Cleavage of tRNA by Fe(II)-Bleomycin*

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We have investigated the action of the chemotherapeutic agent Fe(II)-bleomycin on yeast tRNA<sup>Ph</sup>*, an RNA of known three-dimensional structure. In the absence of Mg<sup>2+</sup> ions, the RNA is cleaved preferentially at two major positions, A31 and G53, both of which are located at the terminal base pairs of hairpin loops, and coincide with the location of tight Mg<sup>2+</sup>-binding sites. A fragment of the tRNA (residues 47-76) containing the T stem-loop is also cleaved specifically at G53. Cleavage of both the intact tRNA and the tRNA fragment is abolished in the presence of physiological concentrations of Mg<sup>2+</sup> (>0.5 mM). Since Fe(II) is not displaced from bleomycin under these conditions, we infer that tight binding of Mg<sup>2+</sup> to tRNA excludes the RNA. These results also show that loss of cleavage is not due to Mg<sup>2+</sup>-dependent formation of tertiary interactions between the D and T loops. In contrast, cleavage of synthetic DNA analogs of the anticodon and T stem-loops is not detectably inhibited by Mg<sup>2+</sup>, even at concentrations as high as 50 mM. In addition, the site specificities observed in cleavage of RNA and DNA differ significantly. From these results, and from similar findings with other representative RNA molecules, we suggest that the cleavage of RNA by Fe(II)-bleomycin is unlikely to be important for its therapeutic action.

The bleomycins (BLMs) are a family of glycopeptide antibiotics that are used in combination chemotherapy against many types of cancer (Blum et al., 1971; Sugiuira et al., 1985; Suzuki et al., 1970). These antibiotics require dioxygen and a metal ion such as Fe<sup>2+</sup> to complete their drug action (Stubbe and Kozarich, 1987). The metal chelates of the drug (metal-bleomycins, M-BLMs) are believed to affect their antineoplastic activity by oxidative cleavage of DNA (Hecht, 1986). The dioxygen requirement has been established for both the iron and copper complexes of BLM (Dabrowiak, 1983). The metal-oxo species formed in close proximity to the metal ion such as Fe<sup>2+</sup> to complete their drug action (Stubbe and Kozarich, 1987). The metal chelates of the drug (metal-bleomycins, M-BLMs) are believed to affect their antineoplastic activity by oxidative cleavage of DNA (Hecht, 1986). The dioxygen requirement has been established for both the iron and copper complexes of BLM (Dabrowiak, 1983). The metal-oxo species formed in close proximity to the DNA brings about this cleavage (Petering et al., 1990) (Scheme I).

Recently, it has been reported that tRNA and tRNA precursors are cleaved by Fe(II)-BLM (Magliozzo et al., 1989; Carter et al., 1990). Cleavage of tRNA is accompanied by release of adenine and uracil bases (Magliozzo et al., 1989). However, unlike the DNA cleavage reaction (Umezawa et al., 1984), no base propensities are formed, suggesting that the cleavage of RNA may proceed by a somewhat different mechanism. Carter et al. (1990) have reported cleavage of a tRNA<sup>His</sup> precursor at the invariant uridine corresponding to position 8 of mature tRNA, along with minor cleavage sites adjacent to other pyrimidine residues, while tRNA<sup>Hy</sup> precursor exhibited no such cleavage. In addition, four cleavage sites were determined for the region of human immunodeficiency virus 1 RNA that encodes reverse transcriptase. This has led to the suggestion that RNA may be a therapeutically relevant target for BLM (Carter et al., 1990). However, the cleavage reactions in these studies were performed in the absence of magnesium ions or physiological concentrations of monovalent cations.

We initiated this study in order to assess the action of Fe(II)-BLM on tRNA<sup>Ph</sup> yeast, an RNA whose three-dimensional structure is well known (Kim, 1979). It was anticipated that its pattern of cleavage might provide insight into the stereochemical specificity of Fe(II)·BLM for RNA. Our studies show that tRNA<sup>Ph</sup> yeast is cleaved at specific sites by Fe(II)·BLM in the absence of magnesium ions. However, under ionic conditions where tRNA is biologically active and is known to attain its correct three-dimensional structure, cleavage of tRNA is abolished, although cleavage of DNA is unaffected. The most likely explanation is that, under these conditions, Fe(II)·BLM is prevented from interacting with RNA by tightly bound magnesium ions.

