Sequence-Specific Hydrolysis of Yeast tRNA^{Phe} Mediated by Metal-Free Bleomycin†

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ABSTRACT: Bleomycin A_2 (BLM) was found to mediate sequence specific hydrolysis of tRNA^{Phe} in the absence of added metal ions. BLM A_2 promoted phosphodiester bond hydrolysis 3′ to the pyrimidine residue at all resolved Py-Pu sites not involving modified bases, as demonstrated by high-resolution electrophoretic analysis of 5′- and 3′-^{32}P-end-labeled substrates. The reaction proceeded with surprising facility, approaching in efficiency that of oxidative strand scission mediated by the Fe^{II}•BLM A_2 complex. By the use of a number of BLM congeners, as well as a study of the time, temperature, and salt dependence of the hydrolysis, it was shown that in many respects the hydrolytic reaction parallels the oxidative degradation of RNA and DNA mediated by metallobleomycins. Thus, in contrast to the well-characterized oxidative degradation of DNA and RNA by bleomycin studied for two decades, the present report documents the ability of certain metal-free bleomycins to mediate RNA hydrolysis.

The bleomycins (BLMs,[1] Figure 1) are a family of glycopeptide-derived natural products originally isolated from *Streptomyces verticillus* by Umezawa and co-workers (Umezawa et al., 1966). These compounds have been shown to possess useful antitumor activity. The clinically used mixture of bleomycins, comprised primarily of BLMs A_2 and B_2, has found utility against a variety of cancers, including non-Hodgkins lymphomas, squamous cell carcinomas, and testicular tumors (Umezawa, 1978; Sikic et al., 1985). The therapeutic effectiveness of the bleomycins is believed to derive from their ability to bind and oxidatively cleave cellular DNA, and possibly RNA, in the presence of certain metal ion cofactors (Stubbe & Kozarich, 1987; Natrajan & Hecht, 1993; Kane & Hecht, 1994; Hecht, 1994, 1995). Several metals can support BLM-mediated DNA cleavage including iron (Ishida & Takahashi, 1975; Sausville et al., 1976, 1978a,b), copper (Ehrenfeld et al., 1985, 1987), cobalt (Chang & Meares, 1982, 1984; Saito et al., 1989), manganese (Burger et al., 1984; Ehrenfeld et al., 1984), ruthenium (Subramanian & Meares, 1985), vanadyl (Kuwahara et al., 1985), and nickel (Guan et al., 1993). It is iron, however, that is believed to promote the effects of bleomycin *in vivo*. Additionally, BLM can form complexes with non-redox-active metals such as zinc (Oppenheimer et al., 1979a; Manderville et al., 1994) and cadmium (Petering et al., 1990). It may be noted that the coordination sphere about the metal center has not been defined precisely (Natrajan & Hecht, 1993).

Oxidative DNA cleavage by activated Fe•BLM is initiated by abstraction of the C4′-hydrogen from the deoxyribose ring of DNA; activated Fe•BLM is formed from Fe^{II}•BLM in a process that requires O_2 and an electron (Sausville et al., 1976, 1978a; Kuramochi et al., 1981; Natrajan & Hecht, 1993). Following C4′ H abstraction to form a C4′ deoxyri-

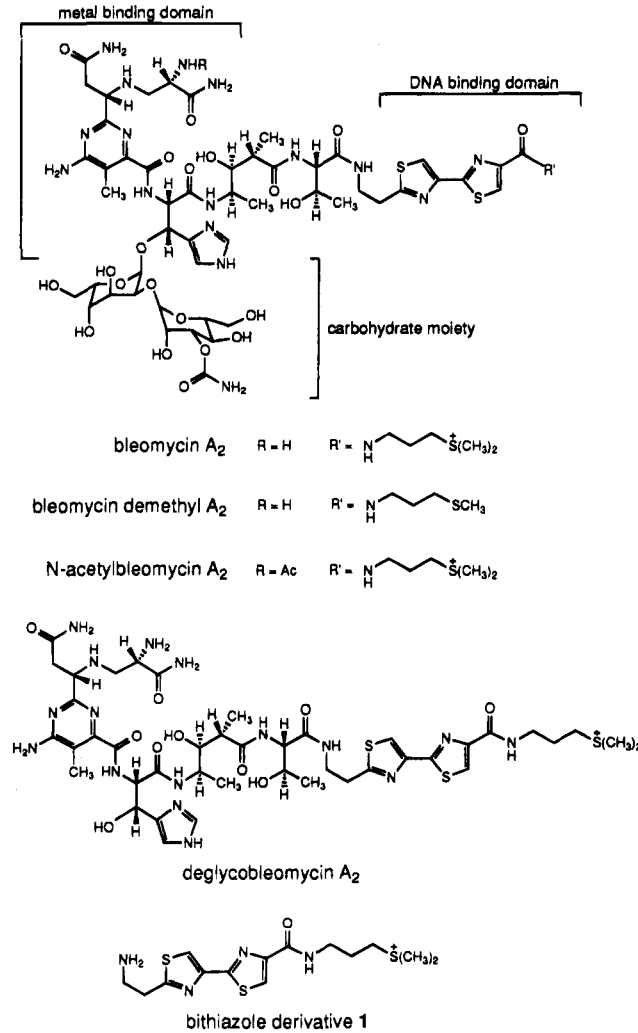

FIGURE 1: Structures of four bleomycin congeners and bithiazole derivative **1**.

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[1] Abbreviations: BLM, bleomycin; Pu, purine; Py, pyrimidine; HPLC, high-pressure liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)-aminomethane; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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bose radical, DNA cleavage can occur by either of two mechanisms, both of which have been studied in some detail (Stubbe & Kozarich, 1987; Natrajan & Hecht, 1993; Kane & Hecht, 1994; Hecht, 1994, 1995). An oxygen-dependent pathway involves combination of the initially formed deoxyribose radical with O_2, forming a C4′-peroxy radical. This reaction is the first in a series of steps which ultimately result in strand breakage and the production of DNA fragments having phosphoroglycolates at their 3′-termini. Alternatively, the initially formed deoxyribose radical can be converted to the respective C-4′ OH sugar; subsequent loss of free nucleobase from the intact strand occurs concomitantly with formation of an abasic site. Treatment of this formed lesion with alkali leads to strand scission and the formation of fragments containing phosphate or hydroxycyclopentenone moieties at their 3′-termini.

