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Binding and Cleavage Characteristics of DNA by Bleomycin-Vanadium Complex

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KEY WORDS: Bleomycin-Vanadium Complex/ DNA Cleavage/ Binding Affinity/ Action Mechanism/

INTRODUCTION

Bleomycins are a family of glycopeptide-derived antitumor antibiotics discovered by Umezawa and co-workers.1) It is well known that bleomycin activates dioxygen by the formation of unique iron complex of the β-aminoalanine-pyrimidine-β-hydroxyhistidine region to generate active species closely related to bleomycin-Fe(III)-O₂²⁻, and binds to guanine base of DNA duplex by the bithiazole-terminal amine moiety (Figure 1).2) Indeed, DNA is selectively cleaved at 5'-guanine-pyrimidine sequences such as GC and GT by bleomycin-iron-oxygen system.3) Under UV irradiation, bleomycin-cobalt(III) complex can also cut DNA strand at G-C(5' → 3') and G-T(5' → 3') sites.4) Metal ion, in particular iron-dependent DNA strand scission is believed to be responsible for antineoplastic action of bleomycin.

Vanadium is an important dietary trace element and has many biological activities.5) It has been reported that vanadium forms a complex with bleomycin and is effectively capable of cleaving DNA in the presence of hydrogen peroxide. In this study, we found that bleomycin-vanadium complex induces DNA breakage not only with hydrogen peroxide but also with dithiothreitol. Sequence-specific DNA cleavage modes between vanadium and iron complex systems of bleomycin were compared. DNase I footprinting experiment also showed that bleomycin-vanadium complex binds to GC-rich region of DNA duplex. In addition, redox mechanism of bleomycin-vanadium complex in the DNA cleaving reaction has also been discussed.

EXPERIMENTAL PROCEDURES

Materials: We used peplomycin (R=−NH(CH₂)₃NHC(CH₃)C₆H₅) as bleomycin antibiotic in this study. Highly purified peplomycin and deferoxamine were kindly provided by Nippon Kayaku and Ciba-Geigy, respectively. Restriction endonuclease FokI was a gift from Prof. M. Takanami and Dr. H. Sugisaki (Kyoto University). Deoxyribonuclease I

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Fully deionized, distilled water was used throughout the experiments, and the labwares were acid-washed and thoroughly rinsed in order to avoid contamination by foreign metal ions. The bleomycin-metal (1:1) complexes were prepared by mixing the antibiotic and metal ion at equimolar amount in Tris-HCl buffer (pH 7.5). All other chemicals used were of commercial reagent grade.

**Sequence-Specific Cleavage Analysis:** DNA cleavage activity was determined on pBR322 DNA by using 1% agarose gel electrophoresis which contained ethidium bromide (0.5 μg/ml). All samples were incubated for 20 min at 37°C. The restriction fragment of 134-base pairs was obtained by digestion of purified pIBI 24 DNA with EcoRI. After treatment by EcoRI, the 5’-end or 3’-end was labeled with ^32^P. Next, the fragments were treated with restriction endonuclease FokI. The obtained EcoRI-FokI DNA fragment (134-base pairs) was used in the sequence analysis. The DNA samples were reacted with 1 μM bleomycin-iron complex for 5 min or with 1 μM bleomycin-vanadium complex for 25 min. The samples were subjected to electrophoresis together with the Maxam-Gilbert sequencing fragments on a 10% polyacrylamide/7M urea slab gel. The autoradiograms were scanned with a laser densitometer (Pharmacia LKB Model 2222 UltraScan XL).

**DNase I Footprinting Analysis:** Each reaction mixture (final volume, 20 μl) contained 20 mM Tris-HCl buffer (pH 7.5), 5 mM CaCl₂, 10 mM MgCl₂, 1:1 bleomycin-metal complex, end-labeled 190-base pairs pBR322 DNA fragment (BamHI-SphI), and sonicated calf thymus DNA (600 pmol). After incubation for 30 min at 20°C, the sample was digested with DNase I (final conc., 1 unit/ml) for 2 min. Then, 5 μl of DNase I stop solution (250 mM NaOH-adjusted Na₂EDTA and 3 M sodium acetate) and 60 μl of cold ethanol were added to stop the DNase I’s reaction. The samples were subjected to electrophoresis together with the Maxam-Gilbert sequencing fragments on a 10% polyacrylamide/7M urea slab gel.

