Fe-Bleomycin Cleave a Transfer RNA Precursor and Its "Transfer DNA" Analog at the Same Major Site*

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Previously, Fe-bleomycin (BLM) has been shown to mediate RNA cleavage in a fashion more highly selective than that of DNA. Because RNAs often assume secondary and tertiary structures not commonly encountered with DNAs, it was not clear whether the greater selectivity of RNA cleavage was a consequence of differences in the mononucleotide constituents of RNA and DNA, or of the three-dimensional structures of the individual substrates. Accordingly, we prepared a "tDNA" identical in sequence with Bacillus subtilis tRNAHis precursor, the latter of which is known to be a good substrate for Fe(II)-BLM Ap and which undergoes oxidative cleavage predominantly at T35. Remarkably, the tDNA underwent cleavage predominantly at T35. At higher concentrations of Fe(II)-BLM Ap, the tDNA was extensively degraded, while the tRNAHis precursor was not. Competition experiments suggested that this was not due to more efficient binding of Fe-BLM to the tDNA; in fact the tRNA precursor appeared to be bound more efficiently. The lesser cleavage of the tRNAHis may be due to limitations in the facility of chemical transformation following Fe-BLM binding, or else to the formation of RNA lesions that do not lead directly to RNA strand scission.

The bleomycins are clinically useful antitumor agents having unique chemical and biochemical properties (1–3). The latter have been studied intensively in an effort to define the biochemical loci at which bleomycin mediates its therapeutic effects, and to characterize its behavior at those loci.

Although a few different therapeutic targets were suggested for BLM in early studies, most efforts have focused on the ability of the drug to mediate the oxidative destruction of DNA (2, 3). This process, which involves an "activated" species formed from BLM, a redox-active metal ion such as iron or copper, and O2, produces lesions in chromosomal DNA that include single- and double-stranded breaks as well as alkali-labile lesions (2–4). These structural alterations, all or most of which can be repaired (5), are believed to form predominantly at a subset of the 5′-GC-3′ and 5′-GT-3′ sequences in DNA (6–8).

Recently, it has been shown convincingly that BLM can also affect RNA strand scission (9–15). Although occurring with much greater selectivity than DNA strand scission, the two processes also have key features in common. In particular, BLM-mediated RNA degradation was also shown to be oxidative in nature and to afford strand scission products some of which can also be envisioned to form via abstraction of the ribose C-4′ H by activated Fe-BLM (10, 14). Relative to DNA, RNA has at least three potential advantages as a therapeutic target for BLM. These include the greater selectivity of RNA cleavage, the greater accessibility of (cytoplasmic) RNA to exogenous agents, and the dearth of cellular mechanisms for RNA repair.

Although the greater selectivity of RNA cleavage by BLM might logically be ascribed to intrinsic differences in the susceptibility of DNA and RNA to BLM-mediated destruction, it may be noted that the RNA substrates studied thus far are believed to have secondary and tertiary structures unlike those found in B-form DNA, the "usual" DNA substrate for BLM. In fact, it has been noted that structural alteration of DNA via ethylation (16–18), platination (19, 20), or the introduction of bulges in the duplex (21) substantially alters the observed pattern of BLM-mediated degradation.

To facilitate a comparison of RNA and DNA cleavage by BLM, we have prepared a single-stranded DNA molecule having the same primary structure as Bacillus subtilis tRNAHis precursor (Fig. 1), the latter of which is a particularly good substrate for cleavage by Fe(II)-BLM and is cleaved predominantly at a single site (10–12). Presently, we demonstrate that the tRNA and "tDNA" substrates are cleaved at the same major site by Fe-BLM, an observation with important implications for the nature of substrate recognition by BLM.

EXPERIMENTAL PROCEDURES

Materials

Blenoxane, obtained from Bristol Laboratories, was fractionated as described to afford bleomycin Ap (22). A SP64 plasmid encoding B. subtilis tRNAHis precursor was kindly provided by Dr. Barbara Vold (SRI International, Menlo Park, CA). Calf intestinal phosphatase and restriction endonuclease EcoRI were purchased from Boehringer Mannheim; T4 DNA ligase and T4 polynucleotide kinase were from Life Technologies Inc. SP6 RNA polymerase was purchased from Promega; [γ-32P]ATP was obtained from ICN Radiochemicals. The protected nucleotides for tDNA synthesis were from Milligen Biosearch.

