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NMR conformational dynamics of an Anthrax Lethal Factor domain studied by multiple amino acid-selective labeling

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ABSTRACT

NMR-based structural biology urgently needs cost- and time-effective methods to assist both in the process of acquiring high-resolution NMR spectra and their subsequent analysis. Especially for bigger proteins (>20 kDa) selective labeling is a frequently used means of sequence-specific assignment. In this work we present the successful overexpression of a polypeptide of 233 residues, corresponding to the structured part of the N-terminal domain of Anthrax Lethal Factor, using *Escherichia coli* expression system. The polypeptide was subsequently isolated in pure, soluble form and analyzed structurally by solution NMR spectroscopy. Due to the non-satisfying quality and resolution of the spectra of this 27 kDa protein, an almost complete backbone assignment became feasible only by the combination of uniform and novel amino acid-selective labeling schemes. Moreover, amino acid-type selective triple-resonance NMR experiments proved to be very helpful.

1. Introduction

Anthrax, a disease caused by the Gram positive bacterium *Bacillus anthracis*, most commonly infects domestic animals and only rarely affects humans. However, it is thought to pose a great danger for public health if it would be ever used as a biological weapon [1]. The host can get infected by various ways, however the severity of the disease is highly dependent on the way of the infection. Infection through inhalation is often fatal for the host [1].

The toxicity of *B. anthracis* is mainly due to two factors, namely its anti-phagocytic, polyglutamic acid capsule [2] and the secretion of an exotoxin, called anthrax toxin [3–5]. This toxin is composed of three separate proteins, (a) the protective antigen (PA), (b) the lethal factor (LF) and (c) the edema factor (EF). When alone, these proteins do not exhibit any toxicity, while when together they manage to disrupt the physiological cell cycle and finally lead to apoptosis of the infected cells. PA creates an octamer and binds to the cell membrane, subsequently creating a transmembrane pore, through which LF and EF enter the cell. LF is a Zn^{2+}-dependent metalloprotease with four domains. It uses domain I (N-terminal; N-ALF) to interact and assemble with PA in order to enter the cell, a compulsory step for LF toxicity [6]. LF exhibits its proteolytic activity through domain IV (C-terminal; C-ALF), which contains the Zn(II) catalytic site and the substrate cavity. C-ALF seems to selectively recognize and catalyze the hydrolysis of the N-terminus of mitogen-activated protein kinase kinases (MAPKK), thus disturbing the physiological cellular signaling, life cycle and cell migration [6].

Here we present the preliminary structural determination of a 233-residue polypeptide derived from the N-terminal domain of Anthrax Lethal Factor (N_ALF_{233}) via solution NMR spectroscopy coupled with amino-acid selective labeling, including the efficient simultaneous labeling of two amino acids in one NMR sample. N-ALF comprises residues 1–263 of the full length protein, however the first 26 residues are invisible in the LF crystal structure and are thought to be disordered [7]. Three interaction sites have been identified between the N-ALF domain and the PA pore, one of them involving some of those unstructured residues (2–5) [8]. To simplify the NMR spectra for structure determination, the N-terminal disordered residues are not included in our NMR construct N_ALF_{233} that encompasses residues 28–260 of LF and has a MW of ~27 kDa. Information about the solution structure and dynamics of N-ALF is important for understanding the structural basis of its interaction with the PA pore.

Solution NMR spectroscopy is a valuable method to determine the three-dimensional structure of proteins up to a molecular

Abbreviations: ALF, Anthrax Lethal Factor; TROSY, transverse relaxation optimized spectroscopy; *IPTG*, isopropyl β-D-1-thiogalactopyranoside; N-/C-ALF, N-/C-terminal domain of Anthrax Lethal Factor.

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weight of ~30 kDa. In a typical workflow protein expression on a mg-scale, sample preparation, data acquisition and processing are followed by the sequence-specific assignment of the protein. The latter step becomes increasingly difficult for proteins above ~20 kDa due to limited chemical shift dispersion and/or spectral resolution. One solution to this problem is the use of different labeling schemes.