**Estimation of DNA Binding Affinity:** Apparent binding constant between metallobleomycin and calf thymus DNA was evaluated in 10 mM Tris-HCl buffer (pH 7.5) by a fluorescence-quenching method. Fluorescence measurements were performed with a Hitachi F-3010 spectrofluorometer.

### RESULTS AND DISCUSSION

Bleomycin antibiotics can bind several metal ions such as iron, cobalt and copper (Figure 1). For the metal coordination environment of these metal complexes, it has been established that four nitrogen atoms from the secondary amine of the β-aminoaalanine portion, the pyrimidine ring, the deprotonated peptide and the imidazole ring of β-hydroxyhistidine residue bind as basal planar ligands and a nitrogen atom from the α-amine as axial donor, and that the metal site is substantially a square-pyramidal structure with four chelate ring of 5-5-5-6 ring members. Vanadyl(IV) ion can also form a 1:1 complex with bleomycin (designated BLM) at pH 6-9 like other transition metal ions.

Figure 2 shows cleavage of replicative form of plasmid pBR322 DNA by the BLM-VO(IV) complex. When the reaction solution containing 0.5 μg DNA, 10 μM BLM-VO(IV) complex, and 20 mM Tris-HCl buffer (pH 7.5) was treated with 2 mM hydrogen...
peroxide or 1 mM dithiothreitol (designated DTT) at 37°C for 10 min, typical agarose (1%) gel electrophoretic pattern revealed distinct change from covalently closed circular (ccc) DNA to nicked and linear DNAs (Figure 2). The lanes 3 and 4 present DNA strand scission with the BLM-VO(IV)-H₂O₂ and BLM-VO(IV)-DTT systems, respectively. To avoid contamination of trace amount of other transition metal ions, especially iron, all experiments were performed with very care. Every reaction sample contains deferoxamine (0.5 μM) which specifically traps ferric ion. Here, the repeated and careful experiments established that DTT can effectively promote DNA breakage by the vanadium complex of BLM as well as hydrogen peroxide.

The nucleotide sequence-specific cleavages of the BLM-VO(IV) complex with DTT or H₂O₂ were determined by the method of Maxam-Gilbert using 10% polyacrylamide/7M urea slab gel electrophoresis. The 5'-end and 3'-end labeled fragments of pIB124 (134 base pairs) were used for these experiments (see Figures 3 and 4, respectively). The histograms of DNA cutting sites are also given in Figure 5. The results indicate that the BLM-
Figure 3. Autoradiograms for sequence analysis of pIBI 24 DNA fragment (134 base pairs) cleaved by BLM-VO(IV) complex plus hydrogen peroxide (or DTT) system. The reaction mixtures contained 20 mM Tris-HCl buffer (pH 7.5), 5'-end labeled DNA fragment, 1 μg carrier calf thymus DNA, and the following additions: lane 3, BLM-Fe(III) complex (10 μM) plus H₂O₂ (1 mM); lanes 4 and 5, BLM-VO(IV) complex (20 μM and 40 μM, respectively), H₂O₂ (1 mM), and deferoxamine (0.5 μM); lane 6, BLM-VO(IV) complex (40 μM) plus DTT (1 mM) and deferoxamine (0.5 μM); lane 7, BLM-VO(IV) complex (40 μM) and deferoxamine (0.5 μM); lane 8, vanadyl sulfate (40 μM) plus H₂O₂ and deferoxamine (0.5 μM). Lane 9 presents intact pIBI 24 DNA fragment. Lanes 1 and 2 show the Maxam-Gilbert sequencing reactions for G+A and C+T, respectively.
DNA Binding and Cleavage of Bleomycin-Vanadium Complex

