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Molecular Domain Organization of BldD, an Essential Transcriptional Regulator for Developmental Process of *Streptomyces coelicolor* A3(2)

Chang-Jin Lee,[1†] Hyung-Sik Won,[2†] Jeong-Mok Kim,[1] Bong-Jin Lee,[3*] and Sa-Ouk Kang[1*]

[1]*Laboratory of Biophysics, School of Biological Sciences, and Institute of Microbiology, Seoul National University, Seoul 151-742, Republic of Korea*

[2]*Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju 380-701, Republic of Korea*

[3]*Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea*

ABSTRACT **A homodimeric protein, BldD is a key regulator for developmental process of *Streptomyces coelicolor* and the *bldD* mutant exhibits severely pleiotropic defects in the antibiotic production and morphological differentiation of the bacterium. In the present work, we approached domain organization of BldD, to structurally and functionally characterize the protein as a DNA-binding protein. We first observed a proteolytic cleavage of BldD by the cytoplasmic extracts of *S. coelicolor*, which was highly dependent on the developmental stage of the bacterium. The resulting fragment of BldD was identified by mass spectrometry as the N-terminal domain resistant to the proteolysis. Recombinant proteins corresponding to the intact BldD, the N-terminal domain (residues 1–79) and the rest part (C-terminal domain; residues 80–167) were used for comparative analyses by several spectroscopic, thermodynamic, and biochemical experiments, respectively. The results of circular dichroism and nuclear magnetic resonance spectroscopies certified each of the two determined domains could be regarded as an individual folding unit possessing an independent thermodynamic cooperativity. Structural interaction between the two domains was little observed in the DNA-free and DNA-bound states. Strikingly, it was revealed by gel permeation chromatography, chemical crosslink, gel mobility shift, and NMR-monitored DNA-binding experiments, that only the N-terminal domain is responsible for the dimerization as well as DNA-binding of BldD. Detailed inspection of the present results suggests that BldD function in a unique and complicated mode to totally regulate the diverse developmental stages of *S. coelicolor*. Proteins 2007;68:344–352.**

Key words: *bld* mutant; morphological differentiation; transcriptional regulator; structural domain organization; DNA-binding domain; dimerizing domain

INTRODUCTION

The gram-positive, spore-forming filamentous bacterial genus *Streptomyces* is a model system for the study of bacterial differentiation and secondary metabolite production. *Streptomyces coelicolor*, a representative organism of the genus, undergoes extensive colonial differentiation that involves the development of spore-forming, multicellular aerial hyphae on a vegetative substrate mycelium.[1] This morphological differentiation is one of the most notable characteristics of the species, since it is critically related with the carbon metabolism[2] and the synthesis of secondary metabolites, such as antibiotics and pigments.[3] In recent decades, a number of mutants that are defective in the aerial mycelium formation from substrate hyphae, namely bld mutants, have been isolated. Among them, the *bldD* mutant is capable of rescuing the developmental defects of the other bld mutants by extracellular complementation.[4,5] Based on this finding, the *bldD* has been suggested to be placed at the bottom of the extracellular signaling cascade, which controls the onset of metabolisms including the colonial differentiation, the production

Abbreviations: A_{600}, absorbance at 600 nm; CD, circular dichroism; DTT, dithiothreitol; HSQC, heteronuclear single quantum coherence; HTH, helix-turn-helix; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; T_d, half-denaturing temperature.

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†Chang-Jin Lee and Hyung-Sik Won contributed equally to this work.

*Correspondence to: Sa-Ouk Kang, Laboratory of Biophysics, School of Biological Sciences, and Institute of Microbiology, Seoul National University, Seoul 151-742, Republic of Korea. E-mail: kangsaou@snu.ac.kr or Bong-Jin Lee, Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea. E-mail: lbj@nmr.snu.ac.kr

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of secondary metabolites, and the regulation of carbon utilization in *S. coelicolor*.[2,6]

The protein BldD is a small DNA-binding protein with 167 amino acids long and occurs in solution predominantly as a homodimer.[7,8] BldD has been suggested to bind to the promoter regions of several sigma factors, including the ECF σ^{BldN}, the sporulation specific σ^{WhiG}, and the stress response σ^{SigH}, which are critically involved in the developmental processes and environmental responses of *S. coelicolor*.[9] Since the transcript levels of these genes in the *bldD* mutant are upregulated in comparison with those of the wild type, BldD has been suggested to act as a repressor of key genes involved in the morphological differentiation.[9,10] However, these genotypic results conflicts with the morphological observation of *bldD* null mutant that suggests an activator-type role of BldD: that is, *bldD* null mutant displays a 'bald' phenotype with the loss of aerial hyphae.[8] It has been also noted that BldD, like other activator-type regulators, binds to its own promoter region.[5,7,8] However, another possibility of dual function cannot be excluded, as observed for some transcriptional regulators, which function as both the transcriptional activator and repressor, dependently on target genes. For example, cyclic AMP receptor protein, a representative transcriptional activator in *Escherichia coli*, roles as a repressor of some target genes as well as its own transcription.[11,12] To describe the ambiguous role of BldD in the morphological differentiation of *S. coelicolor*, two transcriptional regulators AbrB and SinR in *Bacillus subtilis* serve as well-characterized examples that exhibit similar characteristics of BldD. The former controls the cellular transition states, including sporulation, antibiotic synthesis, and catabolite repression, by repressing its own expression and by activating other target genes.[13,14] In contrast, the SinR acts as a developmental switch, by repressing the stage II sporulation genes and by positively regulating several genes for competence and motility.[15] In particular, it is noteworthy that BldD and SinR show a statistically significant sequence similarity, belonging to the HTH-3 family of DNA-binding domains[10] From this close resemblance of BldD with SinR, it can be also speculated that BldD might play a role of developmental switch that acts as a developmental repressor during vegetative growth and then functions as an activator for aerial differentiation. The repression of SinR is overcome through the interaction with a cognate partner, SinI, which disrupts the SinR oligomeric state by forming a SinI-SinR heterodimer.[16] Therefore, to check a likely role of BldD as a developmental switch in *S. coelicolor*, it would be very worthy of investigation whether the BldD undergoes development-dependent modifications at the molecular level. Thus, the developmental stage-dependent molecular modification of BldD was examined in the present work.