The full or partial replacement of the non-exchangeable protons with ^{2}H is a standard approach to improve spectral resolution [9] via an increase of relaxation times. An alternative labeling approach is the amino-acid selective labeling or unlabeling, which assists in the unequivocal assignment of chemical shifts to the nuclei of specific amino acid residues [10–12]. This usually takes place by adding a single labeled/non-labeled amino acid to the culture medium, without or with labeled sources of nitrogen/carbon added as well. The main difficulties of this non-uniform labeling approach are the high cost of labeled amino acids, the number of samples that have to be prepared, and the risk of cross-labeling which is due to the crosslinking of metabolic pathways of the amino acids in the cell [13].

To diminish undesired cross-labeling effects the auxotrophic *Escherichia coli* strain DL39, lacking transaminase activity had to be used. By fine-tuning the quantities of unlabelled amino acids in the medium the selective labeling of a mainly helical 27 kDa protein with two hydrophobic residues (^{15}N-Phe/^{15}N-Leu, ^{15}N-Ile/^{15}N-Val and ^{15}N-Tyr/^{15}N-Ala) was achieved. Additionally, an optimized protocol allowed for the incorporation of ^{15}N-labeled glutamate with limited cross-labeling.

These labeling protocols greatly facilitated the sequence-specific assignment and the solution NMR characterization of a 233-residue polypeptide derived from the N-terminal domain of Anthrax Lethal Factor (N_ALF$_{233}$).

Amino-acid selective labeling combined with transverse relaxation-optimized spectroscopy (TROSY) [14] was crucial to extend the assignment of $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ resonances of N_ALF$_{233}$ from 69% to ~85% and to determine its secondary structure in solution.

2. Materials and methods

The chemical shifts of the assigned N_ALF$_{233}$ $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ resonances have been deposited in the BMRB data bank (http://www.bmrb.wisc.edu; accession code 19803).

2.1. Uniform ^{15}N, $^{15}N/^{13}C$ and $^{2}H/^{15}N/^{13}C$ labeling using the prototrophic E. coli strain DL21(DE3)

E. coli BL21(DE3) cells that had been transformed with the N_ALF$_{233}$ expression vector (pGEX.4T$_1$) were grown at 37 °C and 170 rpm (rounds per minute) in M9 minimal medium containing 1 g $(NH_4)_2SO_4$, 3 g D-glucose, 2 ml solution Q (40 mM HCl, 50 mg/l $FeCl_2{\cdot}4H_2O$, 184 mg/l $CaCl_2{\cdot}2H_2O$, 64 mg/l H_3BO_3, 18 mg/l $CoCl_2{\cdot}6H_2O$, 340 mg/l $ZnCl_2$, 605 mg/l $Na_2Mo_4{\cdot}2H_2O$, 40 mg/l $MnCl_2{\cdot}4H_2O$), 1 ml 1 M $MgSO_4$, 1 ml biotin (0.5 mg/ml), 1 ml thiamin (0.5 mg/ml), 1 ml $^{2}H/^{15}N/^{13}C$ Bioexpress Cell Growth Media™ and 100 μg/ml ampicillin. According to the desired labeling scheme, $^{15}NH_4Cl$ and $^{13}C_6$ D-glucose were used. In case of a perdeuterated sample, H_2O was replaced with 98% D_2O and deuterated $^{13}C_6$ D-glucose was used as a carbon source. At O.D.$_{600\,nm}$ ≈ 0.6, protein expression was induced by addition of 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) (final concentration). Cells were further incubated for 4 h. Finally, cultures were centrifuged at 4 °C and 5000*g* for 10 min, and the cell pellet stored at −20 °C until protein purification.

