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Mainchain NMR Assignments and secondary structure prediction of the C-terminal domain of BldD, a developmental transcriptional regulator from *Streptomyces coelicolor* A3(2)

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Abstract BldD, a developmental transcription factor from *Streptomyces coelicolor*, is a homodimeric, DNA-binding protein with 167 amino acids in each subunit. Each monomer consists of two structurally distinct domains, the N-terminal domain (BldD-NTD) responsible for DNA-binding and dimerization and the C-terminal domain (BldD-CTD). In contrast to the BldD-NTD, of which crystal structure has been solved, the BldD-CTD has been characterized neither in structure nor in function. Thus, in terms of structural genomics, structural study of the BldD-CTD has been conducted in solution, and in the present work, mainchain NMR assignments of the recombinant BldD-CTD (residues 80-167 of BldD) could be achieved by a series of heteronuclear multidimensional NMR experiments on a [^{13}C/^{15}N]-enriched protein sample. Finally, the secondary structure prediction by CSI and TALOS+ analysis using the assigned chemical shifts data identified a β-α-α-β-α-α-α topology of the domain. The results will provide the most fundamental data for more detailed approach to the atomic structure of the BldD-CTD, which would be essential for entire understanding of the molecular function of BldD.

Keywords BldD, Transcriptional regulator, C-terminal domain, NMR backbone assignment, Secondary structure

Introduction

Streptomycetes are gram-positive soil bacteria that undergo a series of regulated developmental processes including germination, vegetative growth, secondary metabolites production and spore-forming. Spores are generated through the multiple septation of multicellular aerial hyphae, which emerge from the colony into air.[1] These morphological differentiations are eventually accompanied by production of secondary metabolites, such as antibiotics and pigments.[2] Genetic studies of differentiation in *Streptomyces coelicolor* have isolated a number of mutants that are incapable of aerial hyphae formation, called bld (bald) mutants. Among them, the bldD mutant is able to trigger the aerial hyphae formation of the other bld mutants by extracellular complementation.[3,4] These findings place the *bldD* at the bottom of the extracellular signaling cascade.[5]

The *bldD* gene product, BldD, is a small DNA-binding protein with 167 amino acids and exists predominantly as a homodimer in solution.[6-8] BldD binds its own promoter and regulatory regions

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of several genes,[6,7,9,10] including *bldN* encoding a sigma factor involved in aerial hyphae formation,[11] *whiG* encoding a sigma factor related in spore formation[12] and *sigH* encoding a sigma factor associated with stress responses.[13] The transcription levels of these target genes in the *bldD* mutant in comparison with those of the wild type have shown that BldD operates as a transcriptional repressor.[9,10,13] BldD has two structurally independent domains, an N-terminal domain (residues 1-79, BldD-NTD), which is responsible for both the DNA-binding and dimerization, and a C-terminal domain (residues 80-167, BldD-CTD), of which function is unknown.[8] Although the crystal structure of BldD-NTD showed a classical helix-turn-helix motif for DNA binding and a remarked structural similarity with lambda repressor,[14] it was not sufficient to fully explain the regulatory mechanism of BldD, without structural information of the BldD-CTD. Unfortunately, intensive attempts for crystallization of the intact BldD or the BldD-CTD have failed, probably due to the low stability of the proteins *in vitro*, prone to degradation during crystallization.[14] Accordingly, an alternative approach by using NMR to the atomic structure in solution is essential for fine understanding of the mode of action of BldD, which is directly linked to the developmental processes in *Streptomyces coelicolor*. Thus, in terms of structural genomics, NMR experiments were conducted in the present study on the recombinant BldD-CTD protein and the secondary structure determination is reported.