**MATERIALS AND METHODS**

Bleomycin—BLM-sulfate was a generous gift of Dr. Bradford Baer, Cetus Corp., Emeryville, CA. tRNA—Yeast tRNA<sup>Ph</sup> was obtained from Boehringer Mannheim. A subfragment of yeast tRNA<sup>Ph</sup> containing residues 47-76 was prepared by sodium borohydride/analine cleavage at m<sup>3</sup>G46 in the presence of 7-methylguanosine according to Zueva et al. (1985). The subfragment was then end-labeled with [5'-<sup>32</sup>P]pCp as described by England et al. (1980). After end-labeling, tRNA<sup>Ph</sup> was purified on a 10% polyacrylamide, 7 M urea gel, and renatured in 10 mM Tris-HCl pH 7.5, by incubation for 5 min at 55 °C in the absence or presence of MgCl<sub>2</sub> (0.1-10 mM, as specified) followed by slow cooling to room temperature in a water bath.

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The abbreviation used is: BLM, bleomycin.
DNA—DNA oligonucleotides were synthesized on a MilliGen/Biosearch 8600 automated DNA synthesizer and purified by preparative gel electrophoresis on 20% polyacrylamide, 7 M urea gels. DNA was visualized by UV shadowing and recovered by elution from the gel into 0.3 M NaOAc buffer, pH 5.2; DNA was subsequently precipitated with 3 volumes of ethanol and dissolved in 10 mM sodium cacodylate buffer, pH 7.5.

DNA oligonucleotides were 5' end-labeled using [γ-32P]ATP and T4 polynucleotide kinase as described by Maniatis et al. (1982) and annealed by heating to 55 °C followed by slow cooling to room temperature in 10 mM sodium cacodylate buffer, pH 7.5.

**BLM Cleavage**—Reaction mixtures (10 μl) contained 5 pmol of RNA or DNA (approximately 100,000 cpm) and 0.5 mM BLM in 10 mM sodium cacodylate buffer, pH 7.5, with or without MgCl₂, as specified. Reactions at 20 °C were initiated by the addition of freshly made 0.45 mM Fe(NH₄)₂(SO₄)₂ in three 1-μl aliquots over a period of 45 min. After the reaction was completed, samples were precipitated with 3 volumes of ethanol in the presence of 10 μg of oyster glycogen and applied to 10% (RNA samples) or 20% (DNA samples) polyacrylamide, 7 M urea gels (60 × 20 × 0.02 cm).

For calibration of gel band positions, end-labeled tRNA was cleaved at G residues by digestion with T₁ ribonuclease or was subjected to alkaline hydrolysis, respectively, as described by Donis-Keller et al. (1985). Full-length tRNA or DNA oligonucleotides and defined cleavage products were located by autoradiography, excised from the gel, and quantitated by Cerenkov counting.

**RESULTS**

**Fe(II)-BLM Causes Specific Cleavage of Yeast tRNA**—Yeast tRNA<sub>Phe</sub> was chosen as a model system for RNA cleavage by Fe(II)-BLM because the availability of a well-defined crystal structure makes it possible to correlate sites of cleavage with stereochemical details of the RNA structure. Fe(II)-BLM cleaves yeast tRNA<sub>Phe</sub> preferentially at two major positions under conditions comparable to those utilized for DNA cleavage.

The sites of cleavage correspond to excision of A31 in the anticodon stem and to excision of G53 in the T stem of the tRNA (Figs. 1 and 2). Both cleavage sites are located at double strand-single strand junctions. In addition to the two main cleavage sites, a number of minor cleavage sites are also observed (Fig. 1, lane 2). Also evident is a consistently reproducible band, running more slowly than that of full-length yeast tRNA, which, based upon its mobility, could represent a stable tRNA-BLM adduct (Fig. 1).