Another requirement to assess the exact function of BldD is structural information of the protein. We have preferentially attempted the three-dimensional structure determination of BldD by X-ray crystallography. Unexpectedly, the single crystal developed from the concentrated recombinant BldD lacked the C-terminal half, probably due to spontaneous proteolytic degradation during the crystallization process. The structure of the crystallizing N-terminal half, most recently solved at 1.8 Å resolution,[17,18] revealed a compact and globular domain fold highly resembling that of the DNA-binding domain of lambda repressor.[19–22] However, the structural information of the N-terminal domain of BldD lacking C-terminal half were insufficient to understand precisely how the BldD regulate genes involved in developmental processes and why the *bldD* null mutant displays defects in morphological differentiation. Parallel to the crystallographic study, in this paper, spectroscopic and biochemical researches have been further forwarded to structurally identify the molecular domain organization and to characterize the functional domain units of BldD in solution.

MATERIALS AND METHODS

Mycelia Collection and DNA Constructs

For the collection of mycelia at different cell stages, spores of *S. coelicolor* A3(2) were inoculated onto the sterile cellophane disks on R2YE[19] agar surface at 30°C. To produce recombinant proteins, DNA fragments encoding the following target proteins were amplified by PCR with a genomic DNA of *S. coelicolor* A3(2), as a template, using the following three pairs of oligonucleotide primers (*Nde*I and *BamH*I restriction sites are underlined): *bldD*-F (5′-CATATGTCCAGCGAATACGCCAAAC-3′) with *bldD*-R (5′-GGATCCTCAGAGCTCGTCGTGGGAC-3′) for the full-size BldD (residues 1–167), *bldD*-F with *bldDn*-R (5′-GGATCCTCAGGCGGCGCCGCCCGGGG-3′) for the N-terminal domain of BldD (residues 1–79), and *bldDc*-F (5′-CATATGGAGCCGCCGCCGAAGCTG-3′) and *bldD*-R for the C-terminal domain of BldD (residues 80–167). The PCR products were digested with *Nde*I and *BamH*I and then ligated into the pET-15b (Novagen) vector, to produce N-terminally 6-histidine tagged proteins (H_6BldD, H_6BldDN, and H_6BldDC). The resultants, pET-BldD, pET-BldDN, and pET-BldDC were verified by DNA sequencing and transformed into *E. coli* BL21-AI™ (Invitrogen) strain.

Preparation of Proteins and Oligonucleotides

E. coli BL21-AI™ harboring the DNA constructs (pET-BldD, pET-BldDN, and pET-BldDC) were grown in LB media[23] containing 50 μg/mL of ampicillin (Duchefa biochemie) and 5 μg/mL of tetracycline (Sigma) at 37°C. When necessary, the cells were grown in M9[24] media containing ^{15}N-NH_4Cl, as a sole nitrogen source, to produce the isotope-enriched proteins. When A_{600} of the cell growth reached about 0.5, expression of the proteins was induced by adding IPTG (Sigma) and L-arabinose (Sigma) at a final concentration of 1 m*M* and 0.2%, respectively, and the induction was prolonged for 15 h at

22°C. Cells were harvested by centrifugation and disrupted by sonication. The cell debris was removed by centrifugation and the supernatant was applied to a nickel-affinity column (Novagen) chromatography for purification. To cleave the N-terminally tagged histidines, the purified solution was concentrated by ultrafiltration (Amicon) and buffer-exchanged by passing a PD-10 column (GE Healthcare) preequilibrated with thrombin cleavage buffer (20 m*M* Tris-HCl containing 150 m*M* NaCl and 2.5 m*M* $CaCl_2$, at pH 8.4). Then, thrombin (Novagen) was mixed and incubated at 22°C for 18 h. Final purification was achieved by removal of thrombin and other impurities, using gel-permeation chromatography (HiLoad 16/60 Superdex^{TM} 75, Pharmacia).

FDP, a 27-bp DNA fragment corresponding to the −27 to −1 region of the *bldD* promoter,[9] was prepared by annealing equimolar amounts of complementary oligonucleotides FDP-1 (5′-AGCAGAGTAACGCTGCGTAACCTCACA-3′) and FDP-2 (5′-TGTGAGGTTACGCAGCGTTACTCTGCT-3′). The annealing mixture was heated to 98°C for 5 min, and then cooled down to 37°C at 0.04°C/s rate in an annealing buffer (20 m*M* Tris-HCl containing 10 m*M* $MgCl_2$ and 50 m*M* NaCl, at pH 7.5).

Growth Stage-Dependent Reaction and Protein Analysis

Ten microgram of purified BldD was incubated with 10 ng of crude extracts of *S. coelicolor* at different growth stage, for 30 min in a reaction buffer (10 m*M* Tris-HCl containing 2 m*M* DTT and 75 m*M* NaCl, at pH 7.5) at 30°C. Immediately after a rapid cooling, the reactants were resolved on a denaturing 15% Tris-tricine polyacrylamide gel electrophoresis. For peptide mass fingerprinting, the excised gel pieces corresponding to the specific BldD fragments were washed with 25 m*M* ammonium bicarbonate (Sigma) in 50% acetonitrile (Fisher scientific), followed by a rehydration with 25 m*M* ammonium carbonate (pH 8.0) containing 1 U of trypsin (Promega). After incubating at 22°C for 12 h, the protein digests were extracted in 5% trifluoroacetic acid (Sigma)/50% acetonitrile, and then analyzed by using a Voyager-DE^{TM} STR Biospectrometry MALDI-TOF system (Applied Biosystems Inc.) operated in a reflector mode. The masses of peptides, internally calibrated with a trypsin peak, were analyzed at Mascot search engine (http://www.matrixscience.com), using the primary sequence database of *S. coelicolor* A3(2). Prediction of secondary structure and tertiary fold of BldD was performed by searching the database, http://www.compbio.dundee.ac.uk/∼www-jpred, from its primary sequence.