Experimental Methods

Protein preparation- The recombinant BldD-CTD was prepared using the pET-15b (Novagen) vector and the *E. coli* BL21(DE3)pLysS (Novagen), as described previously.[8] Briefly, the cells transformed with the recombinant plasmids were grown at 37°C in M9 minimal medium, which was supplemented with [^{15}N]NH_4Cl and [^{13}C]glucose, as the sole source of nitrogen and carbon, respectively. When OD_{600} of the cell growth reached about 0.5, expression of proteins was induced by adding IPTG (Duchefa) at a final concentration of 1 mM, and the induction was prolonged for 15 h at 22°C. Cells were harvested by centrifugation and disrupted by sonication. After centrifugation, the supernatant was applied to a nickel-affinity column (Novagen) chromatography for purification. The eluent was concentrated by ultrafiltration (Amicon) and buffer-exchanged by PD-10 column (GE healthcare). The eluent in thrombin cleavage buffer (20 mM Tris-HCl buffer contacting 150 mM NaCl and 2.5 mM $CaCl_2$, at pH 8.4) was mixed with Thrombin (Novagen) and incubated at 22°C for 18 h to cleave the N-terminally tagged histidines, followed by the removal of thrombin and other impurities via the sequential application of nickel-affinity and gel-permeation chromatography again. For NMR measurements, the purified protein solution was applied to gel permeation chromatography (HiLoad 16/60 Superdex^{TM} 75, GE healthcare) and concentrated to 2 mM in a standard buffer (40 mM sodium phosphate buffer containing 150 mM NaCl and 0.05% (w/v) NaN_3, at pH 6.8).

NMR spectroscopy- NMR spectra were obtained on a Bruker Biospin Avance 500 NMR spectrometer equipped with a cryoprobe, at KBSI (Korea Basic Science Institute). A conventional 2D-[^{1}H/^{15}N]HSQC, 3D HCCH- COSY, 3D ^{15}N-TOCSY-HSQC and a series of triple resonance spectra including HNCACB, HN(CO) CACB, HNCO were recorded on 2 mM of [^{13}C/^{15}N]- BldD-CTD in a standard buffer with 93% H_2O/7% D_2O, at 295 K. DSS (4,4-dimethyl-4-silapentane-1- sulfonic acid) was used externally as a chemical shift reference for ^{1}H. ^{13}C and ^{15}N chemical shifts were then referenced indirectly using the chemical shift ratio values suggested in the BMRB (http://www.bmrb.wisc.edu). All NMR spectra were processed with the NMRPipie/nmrDraw[15] and analyzed with the NMRView[16]. Secondary structure was predicted by CSI (Chemical Shift Index)[17,18] and Talos+ analysis[19] with the assigned $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{13}C'$ and $^{1}H^{\alpha}$ chemical shifts.

Results and Discussion

Our preliminary investigation using far-UV CD and 2D-[^{1}H/^{15}N]HSQC experiments has previously revealed that BldD-CTD would adopt a well-folded helical structure.[8] However, unfortunately, incubation of BldD-CTD at 313K for more than 6 hours caused severe degradation of the protein (data not shown), which was not suitable for 3-dimensional NMR experiments. In the present study, a tolerable stability in NMR measurements could be achieved by conducting the experiments at 295K and using the buffer containing sodium azide. As shown in Fig. 1, 2D-[^{1}H/^{15}N]HSQC spectrum of BldD-CTD showed a good spectral dispersion with a narrow linewidth, which supports the well-folded state of the protein, consistent with the previous observations. Then, sequential backbone NMR assignments for ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{13}C'$ and $^{1}H^{N}$ atoms were performed using the triple resonance spectra, by a standard method verifying and linking peak clusters, according to the inter-residue ^{13}C correlations.[20,21]

As we used the pET-15b vector that confers N-terminally tagged six histidines to the expressing protein for efficient purification, our recombinant BldD-CTD protein finally has four non-native