Methods

Preparation of 5′-32P-End Labeled tRNAHis Precursor—The plasmid encoding tRNAHis precursor (23) was isolated from Escherichia coli JM101 as described (24). The tRNAHis precursor was prepared from the EcoRI-linearized DNA plasmid using SP6 RNA polymerase (25). The RNA transcripts were isolated, dephosphorylated using calf intestinal phosphatase, and 5′-end labeled in the presence of T4 polynucleotide kinase and [γ-32P]ATP essentially as described (14).

Preparation of 5′-32P-End Labeled tRNA Precursor Substrate—The tDNA substrate was prepared by the enzymatic manipulation of chemically synthesized DNA fragments. Initially three DNA oligonucleotides were prepared on a Biosearch 7800 series DNA synthesizer using standard phosphoramidite chemistry, and then purified on a NENSORB...
Reaction mixtures (5 μl total volume) contained 2–8 nM unlabeled ATP (56-nucleotide fragment) or 0.5 nCi of [γ-32P]ATP (62-nucleotide fragment). The three DNA oligonucleotides were heated at 85°C for 1 min in 50 mM NaCl then cooled to room temperature over a period of 2–3 h to effect annealing of the three strands. Ligation of the 62- and 56-nucleotide fragments was accomplished in a reaction mixture (100 μl total volume) containing 50 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1.4 mM ATP, 5 mM 2-mercaptoethanol, 2 mM dithiothreitol, 10 mM MgCl₂, polyethylene glycol (2% w/v, PEG-8000) and 12 units of T4 DNA ligase (1 unit catalyzes the exchange of 1 n mole of 32P-labeled pyrophosphate into ATP in 20 min at 37°C). The reaction mixture was maintained at 0°C for 2 h, then at room temperature for 12 h. The tDNA product was purified by 15% denaturing polyacrylamide gel electrophoresis. The strategy for the elaboration of the tDNA substrate is summarized in Fig. 2.

Cleavage of the tRNA and tDNA Substrates by Fe-Bleomycin —Reaction mixtures (5 μl total volume) contained 2–8 μM (final nucleotide concentrations) radiolabeled tRNA or tDNA in 5 mM sodium phosphate buffer, pH 7.5. The reactions were initiated by the simultaneous addition of freshly prepared solutions containing equal amounts of Fe²⁺ and BLM A₂ to the final concentrations indicated in the figure legends. Where additional components such as Mg²⁺ were included, they were added to the reaction prior to Fe²⁺ and BLM without subsequent renaturation of the RNA or DNA substrate. Reaction mixtures were incubated at room temperature for 15 min, then quenched by the addition of 3 μl of loading buffer (80% (w/v) formamide, 50 mM Tris borate, pH 8.3, 1 mM EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue) and analyzed on denaturing 10% polyacrylamide gels (50 W for 1.5–2 h).

Maxam-Gilbert DNA sequencing was carried out essentially as described (27).

RESULTS

The tRNAHis precursor substrate employed in this study was prepared by in vitro transcription from an EcoRI-linearized DNA plasmid, as described previously (10–12, 25). The tDNA substrate was prepared by ligation of two synthetic DNAs 62 and 56 nt in length, respectively. The ligation was accomplished via the agency of T4 DNA ligase after the fragments had been aligned by annealing to a 27-nucleotide DNA oligonucleotide complementary to the 3'- and 5'-terminal sequences in the appropriate tDNA "fragments." The 118-nucleotide product was then purified by 15% polyacrylamide gel electrophoresis (Fig. 2).

The ability of the tDNA to act as a substrate for oxidative cleavage by Fe-BLM was examined under the reaction conditions employed previously for tRNA (10–12, 14) (Fig. 3). Fe(II)-BLM-dependent cleavage of the tDNA obtained at a BLM concentration as low as 250 nM in the absence of any added reducing agent, affording a single major cleavage band. This same band was also prominent when the substrate was added reducing agent, affording a single major cleavage band.

The major sites of cleavage at U₃⁰ (T₃⁰), denoted by large arrows, other significant sites of tDNA cleavage are indicated by small arrows. Minor sites of cleavage of both substrates are denoted by asterisks.