Gel Permeation Chromatography and Chemical Crosslinking

Purified proteins and LMW molecular mass standards (Amersham Biosciences) were applied on a HiLoad 16/60 Superdex^{TM} 75 (Pharmacia) gel permeation column equilibrated with a 20 m*M* sodium phosphate buffer (pH 6.8) containing 150 m*M* NaCl. Elution profiles for each protein were compared with the profiles of LMW molecular mass standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen *A* (25 kDa), and ribonuclease *A* (13.7 kDa). The standard curve was generated by plotting logarithm of molecular mass against K_{av}, where $K_{av} = (V_e - V_o)/(V_t - V_o)$: V_e, elution volume; V_o, void volume; V_t, total bed volume.

For chemical crosslinking, 20 μ*M* of purified proteins were incubated with 0.05% of glutaraldehyde (Sigma) solution in a 20 m*M* Tris-HCl (pH 7.5) for 30 min at 20°C. The crosslinked products were then separated on a denaturing 15% Tris-tricine polyacrylamide gel electrophoresis.

Gel Mobility Shift Assay

Gel mobility shift assay was performed using the FDP probe labeled with fluorescein at the 5′-end position. The fluorescein-labeled probe (50 ng) was incubated with 4 μ*M* of proteins at 30°C for 15 min in a binding buffer (10 m*M* Tris-HCl containing 150 m*M* NaCl, 2 m*M* DTT, 500 ng of poly-d(I-C) (Roche), and 10% glycerol, at pH 7.8). The reactants were resolved on a nondenaturing 10% polyacrylamide gel, and then the gel was photographed by using a LAS3000 (FUJIFILM) pictography system.

Circular Dichroism and Nuclear Magnetic Resonance Spectroscopy

All of the CD experiments were performed on a JASCO J-715 spectropolarimeter equipped with a temperature-controlling unit, using a 0.2 cm path-length cell, with a 1 nm bandwidth and 4 s response time. The standard far-UV CD spectra of 14 μ*M* proteins dissolved in a standard buffer (40 m*M* sodium phosphate buffer containing 150 m*M* NaCl, at pH 6.8) were collected at 20°C with a scan speed of 50 nm/min and 0.2 nm step resolution. Individual three scans taken from 255 to 195 nm were added and averaged, followed by subtraction of the solvent CD signal. Thermal denaturation was monitored by measuring the CD signals at 222 nm with increasing temperature from 20 to 90°C at 60°C/h rate. The CD signal was recorded at every 0.1°C and the half-denaturing temperature (T_d) was determined as described previously.[25] When the temperature reached 90°C, the standard far-UV spectra were measured to monitor the thermally denatured conformation. Then, the samples were quickly cooled down to 20°C for renaturation, followed by the additional measurements of the standard far-UV CD spectra at 20°C to estimate the reversibility of the thermal denaturation.

For NMR, the conventional 2D-[^{1}H-^{15}N]HSQC spectra[19,26] of 1 m*M* [*u*-^{15}N]proteins dissolved in the standard buffer containing 7% D_2O, in the presence and in the absence of 1 m*M* FDP DNA fragments, respectively, were obtained at 313 K on a Bruker DRX 500 spectrometer. All of the spectra were processed and analyzed using the NMRPipe/NMRDraw software[27] and the NMRView program.[28]

RESULTS AND DISCUSSION

BldD Undergoes Developmental Stage-dependent Degradation

Although the transcription of the target genes of BldD, which are involved in the morphological development of *S. coelicolor*, is upregulated in *bldD* mutant,[9,10] the *bldD* null mutant is arrested at the predevelopmental stage.[8,18] These conflicts lead to a hypothesis that BldD may act as a developmental switch that represses the known target genes at the vegetative growth stage and activates another target genes after an unknown structural modification and/or the binding of an unidentified effector, which responds to a differentiation signal. The above proposed mechanism for the pleiotropic function of BldD could be supported by investigating whether the protein undergoes a post-translational modification in response to signals at a specific developmental stage. Thus, we initially intended to detect BldD on the Western blots of the cytoplasmic proteins from different developmental stages of *S. coelicolor*, by using the anti-BldD antibody. Unfortunately, it could not be clearly detected even with over 100 μg of cytoplasmic proteins (data not shown). As an alternative approach, we incubated excess amount of purified H_6BldD with the cytoplasmic fractions extracted from the mycelia at different developmental stages, respectively. As shown in Figure 1, the H_6BldD was fragmented in a specific manner by the cytoplasmic fractions after 30-min incubation, depending on the developmental stage. When the extract of substrate hyphae (before 36 h) was incubated, the majority of H_6BldD was just slightly fragmented, resulting in the $BldD_{Deg1}$ band. This fragmentation can be referred to be cleaved at the tagged site of N-terminal histidines of H_6BldD, which was verified by a Western blot analysis using antihistidine antibody (data not shown). In contrast, the extracts from the mycelia at the aerial hyphae and/or the spore-forming phases digested significantly the majority of H_6BldD, resulting in a smaller fragment (ca. 8.5 kDa) than $BldD_{Deg1}$, which is named $BldD_{Deg2}$. Thus, it would be suggested that the development of *S. coelicolor* may be modulated by the phase-specific and post-translational modification of BldD, such as proteolytic cleavage. The proteolysis-resistant fragment ($BldD_{Deg2}$) was observed to be maintained until the spore-forming phases, indicating it would be engaged in the late developmental processes of *S. coelicolor*. In the peptide fingerprinting analysis for $BldD_{Deg2}$, all masses of the trypsin-digested peptides of $BldD_{Deg2}$ were matched completely with those of an N-terminal region of BldD (underlined in Fig. 2). Consequently, it can be suggested that the C-terminal part of BldD may be degraded at the time of aerial hyphae formation, while the remaining N-terminal part (proteolysis-resistant N-terminal domain) would be solely play leading roles for the morphological differentiation at the late stage of life cycle.