**Magnesium Ions Abolish Cleavage of tRNA by Fe(II)-BLM**—When tRNA is treated with similar concentrations of Fe(II)-BLM under ionic conditions where tRNA is biologically active, site-specific cleavage is abolished. This inhibition is due to the presence of magnesium ions, as shown in Fig. 3. The site-specific cleavage is strongly inhibited by the addition of 0.1 mM MgCl₂ and is totally inhibited by concentrations above 1 mM. Interestingly, at 0.1 mM MgCl₂, a minor cleavage site at position 68 becomes enhanced and decreases at magnesium concentrations above 0.5 mM. In addition, we observe two minor cleavage sites (at positions 16 and 19, respectively) which increase in response to increasing MgCl₂ concentrations. These appear to be purely magnesium ion-dependent cleavages, since they are identical with cleavage sites that are observed, when only tRNA and MgCl₂ are present (Fig. 3, lane c); this is in agreement with previously published cleavage of yeast tRNA<sub>Phe</sub> by magnesium ions (Cieślińska et al., 1989). No inhibition of Fe(II)-BLM cleavage was noted with increasing amounts of monovalent ions (0–20 mM), such as K⁺, to the reaction mixture (data not shown).

We considered three possible explanations for the magnesium ion-dependent inhibition of tRNA cleavage by Fe(II)-BLM: 1) displacement of Fe<sup>2+</sup> from the BLM complex; 2) formation of Mg<sup>2+</sup>-dependent tertiary interactions that might displace the Fe(II)-BLM complex; and 3) competition of tightly bound Mg<sup>2+</sup> for Fe(II)-BLM binding sites on tRNA.

The electronic absorption spectrum of Fe(II)-BLM in the presence of 0.5 mM MgCl₂ exhibits only minor decreases in the characteristic 291 nm absorption band under conditions

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**FIG. 1. Cleavage of yeast tRNA<sup>Phe</sup> by Fe(II)-BLM.** The 3'-[γ<sup>32</sup>P]GpG end-labeled tRNA (5 pmol) was treated as follows: lane 1, control incubation in the absence of BLM; lane 2, 0.5 mM Fe(II)-BLM; T<sub>1</sub>, partial RNase T<sub>1</sub>; L, partial alkaline hydrolysis ladder. Arrows indicate major cleavage sites at position A31 and position G53 of the tRNA, as well as a BLM-dependent band with a mobility slightly slower than that of the untreated tRNA.

**FIG. 2. Secondary structure of yeast tRNA<sup>Phe</sup>.** Arrows indicate major sites of cleavage by Fe(II)-BLM. Also shown are coordination sites for tightly bound magnesium ions (Teeter et al., 1980).
Cleavage of tRNA by Bleomycin

Inhibition of Fe(II)-BLM-mediated tRNA<sup>Phe</sup> cleavage. The 3'-[32P]pCp end-labeled tRNA<sup>Phe</sup> (5 pmol) was cleaved by 0.5 mM Fe(II)-BLM in the presence of the following concentrations of MgCl<sub>2</sub>: lane 1, no magnesium; lane 2, 0.1 mM; lane 3, 0.5 mM; lane 4, 1 mM; lane 5, 5 mM; lane 6, 10 mM; T, partial RNase T digest; L, partial alkaline hydrolysis ladder; C, tRNA<sup>Phe</sup> incubated under conditions identical with lanes 1-6, but in the absence of Fe(II)-BLM and in the presence of 10 mM MgCl<sub>2</sub>.

where the cleavage reaction is completely inhibited (Fig. 4).<sup>2</sup> This result rules out possible displacement of Fe<sup>3+</sup> from the Fe(II)-BLM complex by magnesium ions.

