Mutation of the N-terminal proline 9 of BLMA from *Streptomyces verticillus* abolishes the binding affinity for bleomycin

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Abstract A gene, *blmA*, from bleomycin (Bm)-producing *Streptomyces verticillus*, encodes a Bm-binding protein, designated BLMA. The expression of BLMA conferred resistance to Bm in the *Escherichia coli* host, whereas a mutant protein, designated Pro-9/Leu, with the N-terminal proline 9 residue in BLMA replaced by leucine, did not. We created a fusion protein between the maltose-binding protein (MBP) and a mutant protein Pro-9/Leu/Leu with Met-94 in Pro-9/Leu replaced by leucine. Pro-9/Leu/Leu from the fusion protein, obtained by digestion with CNBr digestion, did not inhibit DNA-cleaving and antibacterial activities of Bm. Native-polyacrylamide gel electrophoresis (PAGE) and gel filtration column chromatographic analysis showed that the molecular size of Pro-9/Leu/Leu is roughly half of that of BLMA, suggesting that the mutant protein cannot form dimeric structure. Furthermore, Far-UV circular dichroism (CD) spectrum of Pro-9/Leu/Leu was quite different from that of BLMA and similar to the spectra obtained from unordered proteins [Venyaminov, S.Y. and Vassilenko, K.S. (1994) Anal. Biochem. 222, 176–184], suggesting that the secondary structure of Pro-9/Leu/Leu is disrupted. These results indicate that the mutation abolishes not only dimer formation but also the secondary structure of BLMA, which results in the loss of its function as a Bm-resistance determinant.

Key words: Bleomycin-binding protein; Bleomycin resistance; CD spectrum; Site-directed mutagenesis; *Streptomyces verticillus*

1. Introduction

Since bleomycin (Bm) and its related antibiotics cause nucleotide sequence-specific DNA cleavage and inhibit the growth of both bacterial and mammalian cells [1], they are used to treat human malignancies. The DNA-cleaving process seems to be mediated via a Fe^{2+} chelate of Bm that is capable of generating a reduced form of oxygen in proximity to susceptible site(s) in the DNA [2]. To understand how Bm-producing micro-organism are protected from the lethal effect of their own product, we have cloned genes encoding Bm-resistance determinants from Bm-producing *Streptomyces verticillus* ATCC15003 [3]. We have found that the two independent cloned genes, designated *blmA* and *blmB*, encode a Bm-binding protein and a Bm *N*-acetyltransferase, respectively [3,4]. The *blmA* consists of a 366 bp open reading frame and encodes an acidic protein consisting of 122 amino acids with a calculated molecular size of 13179 daltons. We have overproduced and physico-chemically characterized the *blmA* gene product, designated BLMA [5]. Moreover, we have crystallized BLMA [6] and determined in detail the X-ray crystal structure at a high resolution of 1.5 Å (submitted), showing that BLMA forms a dimeric structure through N-terminal arm-exchange. The resulting concavity and groove may contribute to trapping two Bm molecules. That is, the formation of dimeric structure may be necessary to retain an affinity for Bm.

Proline appears to play a key role in the β-strand of the hinge peptide that in bovine seminal ribonuclease A [7] and tumor necrosis factor [8] links the exchangeable N-terminal segment to its subunit body. Therefore, we hypothesized that the N-terminal proline 9 residue in BLMA may play a role as a hinge to form its dimeric structure. In this study we created mutant BLMAs, with Pro-9 of BLMA replaced by one of several amino acids and examined whether the mutant protein loses function as a Bm-resistance determinant.

2. Materials and methods

2.1. Bacterial strains and plasmid

E. coli TB1 and plasmid pMAL-c2 [9] were used for the expression of *blmA* or its mutant gene fused to a maltose-binding protein (MBP) gene, *malE*. *E. coli* strains DH5α and BMH71-18 were used for site-directed mutagenesis.

2.2. Site-directed mutagenesis and DNA sequencing

The gene *blmA*, contained in the *Eco*RI-*Bam*HI fragment of plasmid pKM10 [6], was subcloned into the pUC18 to generate pUC-*blmA*. The chimeric plasmid was used for site-directed mutagenesis using transformer site-directed mutagenesis kit (Clonetech, USA). To confirm the mutation in *blmA*, DNA sequence was determined by the dideoxy chain termination method [10] with the ALF-II automatic sequencer (Pharmacia Biotech, Sweden) using the AutoRead Sequencing Kit (Pharmacia Biotech).