Fig. 1. Structures of B. subtilis tRNAHis precursor and a tDNA arbitrarily folded in the same secondary structure. In addition to the major sites of cleavage at U₃⁰ (T₃⁰), denoted by large arrows, other significant sites of DNA cleavage are indicated by small arrows. Minor sites of cleavage of both substrates are denoted by asterisks.

Fig. 2. Scheme employed for elaboration of the tDNA substrate. Two chemically synthesized tDNA fragments were 5'-phosphorylated, then annealed to a 27-nucleotide DNA oligonucleotide complementary to the 3'- and 5'-terminal sequences in the 62- and 56-nucleotide fragments, respectively. The fragments were then ligated with T4 DNA ligase to afford the requisite tDNA substrate.
cleavage site to be T35 (Figs. 1 and 3), i.e. corresponding exactly to the major site of cleavage of the corresponding tRNA (Fig. 1) (10–12, 14). In addition to this unexpected finding, BLM also cleaved the tDNA at five sites (C23, G37, G37, A40, and T43) corresponding to five of the six minor cleavage sites in the tRNAHis precursor substrate. The tDNA substrate was also cleaved at several additional sites that did not correspond to sites of cleavage in the tRNAHis substrate. Interestingly, many of these sites were clustered at the junction between the acceptor stem and dihydrouridine stem-loop structures. While the basis for this selectivity is unclear, it may be noted that the majority of cleavage sites in the tRNAHis substrate also occurred in this region.

Relative Efficiencies of tRNA and tDNA Binding and Cleavage—The relative efficiencies of tRNA and tDNA cleavage by Fe(II)-BLM A2 were evaluated using the two substrates at comparable concentrations. As illustrated in Fig. 4, cleavage of the tDNA was readily detectable using 500 nM Fe(II)-BLM, i.e. at a 15:1 nucleotide:BLM ratio. Substantial cleavage of the tDNA substrate was apparent using 1.25 μM Fe(II)-BLM, and the substrate was completely consumed at higher concentrations of Fe(II)-BLM (lanes 3–8). In contrast, cleavage of tRNAHis was detectable in the presence of 1.25 μM Fe(II)-BLM, but proceeded readily only at 2.5 μM Fe(II)-BLM. In contrast with the results obtained for the tDNA, higher concentrations of Fe(II)-BLM did not produce significantly greater cleavage of the tRNAHis precursor substrate.

To assess whether the relative efficiencies of tRNA and tDNA cleavage were related to the relative affinities of Fe(II)-BLM for the two substrates, competition experiments were carried out using radiolabeled tRNA or tDNA substrates in the presence of the unlabeled substrates. As shown in Fig. 5, the cleavage of radiolabeled tRNAHis (7–8 μM nucleotide concentration) was little affected by unlabeled tDNA at any tested concentration up to 80 μM. In contrast, admixture of 80 μM unlabeled tRNAHis to the labeled tRNA dramatically diminished cleavage of the labeled substrate. Analogously, cleavage of radiolabeled tDNA...
(7–8 μM nucleotide concentration) by 1.25 μM Fe(II)-BLM was inhibited by 99 μM unlabeled tDNA, but greater inhibition of tDNA cleavage was obtained by admixture of unlabeled tRNAHis. These data argue that Fe(II)-BLM A2 is bound to tRNAHis more strongly than to the corresponding tDNA.

Effect of Mg²⁺ on tDNA and tRNA Cleavage—Previous studies have suggested that Fe(II)-BLM-mediated cleavage of some RNA substrates may be more readily inhibited by added Mg²⁺ than cleavage of B-form DNA substrates (13, 14). In order to explore the basis for this effect, the cleavage of tRNAHis and tDNA substrates was studied in the presence of varying concentrations of Mg²⁺. As shown in Table I, both substrates were cleaved more efficiently in the absence of Mg²⁺ than in its presence. However, the diminution of cleavage was comparable for both substrates. Furthermore, cleavage of both substrates was readily apparent even at 5 mm Mg²⁺ concentration. These data suggest that the ability of Mg²⁺ to inhibit cleavage of a polynucleotide substrate by Fe(II)-BLM is related primarily to the three-dimensional structure of that substrate, not to whether it is constituted from ribonucleotides or deoxynucleotides.