Tertiary base-base interactions between the D and T loops of tRNA are stabilized by Mg<sup>2+</sup> ions (Heerschap et al., 1983). To test whether loss of cleavage at G53 in the T loop could be due to formation of tertiary interactions with the D loop, we prepared a fragment of tRNA<sup>Phe</sup> yeast (residues 47-76) that contains only the T loop. Treatment of this fragment with Fe(II)-BLM again results in cleavage at G53 and is inhibited by Mg<sup>2+</sup> in a manner similar to that observed for the complete tRNA (Fig. 5). This result eliminates the possibility that the D loop-T loop interactions displace the Fe(II)-BLM complex from the RNA and leaves direct displacement by tight-binding magnesium ions as the most likely explanation for the observed inhibition. In addition, in the presence of Fe(II)-BLM, we observe two bands running slightly slower than the untreated RNA fragment (Fig. 5, lane 6). Significantly, the intensity of these slow migrating bands decreases in the presence of added Mg<sup>2+</sup> ions (Fig. 5, lanes 7-9).

Cleavage of DNA Analogs of tRNA Stem-Loops by Fe(II)-BLM—To compare directly the cleavage specificity of Fe(II)-

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<sup>2</sup>In contrast, addition of reagents like EDTA which displace Fe<sup>3+</sup> from Fe(II)-BLM causes significant diminution of the absorption at 291 nm.

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BLM for DNA and RNA, we synthesized DNA analogs of the anticodon stem-loop (residues 27-43) and of the 3’-terminal fragment (residues 47-76) studied above, containing the T loop clearly occurs one position 5’ to the site of cleavage of tRNA. Both DNA sites follow the general rule of Fe(II)-BLM cleavage at double-stranded 5’-G-N3’ sequences, in which N, the site of cleavage, can be A, T, or C (Umezawa et al., 1984), and are in fact the only two sites in the DNA fragments that fulfill this criterion. This suggests that cleavage of both the RNA and DNA at the same position of the anticodon stem is coincidental. This interpretation is supported by experiments using mutant versions of the DNA anticodon stem analog. Cleavage occurred at position 31 whether the base at position 31 was A, C, or T (Fig. 6). When the G30-C40 base pair was changed to C30-G40, cleavage at position 31 was abolished, and, instead, cleavage occurred at T41 (Fig. 6). These results are in good agreement with the rules proposed by Umezawa et al. (1984) for Fe(II)-BLM cleavage of DNA.

Another difference between the DNA and RNA cleavage reactions is the insensitivity of DNA cleavage to the presence of Mg<sup>2+</sup> ions. Even at concentrations of Mg<sup>2+</sup> ions as high as 50 mM, efficient cleavage of the DNA fragments is observed (Fig. 7, A and C, lane 7).

Finally, the relative cleavage efficiencies of DNA and RNA differ significantly. Cleavage of DNA by Fe(II)-BLM is about 15-fold more efficient than that of RNA under similar conditions (Table I). Taken together, these results show that there are major differences in the mode of recognition and cleavage of DNA and RNA by Fe(II)-BLM.

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<sup>3</sup>A. Hüttenhofer, S. Hudson, H. F. Noller, and P. K. Mascharak, unpublished results.
Cleavage of tRNA by Bleomycin