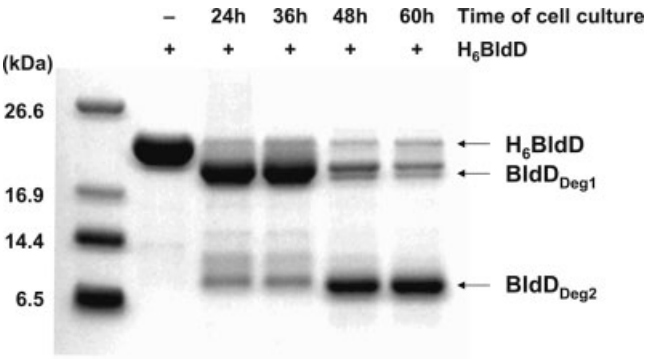

Fig. 1. Developmental stage-dependent degradation pattern of BldD. Ten microgram of His_6-tagged recombinant BldD was incubated for 30 min at 30°C with 10 ng of cytoplasmic extracts from *S. coelicolor* cells cultured for different time periods (24, 36, 48, and 60 h, respectively) after inoculation onto R2YE agar plate. The reactions and the polypeptide molecular weight standards (left lane) were then resolved on 15% Tris-tricine gel electrophoresis.

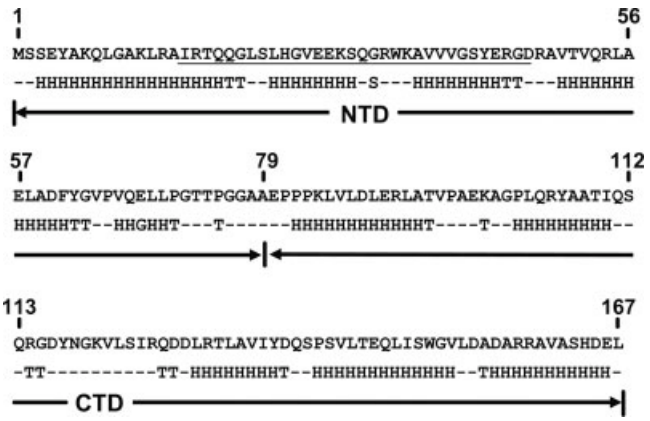

Fig. 2. Prediction of secondary structure and domain composition of BldD. A web-based database search was employed for the prediction (http://www.compbio.dundee.ac.uk/~www-jpred/). The abbreviations H, G, T, and S represent an α-helix, a 3_{10}-helix, a turn, and a strand structure element, respectively. The underlined sequence matched with the peptide fingerprinting results of $BldD_{Deg2}$. Each region indicated by arrows designates the constructed two domains, NTD and CTD.

BldD Is Composed of Two Structural Domains Stably Well-folded

Regarding the domain composition of BldD, the approximately 79 residues at the N-terminal region was judged as the N-terminal domain of BldD based on the predicted secondary structure of BldD (Fig. 1) and the molecular mass of $BldD_{Deg2}$ which was mutually estimated by the gel electrophoresis and MALDI-TOF MS spectrometry. Accordingly, the other part (residues 80–167) was tentatively assigned to the C-terminal domain of BldD. To make sure this provisional determination of BldD domains, recombinant proteins corresponding to each domain were constructed. The two constructs and the intact BldD were successfully overproduced, purified, and then applied to the far-UV CD and the [^{1}H-^{15}N]HSQC NMR experiments, to evaluate their structural completeness and characteristics. The results