2.3. Assay of Bm resistance

E. coli HB101 carrying *blmA* or its mutant gene, inserted into plasmid pKK*trp* [5], was cultured in M9-casamino acid medium [11] supplemented with Bm (100 μg/ml) and 3-indoleacrylic acid (25 μg/ml) as an inducer of the *trp* promoter. *E. coli* TB1 harboring *blmA* or its mutant gene, fused to *malE*, was cultured in Luria-Bertani (LB) medium [11] supplemented with Bm (100 μg/ml). The induction of the *tac*-controlled gene expression was done by addition of 1 mM isopropyl-β-thiogalactopyranoside (IPTG).

2.4. Western blotting

E. coli HB101 harboring *blmA* or the mutant gene was washed twice with buffer A (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 2 mM 2-mercaptoethanol) and suspended in the same buffer. The washed cells were sonicated (Bioruptor, Cosmo Bio, Japan) and centrifuged at $10\,000\times g$ for 20 min. The protein concentration of the resulting supernatant fluid was determined according to the method

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Abbreviations: Bm, bleomycin; CD, circular dichroism; CNBr, cyanogen bromide; MBP, maltose-binding protein; IAA, 3-indoleacrylic acid; IPTG, isopropyl-β-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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[12], and subjected to Tricine-SDS-PAGE [13]. The proteins in the gel were electrophoretically transblotted onto a nitrocellulose membrane (Hybond-C super, Amersham) and detected with Elite ABC kit (Vectastain, USA) using an anti-BLMA monoclonal antibody described previously [5].

2.5. CD spectrum

After BLMA or Pro-9/Leu/Leu was dissolved in 1 mM Tris-HCl (pH 7.5) at a concentration of 5 μM, its CD spectrum was measured using a spectrophotometer (Model J-720, Jasco, Japan) at the range between 250 and 185 nm.

2.6. Native-PAGE

Native-PAGE of the protein was performed according to [14]. The proteins were stained with Coomassie brilliant blue R-250.

3. Results and discussion

3.1. Bm resistance of E. coli harboring the mutant blmA

The monomeric form of BLMA consists of three α-helices and two anti-parallel β-strands, in addition to two short β-strands (β1 and β2) twisting at its N-terminal proline 9, designated Pro-9 (submitted). The dimeric form, generated by the alternate arm-exchange of the monomeric BLMA molecule, results in a large concavity and a long groove which may trap two molecules of Bm (Fig. 1). The X-ray crystallography suggested that Pro-9 plays a role as a hinge to support the dimeric structure. To confirm this hypothesis, we generated several mutant proteins with Pro-9 in BLMA replaced by some amino acids using the site-directed mutagenesis technique. A codon CCC for Pro was replaced by GGC, CAG, CTG, TAC, or GCT which is corresponded to Gly, Gln, Leu, Tyr, and Ala, respectively, according to the codon usage of *E. coli*. Each mutant *blmA*, confirmed by the nucleotide sequence determination, was placed under control of the *trp* promoter in the plasmid pKK*trp* [5] and introduced into *E. coli* HB101. *E. coli* producing BLMA or Pro-9/Ala was resistant to 100 μg/ml of Bm, but the same hosts producing other mutant proteins were not (Fig. 2A). The minimum inhibitory concentration (MIC) of Bm against *E. coli* carrying each mutant *blmA* except Pro-9/Ala-encoding gene was the same as that of the host cell. Although *E. coli* carrying *blmA* overproduced BLMA, the same cells transformed with the mutant *blmA*, even the Pro-9/Ala-encoding gene, produced at a lower level, as detected by Western blot analysis (Fig. 2B).

Fig. 1. A ribbon model of the BLMA dimer. Two monomers were indicated by red and green color. The dimeric structure was formed through the alternate arm-exchange. The N-terminal proline 9 indicated by an arrow is positioned at the hinge between two monomers.