Interestingly, oxidative DNA cleavage by metallobleomycins exhibits substantial sequence specificity, resulting predominantly in destruction of the pyrimidine nucleotides within 5′-GT-3′ and 5′-GC-3′ sequences. Modifications that alter DNA structure, however, have been observed to affect both the selectivity and the efficiency of DNA cleavage. Examples include platinated (Mascharak et al., 1983; Gold et al., 1988) or methylated DNA (Hertzberg et al., 1985; Long et al., 1990), DNA containing bulges (Williams & Goldberg, 1988) and poly -(AT)$_n$- runs (Nightingale & Fox, 1993), and RNA·DNA heteroduplexes (Morgan & Hecht, 1994). Substrate structure, therefore, clearly plays a major role in directing the sites of BLM-mediated oxidative DNA cleavage. The structural interplay between the DNA and BLM that is responsible for these effects is poorly understood, although much effort is currently being focused in this direction (Manderville et al., 1994, 1995; Wu et al., 1994).

For many years, RNA was believed not to be a target for BLM degradation. Following the first reports (Magliozzo et al., 1989; Carter et al., 1990a) of Fe^{II}·BLM mediated tRNA cleavage, such transformations have been demonstrated for several different RNA substrates, including various messenger, transfer, and ribosomal ribonucleic acids (Carter et al., 1990a, 1991; Hüttenhofer et al., 1992; Holmes et al., 1993; Hecht, 1994) as well as the RNA strand of an RNA·DNA heteroduplex (Morgan & Hecht, 1994). The data indicate that RNA cleavage proceeds via oxidative mechanisms analogous to those observed during DNA strand scission (Duff et al., 1993; Holmes et al., 1993). Interestingly, while RNA substrates do show cleavage at G-Py sites, in analogy to DNA degradation by BLM, the sequence selectivity is not entirely the same. For example, *Bacillus subtilus* $tRNA^{His}$ precursor and mature *Escherichia coli* $tRNA_1^{His}$ are cleaved at a variety of sites, most of which are nominally single stranded (Holmes et al., 1993). Yeast $tRNA^{Phe}$ exhibits BLM cleavage at three major sites that exist in loop regions or at loop−stem junctions (Hüttenhofer et al., 1992; Holmes, 1993). Thus, as is true for DNA, it appears that the local structure, rather than the nucleic acid sequence, contributes importantly to the determination of the sites of cleavage of these substrates (Holmes et al., 1993).

During our studies of RNA−BLM interaction, we found that BLM was also capable of effecting RNA strand scission in the absence of any added metal ion. This process, which occurs with surprising facility, affords products whose formation is consistent with a hydrolytic process. The sequence selectivity of RNA cleavage by metal-free BLM is also completely different than that noted for metallobleomycins. Presently, we report experiments that demonstrate RNA cleavage by metal-free BLM and define key parameters of this novel process.

EXPERIMENTAL PROCEDURES

Materials. Fractionation of blenoxane, obtained as a gift from Bristol Laboratories, afforded bleomycins A_2 and B_2 (Chien et al., 1977; Oppenheimer et al., 1979b). Bleomycin A_5 was obtained through the courtesy of Dr. Li-Ho Chang, Beijing Medical University. *N*-Acetyl-bleomycin A_2 (Oppenheimer et al., 1980) and deglycobleomycin A_2 (Kenani et al., 1988) were prepared from bleomycin A_2 as described and purified by C_{18} reversed-phase HPLC using Vydac column 218TP1010 with aqueous ammonium acetate/acetonitrile mobile phases. Bithiazole derivatives were prepared by known methods (Kane et al., 1994). $Fe^{II}(NH_4)_2(SO_4)_2$ was purchased from Alfa Biochemicals. Yeast $tRNA^{Phe}$ was obtained from Boehringer Mannheim Biochemicals. [γ-^{32}P]-ATP (7000 Ci/mmol, labeling grade) was purchased from ICN Biomedicals. T4 polynucleotide kinase was obtained from New England Biolabs; T4 RNA Ligase was from Bethesda Research Laboratories. Shrimp alkaline phosphatase was purchased from United States Biochemicals. Cytidine 3′-monophosphate and Chelex 100 resin were purchased from Sigma Chemical Co.; the latter was batch washed with sodium hydroxide and then neutralized to pH 7 with sodium acetate buffer and autoclaved.

Substrate Labeling. Yeast $tRNA^{Phe}$ was 3′-^{32}P-end-labeled essentially as described (Uhlenbeck & Gumport, 1982). Briefly, [5′-^{32}P]pCp, prepared from cytidine 3′-monophosphate (22.5 μmol) and [γ-^{32}P]ATP (500 μCi) via the agency of 30 units of T4 polynucleotide kinase, was incubated with the tRNA (~4 μg) in the presence of 80 units of T4 RNA ligase in a reaction mixture (50 μL total volume) containing 50 mM Hepes buffer, pH 7.5, 5 mM DTT, 2 mM ATP, and 12.5 mM $MgCl_2$ at 4 °C for 12−16 h. The labeled RNA was purified by 10% denaturing PAGE. Alternatively, the RNA was 5′-^{32}P-end-labeled in two steps as follows. First, 4 μg of $tRNA^{Phe}$ was incubated for 2 h at 37 °C with 20 units of shrimp alkaline phosphatase in a reaction mixture (30 μL total volume) containing 25 mM Tris-HCl, pH 7.4, and 20 mM $MgCl_2$. The solution was then incubated at 70 °C for 45 min, cooled, and combined with 5 μL of 100 mM β-mercaptoethanol, 5 μL of water, 500 μCi of [γ-^{32}P]ATP, and 30 units of T4 polynucleotide kinase. This mixture (42 μL total volume) was incubated for 2 h at 37 °C and then purified by 10% denaturing PAGE.

BLM Reactions. Except as indicated, BLM-mediated $tRNA^{Phe}$ hydrolysis reactions were carried out for 1 h at 45 °C. Reaction volumes were 5−10 μL and contained 5 mM sodium phosphate buffer, pH 7.4, 500 μM BLM A_2, and ~50 000 cpm (10−15 pmol) of $tRNA^{Phe}$. Cleavage by Fe^{II}·BLM, carried out in the same buffer but containing 5 mM DTT, was initiated by simultaneous addition of Fe^{2+} and BLM to 500 μM concentration, followed by incubation at 37 °C for 15 min. When required, salts or other exogenous agents were added from concentrated stock solutions prior to BLM addition.