DISCUSSION

Earlier studies of Fe(II)-BLM-mediated RNA strand scission indicated that RNA cleavage occurred in a more highly selective fashion than DNA cleavage. Furthermore, while 5′ G-pyr 3′ sites were well represented among RNA cleavage sites, as is also true for DNA, sites of RNA cleavage were noted frequently at the junction between double- and single-stranded regions of the RNA substrate (10, 14). The last of these observations seemed to constitute a sharp distinction between the cleavage of RNA and DNA by BLM. Detailed analyses of DNA substrates amenable to cleavage by BLM indicated that normal DNA duplexes were generally good substrates, but that single-stranded regions of DNAs (28, 29) or Z-DNA (17) were not. These observations notwithstanding, it should also be noted that several studies have suggested that the recognition of DNA by BLM must also have a conformational component. For example, Hadfield and co-workers (30) found that treatment of linearized plasmid DNAs with a limited amount of BLM resulted in small numbers of breaks at discrete sites highly susceptible to cleavage. Likewise, Mirabella et al. (31) compared the cleavage of Form I and Form III pBR322 DNA. In an analogous fashion, local alteration of DNA conformation by platination (19, 20), methylation (16–18), or the introduction of bulges (21) altered the pattern of DNA cleavage substantially.

In view of the dependence of DNA cleavage patterns on the conformation of the individual substrates, it seemed likely that the observed patterns of RNA cleavage should also reflect the conformations of individual RNA substrates. Therefore, we wondered whether the differences in sites of cleavage noted for RNA versus DNA primarily reflected differences in the constituent nucleotides present in the two polymers, or else differences in conformation between the two. Accordingly, we prepared a DNA molecule having the same primary sequence as B. subtilis tRNAHis precursor (25) and studied it as a substrate for cleavage by Fe(II)-BLM. As shown in Figs. 1, 3, and 4, both the RNA and DNA were cleaved predominantly at the same site, believed to be at the junction between single- and double-stranded regions of the molecules. This observation suggested that shape, rather than sequence, may be the primary determinant of sites of oligonucleotide cleavage by Fe(II)-BLM. In fact, Huttenhofer et al. (13) found that BLM-mediated cleavage of a DNA fragment having the same sequence as the anticodon loop of tRNAHis was cleaved in the same unique position, but concluded that the result was coincidental.

The comparison of sites of BLM-mediated cleavage in the tRNAHis precursor substrate and its corresponding tDNA rests on the assumption that these two species have similar secondary and tertiary structures. Although no information is presently available concerning the three-dimensional structure of either molecule, the fact that both are cleaved predominantly at the same position by Fe(II)-BLM A2 suggests strongly that they must share structural features in common.

Additional supporting evidence for structural similarities may be inferred from studies of two related systems. Paquette et al. (32) analyzed the structures of yeast tRNAHis and its corresponding tDNA; treatment with nucleases specific for single- and double-stranded structural elements indicated that the two species adopted cloverleaf secondary structures and that the tertiary structures were also similar, although that of the tDNA was more labile. Consistent with this analysis, both E. coli tRNAHis and the corresponding tDNA having a 3′-terminal riboadenosine were found to be substrates for the cognate aminoaeryl-tRNA synthetase (33). Likewise, Kahn and Roe (34) prepared tDNA analogs of E. coli tRNAHis and tRNAAsp. Each of the tDNAs specifically inhibited activation of the corresponding tRNA by its aminoaeryl-tRNA synthetase; tDNAHis containing a 3′-terminal riboadenosine and tDNAAsp were also substrates for aminoacylation by the cognate synthetase. While the ability of BLM to recognize and cleave a variety of DNA structures has been demonstrated previously (16–21), the substrates involved have all retained essential elements of a DNA double helix. That single-stranded regions of DNA are largely refractory to BLM-mediated cleavage was supported by studies of a 168-nt single-stranded DNA fragment of the lac p-o region, which could be cleaved by BLM only in regions potentially capable of forming double-stranded structures (28). Likewise, cleavage of a single-stranded DNA corresponding to the bacteriophage G4 origin was largely limited to those nucleotides within inverted repeat sequences, i.e. capable of forming duplex structures (29). In contrast, if the secondary structures for tRNAHis and its corresponding tDNA are correctly represented in Fig. 1, then Fe(II)-BLM must cleave both molecules predominantly at a 5′ G-pyr 3′ site that is not part of a duplex structure. Numerous additional RNA cleavage sites in tRNAs and tRNA precursors would also seem to reside in regions that are nominally single-stranded (14). It seems likely that BLM-mediated cleavage of single-stranded RNA and DNA may be possible if the single-stranded regions affected are part of a higher order structure conformationally constrained in a fashion conducive to cleavage.