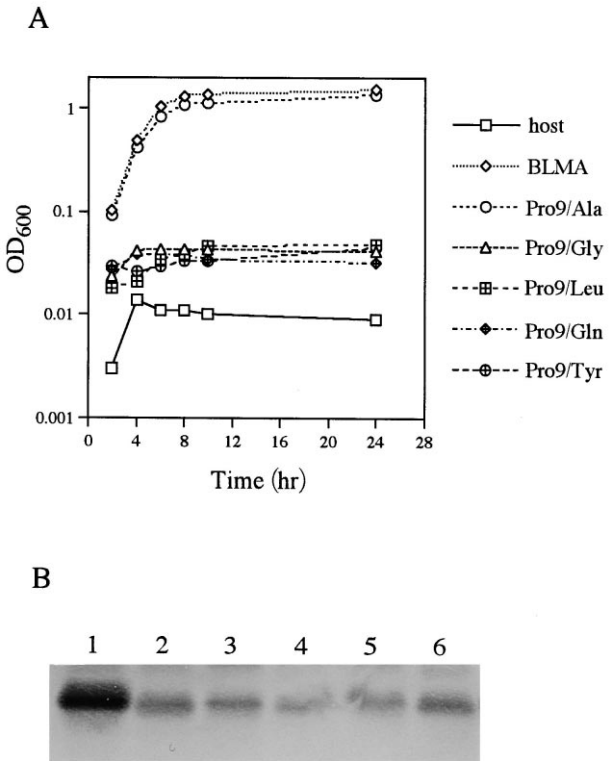

Fig. 2. Bm-resistance profile of *E. coli* harboring each mutant *blmA* expressed under control of the *trp* promoter (A) and production of the corresponding proteins (B). A: *E. coli* harboring wild-type and each mutant *blmA* under control of the *trp* promoter were cultured in M9-casamino acid medium supplemented with Bm (bleomycin A_2 sulfate, 100 μg/ml) and IAA (25 μg/ml). The cell growth was monitored by the absorbance at 600 nm at the given times. B: Western blot analysis of the proteins produced by *E. coli* harboring wild-type or each mutant *blmA* under control of the *trp* promoter. lanes: 1, wild-type; 2, Pro-9/Gly; 3, Pro-9/Ala; 4, Pro-9/Gln; 5, Pro-9/Leu; 6, Pro-9/Tyr.

Fig. 3A shows that each mutant *blmA*, replacing Pro-9 in BLMA by Gly, Gln, Leu, Tyr, or Ala, was overexpressed at the same level when fused to *malE* as a reporter gene. The Pro-9/Ala-producing *E. coli* exhibited the same Bm resistance as in the case of the direct expression manner under the control of *trp* promoter (Fig. 3B), whereas Pro-9/Gly, Pro-9/Gln and Pro-9/Tyr mutant-producing cells showed moderate resistance to Bm. Although the solubility of BLMA derivatives might affect the Bm-resistance phenotype, all mutant BLMAs were similarly soluble. These suggest that the binding affinity of these mutant proteins for Bm might be lower than that of the wild-type. Interestingly, the growth of *E. coli* expressing Pro-9/Leu was completely inhibited by 100 μg/ml of Bm, indicating that this protein had lost function as a Bm-resistance determinant.