For experiments involving purification of reagents with Chelex resin, batch treatments were performed. Water and

sodium phosphate buffer were stored over the resin for at least 2 days at 4 °C, while BLM and RNA were incubated in the presence of the resin on ice for varying periods of time. Enzymatic RNA sequencing and alkaline hydrolysis reactions were carried out using a USB nuclease sequencing kit according to the manufacturer's protocol. Fe•EDTA-DTT oxidative RNA cleavage reactions were carried out as described (Latham & Cech, 1989). RNA cleavage was analyzed by 10−25% sequencing PAGE; the gels were visualized by autoradiography. Cleavage yields were calculated from data obtained through phosphorimager analysis using Imagequant software.

Neutron activation analysis was carried out at the University of Virginia Nuclear Reactor Facility using a nuclear research reactor operating at a power of 2 MW and providing a neutron flux of approximately 8×10^{16} neutrons•m^{-2}•s^{-1}. Detection was carried out using a lithium doped germanium detector coupled to a multichannel analyzer.

RESULTS

Incubation of 3′-^{32}P-end-labeled yeast tRNA^{Phe} with 500 μM BLM A_2 in the absence of added metal ions afforded a set of cleavage products which were different from those obtained in the presence of one equivalent of Fe^{II}. This result is demonstrated clearly in Figure 2. Lane 2 shows the products of incubation of the tRNA with Fe^{II}•BLM. Major cleavage sites were observed at positions A_{31}, U_{52}, and A_{66} (Figure 3, bold), as has been shown previously (Hüttenhofer et al., 1992; Holmes, 1993). Lane 3 indicates the sites of BLM-mediated cleavage of this substrate in the absence of added metal ion. Strong cleavage was observed to occur at positions C_{13} (7.9%), C_{28} (9.0%), C_{61} (4.0%), C_{63} (6.6%), and C_{72} (10.1%); the numbers in parentheses refer to cleavage yields. These sites are indicated by the numbers in boxes in Figure 3. Strikingly, all five of these sites represent cleavage following the cytidine present at a 5′-CA-3′ site. A sixth strong cleavage site, better resolved using a 5′-^{32}P-end-labeled substrate (*vide infra*), occurred at U_8 (21%), a nucleotide also followed in the tRNA sequence by adenosine. Additionally, upon longer incubation five minor cleavage sites could also be observed (*vide infra*). These occurred at positions U_{41} (2.4%), U_{50} (1.6%), U_{52} (1.0%), C_{56} (0.8%), and C_{70} (3.0%), as indicated by the numbers in circles in Figure 3. Significantly, all five of these residues are pyrimidines followed by a guanosine in the tRNA^{Phe} sequence.

It is readily apparent from Figure 3 that all RNA cleavages mediated by metal-free bleomycin A_2 occurred after the pyrimidine nucleotide in a pyrimidine−purine sequence. In fact, with the possible exception of two Py-Pu sites present at the two ends of the molecule, and therefore not resolved in these gels, all such sites present in tRNA^{Phe} not involving modified bases were cleaved.

The data in Figure 2 were also consistent with the suggestion that the cleavage occurred via a hydrolytic mechanism. This conclusion can be drawn because oxidative and hydrolytic cleavage processes produce fragments with different end groups which are distinguishable by high-resolution polyacrylamide gel electrophoresis. During hydrolysis, the 2′-hydroxyl group of the nucleoside 5′ to the cleaved phosphodiester bond participates in a nucleophilic attack on the phosphorus atom, resulting in strand breakage and the formation of 5′-hydroxyl and 2′,3′-cyclic phosphate

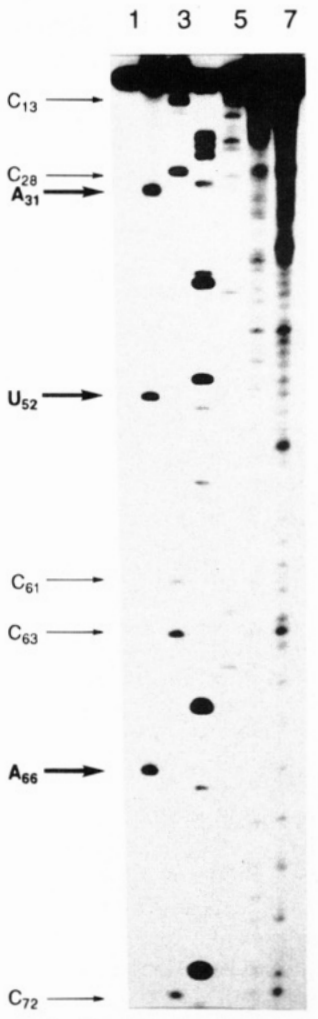

FIGURE 2: Polyacrylamide gel electrophoretic analysis of the cleavage of 3′-^{32}P-end-labeled yeast tRNA^{Phe} by Fe^{II}•BLM (bold arrows) and metal-free BLM (arrows). Lane 1, RNA alone; lane 2, 500 μM Fe^{II}•BLM A_2 + 5 mM DTT, 37 °C, 15 min; lane 3, 500 μM BLM A_2, 45 °C, 1 h; lane 4, RNase T1 sequencing reaction (G); lane 5, RNase Phy M sequencing reaction (U+A); lane 6, partial alkaline hydrolysis; lane 7, Fe•EDTA-DTT oxidative cleavage ladder.

termini at the cleavage site (Adams et al., 1986). Alternatively, during oxidative cleavage the ribose ring is destroyed, typically resulting in the loss of one nucleotide and the production of 5′-phosphate and 3′-phosphate or phosphoroglycolate termini (Hertzberg & Dervan, 1984; Natrajan & Hecht, 1993).

