ion by BLM (Table III) and possibly also by electron transfer from reduced metal ions to preformed metallobleomycins (data not shown). The reasons for the differences in mode of enzymatic activation of Cu·BLM and Fe·BLM are not altogether clear but may well relate to differences in redox potential for the two complexes (Dabrowiak et al., 1979; Melnyk et al., 1981; Freedman et al., 1982).

The ability of enzymatically activated Cu·BLM to effect DNA strand scission is consistent with an earlier report from this laboratory (Murugesan et al., 1982) that demonstrated DNA cleavage by Cu(II)·BLM + C_6H_5IO and with the suggestion (Sugiura, 1979; Oppenheimer et al., 1981; L. O. Rodriguez, G. M. Ehrenfeld, S. M. Hecht, C. Chang, V. J. Basus, and N. J. Oppenheimer, unpublished results) that Cu(I)·BLM + O_2 may also produce a species that can degrade DNA. It seems reasonable to conclude the Cu·BLM can no longer be regarded (Suzuki et al., 1969; Shirakawa et al., 1971; Umezawa, 1974; Asakura et al., 1975; Sausville et al., 1976, 1978a; Takahashi et al., 1977; Umezawa et al., 1979; Antholine et al., 1982; Freedman et al., 1982) as an inactive metallobleomycin, although the experimental parameters for its activation as well as its mode of DNA degradation differ from those of Fe·BLM. It is interesting to note that Lown & Sim (1977) have observed DNA nicking by 16 μM Cu(II) in the presence of 2 mM NADPH; the system described here differs in that it is both BLM and enzyme dependent (Table I).

The mechanism of activation of Fe(III)·BLM by NADPH–cytochrome P-450 reductase might simply involve enzyme-mediated reduction to Fe(II)·BLM, the latter of which could react chemically with O_2 to produce the activated metallobleomycin. Recently, however, it has been suggested that direct chemical activation of Fe(III)·BLM, i.e., without initial reduction to Fe(II)·BLM, may be possible (Burger et al., 1981; Kuramochi et al., 1981; Murugesan et al., 1982; Sugiura et al., 1982), and direct enzymatic activation of Fe(III)·BLM also seems plausible. For example, Fe(III)·BLM might be activated directly by superoxide anions produced by NADPH–cytochrome P-450 reductase (Pederson & Aust, 1972; Grover & Piette, 1981), a suggestion consistent with the observation that 4,5-dihydroxy-*m*-benzenedisulfonic acid [Tiron, an agent known to destroy $O_2^{-}\cdot$ (Miller, 1970; Strobel & Coon, 1971; Greenstock & Miller, 1975)] inhibited DNA strand scission by Cu and Fe·BLM in the presence of NADPH–cytochrome P-450 reductase (Figure 4, right panel).

The finding that activated Fe·BLM and Cu·BLM can be produced by NADPH–cytochrome P-450 reductase is consistent with observations that microsomal preparations facilitate DNA cleavage by bleomycin (Yamanaka et al., 1978; Trush et al., 1982a,b) and has obvious and important implications for the mechanism of activation of bleomycin in situ and the active form of the drug. A scheme currently postulated for bleomycin activation (Umezawa, 1977, 1979; Takahashi et al., 1977; Freedman et al., 1982) assumes intracellular conversion of administered (metal-free) bleomycin to Cu(II)·BLM, followed by enzymatic conversion to Cu(I)·BLM, metal-free bleomycin, and finally Fe(II)·BLM, only the last of which may be activated for DNA strand scission. Given that bleomycin has been reported to accumulate in the nuclear membrane of tumor cells of mice to which BLM had been

administered (Fujimoto, 1974), that DNA is associated with the nuclear membrane (Hildebrand & Tobey, 1973; Infante et al., 1973, 1976), and that NADPH–cytochrome P-450 reductase is present within the nucleus or nuclear membrane (Khandwala & Kasper, 1973; Vaught & Bresnick, 1976; Rogan et al., 1976; Bresnick et al., 1976), the present finding that activated Cu·BLM and Fe·BLM can be produced enzymatically by NADPH–cytochrome P-450 reductase suggests a fundamentally simpler pathway that could be utilized for activation of this therapeutic agent in situ. It should be noted, however, that the data presently available do not permit an assessment of the extent to which this mode of activation may obtain in vivo, in part because the effective in vivo concentrations of the several components required for such activation have not been defined as yet.