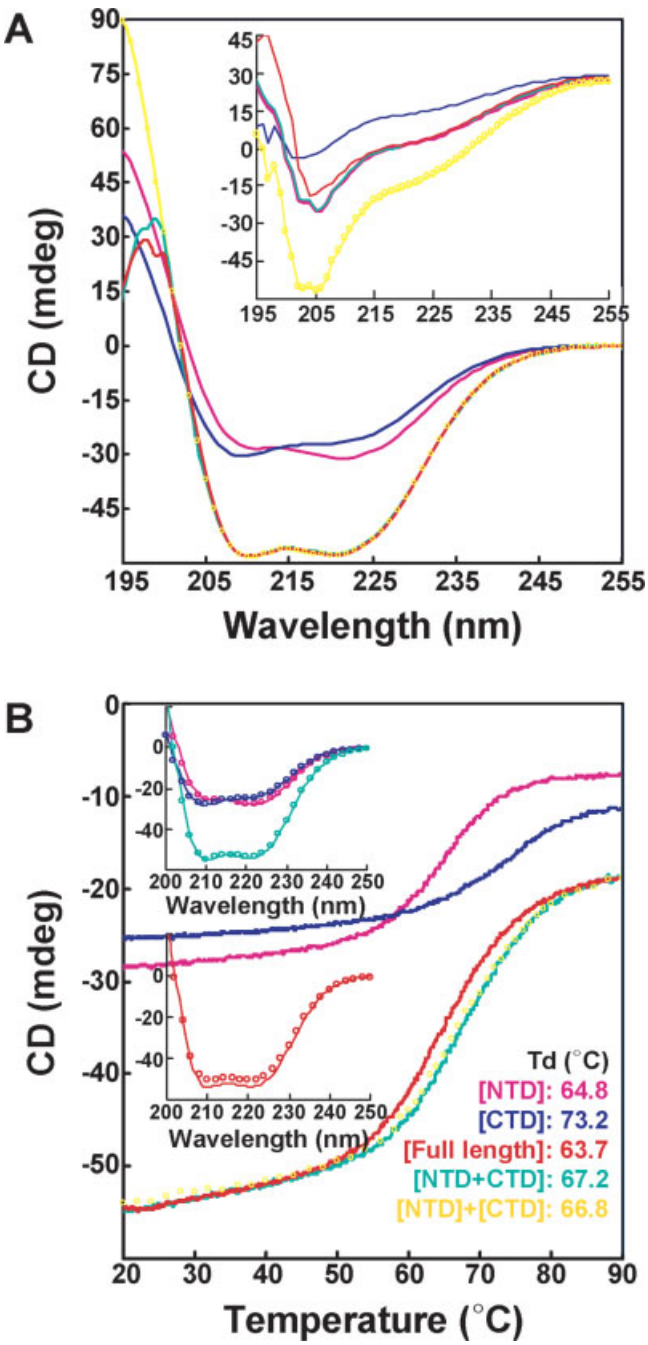

Fig. 3. CD results of BldD and its individual domains. Colors are designated as follows: pink, CD^{NTD}; blue, CD^{CTD}; green, $CD^{CTD+NTD}$ (1:1 mixture); red, CD^{intact}; yellow, $CD^{NTD}+CD^{CTD}$ (numerical summation). The protein concentration was kept at 14 μM. (**A**) Standard far-UV CD spectra at 20°C (main panel) and 90°C (inset). (**B**) Thermal denaturation profile monitored by CD at 222 nm upon increasing temperature at 1°C/min. As soon as the temperature reached 90°C, the protein samples were renatured by a quick cooling to 20°C. In the insets, the far-UV CD spectra before denaturation (at 20°C; lines) are compared with those after renaturation (at 20°C; circles).

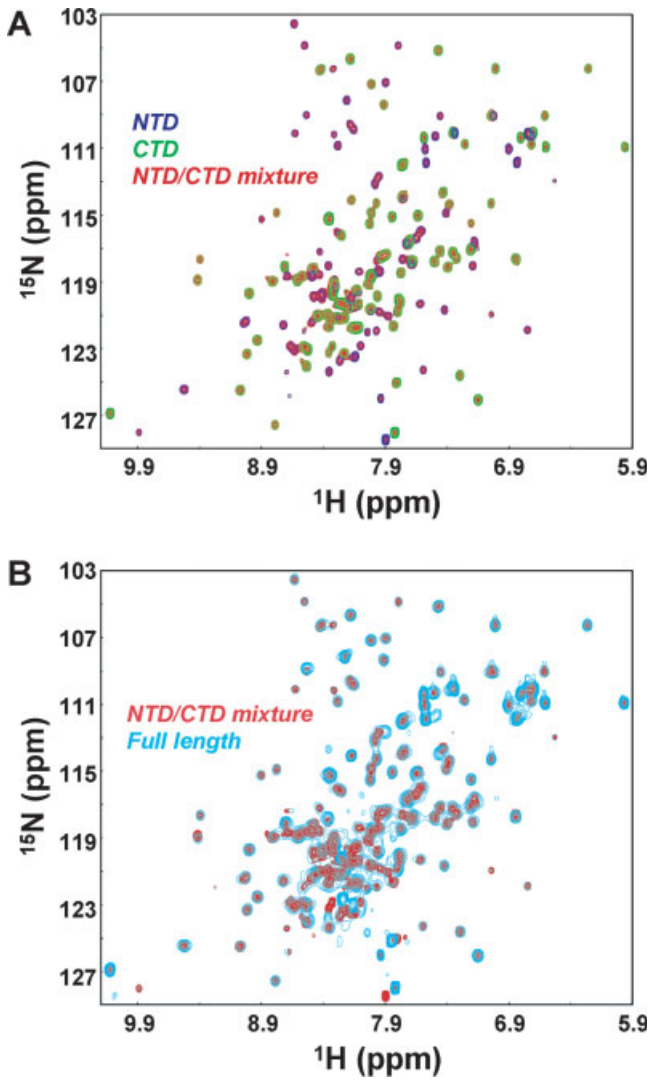

Fig. 4. [^{1}H-^{15}N]HSQC results of uniformly ^{15}N-labeled BldD and its individual domains, in the absence of DNA. The protein concentration was kept around 1 m*M*. The $HSQC^{NTD}$ (blue peaks) and the $HSQC^{CTD}$ (green peaks) are superimposed on the $HSQC^{NTD+CTD}$ (1:1 mixture; red peaks) in (**A**). $HSQC^{intact}$ (cyan peaks) is superimposed on $HSQC^{NTD+CTD}$ (red peaks) in (**B**). Most of the some unmatched peaks in each panel are actually well-matched at certain lower contour levels (data not shown).

indicated that both the two individual domains as well as the intact BldD adopt a well-folded structure. First, in the far-UV CD spectra [main panel in Fig. 3(A)], the strong negative bands centered near 208 and 222 nm indicate that the secondary structure contents of the proteins are as high as those generally observed for compact globular proteins. In addition, consistent with the secondary structure prediction (Fig. 2), mainly α-helical conformation of all of the three (NTD, CTD, and intact form) proteins are evidenced from the double minima at 208 and 222 nm.[29] Second, in the [^{1}H-^{15}N]HSQC spectra (Fig. 4), the wide dispersion of amide (^{1}H and ^{15}N) resonances are indicative of a well-ordered tertiary structure and the overall peak distribution is similar to those typically observed for helical proteins. Finally, in the thermal denaturation profiles monitored by CD [Fig. 3(B)], all of the three proteins clearly exhibited cooperative

denaturation with the half-denaturing temperature (T_d) over 60°C, which means that the proteins are folded into a stable or compact conformation. All these results indicated that both the constructed NTD and CTD are well-folded and thus supported the physiological relevance of the determined domain composition of BldD.