The 3′-^{32}P-end-labeled substrate used in Figure 2 permitted analysis of the 5′-termini created at the cleavage sites. The products of cleavage by metal-free BLM (lane 3) comigrated exactly with the nuclease sequencing bands (lanes 4 and 5) and the alkaline hydrolysis ladder (lane 6), demonstrating that 5′-hydroxyl termini were produced. This result is consistent with hydrolytic chemistry. In contrast, the bands resulting from cleavage by Fe^{II}•BLM (lane 2) migrated at positions in between the bands of the alkaline hydrolysis ladder but at the same positions as bands produced by Fe•EDTA-DTT treatment (lane 7), consistent with the formation of 5′-termini ending in phosphate, the expected result of oxidative RNA cleavage (Holmes et al., 1993).

Further study of the cleavage promoted by metal-free BLM was accomplished by the use of a 5′-^{32}P-end-labeled tRNA^{Phe} substrate. This substrate permitted analysis of the 3′-termini

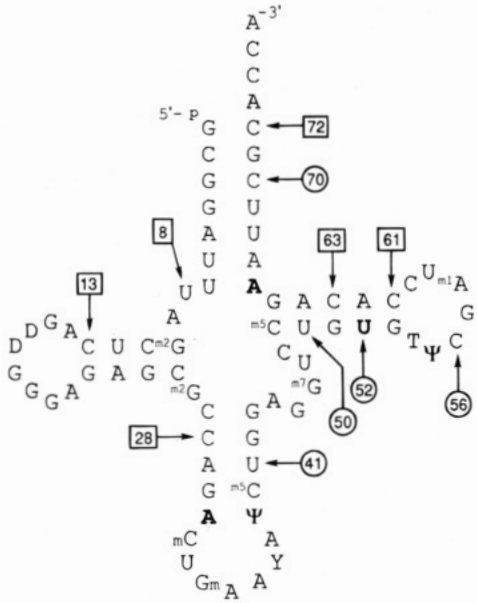

FIGURE 3: Cloverleaf representation of the secondary structure of yeast $tRNA^{Phe}$ showing sites of cleavage produced by Fe^{II}•BLM and metal-free BLM. Numbers indicate position in the nucleotide sequence. Fe^{II}•BLM cleavage sites are denoted by bold type. The major cleavage sites induced by metal-free BLM are denoted by boxed numbers, while the minor sites are denoted by circled numbers.

produced at the cleavage sites. As described above, hydrolysis produces 2′,3′-cyclic phosphates, while oxidative cleavage typically produces 3′-phosphate and 3′-phosphoroglycolate termini, depending on the specific nature of the oxidative chemistry involved. For short RNA fragments, products containing these three termini can be resolved by high-resolution PAGE.

The results of this experiment are presented in Figure 4. Here, the strong uridine$_8$ cleavage band (lane 3) was clearly observed to comigrate with the alkaline hydrolysis band known to contain a 2′,3′-cyclic phosphate (lane 4). Further, these bands migrated slightly more slowly than the phosphate and phosphoroglycolate termini generated by Fe•EDTA oxidative cleavage (lane 5). This experiment thus provides unequivocal evidence for products consistent with the hydrolytic nature of the cleavage reaction.

Critically, comparison of the position of the cleavage bands for the 5′- and 3′-^{32}P-end-labeled substrates relative to the sequencing lanes indicates that no nucleotide was destroyed during the cleavage process. This result is also consistent with the hydrolysis of a phosphodiester bond but inconsistent with oxidative chemistry.

Several additional experiments were carried out to further characterize the nature of this putative BLM-mediated RNA hydrolysis. Figure 5 shows the time and temperature dependence of the reaction. Reaction times covered a 2 h period at temperatures of 37 and 45 °C. Under no condition was RNA cleavage observed in the absence of BLM. In its presence, cleavage intensities at the major and minor sites increased with time and were stronger at the higher temperature. Notably, some cleavage was observed even at incubation times of only 10 min, indicating that this reaction is quite facile. In fact, the efficiency of this reaction approaches that of Fe^{II}•BLM for this substrate, a result that is also clear from Figure 2. Additional experiments have shown that lesser amounts of hydrolysis also occurred at 25 °C (supporting information, Figure 1).

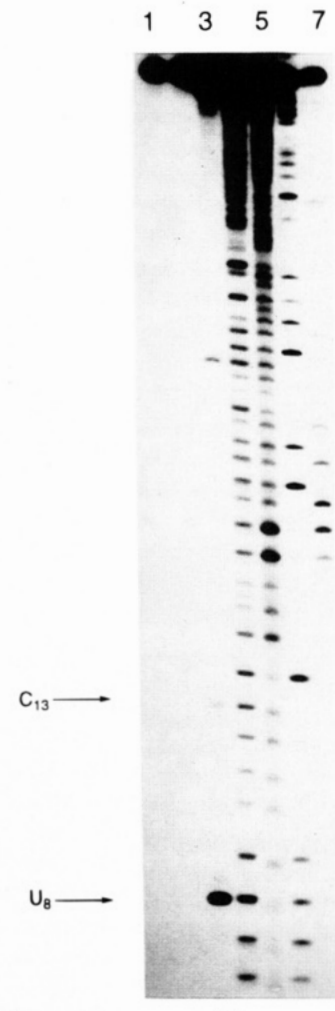

FIGURE 4: Products of cleavage of 5′-^{32}P-end-labeled $tRNA^{Phe}$ by metal-free BLM A_2. Lane 1, RNA alone, untreated; lane 2, RNA alone, 45 °C, 1 h; lane 3, 500 μM BLM A_2, 45 °C, 1 h; lane 4, alkaline hydrolysis; lane 5, Fe•EDTA-DTT oxidative cleavage ladder; lane 6, RNase Phy M sequencing reaction (U+A); lane 7, RNase T1 sequencing reaction (G).