On the basis of in vitro Fe(II)-BLM-mediated cleavage of tRNA precursors, it has been proposed that cleavage of cellular RNA may be important for the anti-tumor activity of this drug (Carter et al., 1990). This proposal was based on experiments carried out in the absence of Mg\(^{2+}\) ions (Carter et al., 1990; Magliozzo et al., 1989). Here, we confirm the ability of Fe(II)-BLM to cleave RNA; however, we also show that, under ionic conditions more closely resembling intracellular Mg\(^{2+}\) ion levels, RNA cleavage is abolished. This is true not only for tRNA\(^{Phe}\), as shown here, but also for cleavage of *Escherichia coli* tRNA\(^{Amp}\), an *E. coli* tRNA\(^{Amp}\) precursor transcript and RNase P RNA; in all cases, specific cleavage is observed in the absence, but not in the presence, of even low levels of Mg\(^{2+}\) ions. Thus, we believe that the absence of Mg\(^{2+}\) ions for cleavage of RNA by Fe(II)-BLM is likely to be a general requirement. If this is the case, cleavage of RNA is unlikely be of significance for the therapeutic action of BLM. The three-dimensional structure of yeast tRNA\(^{Phe}\) (Kim, 1979) provides a basis for further insight into the structural specificity of BLM-RNA interactions. The two main sites of cleavage of tRNA\(^{Phe}\) coincide with regions of the RNA structure that contain strong binding sites for Mg\(^{2+}\) ions (Fig. 2; Teeter et al. (1980)). The D and T loop sites are also close to a cluster of complex tertiary interactions, some of which involve base-base interactions between the two loop regions. The fact that the T loop is cleaved efficiently, even in the absence of the D loop (Fig. 5), shows that, at least for this loop, cleavage does not depend on these long-range tertiary interactions. More likely is the possibility that Fe(II)-BLM selectively binds to certain three-dimensional features of tRNA that also favor tight binding of Mg\(^{2+}\) ions. This would account for the inhibition of cleavage by low concentrations of Mg\(^{2+}\) ions. In fact, the observed binding constants for Fe(II)-BLM and the tightly bound class of Mg\(^{2+}\) ions to tRNA are similar (on the order of 0.9 \(\times \) 10\(^5\) M\(^{-1}\); Magliozzo et al. (1989), Schimmel and Redfield (1989)). Thus, displacement of Fe(II)-BLM (at a concentration of 0.5 mM) from tRNA by Mg\(^{2+}\) ions at concentrations greater than 0.5 mM is entirely consistent with the known binding constants of the two cations. Alternatively, Mg\(^{2+}\) might act by stabilizing a conformation of tRNA that is no longer susceptible to attack by Fe(II)-BLM. The absence of single-stranded loop structures in cellular DNA, which appear to be important in providing the environment for tight binding of Mg\(^{2+}\) ions to tRNA, is also in accord with the idea that BLM recognizes quite different binding sites in DNA and RNA. There may be some secondary structural specificity in the preferred sites of cleavage of RNA. In the examples we have seen so far, and in those reported by Carter et al. (1980), Fe(II)-BLM appears to favor sites at the junction of single-

Fig. 5. Cleavage of yeast tRNA\(^{Phe}\) and its 3'-terminal fragment (bases 47-76; see Fig. 6). Lanes 1-4 and 5-9 display 3'-\([\gamma^{32}P]pCp\) end-labeled tRNA\(^{Phe}\) or its 3'-terminal fragment (residues 47-76), respectively, treated under Fe(II)-BLM cleavage conditions with the following additions: lane 1, no addition; lane 2, 0.5 mM Fe(II); lane 3, 0.5 mM BLM; lane 4, 0.5 mM Fe(II)-BLM; lane 5, no addition; lane 6, 0.5 mM Fe(II)-BLM; lane 7, 0.5 mM Fe(II)-BLM plus 0.1 mM MgCl\(_2\); lane 8, 0.5 mM Fe(II)-BLM plus 2 mM MgCl\(_2\). T1, partial RNase T1 digest; L, partial alkaline hydrolysis ladder.

Fig. 6. RNA and DNA oligonucleotides corresponding to segments of tRNA\(^{Phe}\). Arrows indicate sites of specific cleavage by Fe(II)-BLM (see Figs. 1, 3, 5, and 7). a, tRNA\(^{Phe}\); b, 3'-terminal fragment (residues 47-76) of tRNA\(^{Phe}\); c, DNA analog of the tRNA anticodon stem-loop; N, A (wild type), T, C (base mutations); d, mutant DNA analog of the anticodon stem-loop; change of the G30-C40 base pair to C30-G40; e, DNA analog of the tRNA T stem-loop and 3'-terminal region.
A DNA analog of the 3' terminal (residues 47-76) fragment of tRNA\textsubscript{Phe}. C, DNA analog of the tRNA\textsubscript{Phe} anticodon stem-loop (residues 27-43). Oligomers were treated with 0.5 mM Fe(II).BLM, except where indicated: lanes 1, no Fe(II)-BLM; lanes 2, Fe(II)-BLM without MgCl\textsubscript{2}; lanes 3, with 0.1 mM MgCl\textsubscript{2}; lanes 4, with 0.5 mM MgCl\textsubscript{2}; lanes 5, with 1 mM MgCl\textsubscript{2}; lanes 6, with 5 mM MgCl\textsubscript{2}; lanes 7, with 50 mM MgCl\textsubscript{2}. The arrow in C indicates the position of a Fe(II)-BLM dependent slowly migrating band. B, sequence analysis of 5'-32P-end-labeled DNA oligonucleotides, cleaved with 0.1 mM Fe(II).BLM, lane 4, no Fe(II)-BLM; lane 5, no Fe(II)-BLM; lane 6, chemical cleavage at G residues; lanes 4-6, DNA analog of anticodon stem-loop fragment of tRNA\textsubscript{Phe}; lane 7, with 0.1 mM Fe(II)-BLM; lane 5, no Fe(II)-BLM; lane 6, chemical cleavage at G residues.