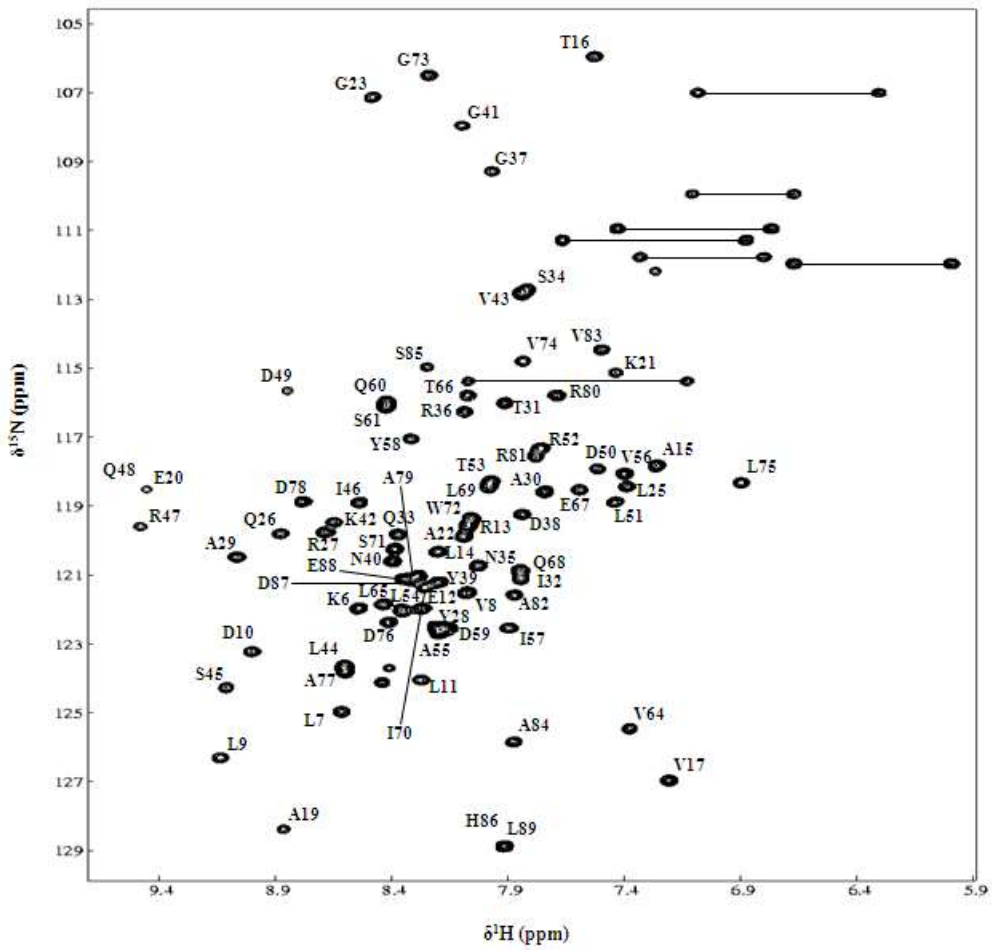

Figure 1. 2D-[^{1}H/^{15}N]HSQC spectrum of BldD-CTD. Each resonance in the spectrum is labeled with the corresponding residue finally assigned. Sidechain signals from glutamine and asparagine residues are indicated by horizontal lines.

residues (Gly-Ser-His-Met-) at its N-terminus, as a resultant of the His-tag cleavage by thrombin. For convenience, the non-native methionine of the N-terminus was designated as the first residue in the numbering of amino acid sequence and the assignment results are summarized in Fig. 1 and Table 1. The N-terminal five residues including the non-native sequence and its neighboring E2 residue didn't show obvious signals in 3D NMR spectra, probably due to dynamic properties at the N-terminus, as generally observed for proteins. Additionally, the amide signal from a serine residue, S63, was not detected, also implying an intermediate rate of chemical exchange. In the 2D-[^{1}H/^{15}N]HSQC spectrum (Fig. 1), the seven pairs of NH_2 signals from six glutamines and one asparagine could be clearly distinguished from the backbone amide resonances (indicated by lines in Fig. 1). However, a few signals without labeling in Fig. 1, which might originate from the non-assigned backbone amides or from the arginine guanidine groups, could not be assigned due to the absence of corresponding signals in the 3D NMR spectra. Finally, extents of the present sequence-specific assignments of native sequence correspond to 97.6% for $^{1}H^{N}$, 90.9% for ^{15}N, 96.6% for $^{13}C^{\alpha}$, 95.3% for $^{13}C^{\beta}$, and 90.8% for $^{13}C'$ atoms. Then, for more accurate prediction of secondary structure, total 85 of $^{1}H^{\alpha}$ atoms were