Although this putative hydrolytic RNA chemistry occurred in the absence of added metal, it was important to verify that the cleavage was not due to a complex of BLM with some adventitious metal contaminant. Several experiments were carried out in an effort to establish this point. First, incubation of the reaction components in the presence of Chelex resin for limited periods of time (1−2 h) did not inhibit the reaction. Prolonged Chelex treatment was found to cause BLM to bind to the resin, as determined by spectrophotometric quantitation of BLM in the supernatant. In a related experiment, the presence of the metal chelator EDTA in the reaction mixture was found to be inhibitory only at high EDTA concentrations, i.e., greater than 10 mM. At concentrations above this level, the degree of inhibition was found to vary for the different cleavage sites. Specifically, the presence of EDTA at a concentration of 25 mM diminished cleavage more strongly at C_{28} and C_{61} than at C_{13} and C_{63}. The trend continued when the EDTA concentration was increased to 50 mM. Under these conditions, the C_{28} and C_{61} cleavage bands were essentially absent. When the concentration of the chelator in the reaction mixture reached 100 mM, all BLM-dependent cleavage was inhibited (supporting information, Figure 2). This behavior paralleled that observed upon addition of NaCl (see below) and is thus

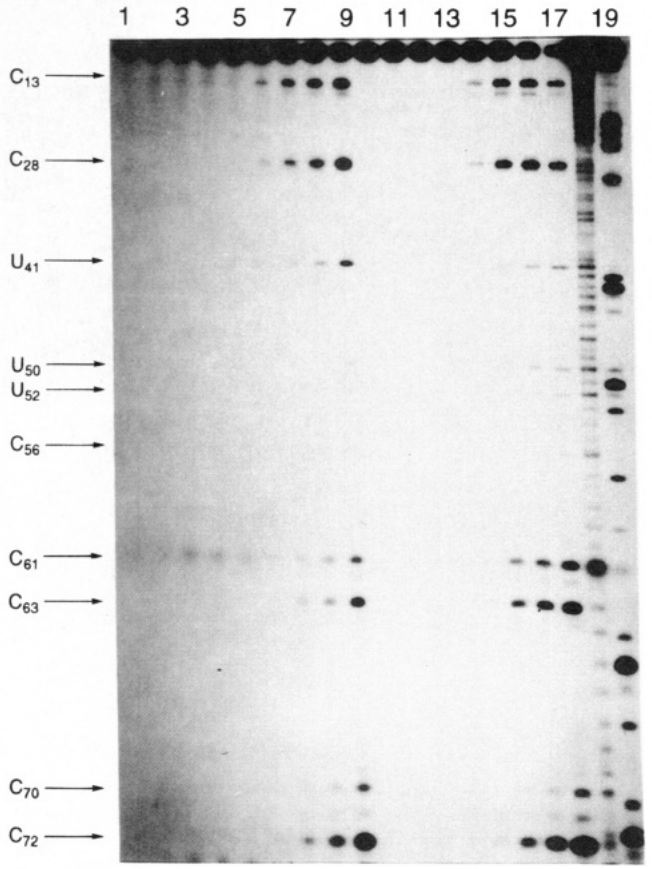

FIGURE 5: Time and temperature dependence of the BLM hydrolysis reaction. Lane 1, 3′-^{32}P-end-labeled yeast tRNA^{Phe} control; lanes 2−5, incubation of the RNA at 37 °C for 10, 30, 60, and 120 min, respectively; lanes 6−9, incubation at 37 °C in the presence of 500 μM BLM A_2 for 10, 30, 60, and 120 min, respectively; lanes 10−13, incubation of the RNA at 45 °C for 10, 30, 60, and 120 min, respectively; lanes 14−17, incubation at 45 °C in the presence of 500 μM BLM A_2 for 10, 30, 60, and 120 min, respectively; lane 18, alkaline hydrolysis; lane 19, RNase T1 sequencing reaction (G).

Table 1: List of Stability Constants for M·BLM[a] and M·EDTA[b] Complexes

metal	log K_{BLM}	log K_{EDTA}
Cu(II)	18	18
Fe(III)	16	25
Fe(II)	<9.7	14
Zn(II)	9.7	16
Cd(II)	7.2	16

[a] Petering et al. (1990). [b] Martell and Smith (1974).

most likely an electrostatic effect rather than one related to metal binding.

The relative stability constants of the M·BLM and M·EDTA complexes (K_{BLM} and K_{EDTA}, respectively) can be used to estimate the maximum amount at equilibrium of a given metallobleomycin in solution based on these experiments. Table 1 lists M·BLM [Petering et al., 1990; see also Sugiura et al. (1979)] and M·EDTA (Martell & Smith, 1974) complex stability constants for metals for which both of these values are known. For a competition between BLM and EDTA for the binding of a given metal, the equilibrium is described by

$$\text{M·BLM} + \text{EDTA} \rightleftharpoons \text{M·EDTA} + \text{BLM} \qquad (1)$$

$$K_{eq} = \frac{[\text{M·EDTA}][\text{BLM}]}{[\text{M·BLM}][\text{EDTA}]} = \frac{K_{EDTA}}{K_{BLM}} \qquad (2)$$

The EDTA experiment described above indicated that the hydrolysis reaction proceeded readily in the presence of 10 mM EDTA and 500 μM BLM concentrations, respectively. Using these values eq 3 is obtained, which is a good approximation at low metal ion concentrations.

$$\frac{[\text{M·EDTA}]}{[\text{M·BLM}]} = \frac{20K_{EDTA}}{K_{BLM}} \qquad (3)$$

If it is assumed that the contaminating metal is present at a concentration of 10 μM, which represents very high estimate (*vide infra*), an extreme upper limit for metal ion participation in the putative BLM-mediated hydrolysis can be defined. If all of the assumed metal ions present existed as a complex of either EDTA or BLM, then eq 4 would obtain. Combining eqs 3 and 4 gives an expression that can be approximated by eq 5 (see supporting information for derivation), from which the equilibrium concentration of the metallobleomycin can be calculated.

$$[\text{M·EDTA}] + [\text{M·BLM}] = 10^{-5} \qquad (4)$$

$$[\text{M·BLM}] = \frac{(5 \times 10^{-7})K_{BLM}}{K_{EDTA}} \qquad (5)$$

In the case of Fe^{III}, where the M·EDTA complex is several orders of magnitude more stable than the M·BLM complex, the maximum equilibrium concentration of Fe^{III}·BLM achievable would be 0.5 fM. In the case of Cu^{II}, where the metal binding constants of the two ligands are very similar, the maximum metallobleomycin concentration attainable would still be only 50 nM. Notably, this concentration is much lower than that required for any metal complex found so far to be capable of effecting tRNA^{Phe} hydrolysis (Dock-Bregeon & Moras, 1987; Ciesiolka et al., 1989; Hayashi et al., 1993). Thus, the lack of inhibition of this hydrolysis reaction in the presence of 10 mM EDTA strongly supports the conclusion that the reaction is not metal ion mediated.