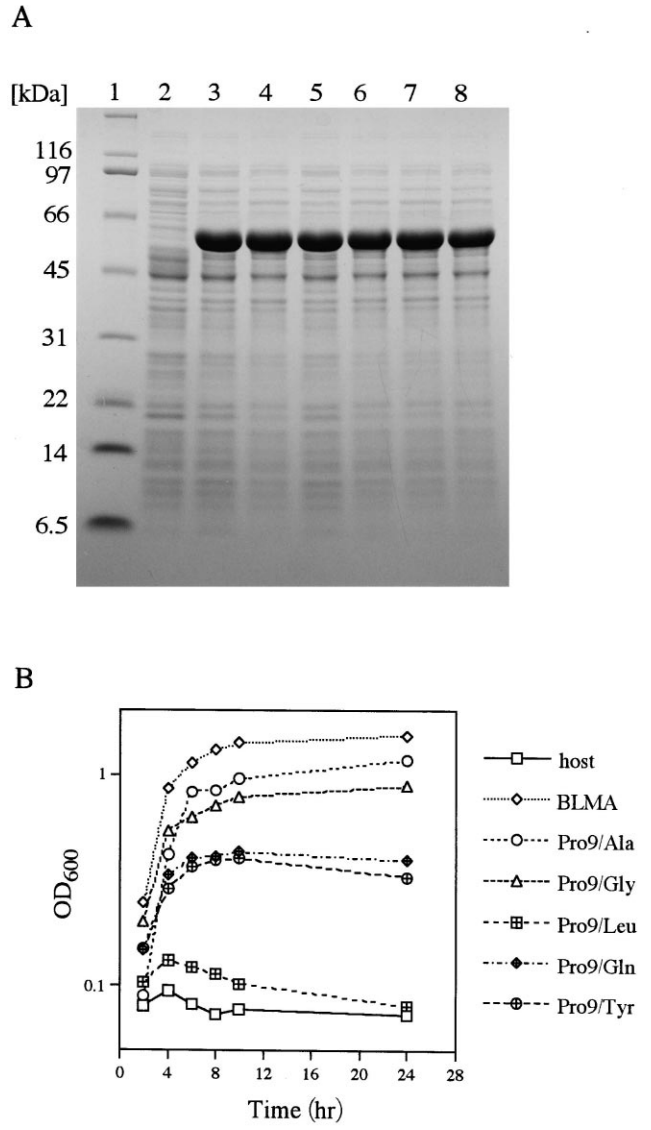

Fig. 3. Overproduction of mutant BLMAs fused with maltose-binding protein and Bm-resistance profile of *E. coli* producing the fusion proteins. A: Tricine-SDS-PAGE of the proteins from *E. coli* harboring wild-type and each mutant *blmA* fused with *malE* gene. lanes: 1, molecular size marker; 2, host; 3, wild-type; 4, Pro-9/Gly; 5, Pro-9/Ala; 6, Pro-9/Gln; 7, Pro-9/Leu; 8, Pro-9/Tyr. B: *E. coli* harboring wild-type or each mutant *blmA* fused with *malE* were grown in LB medium supplemented with Bm (100 μg/ml) and IPTG (1 mM). The cell growth was monitored by the absorbance at 600 nm at the given times.

3.2. Physico-chemical properties of the Pro-9/Leu protein

A fusion protein composed of the MBP and Pro-9/Leu, contains a recognition site for cleavage by a blood coagulation factor Xa protease. But the fusion protein was unexpectedly not cleaved into the two domains. Since BLMA has methionine residues at 1st and 94th position from the N-terminus, we designed a mutant fusion protein between the MBP and Pro-9/Leu cleaved by digestion with cyanogen bromide (CNBr): that is, the double mutant protein, designated Pro-9/Leu/Leu, replacing Met-94 in Pro-9/Leu by Leu was generated by site-directed mutagenesis and fused to MBP. The Pro-9/Leu/Leu-encoding gene fused to *malE* was overexpressed in *E. coli* TB1. We preliminarily confirmed that *E. coli* transformed with a mutant protein-encoding gene, replacing Met-94 in BLMA by Leu, overproduced the protein under control of the *trp* promoter and exhibited resistance to Bm at the same level as the cell transformed with *blmA*. Since the MBP and Pro-9/Leu/Leu domains in the fusion protein have six Met and one N-terminal Met, respectively, Pro-9/Leu/Leu can be cut out by digestion of the fusion protein with CNBr. The digests were dialyzed against 20 mM Tris-HCl (pH 8.0), subjected to a ResourceQ ion-exchange HPLC column (Pharmacia Biotech) and eluted with 20 mM Tris-HCl (pH 8.0) containing 0–0.5 M NaCl to purify to homogeneity (Fig. 4). Although, Pro-9/Leu/Leu in the CNBr-treated protein mixture (Fig. 4, lane 4) migrated more slowly than the purified protein (Fig. 4, lane 5), Western blot analysis confirmed their identity. The N-terminal amino acid sequence of Pro-9/Leu/Leu was determined and confirmed using Model A490 automatic protein sequencer (PE Applied Biosystems, USA).