Independent Domains Scarcely Interacting with Each Other

To begin with, no or little interaction between the two individual domains was evidenced in their mixture. The far-UV CD spectrum of the NTD/CTD (1:1) mixture was well consistent with the spectrum obtained by numerical summing of the NTD and CTD spectra [Fig. 3(A)], suggesting no significant interaction that could lead to a conformational change. More clearly, the HSQC spectra of the ^{15}N-NTD, the ^{15}N-CTD, and the ^{15}N-NTD/^{15}N-CTD (1:1) mixture were superimposed in Figure 4(A). In this spectral superimposition, the specific interaction between the domains in their mixture would lead to a spectral perturbation, so that the spectrum of the mixture would not match, in many resonances, with the spectrum of each domain. However, the spectrum of the ^{15}N-NTD/^{15}N-CTD (1:1) mixture was entirely matched with the superimposed spectra of the ^{15}N-NTD and ^{15}N-CTD. In addition, the HSQC spectrum of ^{15}N-NTD was never changed by the addition of ^{14}N-CTD and *vice versa* (data not shown). These results clearly prove that the isolated NTD and CTD do not interact with each other in their mixture.

The present CD and NMR results also certified that the three-dimensional structures of the isolated domains are nearly identical to those in the intact BldD. The far-UV CD spectrum of the intact BldD was consistent with the spectrum obtained by numerical summing of the NTD and CTD spectra [Fig. 3(A)]. Even in the HSQC experiments, the ^{15}N-NTD/^{15}N-CTD (1:1) mixture spectrum, which is identical to the superimposition of the ^{15}N-NTD and ^{15}N-CTD spectra [Fig. 4(A)], was in good agreement with the spectrum of the intact ^{15}N-BldD [Fig. 4(B)]. In this spectral comparison, only a few peaks were observed to be unmatched and these peaks are necessarily expected from the different termini; that is the interdomain linker region of the intact form is reconstituted as the C-terminus in NTD and the N-terminus in CTD. Thus, it can be reasonably concluded that the specific interdomain interaction in BldD does not exist or is localized at the linker region.

The interdomain interaction in BldD was additionally inspected by comparing the thermal denaturing profiles. The thermal denaturation of the NTD, the CTD, and the NTD/CTD mixture was so fully reversible that their far-UV CD spectra after renaturation were nearly (more than 99%) identical to those before denaturation [insets in Fig. 3(B)]. Thus, their thermal denaturation can be regarded as a typical two-state (all-or-none) unfolding model without any intermediate conformation. This also suggests that each domain would be a single folding unit. The thermal denaturation profile of the NTD/CTD (1:1) mixture was in good agreement with the numerical summing of the denaturation profiles of the individual domains; that is the calculated T_d differed just by about 0.4°C. This also indicates that the interaction between NTD and CTD in their mixture was so negligible that it could not significantly affect the cooperativity in their thermal unfolding. The intact BldD also showed a highly (more than 94%) reversible denaturation, which suggests reciprocally that the structural interaction between the two domains in the intact BldD is little. Although the T_d of the intact BldD is lower by approximately 3.1°C than that of the numerically summed profile of the NTD and CTD denaturations, this degree of difference does not seem to be significant. Considering that the two domains are covalently linked in the intact form, nonspecific contacts would likely occur during the denaturing period. This assumption is supported by the spectral inconsistency in the denatured state [at 90°C; inset in Fig. 3(A)] between the intact form and the NTD/CTD (1:1) mixture. Generally, thermal denaturation of proteins exposes hydrophobic residues more onto the surface. Thus, it is reasonable to assume that the denatured domains in intact form, which are closely joined by the covalent linkage, would readily confer nonspecific hydrophobic contact to each other. This undesirable contact during the denaturing process could slightly affect the denaturing cooperativity and decrease the reversibility. In summary, the structural characteristics and the thermodynamic behaviors of BldD, monitored by CD and NMR, showed that the protein is composed of two independent structural domains, which scarcely interact with each other.

DNA-Binding Mediated Solely by the NTD

DNA-binding activity of BldD has been demonstrated previously by gel mobility shift assays of *bldD* promoter.[7,8,18] Elliot et al.[5] have suggested the DNA-binding property of BldD would be mediated by the C-terminal domain, which was based on the structural prediction of LysR-family helix-turn-helix motif in its C-terminal chain. In addition, our web-based (http://www.compbio.dundee.ac.uk/~www-jpred) prediction of secondary (Fig. 2) and tertiary structure suggests that the N-terminal region could adopt a lambda repressor-like DNA-binding fold, which was finally confirmed by our crystallographic study.[17,18] Thus, to elucidate definitely which part of BldD is responsible for the DNA-binding, the individual domains were incubated respectively with the fluorescein-labeled, 27-bp *bldD* promoter (FDP). The result of gel mobility shift assay (Fig. 5) clearly indicated that the NTD could bind to the FDP as strongly as do the intact BldD, while the CTD never shifted the band. The [^{1}H-^{15}N]HSQC experiments were also employed to confirm more precisely the specific DNA-binding of BldD [Fig. 6(A, B)] in solution. Upon the addition of DNA, an overall perturbation in the HSQC spectra of the ^{15}N-NTD [blue in Fig. 6(A)] and the intact ^{15}N-BldD [cyan in