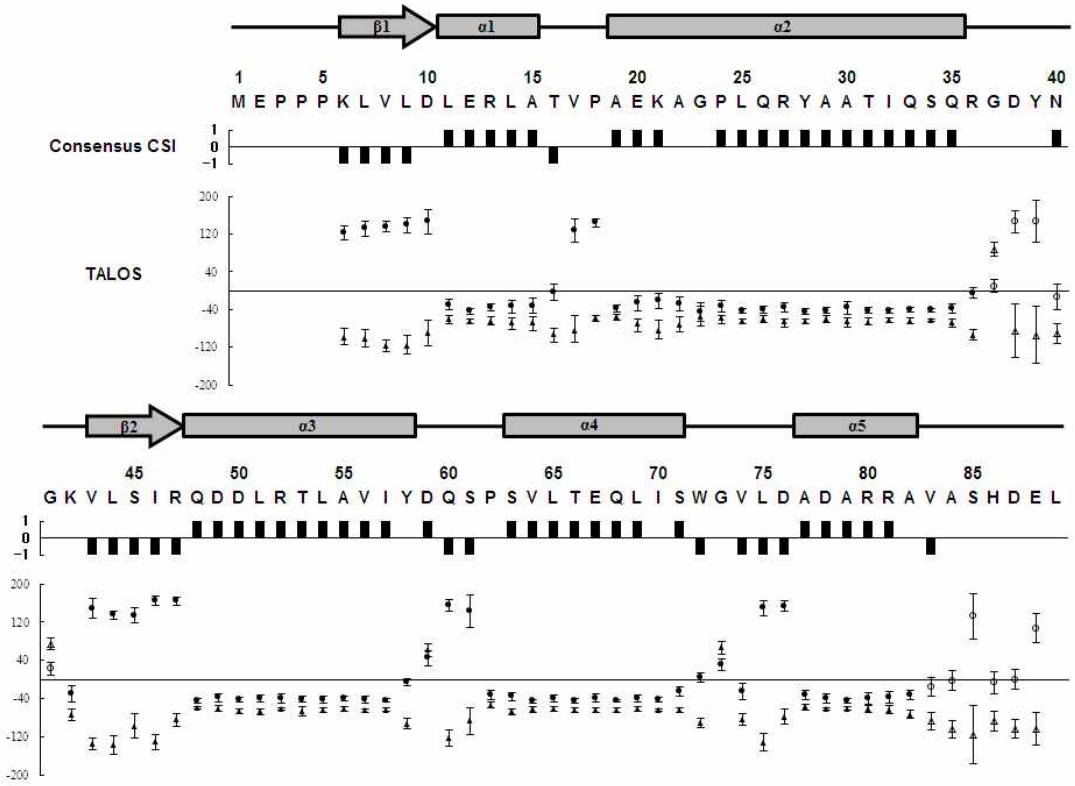

Figure 2. Secondary structure prediction of BldD-CTD using chemical shift data sets. In the CSI results, the mark "1" represents the α-helical tendency of the residue (downfield shifts of $^{13}C^{\alpha}$ or $^{13}C'$ resonances, and upfield shifts of $^{13}C^{\beta}$ or $^{1}H^{\alpha}$ resonances from their reference value ranges),[20] while "-1" represents the opposite pattern (β-strand tendency). The chemical shift within the reference value range was marked as a "0". The length of error bars with the TALOS+-predicted backbone dihedral angles, ϕ (triangles) and φ (rectangles), indicate the standard deviation from the average.[20] Predicted secondary structure elements are indicated by boxes for α-helices and arrows for β-strands, along the amino acid sequence.

unambiguously assigned based on the $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ correlations observed in the 3D HCCH-COSY and $^{1}H^{N}$-^{15}N-$^{1}H^{\alpha}$ correlations in 3D ^{15}N-TOCSY-HSQC spectra.