2.2. Partial ^{2}H and uniform $^{15}N/^{13}C$ labeling using the prototrophic E. coli strain BL21(DE3)

E. coli BL21(DE3) cells containing the expression vector pGEX.4T$_1$ were grown in LB medium made with 50% D_2O until O.D.$_{600\,nm}$ reached 1.0. Cells were harvested and stored at −20 °C. On the following day, 1 l M9 minimal medium in 70% D_2O and 30% H_2O was prepared, containing 1 g $(^{15}NH_4)_2SO_4$, 3 g $^{13}C_6$ D-glucose, 2 ml solution Q (40 mM HCl, 50 mg/l $FeCl_2{\cdot}4H_2O$, 184 mg/l $CaCl_2{\cdot}2H_2O$, 64 mg/l H_3BO_3, 18 mg/l $CoCl_2{\cdot}6H_2O$, 340 mg/l $ZnCl_2$, 605 mg/l $Na_2Mo_4{\cdot}2H_2O$, 40 mg/l $MnCl_2{\cdot}4H_2O$), 1 ml 1 M $MgSO_4$, 1 ml biotin (0.5 mg/ml), 1 ml thiamin (0.5 mg/ml), 1 ml $^{2}H/^{15}N/^{13}C$ Bioexpress Cell Growth Media™ and 1 ml ampicillin (0.1 g/ml). Cells stored at −20 °C were added to this medium till O.D.$_{600\,nm}$ reached 0.3. The culture was incubated at 37 °C and 180 rpm. When O.D.$_{600\,nm}$ reached 0.6–0.9, protein expression was induced by adding IPTG to final concentration of 1 mM, and cells were harvested 4 h later.

2.3. Multiple selective ^{15}N-labeling with ^{15}N-Phe/^{15}N-Leu,^{15}N-Ile/^{15}N-Val and ^{15}N-Tyr/^{15}N-Ala using the auxotrophic E. coli strain DL39

E. coli DL39 cells were transformed by the expression vector pGEX.4T$_1$. A 1 l culture of LB medium was inoculated with 10 ml of LB preculture, which had been incubated at 37 °C overnight in the presence of ampicillin (100 μg/ml). When O.D.$_{600\,nm}$ reached ≈1, the cells were harvested through centrifugation at 4 °C and 8000 rpm for 20 min, and stored at −20 °C overnight. M9 minimal medium was prepared on the following day, containing 1 g $(NH_4)_2SO_4$, 5 g D-glucose, 4 ml solution Q (see above), 1 ml 1 M $MgSO_4$, 2 ml biotin (0.5 mg/ml), 2 ml thiamin (0.5 mg/ml), 1 ml ampicillin (0.1 g/ml), 150 mg Phe, 200 mg Leu, 90 mg Tyr, 400 mg Asp, 200 mg Ile, 200 mg Val, 400 mg Ala, 200 mg Asn, 500 mg Gly,100 mg Met and 210 mg Lys per liter. According to the planned labeling scheme either Phe and Leu, Val and Ile, or Tyr and Ala were added as ^{15}N labeled compounds. This medium was inoculated with *E. coli* DL39 cells containing the expression vector pGEX.4T$_1$ (stored at −20 °C) up to O.D.$_{600\,nm}$ ≈ 0.3 and the culture was incubated at 37 °C and 180 rpm. At O.D.$_{600\,nm}$ ≈ 0.6–0.7 IPTG was added to a final concentration of 1 mM to induce protein expression. The cells were further incubated for 4 h, after which the cells were harvested as above and stored at −20 °C till cell lysis.

2.4. Selective ^{15}N-Glu labeling using the auxotrophic E. coli strain DL39

The procedure for selective labeling of N_ALF$_{233}$ with ^{15}N-Glu was the same as described above for the simultaneous selective labeling with two hydrophobic amino acids except for the composition of the M9 minimal medium: it contained 1 g $(NH_4)_2SO_4$, 5 g D-glucose, 4 ml solution Q (see above), 1 ml 1 M $MgSO_4$, 2 ml biotin (0.5 mg/ml), 2 ml thiamin (0.5 mg/ml), 1 ml ampicillin (0.1 g/ml), 1067 mg ^{15}N–Glu, 150 mg Phe, 90 mg Tyr, 400 mg Leu, 400 mg Asp, 200 mg Ile, 200 mg Val, 400 mg Asn, 400 mg Ala, 500 mg Gly, 100 mg Met, 210 mg Lys, 220 mg Arg, 430 mg Ser and 198 mg Thr.

2.5. Purification and sample preparation of N_ALF$_{233}$

After thawing and resuspending the cell pellet in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing bacterial protease inhibitor cocktail (Sigma Aldrich®), the suspension was sonicated (Misonix®, Sonicator 4000) and centrifugated at 4 °C and 40,000 rpm (Beckman 60 Ti rotor) for 10 min. The soluble fraction containing the GST-tagged recombinant protein was loaded onto a GSTrap FF 5 ml affinity column (Amersham Biosciences) that had been previously equilibrated with PBS buffer.

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