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Registry No. NADPH–cytochrome P-450 reductase, 9039-06-9; Fe(III)–bleomycin A_2, 72028-04-7; Cu(II)–bleomycin A_2, 71794-63-3.

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Elongation Factor Tu Ternary Complex Binds to Small Ribosomal Subunits in a Functionally Active State†

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ABSTRACT: A complex between elongation factor Tu (EF-Tu), GTP, phenylalanyl-tRNA (Phe-tRNA), oligo(uridylic acid) [oligo(U)], and the 30S ribosomal subunit of *Escherichia coli* has been formed and isolated. Binding of the EF-Tu complex appears to be at the functionally active 30S site, by all biochemical criteria that were examined. The complex can be isolated with 0.25–0.5 copy of EF-Tu bound per ribosome. The binding is dependent upon the presence of both the aminoacyl-tRNA and the cognate messenger RNA. Addition of 50S subunits to the preformed 30S–EF-Tu–GTP–Phe-tRNA–oligo(U) complex ("30S–EF-Tu complex") causes a rapid hydrolysis of GTP. This hydrolysis is coordinated with the formation of 70S ribosomes and the release of EF-Tu. Both the release of EF-Tu and the hydrolysis of GTP are stoichiometric with the amount of added 50S subunits. 70S ribosomes, in contrast to 50S subunits, neither release EF-Tu nor rapidly hydrolyze GTP when added to the 30S–EF-Tu complexes. The inability of 70S ribosomes to react with the 30S–EF-Tu complex argues that the 30S–EF-Tu complex does not dissociate prior to reaction with the 50S subunit. The requirements of the 30S reaction for Phe-tRNA and oligo(U) and the consequences of the addition of 50S subunits resemble the reaction of EF-Tu with 70S ribosomes, although EF-Tu binding to isolated 30S subunits does not occur during the elongation microcycle. This suggests that the EF-Tu ternary complex binds to isolated 30S subunits at the same 30S site that is occupied during ternary complex interaction with the 70S ribosome. These data also suggest that crucial parts of the 70S binding site for the EF-Tu complex may be on the 30S ribosomal subunit in locations where they do not significantly interfere with subunit association. Because this 30S–EF-Tu complex can be isolated from sucrose gradients, three-dimensional immune mapping of the EF-Tu binding site directly on 30S subunits is feasible.

During protein synthesis, a ternary complex of elongation factor Tu, aminoacyl-tRNA (aa-tRNA),[1] and GTP binds to the 70S ribosome. The binding of this complex is the initial step for the translation of a particular codon [for reviews, see Lengyel (1974), Kaziro (1978), and Lake (1981)]. Immediately following binding of the complex containing the correct aminoacyl-tRNA, GTP hydrolysis occurs, EF-Tu is released, and peptidyl transfer occurs. Hence the binding of the complex to its initial site on the 70S ribosome, the recognition or R site (Lake, 1977; Johnson et al., 1977), is transient. In this paper we show that if only 30S subunits are present, it is possible to obtain stable binding of the EF-Tu complex. Unlike a similar 30S–EF-Tu complex previously reported and characterized (Brot et al., 1970; Weissbach et al., 1972a), GTP is rapidly hydrolyzed when 50S subunits are added to this 30S–EF-Tu complex. In other respects as well, the functional properties of our 30S–EF-Tu complex are like those of the interaction of the complex with 70S ribosomes. Although the 30S–EF-Tu complex plays no role in the elongation microcycle

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[1] Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; GMPPCP, guanosine 5′-(β,γ-methylenetriphosphate); oligo(U), oligo(uridylic acid); Phe-tRNA, phenylalanyl-tRNA; Tris, tris(hydroxymethyl)aminomethane.

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