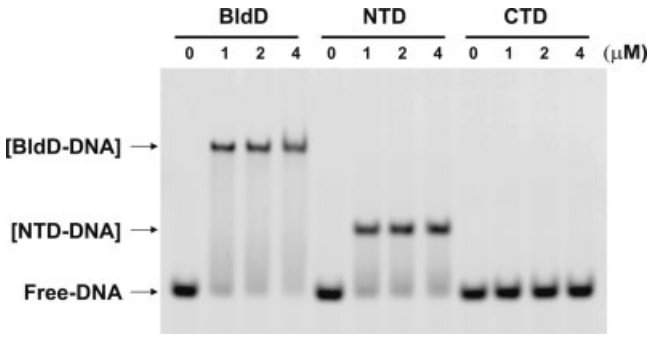

Fig. 5. Gel mobility shift assays of BldD and its individual domains with the FDP (a 27-bp DNA fragment of the *bldD* promoter). Fluorescein-labeled FDP (50 ng) was incubated with 0 to 4 μ*M* of BldD, NTD, and CTD, respectively, in the presence of the ~ 500 ng of unlabeled, nonspecific competitor DNA (poly-d(I-C)) at 30°C for 15 min, and then, resolved on a 10% nondenaturing polyacrylamide gel electrophoresis.

Fig. 6(B)] was observed; that is many peaks either disappeared by severe peak broadening or largely shifted in their chemical shifts. In contrast, the ^{15}N-CTD spectrum remained without any significant change in the presence of DNA [green in Fig. 6(A)]. In addition, little or no structural interaction between the two domains was observed even in the presence of DNA; that is the [^{1}H-^{15}N]HSQC spectrum of intact ^{15}N-BldD is well matched with the spectra of the ^{15}N-NTD, the ^{15}N-CTD, and the ^{15}N-NTD/^{15}N-CTD mixture, in the presence of DNA [Fig. 6(A, B)]. Combining the present gel shift assay and the NMR results, it is now definite that the DNA-binding of BldD, at least at the used promoter region (−27 to −1), is never mediated by the CTD, but near fully mediated by the NTD.

Dimerization Mediated by the NTD

Since the physiologically relevant form of BldD is known to be a dimeric,[8] gel permeation chromatography (Fig. 7) was employed to identify its dimerizing domain. On the basis of the elution profiles, the apparent molecular weight of NTD and intact BldD clearly supported their dimeric states in solution. In contrast, the K_{av} value of CTD, which was definitely increased relatively to that of the NTD, indicated its monomeric behavior, even if we consider a potential difference in the molecular shape or compactness between the two domains. A supplementary verification of the oligomeric state was attempted by a chemical crosslink using glutaraldehyde,[30] which can produce amine–amine coupled adducts of oligomeric proteins in solution. Consistent with the results of gel permeation chromatography, dimeric forms of the intact BldD and the NTD were detected in the presence of glutaraldehyde, whereas neither a dimeric CTD nor an NTD/CTD-heterodimer was observed (Fig. 8). Moreover, it was detected that BldD could adopt a heterodimer with NTD, but not with CTD, in the presence of the crosslinker. It remains to be further investigated whether this observation of heterodimer is related to physiological properties such as intermolecular cooperativity. However, it could be certain that the NTD is mainly responsible for the dimerization of BldD as well as its DNA-binding. This is a unique characteristic of BldD that has been rarely observed from known transcription regulators. In particular, most proteins in the lambda repressor-like family, where BldD belong, forms a dimer by accompanying another nonconserved, dimerizing domain that sometimes serves as an effector/signal receiver. Additionally, other related DNA-binding proteins such as the bacteriophage lambda *cro* repressor and the *E. coli* catabolite-gene activator protein (or cyclic AMP receptor protein) also make dimeric contacts at the non-DNA binding domains.[19–22] Based on these insights,

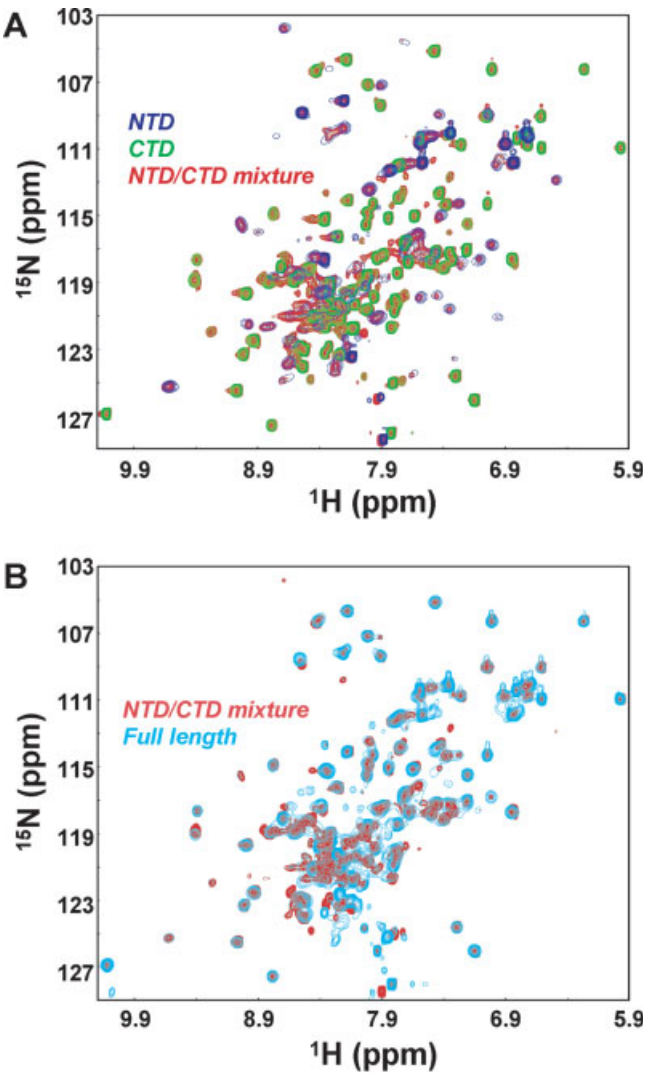

Fig. 6. [^{1}H-^{15}N]HSQC results of uniformly ^{15}N-labeled BldD and its individual domains, in the presence of DNA. The protein concentration was kept around 1 m*M*. The HSQC^{NTD} (blue peaks) and the HSQC^{CTD} (green peaks) are superimposed on the HSQC$^{NTD+CTD}$ (1:1 mixture; red peaks) in (**A**). HSQC^{intact} (cyan peaks) is superimposed on HSQC$^{NTD+CTD}$ (red peaks) in (**B**). Most of the some unmatched peaks in each panel are actually well-matched at certain lower contour levels (data not shown).