All of the assigned chemical shift data sets were applied to CSI and TALOS+ analysis to determine the secondary structure (Figure 2). The determined secondary structure by combining the CSI and TALOS+ results indicated that BldD-CTD is predominantly α-helical, similar to the previous far-UV CD results.[8] However, the present results additionally revealed the presence of two short β-strands, therby forming a β-α-α-β-α-α-α topology. The two β-strands are expected to form a β-sheet since their lengths are almost the same.

As introduced above, the BldD-NTD showed a remarked structural homology to the N-terminal DNA-binding domain of lambda repressor. In contrast, the C-terminal part of lambda represor, which is primarily responsible for dimerization of the protein, is formed by a highly twisted seven-stranded β-sheet.[22,23] In addition, dimerization of other related DNA-binding proteins such as the bacteriophage lambda *cro* repressor and the *E. coli* catabolite-gene activator protein is also mediated by the non-DNA binding domain, which consists primarily of β-strands.[24-27] However, previous biochemical analysis revealed that the DNA-binding NTD of BldD sufficiently forms a dimer in solution, while the isolated BldD-CTD behaves as a monomer.[8] Therefore, it is expectable that the BldD-CTD would play a distinct role in the molecular function of intact BldD. The present results also suggest a unique functional fole of the BldD-CTD, since its secondary structure adopting the β-α-α-β-α-α-α topology is quite different from that of the C-terminal domain of lambda repressor. Altogether, a high-resolution atomic structure of the BldD-CTD is now required for clear understanding of the functional mechanism of BldD, and the present NMR assignment results

Table. 1. Chemical shifts of $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{13}C'$ and $^{1}H^{\alpha}$ atoms of BldD-CTD, assigned at 295 K and pH 6.8 (NA, not available; ND, not detected).

Residue	$^{1}H^{N}$	^{15}N	$^{13}C^{\alpha}$	$^{13}C^{\beta}$	$^{13}C'$	$^{1}H^{\alpha}$
M1	ND	ND	ND	ND	ND	ND
E2	ND	ND	ND	ND	ND	ND
P3	NA	NA	ND	ND	ND	ND
P4	NA	NA	ND	ND	ND	ND
P5	NA	NA	62.783	32.354	176.334	4.401
K6	8.542	121.974	56.172	33.270	175.715	4.374
L7	8.615	124.971	54.050	43.171	174.994	4.523
V8	8.078	121.529	61.206	34.596	175.042	4.547
L9	9.136	126.306	56.546	44.233	175.831	4.940
D10	9.000	123.224	53.593	40.820	176.685	4.848
L11	8.272	124.049	57.013	40.850	180.169	4.066
E12	8.270	121.969	59.628	29.560	179.719	4.174
R13	8.075	119.582	57.311	29.555	179.909	4.172
L14	8.200	120.325	57.574	41.798	177.166	3.947
A15	7.262	117.824	54.320	18.712	178.500	4.125
T16	7.529	105.954	61.642	69.957	174.639	4.378
V17	7.206	126.971	60.535	32.388	ND	3.985
P18	NA	NA	63.848	32.711	178.094	4.268

would provide the most fundamental and essential data for the progressing investigation of the 3-dimensional structure.

Table. 1. (Continued)