In an effort to quantify the amounts of any adventitious metals that might actually be present, neutron activation analysis was carried out on the reaction samples. Manganese was the only metal detected which is known to bind BLM with any affinity. It was detected at a level of approximately 0.02 ppm, or about 1 μM. The lack of a known Mn·BLM stability constant precludes a direct estimate of its concentration in the manner described above. However, there is no metal ion known whose affinity for BLM is greater than its affinity for EDTA (Table 1). Therefore, if it is assumed that $K_{Mn\cdot BLM} \leq K_{Mn\cdot EDTA}$, then [Mn·BLM] ≤ 5 nM.

Also arguing against adventitious metal ion participation was the observation that addition of various metal ions such as Fe^{II}, Fe^{III}, Cu^{II}, or Zn^{II} to the reaction mixture actually inhibited the hydrolysis reaction (supporting information, Figure 3). The presence of metals that bind to BLM, therefore, seems to hinder rather than promote the putative hydrolysis reaction. The mechanistic implications of this observation are not entirely clear at present but would be consistent with the interpretation that the metal binding domain of BLM participates in the hydrolysis reaction.

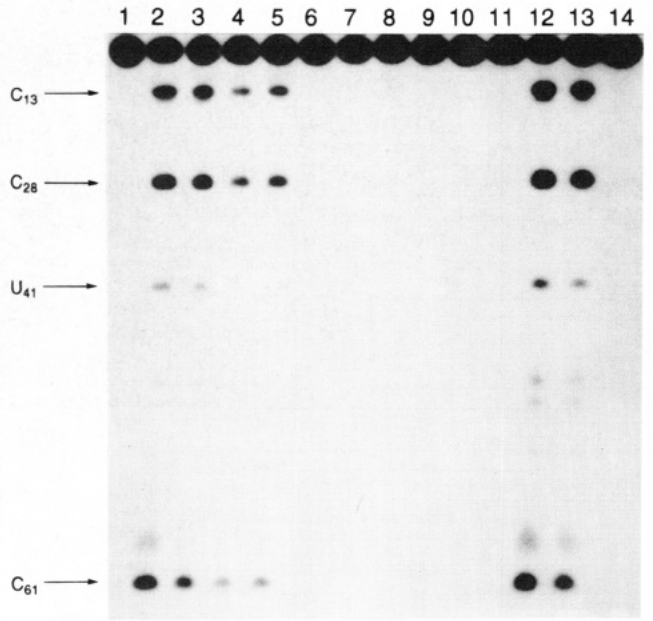

FIGURE 6: Hydrolysis of 3′-^{32}P-end-labeled tRNA^{Phe} mediated by various BLM congeners and fragments. All reactions were carried out for 1 h at 45 °C. Lane 1, RNA alone; lane 2, 500 μM BLM A_2; lane 3, 250 μM BLM A_2; lane 4, 500 μM deglyco-BLM A_2; lane 5, 250 μM deglyco-BLM A_2; lane 6, 500 μM BLM demethyl A_2; lane 7, 250 μM BLM demethyl A_2; lane 8, 500 μM bithiazole **1**; lane 9, 250 μM bithiazole **1**; lane 10, 500 μM *N*-acetyl-BLM A_2; lane 11, 250 μM *N*-acetyl-BLM A_2; lane 12, 500 μM BLM A_2; lane 13, 250 μM BLM A_2; lane 14, RNA alone.

In an effort to determine which part(s) of the bleomycin molecule might actually be responsible for the observed hydrolysis, the RNA cleaving ability of a series of BLM fragments and congeners (Figure 1) was studied. The results are shown in Figure 6. Deglycobleomycin A_2, which lacks the sugar residues present in BLM A_2, exhibited hydrolytic activity slightly less than, but comparable to, that of BLM A_2 (cf. lanes 4 and 5 with lanes 2 and 3). The similar activities of these two derivatives parallel the pattern observed for oxidative cleavage by their respective Fe^{II} complexes and demonstrate that the sugars are not essential for the hydrolysis reaction. In contrast, bleomycin demethyl A_2 (lanes 6 and 7), which lacks a methyl group on the C-terminal sulfur atom, and the associated positive charge, demonstrated minimal cleavage capability. The same result was observed for *N*-acetyl-bleomycin A_2 (lanes 10 and 11), in which the primary amino group in the metal binding domain is acetylated. A BLM "fragment" containing only the bithiazole and C-terminal substituent was also inactive (**1**, lanes 8 and 9). The lack of activity noted for bleomycin demethyl A_2 may simply reflect its lower binding affinity for nucleic acids (Natrajan & Hecht, 1993).

Because certain cations, in particular magnesium, are known to be important for maintaining tRNA tertiary structure (Saenger, 1984), the dependence of the hydrolysis reaction on added $MgCl_2$ and NaCl was also examined. Indeed, previous experiments have demonstrated that for some RNA substrates, cleavage by Fe^{II}·BLM can be inhibited by rather modest magnesium concentrations (Hüttenhofer et al., 1992; Holmes et al., 1993). The dependence of the BLM-mediated hydrolysis reaction on Mg^{2+} is shown in lanes 1−9 of Figure 7. Cleavage was inhibited in an abrupt fashion at magnesium concentrations greater than 200 μM. This effect is similar to the behavior seen during Fe^{II}·BLM-mediated