Figure 4. Autoradiograms for sequence analysis of pIBI 24 DNA fragment (134 base pairs) cleaved by BLM-VO(IV) complex plus hydrogen peroxide (or DTT) system. The reaction mixtures contained 20 mM Tris-HCl buffer (pH 7.5), 3'-end labeled DNA fragment, 1 μg carrier calf thymus DNA, and the following additions: lane 4, BLM-Fe(III) complex (1 μM) plus H₂O₂ (1mM); lanes 5, BLM-VO(IV) complex (40 μM) plus H₂O₂ (1 mM) and deferoxamine (0.5 μM); lane 6, BLM-VO(IV) complex (40 μM) plus DTT (1 μM) and deferoxamine (0.5 μM). Lane 1 presents intact pIBI 24 DNA fragment. Lanes 2 and 3 show the Maxam-Gilbert sequencing reactions for G+A and C+T, respectively.
VO(IV)-DTT activation system cleaved DNA at a nucleotide adjacent to the 3’ side of guanine such as G-C and G-A (5’ → 3’) sequences. The nucleotide cleavage pattern is quite similar to that by the VO-BLM-H2O2 system and did not change in range of concentration from 10 μM to 160 μM. Furthermore, there is no significant alteration of cleavage specificity between the BLM-VO(IV) complex and the BLM-Fe(III) complex, despite large difference of their drug concentrations. Previous study suggested that the BLM-VO(IV)-H2O2 system more frequently attacks G-A site in the GGAA (5’ → 3’) sequences compared to the BLM-Fe(III)-H2O2 system. Unfortunately, we could not confirm the elevated G-A (5’ → 3’) preference might be due partly to sequence-dependent modulation of local structure around the XGAX (5’ → 3’) sequences (X = any nucleotide).

In Figure 3, the 5’-32P end-labeled DNA fragments digested by the BLM-VO(IV) complex with DTT or H2O2 migrated slightly faster than the corresponding bands for the Maxam-Gilbert chemical reactions and comigrated with the fragments cut by the BLM-iron complex. In the case using 3’-end labeled DNA (Figure 4), VO-BLM-treated fragments ran identically with the corresponding chemical reactions. These results strongly indicate that the BLM-VO(IV) complex with DTT or H2O2 produces 3’-phosphoglycolate and 5’-phosphate termini at its cleavage site.

To determine the binding site of VO-BLM, DNase I footprinting experiment was performed. Three major DNase I footprints, namely 5’-GCATCGTGCCGCGC-3’, 5’-GGCGCCACA-3’ and 5’-GTTGCGG-3’, were gradually and clearly observed as increase of
Figure 6. DNase I footprints for Co(III) and VO(IV) complexes of BLM to pBR322 DNA fragment (184 base pairs). Lanes 4 – 10 show DNase I digests containing DNA and the following additions: lane 4, none; lanes 5 and 6, 10 μM and 20 μM BLM-Co complexes, respectively; lanes 7, 8, 9 and 10, 20 μM, 40 μM, 80 μM and 160 μM BLM-VO complexes, respectively. Lanes 11 and 12 give DNA cleavage by 80 μM and 160 μM BLM-VO complexes, respectively. Lane 1 presents intact DNA alone. Lanes 2 and 3 show the Maxam-Gilbert sequencing reactions for G+A and C+T, respectively.
the concentration of the BLM-VO complex (lanes 7–10 of Figure 6). In addition, we can see several cutting sites by the BLM-VO(IV) complex itself within these footprints when high concentration of the complex was added (lane 10 of Figure 6). These cutting bands are easily discriminated from DNase I-treated ones, because migration of DNase I-digested fragment which gives rise to 3'-hydroxyl terminus is retarded as compared to that of the corresponding Maxam-Gilbert standard, while BLM-induced fragment bearing 3'-phosphoglycolate end migrates slightly ahead of the position of the sequencing standard. Each footprint, which centers the base cut by VO-BLM, spans three to four bases pairs. The BLM-Co(III) complex gave very similar footprints at 8–16 times less amount of the vanadium complex. Significant difference of DNA affinity of BLM accompanied by metal substitution is strongly supported by estimation of the DNA binding constant for these metal complexes using fluorescence quenching method (Table 1). Apparent association constant of the BLM-VO(IV) complex to calf thymus DNA \((K_a = 3.87 \times 10^4 \text{ [M}^{-1}\text{]})\) is obviously smaller than the value of the Co(III) complex \((K_a = 4.97 \times 10^5 \text{ [M}^{-1}\text{]})\). These results indicate that change of bound metal from Co(III) ion to VO(IV) ion significantly lowers DNA binding ability of metallobleomycin but has a little effect on sequence specificity for its binding and cleavage. Low DNA affinity of the BLM-VO(IV) complex might be explained by the following reasons. Firstly, decrease of positive charges from Co\(^{3+}\) (or Fe\(^{3+}\)) to VO\(^{2+}\) would weaken electrostatic interaction of metallobleomycin with sugar-phosphate backbone of DNA. Secondly, change of coordination geometry by vanadyl ion-chelation may make somewhat unfavorable configuration for DNA binding.