Residue	$^{1}H^{N}$	^{15}N	$^{13}C^{\alpha}$	$^{13}C^{\beta}$	$^{13}C'$	$^{1}H^{\alpha}$
A19	8.865	128.379	55.317	18.914	180.008	3.944
E20	9.453	118.516	59.636	28.838	177.573	4.114
K21	7.437	115.128	56.987	33.952	177.045	4.652
A22	8.096	119.888	53.181	20.231	177.520	4.144
G23	8.482	107.133	49.012	NA	ND	4.138
P24	NA	NA	65.953	31.371	179.318	4.174
L25	7.390	118.433	57.935	42.350	177.871	4.356
Q26	8.879	119.802	59.771	27.554	179.865	3.894
R27	8.688	119.765	59.636	30.416	178.950	4.141
Y28	8.214	122.557	62.278	39.713	177.843	4.171
A29	9.065	120.477	55.185	17.929	178.739	3.762
A30	7.741	118.587	55.076	18.086	180.895	4.287
T31	7.912	116.012	66.452	68.867	176.687	3.995
I32	7.844	121.104	64.153	36.940	178.583	3.815
Q33	8.373	119.820	59.858	29.365	178.264	3.839
S34	7.813	112.712	60.915	63.235	176.817	4.333
Q35	8.029	120.724	58.109	29.188	177.930	4.194
R36	8.088	116.266	56.424	31.418	177.013	4.398
G37	7.968	109.285	46.270	NA	173.661	3.880
D38	7.838	119.238	53.171	41.640	175.405	4.715
Y39	8.199	121.216	58.128	38.588	176.937	4.527
N40	8.391	120.603	54.383	39.506	175.853	4.603
G41	8.097	107.958	45.930	NA	173.926	3.929
K42	8.647	119.465	57.709	34.345	176.201	4.489
V43	7.838	112.814	59.264	35.953	174.906	5.330
L44	8.604	123.700	53.876	47.498	175.260	4.789
S45	9.110	124.269	58.852	63.509	174.264	5.052
I46	8.540	118.903	59.602	41.595	174.199	4.780
R47	9.481	119.594	54.508	33.318	178.511	4.865
Q48	9.345	122.794	60.527	28.284	178.972	3.818
D49	8.848	115.657	57.357	40.483	178.758	4.360
D50	7.515	117.921	58.075	41.639	177.865	4.612
L51	7.441	118.888	58.129	41.397	178.254	3.912
R52	7.758	117.335	60.285	29.818	178.886	3.910

Table. 1. (Continued)

Residue	$^{1}H^{N}$	^{15}N	$^{13}C^{\alpha}$	$^{13}C^{\beta}$	$^{13}C'$	$^{1}H^{\alpha}$
Y58	8.317	117.051	60.764	39.027	174.728	4.131
D59	8.173	122.557	55.113	39.812	174.853	4.350
Q60	8.422	116.038	53.513	34.384	174.853	4.719
S61	8.422	116.055	56.443	62.811	ND	4.783
P62	NA	NA	66.496	31.780	179.161	4.036
S63	ND	ND	62.369	62.293	176.141	4.838
V64	7.378	125.462	66.432	32.017	178.754	3.561
L65	8.438	121.853	57.021	41.255	178.836	3.899
T66	8.074	115.786	69.056	67.992	175.591	3.321
E67	7.593	118.529	59.225	28.921	179.342	3.841
Q68	7.845	120.875	58.394	28.374	177.403	3.100
L69	7.982	118.379	58.413	40.971	180.177	3.473
I70	8.258	121.358	65.050	38.345	180.910	4.160
S71	8.386	120.256	62.544	62.839	177.045	4.273
W72	8.061	119.364	55.764	31.264	176.839	4.926
G73	8.239	106.500	45.403	NA	174.697	4.167
V74	7.835	114.795	63.113	32.849	174.015	4.197
L75	6.897	118.321	52.632	47.158	175.022	4.911
D76	8.412	122.380	54.383	43.227	176.384	4.603
A77	8.600	123.798	54.731	18.749	179.068	4.054
D78	8.784	118.871	56.278	40.908	174.868	4.513
A79	8.287	121.034	54.391	19.920	178.803	4.001
R80	7.688	115.788	59.229	30.108	178.254	3.924
R81	7.781	117.571	57.693	30.242	177.214	4.206
A82	7.872	121.588	53.255	19.563	177.798	4.279
V83	7.500	114.464	62.041	32.781	175.572	4.141
A84	7.874	125.848	52.771	19.512	177.696	4.321
S85	8.248	114.966	58.393	63.915	ND	4.396
H86	7.971	125.420	56.321	30.312	174.868	4.666
D87	8.322	121.116	54.577	41.256	175.917	4.603
E88	8.344	121.117	56.372	30.373	175.455	4.317
L89	7.916	128.878	57.150	43.446	ND	4.165

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