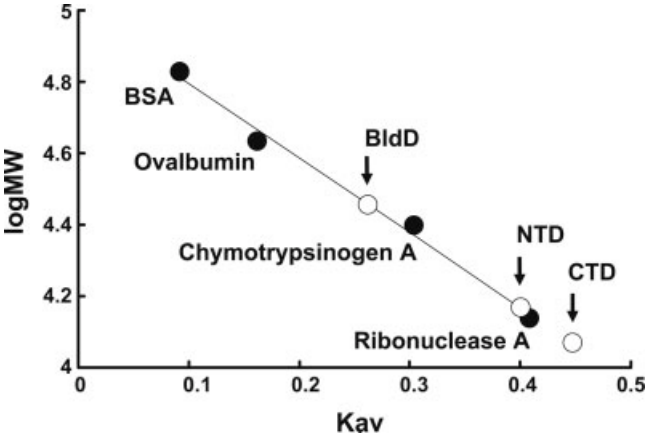

Fig. 7. Oligomeric states of BldD and its individual domains. Gel permeation chromatography analysis of BldD, NTD, and CTD. The K_{av} values of the proteins are indicated on a standard curve generated by plotting logarithm of molecular mass against K_{av}. Filled circles indicate the protein standards used. Open circles and arrows designate the K_{av} values of BldD, NTD, and CTD, respectively.

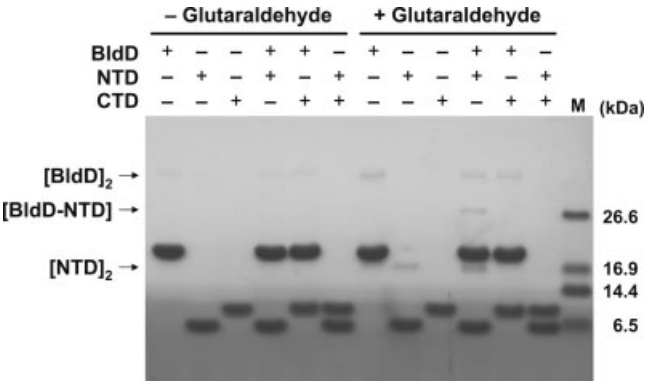

Fig. 8. Chemical crosslinking experiment of BldD and its individual domains. Reaction mixtures containing 20 μM of proteins were crosslinked by 0.05% of glutaraldehyde and then resolved on a 15% denaturing Tris-tricine gel, together with polypeptide molecular weight standards (M). The positions of the dimeric BldD and the dimeric NTD, and the heterodimer of BldD with NTD are indicated by arrows.

it can be also suggested that BldD could function in a more complicated manner, by forming a unique conformation upon binding to its target DNAs.

Concluding Remarks

It is generally accepted that BldD is a central regulator of the developmental process of *S. coelicolor*. However, many lines of cell-based studies have led to contradictory conclusions regarding the cellular function of BldD, whether it acts as a developmental activator or as a repressor: that is, the transcript levels of the essential genes for morphological differentiation are upregulated in the *bldD* mutant (Y62 to C) strain, but nevertheless the Y62C mutant and the *bldD* null mutant are defective in morphological differentiation.[5,8] Thus, an alternative approach to distinguish the exact function of BldD would be a molecular characterization, which was sought to do in this work. The results revealed that this homodimeric protein consists of two structurally independent domains in each subunit. The N-terminal domain (residues 1–79) was particularly responsible for both the DNA-binding and dimerization of the protein. This is a unique characteristic that has not been observed from other known transcription regulators similar to BldD.

The C-terminal domain (residues 80–167) of BldD was observed to be selectively degraded dependently on the developmental stage. This observation suggests that BldD likely play a role of developmental switch in *S. coelicolor* by the selective degradation of its C-terminal domain. However, the exact molecular function of the C-terminal domain remains to be identified, since the CTD was involved neither in DNA-binding nor in dimerization. Regarding the role of the CTD, the following two mechanisms are presumable, on the basis of the literatures and the present results. First, the CTD might be able to bind to DNA at other regions not identified yet. If this is the real case, then a DNA bending, due to cooperative binding of NTD and CTD, is expectable and BldD would likely act as a developmental repressor at the pre-differentiating, vegetative stage of *S. coelicolor*. Then, the specific proteolysis of BldD dependently on developmental signals would trigger next stage and the remaining fragment, defined as NTD, would function as an essential activator to promote and complete the developmental processes, including aerial differentiation and sporulation. Alternatively, if the CTD is specifically responsible for intermolecular interactions with an effector molecule or another transcription factor, their interactions at the time of morphological differentiation would work as transcriptional signals to promote aerial hyphae formation.

The present results constitute the first detailed information based on the physicochemical features of BldD in solution and also encourage determining the solution-state structure of the protein and its complexes with target DNA. Along with this study and crystal structure of the N-terminal domain of BldD,[18] the further structural studies using X-ray crystallography and NMR spectroscopy are presently in progress, as well as the functional studies, especially on the C-terminal domain.

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