ESR parameters \((g_0 = 1.982, A_0 = 93.5 \text{ G})\) of the BLM-VO(IV) complex reveal metal chelation by an axial nitrogen in addition to 4 donor set. The same ligand set as Co- and Fe-BLMs is possibly utilized in the VO complex and thus coordination sphere around the vanadium atom will be saturated and distorted as found in the VO(O\(_2\))(Pic)(2H\(_2\)O) complex (Pic = pyridine-2-carboxylate). With regard to redox chemistry of the VO-BLM complex, a plausible mechanism is proposed. The reaction of the BLM-VO(IV) complex with \(\text{H}_2\text{O}_2\) may form BLM-V(V)-OOH complex and may subsequently give an electrophilic reactive BLM-V(IV)-OO• species which abstract C4'-hydrogen atom of the nucleotide adjacent to the 3'-side of guanine. The mechanism will be supported by several lines of evidences: (1) the vanadyl(IV) ESR signal gradually disappeared by addition of hydrogen peroxide and then appeared again by the presence of Na\(_2\)S\(_2\)O\(_4\), (2) the concentrated BLM-VO(IV) complex (> 100 \(\mu\text{M}\)) in aerobic condition can oxidatively cleave DNA by itself, and (3) electrophoretic behavior of DNA product treated with the BLM-VO(IV) complex in the presence of \(\text{H}_2\text{O}_2\) or DTT suggests 3',4' carbon-carbon bond scission of the deoxyribose ring initiated by abstraction of C4'-hydrogen atom as a trigger reaction. On the other hand, the reaction between the BLM-VO(IV) complex and DTT may form an active BLM-V(III) complex species. Although vanadium redox appears to be essential to the present DNA cleavage, further investigation is required to establish exact reaction mechanism.

In conclusion, the BLM-VO(IV) complex can effectively cleave DNA at a nucleotide adjacent to the 3'-side of guanine such as GC(5'→3') and GA(5'→3') sequences in the presence of reductant, DTT. Metal substitution greatly affects DNA affinity of metallobleomycin but has a little influence on its nucleotide-specific recognition and cleavage.
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DNA cleavage chemistry by the BLM-VO(IV) complex is similar to the case of the BLM-Fe complex which is according to C4'-chemistry.

REFERENCES


<p>| Table 1. Binding parameters for Co(III) and VO(IV) complexes of bleomycin(BLM) to calf thymus(C.T.) DNA |
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<th>K(M⁻¹) x 10⁴</th>
<th>n(base) x 10²</th>
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<td>BLM - C.T. DNA</td>
<td>7.92 ± 2.55 x 10²</td>
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<tr>
<td>BLM(Co) - C.T. DNA</td>
<td>4.97 ± 7.46 x 10⁴</td>
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<tr>
<td>BLM(VO) - C.T. DNA</td>
<td>3.87 ± 4.92 x 10³</td>
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*1 apparent association constant
*2 number of bases per binding site