The Pro-9/Leu/Leu did not inhibit the DNA-cleaving and antibacterial activities of Bm (data not shown), suggesting that the mutant protein may have lost the binding ability for Bm. The lack of the Bm-binding ability of Pro-9/Leu/Leu was not due to the denaturation of the protein, because fully active BLMA was purified from the fusion protein by the same method as used for Pro-9/Leu/Leu. Although Tricine-SDS-PAGE analysis showed that BLMA and Pro-9/Leu/Leu migrate equally, native-PAGE analysis revealed that Pro-9/Leu/Leu migrated more quickly on the gel than that of BLMA (data not shown). The molecular size of BLMA, deduced from the nucleotide sequence of *blmA*, is 13 197 daltons. A gel filtration analysis by *f*KTA explorer system (Amersham Pharmacia Biotech) using a HiLoad 26/60 Superdex 75 column showed that the molecular size of BLMA is approximately 30 kDa, apparently forming dimeric structure, whereas that of Pro-9/Leu/Leu is approximately 20 kDa. These results suggest that the mutant protein can not form the dimeric structure. Fig. 5 shows CD spectra of Pro-9/Leu/Leu and BLMA. The spectrum of BLMA showed two nega-

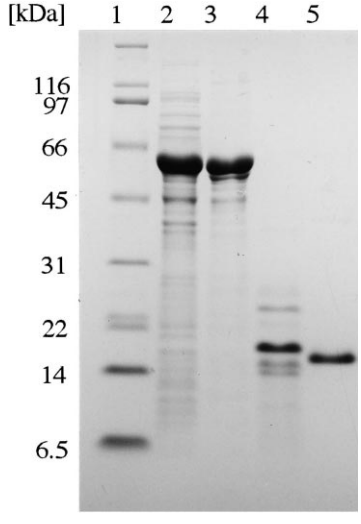

Fig. 4. Purification of Pro-9/Leu/Leu. The proteins at several purification steps of Pro-9/Leu/Leu were analyzed by Tricine-SDS-PAGE. Lanes: 1, molecular size marker; 2, the cell extracts from *E. coli* harboring the double mutant *blmA* (Pro-9/Leu/Leu) fused with *malE*; 3, the fusion protein; 4, the fusion protein cleaved with CNBr; 5, the purified Pro-9/Leu/Leu.

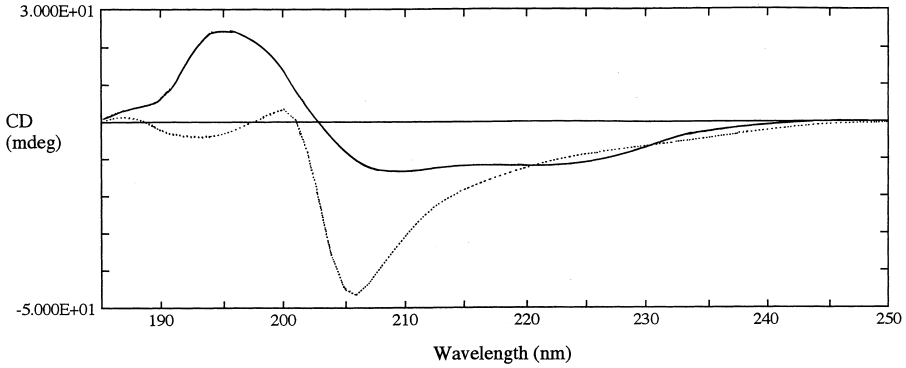

Fig. 5. CD spectra of BLMA and Pro-9/Leu/Leu. CD spectra of BLMA and Pro-9/Leu/Leu (5 μM) were recorded in 1 mM Tris-HCl (pH 7.5). Solid and dotted lines indicate BLMA and Pro-9/Leu/Leu, respectively.

tive bands at 222 and 208–210 nm and one positive band near 195 nm. This is typical to the spectra of α+β- or α/β-proteins [15]. On the other hand, the spectrum of Pro-9/Leu/Leu was quite different from that of BLMA. A strong negative band near 205 nm characterizing the mutant protein was similar to those of unordered proteins [15], suggesting that the secondary structure of Pro-9/Leu/Leu may be disrupted. This result indicates that the replacement of Pro-9 by Leu in BLMA may affect not only the dimeric formation but also the folding of the protein.

The crystal structure of BLMA forms dimeric structure through an alternate arm-exchange composing of the N-terminal eight amino acid residues in BLMA monomeric protein (submitted). Moreover, the N-terminal Pro-9 may play a role as a hinge to support the dimeric structure. The present study shows that the replacement of Pro-9 in BLMA by Leu abolishes the binding affinity for Bm due to the disruption of the dimeric formation. Since the crystal structure of BLMA predicts that small amino acids, such as Ala and Ser, can fit into the space of Pro-9, it is easy to understand how Pro-9/Ala can retain an affinity for Bm, but Pro-9/Leu does not.

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