**FIG. 7. Cleavage of 5'\textsuperscript{32}P-end-labeled DNA oligomers (5 pmol) by Fe(II)-BLM.** A, DNA analog of the 3' terminal (residues 47-76) fragment of tRNA\textsubscript{Phe}. C, DNA analog of the tRNA\textsubscript{Phe} anticodon stem-loop (residues 27-43). Oligomers were treated with 0.5 mM Fe(II)-BLM, except where indicated: lanes 1, no Fe(II)-BLM; lanes 2, Fe(II)-BLM without MgCl\textsubscript{2}; lanes 3, with 0.1 mM MgCl\textsubscript{2}; lanes 4, with 0.5 mM MgCl\textsubscript{2}; lanes 5, with 1 mM MgCl\textsubscript{2}; lanes 6, with 5 mM MgCl\textsubscript{2}; lanes 7, with 50 mM MgCl\textsubscript{2}. The arrow in C indicates the position of a Fe(II)-BLM dependent slowly migrating band. B, sequence analysis of 5'\textsuperscript{32}P-end-labeled DNA oligonucleotides, cleaved by Fe(II)-BLM; lanes 1-3, DNA analog of the 3' terminal fragment of tRNA\textsubscript{Phe} (residues 47-76); lane 1, with 0.1 mM Fe(II)-BLM; lane 2, no Fe(II)-BLM; lane 3, chemical cleavage at G residues; lanes 4-6, DNA analog of anticodon stem-loop fragment of tRNA\textsubscript{Phe}; lane 4, with 0.1 mM Fe(II)-BLM; lane 5, no Fe(II)-BLM; lane 6, chemical cleavage at G residues.

**TABLE I**

Relative cleavage efficiency of RNA and DNA oligomers and tRNA by Fe(II)-BLM

| Substrate                   | A51 | T52 | G53 | % cleavage |
|----------------------------|-----|-----|-----|-----------|
| tRNA                       | 4.1 | 4.7 | 5.6 |           |
| RNA T stem-loop            | 98.5|     |     |           |
| DNA T stem-loop            | 98.5|     |     |           |
| DNA anticodon stem-loop    | 82.2|     |     |           |

* Full-length as well as cleavage product bands were excised from the gels and Cerenkov-counted (see "Materials and Methods"). Cleavage efficiency was determined by the ratio of cleavage products to total RNA or DNA.

and double-stranded structure, at the 3' side of the ends of helices (Fig. 2). If this turns out to be general, it would suggest that some features of the double helix may be important for recognition. Failure to recognize some 3' helical ends could be due to an additional requirement for those structural features that give rise to tight Mg\textsuperscript{2+} binding.

Finally, we note the presence of an unexplained band migrating slightly more slowly than the corresponding full-length RNA or DNA band on denaturing gels, in samples that have been exposed to BLM. (Figs. 1, 3, 5, and 7); a similar slowly migrating band also seems to be apparent in a previously published study (Magliozzo et al. (1989); see Fig. 3, lane 6). The mobilities of these unexplained bands are consistent with the possible formation of a covalent BLM-RNA adduct, possibly an intermediate in the cleavage reaction.

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