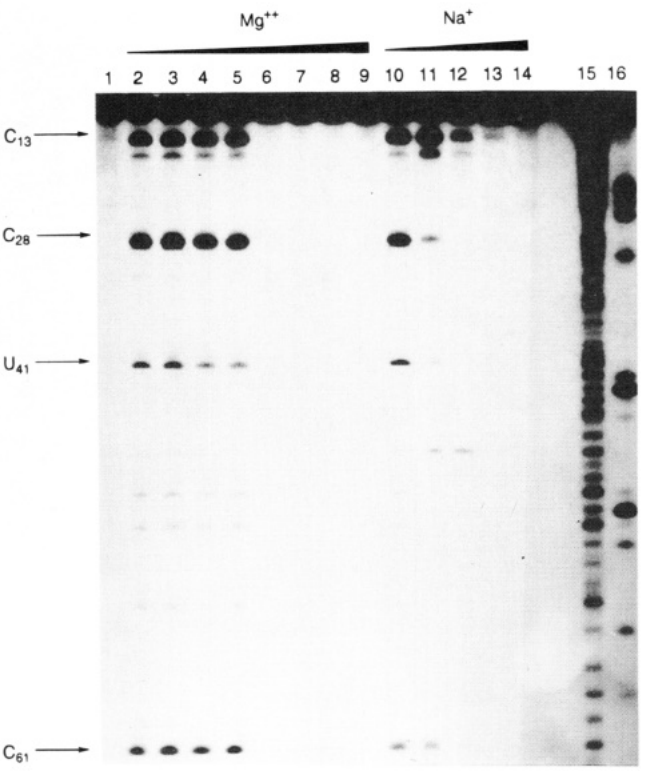

FIGURE 7: Effect of $MgCl_2$ and NaCl on the BLM A_2-mediated hydrolysis reaction. Reactions were carried out in the presence of 500 μM BLM A_2 for 1 h at 45 °C. Lane 1, 3′-^{32}P-end-labeled tRNA^{Phe} alone; lanes 2−14 contain the following salts at the indicated concentrations: lane 2, no salt; lane 3, 50 μM $MgCl_2$; lane 4, 100 μM $MgCl_2$; lane 5, 200 μM $MgCl_2$; lane 6, 300 μM $MgCl_2$; lane 7, 500 μM $MgCl_2$; lane 8, 750 μM $MgCl_2$; lane 9, 1 mM $MgCl_2$; lane 10, 10 mM NaCl; lane 11, 50 mM NaCl; lane 12, 100 mM NaCl; lane 13, 250 mM NaCl; lane 14, 500 mM NaCl; lane 15, alkaline hydrolysis; lane 16, RNase T1 sequencing reaction.

oxidative cleavage; although not addressed experimentally in the present study, it likely reflects a conformational change in the substrate which makes it resistant to cleavage, either by enforcing a more rigid structure or by rendering the phosphodiester moieties inaccessible to the reagent.

The effect of NaCl on the cleavage reaction was quite different (Figure 7, lanes 10−14). Much higher sodium concentrations were required to effect inhibition, relative to magnesium, and the inhibition was more gradual. No inhibition was observed when the concentration of NaCl was less than 10 mM (not shown). Above this value, cleavage intensity started to diminish, but each site was affected differently. The C_{28} and C_{61} sites were essentially absent at salt concentrations of 50 mM, while the cleavage at C_{72} (not shown) was abolished only when NaCl concentrations reached 100 mM. In contrast, the cleavage sites at C_{13} and C_{63} (not shown) persisted until salt concentrations of 250 mM were attained. Essentially all cleavage was inhibited above this concentration. As in the case of magnesium, the pattern observed was similar to that observed for oxidative cleavage of RNA by Fe^{II}·BLM (Holmes et al., 1993). Importantly, this result parallels what was observed upon addition of EDTA to the reaction (*vide supra*). In this regard, it should be noted that for the EDTA experiments the disodium salt of the chelator was used. Thus, the sodium ion concentrations in the EDTA reactions were very similar to the NaCl concentrations described here when similar inhibition occurred. This correlation again suggests strongly

that the inhibition of hydrolysis demonstrated in the presence of EDTA is, in fact, an electrostatic effect.

Finally, it should be noted that the hydrolysis sites observed in these experiments were similar to those that would be expected to result from contamination by a ribonuclease such as RNase A, which shows a similar sequence specificity (Richards & Wickoff, 1971). Several lines of evidence, however, indicate that the present observations were not due to a contaminating ribonuclease. First, the variation in cleavage observed for the various BLM derivatives strongly suggests that the effect is related to BLM and not to a contaminant. Second, the salt dependence and the metal ion inhibition observed in these experiments is not consistent with ribonuclease behavior. Third, removal of the BLM by adsorption onto Chelex resin abolished all cleavage activity. Further, BLM samples retained their hydrolytic activity even after boiling for 30 min (data not shown). Finally, as has been referred to repeatedly above, the behavior of the hydrolytic activity with respect to the effects of exogenous agents and changes in drug structure parallels the behavior of the oxidative cleavage reactions of iron bleomycin. Taken together, these observations indicate that the hydrolytic chemistry described herein is BLM mediated.

DISCUSSION

The development of catalysts for RNA hydrolysis is a goal that has recently received considerable attention. Such compounds might be useful, for example, as research tools, analogous to the restriction enzymes that are currently used for manipulation of DNA structure. In fact, several different types of catalysts capable of RNA hydrolysis have been reported. These include compounds such as simple oligoamines (Yoshinari et al., 1991) or more complex species containing imidazole or guanidine functional groups specifically designed to effect RNA hydrolysis (Breslow & Huang, 1990; Tung et al., 1992; Smith et al., 1993). While these compounds clearly facilitate phosphodiester bond hydrolysis, most do so without any sequence selectivity; however, attachment to an oligonucleotide probe can direct specificity of cleavage (Komiyama et al., 1995). Transition metal complexes have also received considerable attention recently, as they have obvious mechanistic advantages in RNA hydrolysis (Stern et al., 1990; Morrow et al., 1992; Amin et al., 1994). While several complexes of this type have been shown to promote RNA hydrolysis, to date little has been achieved in the way of sequence-specific cleavage.

Interestingly, some metal ions have been shown to promote the hydrolysis of tRNA^{Phe} in a nonrandom fashion. These include Mg^{II} (Wintermeyer & Zachau, 1973), Eu^{III} (Ciesiolka et al., 1989), and Pb^{II} (Brown et al., 1983) as well as other lanthanides and some of their complexes with macrocyclic ligands (Hayashi et al., 1993). In these reactions tRNA cleavage is nonrandom, but it is not sequence specific. Strand scission appears instead to be structure specific, rather than sequence specific, since all cleavage occurs in the anticodon and D loops, which bind these ions.

