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^1H , ^{13}C and ^{15}N resonance assignments of Ca^{2+} bound collagen-binding domain derived from a clostridial collagenase

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Abstract

^1H , ^{15}N , and ^{13}C NMR assignments for 116 amino acids (Gly893-Lys1008) of a bacterial collagen-binding domain (CBD) derived from *Clostridium histolyticum* class I collagenase were accomplished. Clostridial collagenases hydrolyze insoluble collagen. One to three copies of collagen-binding domains (CBDs) are present at their C-termini, each of which is the minimal segment required for the binding to the insoluble substrate. CBD has been shown to be able to anchor fused growth factors for up to 10 days in vivo. Structural analysis of the small domain with the unique function provides insights into designing a novel drug delivery vehicle by the rational drug design.

Keywords

Collagen; Collagen-binding domain; NMR assignments

Biological context

Gas gangrene is caused by a group of anaerobic Gram-positive bacteria, Histotoxic clostridia. They produce collagenases responsible for the connective tissue destruction by hydrolyzing insoluble collagen fibrils. *Clostridium histolyticum* produces two classes of collagenases encoded by two structural genes, *colG* and *colH* (Matsushita et al. 1999; Yoshihara et al. 1994). Similar but different segmental structures were observed in the two enzymes (S1, S2, S3a, S3b for ColG; and S1, S2a, S2b, S3 for ColH). The C-terminal segments, S3's are homologues possessing approximately 120 amino acid residues and bind collagen (CBD) (Matsushita et al. 2001). CBD binds not only to insoluble collagen fibril but also to collagenous peptides by specifically recognizing the triple-helix conformation (Matsushita et al. 2001). It does not bind to gelatin. Since collagens are the major constituent of extracellular matrix, the function could be exploited for a novel drug delivery method. Growth factors fused to CBD

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were indeed anchored at the site of injection (Nishi et al. 1998). For a site-specific drug delivery, precise orientation of collagen on CBD need to be known. X-ray crystal structures of monomeric CBD (S3b, Gly893-Ly1008) were solved at atomic resolution in the presence and absence of Ca^{2+} (Wilson et al. 2003). Crystallization attempts for CBD-collagen complex did not result in any single crystal. Although CBD binds irreversibly to insoluble collagen fibril and immobilized collagenous peptides, it has not been shown that CBD could bind to a soluble triple helical substrate in solution. Hence, solution NMR studies became an important tool to study CBD-collagen interactions. As a first step towards the attainment of this objective we report the assignment of ^1H , ^{13}C , and ^{15}N resonances of CBD in the presence of Ca^{2+} .

Methods and experiments

Expression, purification and NMR sample preparation of C-terminal collagen-binding domain

Monomeric CBD (S3b, Gly893-Lys1008) derived from the *Clostridium histolyticum* class I collagenase (ColG) was expressed as a glutathione S-transferase (GST)-fusion protein essentially in the same way as described previously (Matsushita et al. 2001). About 30 ml of LB medium supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin was inoculated with the recombinant *E. coli* BL21 strain, and cells were grown at 37°C with shaking overnight. Cells were harvested from 20 ml of this preculture, and resuspended in the same volume of a minimal medium, which contains 34 mM NaHPO_4 , 64 mM K_2HPO_4 , 40 mM NH_4SO_4 , 0.3 mM MgSO_4 , 1 μM ZnCl_2 , 1 μM FeSO_4 and 0.4% glucose supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin. The ammonium sulfate and/or glucose were replaced with the same concentration of ^{15}N - NH_4Cl and/or ^{13}C -glucose for single or double labeling. The cell suspension was diluted 100 times with the same medium, and cells were grown in four 2-l flasks at 37°C with vigorous shaking to an optical density at 600 nm of 0.7. Isopropyl-1-thio- β -D-galactopyranoside (IPTG) was added to a final concentration of 1 mM, and cells were grown at 37°C for further 14 h.

Purification of the fusion protein was performed with a glutathione-Sepharose 4B column (bed volume, 10-ml; GE Healthcare) as described by the manufacturer. The protein was cleaved by incubation with thrombin (GE Healthcare, 5 units/mg of fusion protein) for 14 h at room temperature. The protein fraction was dialyzed against 50 mM TrisHCl (pH 7.5) and applied serially to a glutathione-Sepharose 4B column (bed volume, 12 ml) and a Q-Sepharose FF column (bed volume, 2 ml). The flow-through fractions were pooled, concentrated by ultrafiltration using a Centricon 3 device, and stored at -80°C till use.

Estimation of the labeling efficiency

^{15}N -labeled and unlabeled CBDs were diluted with distilled water, mixed with 3,5-dimethoxy-4-hydroxycinnamic acid solution (10 mg/ml of 50% ethanol), and analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (model Voyager-DE STR; Applied Biosystems) in the positive linear mode with time-delayed extraction. The labeled protein was purified to homogeneity as determined by SDS-PAGE. Typical recovery of CBD was approx. 10 mg from 2-l culture, which was determined by using a BCA protein assay kit (Pierce). Molecular mass of the unlabeled CBD was determined to be 13789.18 by mass spectrometry that is consistent with the calculated value of 13786.92. The molecular mass of the ^{15}N -labeled CBD was determined to be 13952.57, which is larger than the unlabeled CBD by 163.31 Da. Since 1 mole of CBD contains 164 mole of nitrogen atoms, the labeling efficiency was estimated to be 99.6%. Labeling efficiency of ^{13}C -CBD was determined in the same manner to be 98.6%.

NMR experiments

About 0.75 mM of NMR samples of purified isotopically labeled (^{15}N or $^{15}\text{N}/^{13}\text{C}$ or ^{13}C) CBD were prepared in 10 mM Tris buffer (pH 7.5 in 90% H_2O and 10% D_2O) containing 100 mM

NaCl and 20 mM CaCl₂. NMR data were acquired at 25 ± 0.1°C using Bruker AVANCE DMX 700 MHz spectrometer equipped with cryoprobe. All NMR data were processed using TOPSPIN 1.2 and analyzed by SPARKY (Goddard and Kneller 1993). Backbone assignments were obtained using the following 3D experiments: HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO. Side chain chemical shifts were obtained from 3D ¹⁵N-TOCSY-HSQC, ¹⁵N-NOESYHSQC, HBHA(CO)NH, HCCC(CO)NH and HCCHTOCSY experiments (Cavanagh et al. 1996).

Assignments and deposition

2D ¹H-¹⁵N HSQC spectrum of the CBD is well-dispersed suggesting that the protein is structured. The backbone ¹H-¹⁵N resonances of all non-prolyl residues except K896, G925, D926, K937, L958, R967, I968, and Y970 have been assigned (Fig. 1). The regions near these sites exhibit weakened and broadened signals in all the spectra collected. 98.2% of the C^α, 99% of C^β, 92% of C^αH and 95% of C^βH resonances in the protein has been unambiguously assigned. The chemical shifts of the backbone and side chain assignments of collagen-binding domain have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number BMRB-15722.

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Fig. 1. ^1H - ^{15}N HSQC spectrum of uniformly ^{15}N labeled collagen-binding domain (CBD) in 90% $\text{H}_2\text{O}/10\%$ D_2O at pH 7.5 and 25°C . Backbone amide assignments are represented in the single letter amino acid code. Side chains NH_2 resonances for asparagine and glutamine residues are connected by horizontal lines