**Inhibition of RNA and DNA Cleavage by Mg²⁺**—Unlike B-form DNA, the cleavage of some RNAs is very susceptible to inhibition by added Mg²⁺ and these species could probably not

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**Table I**

| Mg²⁺ concentration | tDNAHis | tRNAHis |
|---------------------|---------|---------|
| 1.0 μM              | 10.4%   | 13.5%   |
| 0.25 μM             | 8.4%    | 7.2%    |
| 0.5 μM              | 4.4%    | 4.3%    |
| 2.0 μM              | 3.5%    | 1.8%    |
| 5.0 μM              | 1.9%    | 1.8%    |

* Represents % conversion of total RNA or DNA to the major cleavage product (1 gpm). Control reactions lacking Fe²⁺ or BLM gave negligible (<0.5%) conversion to product.
be cleaved under physiological conditions (13, 14). In contrast, other RNAs such as B. subtilis tRNAHis precursor and yeast 5 S ribosomal RNA were refractory to Mg2+ inhibition at physiological concentrations. The data in Table 1, which illustrate comparable cleavage of tRNAHis and tDNAHis by Fe(II)-BLM at each of four Mg2+ concentrations, suggest that the three-dimensional structure of the substrate molecule is an important determinant of the ability of Mg2+ to inhibit cleavage by BLM. This is in sharp contrast with the results of Huttenhofer et al. (13), who compared the inhibitory effects of Mg2+ on BLM-mediated cleavage of yeast tRNAphe and short DNA fragments corresponding to parts of the tRNA structure. The dramatic difference in the susceptibility of tDNAHis and B-form DNA to inhibition by Mg2+ also reinforces the earlier conclusion (13, 14) that Mg2+ inhibition must result from polynucleotide binding by the metal ion, rather than competition for the metal binding domain of BLM.

Efficiency of RNA Binding and Cleavage by Fe(II)-BLM—Previous studies of the relative affinities of BLM for RNA and DNA have employed radiolabeled tRNAHis precursor in the presence of non-identical RNA and DNA substrates (10, 11); these studies suggested that BLM is bound more tightly to B-form DNA than to tRNAHis precursor. In the present case, experiments using radiolabeled tRNAHis or tDNAHis in competition with the unlabeled substrates suggested strongly that Fe(II)-BLM is actually bound more avidly to tRNAHis than to tDNAHis. As illustrated clearly in Fig. 4, the binding results were not reflected in the relative extents of tRNAHis and tDNAHis cleavage, since cleavage of the tRNA required somewhat higher concentrations of added Fe(II)-BLM and generally resulted in conversion of no more than 18% of the tRNAHis precursor to cleavage products (Table 1).2

This apparent anomaly can be rationalized in either of two ways. One possibility is that Fe(II)-BLM binds to RNA in an orientation not conducive to the initiation of a subsequent chemical event that leads to alteration of RNA structure. Alternatively, it seems possible that the chemical alteration of RNA by Fe(II)-BLM may lead in part to one or more products that do not involve strand scission, and which would not be detectable by electrophoretic analysis.

To date, it has not been possible to define the products resulting from tRNA cleavage per se. However, analysis of the products derived from the chimeric oligonucleotides C3-rib0 CGCTAGCG and C3-ara CGCTAGCG following treatment with Fe(II)-BLM indicated that two sets of products were formed in each case. One of these resulted in strand scission with oxidative destruction of ribotara cytidine2, the products were closely analogous to those formed from DNA following abstraction of C-4' H (10). The second set of products did not involve strand scission, but subsequent treatment with dianimobenzene gave a product whose formation could be rationalized via initial abstraction of C-1' H (15). At present, it is not known whether RNA itself undergoes analogous oxidative transformation by abstraction of C-1' H. If this pathway were operative, the disparity in the extents of RNA and DNA cleavage by Fe-BLM, relative to the respective efficiencies of drug binding, could be rationalized.

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