In contrast, the hydrolysis reaction mediated by bleomycin is observed to occur specifically at pyrimidine−purine sites. Similar specificity of RNA hydrolysis has been reported by others. Giegé and co-workers have synthesized imidazole−intercalator conjugates, designed as RNase mimics, that cleave tRNA transcripts primarily at 5′-UA-3′ and 5′-CA-3′ sites (Podyminogin et al., 1993). Kierzek found that single-stranded oligoribonucleotides could be hydrolyzed selectively by several different organic reagents, including polyvinylpyrrolidone, poly(ethylene glycol), and dextrin (Kierzek, 1992a,b). The order of reactivity in these experiments was UpA > CpA > PypG, i.e., essentially in the same order as that reported here for BLM. In this case, although no specific chemical mechanism was determined, it was suggested that substrate flexibility was necessary to allow the tRNA to assume a conformation favoring deprotonation of the critical ribose 2′-OH group by a properly oriented basic species. This conformation was speculated to involve a hydrogen bond between the 6-amino group of the adenosine or the 4-amino group of cytidine, and possibly also to involve a water bridge.

Interestingly, a similar mechanism was proposed by Dock-Bregeon and Moras (1987) to account for the spontaneous hydrolysis reactions observed to occur for a number of tRNA molecules, including yeast tRNA^{Phe}. These authors reported site-specific, spontaneous hydrolysis of this substrate over time at positions U_8, C_{13}, C_{28}, Y_{37}, C_{56}, C_{61}, and C_{63}, a result which implies that these sites are inherently fragile. Notably, these sites are strikingly similar to those induced by metal-free bleomycin (cf. Figure 3). In fact, all major BLM sites are included in this list except for C_{72}, which was not examined. Dock-Bregeon and Moras considered the reactions to be the result of a self-cleavage process in which substrate flexibility and hydrogen bonding interactions are important. Consistent with this interpretation, the addition of magnesium ion inhibited cleavage in stem regions but enhanced cleavage in the loops; loop regions are still able to maintain some degree of flexibility and solvent accessibility in the presence of Mg^{2+}. Further, cleavage of crystalline tRNA molecules was shown to occur at sites different from those reported above and at which more flexibility seemed to be available in the lattice.

In the case of the BLM-induced hydrolysis of tRNA^{Phe} reported here, two different types of mechanisms could explain the observed catalysis at these apparently labile tRNA sites. One possibility is that BLM binds to the RNA substrate and actually provides the basic functional group responsible for deprotonation of the critical ribose 2′-hydroxyl group. Bleomycin is a relatively large molecule which contains a number of functional groups potentially capable of acting in such a manner. From this perspective, it is interesting to note that the drug contains an imidazole moiety, namely, the β-hydroxyhistidine residue of the metal-binding domain. Imidazole residues have been shown to function well in this role both in synthetic systems (Breslow & Huang, 1990; Tung et al., 1992; Podyminogin et al., 1993; Smith et al., 1993) and in the active site of RNase A (Richards & Wickoff, 1971). In this context it may be pertinent that the metallobleomycins studied here all failed to effect tRNA^{Phe} hydrolysis (supporting information, Figure 3); studies of metal ion ligation by BLM all suggest that the β-hydroxyhistidine moiety is either a metal ligand or in close spatial proximity to the metal ion (Natrajan & Hecht, 1993), thus potentially rendering the imidazole functionality unavailable for RNA hydrolysis. It is perhaps no coincidence that the metal-binding domain in Fe^{II}·BLM is now believed to be a major determinant of the sequence specificity observed in DNA cleavage (Carter et al., 1990b; Guajardo et al., 1993; Kane et al., 1994), a role that implies essential interactions between this domain and the nucleic acid target.

It should be noted, however, that although a role for the imidazole moiety is an attractive hypothesis based on what is known about other RNA cleaving agents, its presence is clearly not sufficient for the hydrolytic activity to occur, given the results observed with the acetylated and demethylated BLM derivatives. Both of these contained the imidazole functionality, yet failed to induce RNA hydrolysis, indicating that other structural elements in bleomycin are essential for the observed hydrolysis reaction.

A second possible mechanism involves the binding of bleomycin to the tRNA substrate such that the resulting complex adopts a conformation more conducive to strand scission at these fragile sites. This hypothesis parallels those of Dock-Bregeon and Moras (1987) and of Kierzek (1992a,b) and may imply an explanation as simple as a drug-induced alteration of the flexibility of the RNA at the affected sites. According to this mechanism, BLM would simply permit the substrate to find the reactive conformation with greater facility. This interpretation is supported by the ability of Mg^{2+} to inhibit BLM-mediated hydrolysis at rather modest concentrations; it would also accommodate the observed importance of the metal-binding domain. It is obviously also possible that elements of both mechanisms are important in BLM-mediated RNA hydrolysis.

The present experimental results cannot distinguish between the two types of mechanisms discussed above. Further, because ongoing studies (Hecht, 1994; Manderville et al., 1994, 1995; Wu et al., 1994) of BLM binding to nucleic acids suggest a rather complex interplay of the individual structural domains in BLM with the nucleic acid target, it is not possible to draw firm conclusions from the available data regarding the structural elements in BLM that are essential for hydrolytic activity. However, the accumulated data do suggest an important role for the metal-binding domain of bleomycin.

It is undoubtedly not a fortuitous observation that several chemical agents, including BLM, have been found to target Py-Pu sites for hydrolytic cleavage in RNA substrates. The fact that nature also chose to cleave these sites with several ribonucleases suggests that it is a particularly attractive target for such chemistry, perhaps because it is particularly susceptible as has been suggested previously (Dock-Bregeon & Moras, 1987; Kierzek, 1992a,b). The origin of this preference is still unclear, but one explanation might be that the sequences 5′-UA-3′ and 5′-CA-3′ form localized structural motifs which are predisposed to such cleavage. BLM would then be one of several chemical nucleases that targets this motif, either by direct binding or by induction of some conformational rearrangement. Importantly, this site-specific hydrolysis of $tRNA^{Phe}$ represents a new facet of the complex chemistry of BLM. As already noted for the Fe(II)·BLM-mediated oxidative RNA cleavage, the therapeutic relevance of this chemistry is for the moment an intriguing possibility that remains to be tested.

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SUPPORTING INFORMATION AVAILABLE

Derivation of eq 5 and three figures showing the time dependence of the hydrolysis reaction at 25 °C, the effect of EDTA on the BLM-mediated hydrolysis of $tRNA^{Phe}$, and the inhibition of hydrolysis reaction upon addition of various metal ions (6 pages). Ordering information is given on any current masthead page.

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