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NMR conformational properties 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.

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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Solution NMR spectroscopy is a valuable method to determine the three-dimensional structure of proteins up to a molecular 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 180 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 $O.D._{600\,nm} \approx 0.6$, 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 (see above), 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 1l 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 $O.D._{600\,nm} \approx 0.3$ and the culture was incubated at 37 °C and 180 rpm. At $O.D._{600\,nm} \approx 0.6$–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. The column was washed with 3 column volumes of PBS, in order

to remove all the proteins that were non-specifically bound to the column. Then, 4 ml PBS containing 50 cleavage U of thrombin protease (Amersham Biosciences® were loaded onto the column. Proteolytic cleavage of N_ALF$_{233}$ from the GST fusion was achieved by incubation with thrombin at room temperature for 16 h. The cleaved N_ALF$_{233}$ polypeptide was eluted by washing the column with 10 ml PBS. The protein sample was further purified in 50 mM KPi buffer (pH 7) using an Äkta *FPLC* system equipped with a size exclusion column (Superose 12_10/300). The purity of the polypeptide was checked by Tris–glycine SDS–PAGE in a 17% polyacrylamide gel, followed by visualizing the protein bands with Coomassie Blue R-250 (Fig. 1).

The NMR sample was subsequently concentrated to 500 µl in 50 mM KPi buffer (pH 7) using an Amicon® Ultra 15 ml Centrifugal Filter membrane (nominal molecular weight limit 10,000 Da). The concentration of the polypeptide was determined photometrically using an extinction coefficient of 22,350 at 280 nm as estimated by the software PROTPARAM (www.expasy.org/tools/protparam.html).

2.6. NMR spectroscopy

Heteronuclear NMR spectra of N_ALF$_{233}$ were recorded at 298 K on a Bruker Avance III High-Definition, four-channel 700 MHz NMR spectrometer equipped with a cryogenically cooled 5 mm ^{1}H-^{13}C/^{15}N/D Z-gradient probe. Sequence specific assignments were obtained from the following experiments: 2D [^{1}H–^{15}N]-HSQC and TROSY, 3D TROSY-HNCA, 3D TROSY-HN(CO)CA, 3D TROSY-CBCA(CO)NH, 3D TROSY-CBCANH, 3D TROSY-HNCO, 3D TROSY-HN(CA)CO, 3D TROSY-HBHA(CO)NH, 3D HNHA, 3D ^{15}N-edited NOESY and modified versions of the 3D CBCA(CO)NH experiment for the effective correlation of [NH](i) and [CBCA](i-1) when the (i-1) residue lacks an aliphatic Cγ atom (Ala, Asn, Asp, Cys, Gly, Ser and aromatic residues) or a γCO (Ala, Cys, Ser and aromatic residues; both experiments are included in the Bruker pulse sequence library [15]). Internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as a chemical shift reference for ^{1}H. All NMR data were processed with the TOPSPIN 3.2/3.3 software and analyzed with CARA [16].

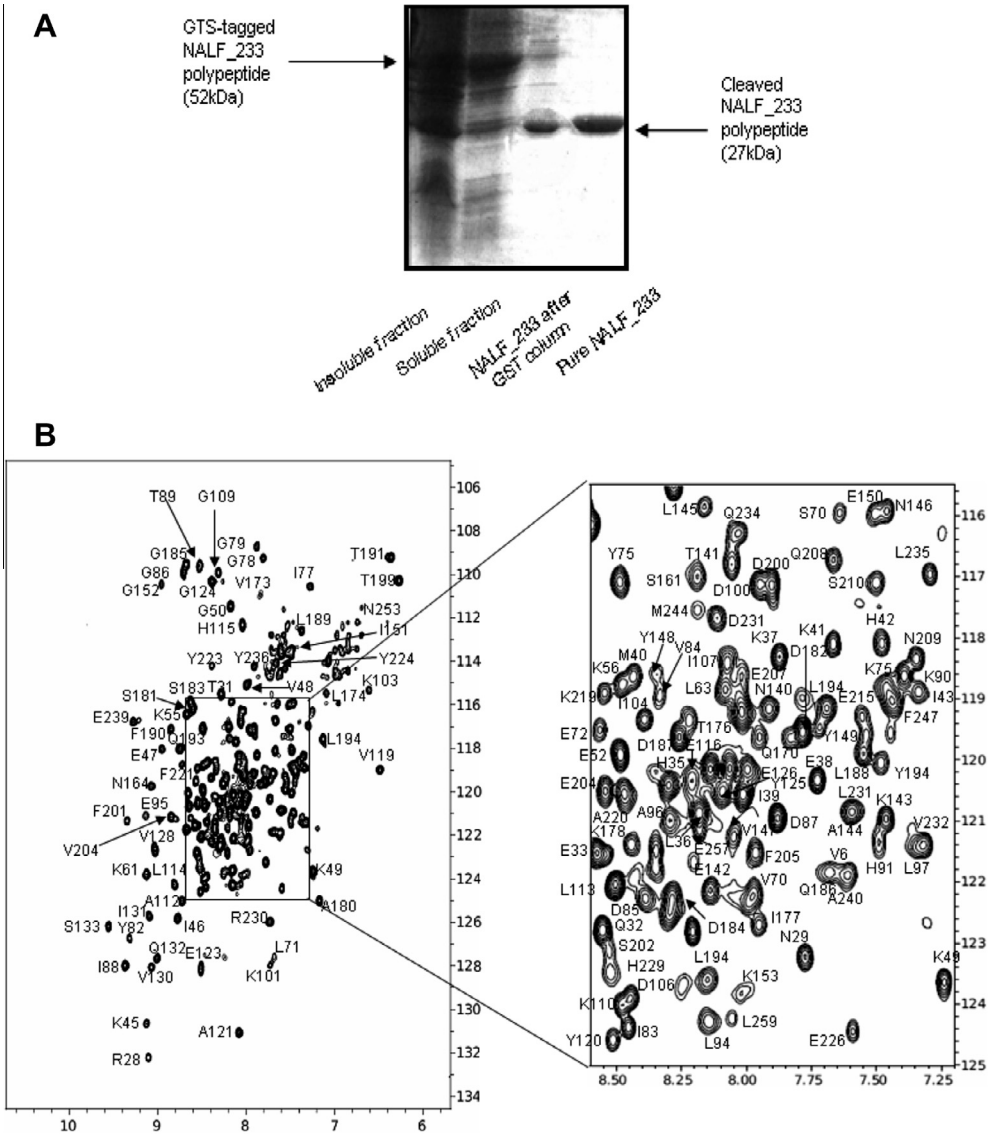

Fig. 1. (A) SDS PAGE analysis of various stages of the expression and purification of N_ALF$_{233}$, including samples from the soluble and insoluble fractions of the cells after sonication, the elution of the thrombin-cleaved polypeptide and the final protein sample after FPLC size exclusion chromatography. (B) 700 MHz ^{1}H–^{15}N TROSY spectrum of a perdeuterated ^{2}H/^{15}N/^{13}C sample N_ALF$_{233}$ at 298 K. The sequence-specific assignment is indicated.

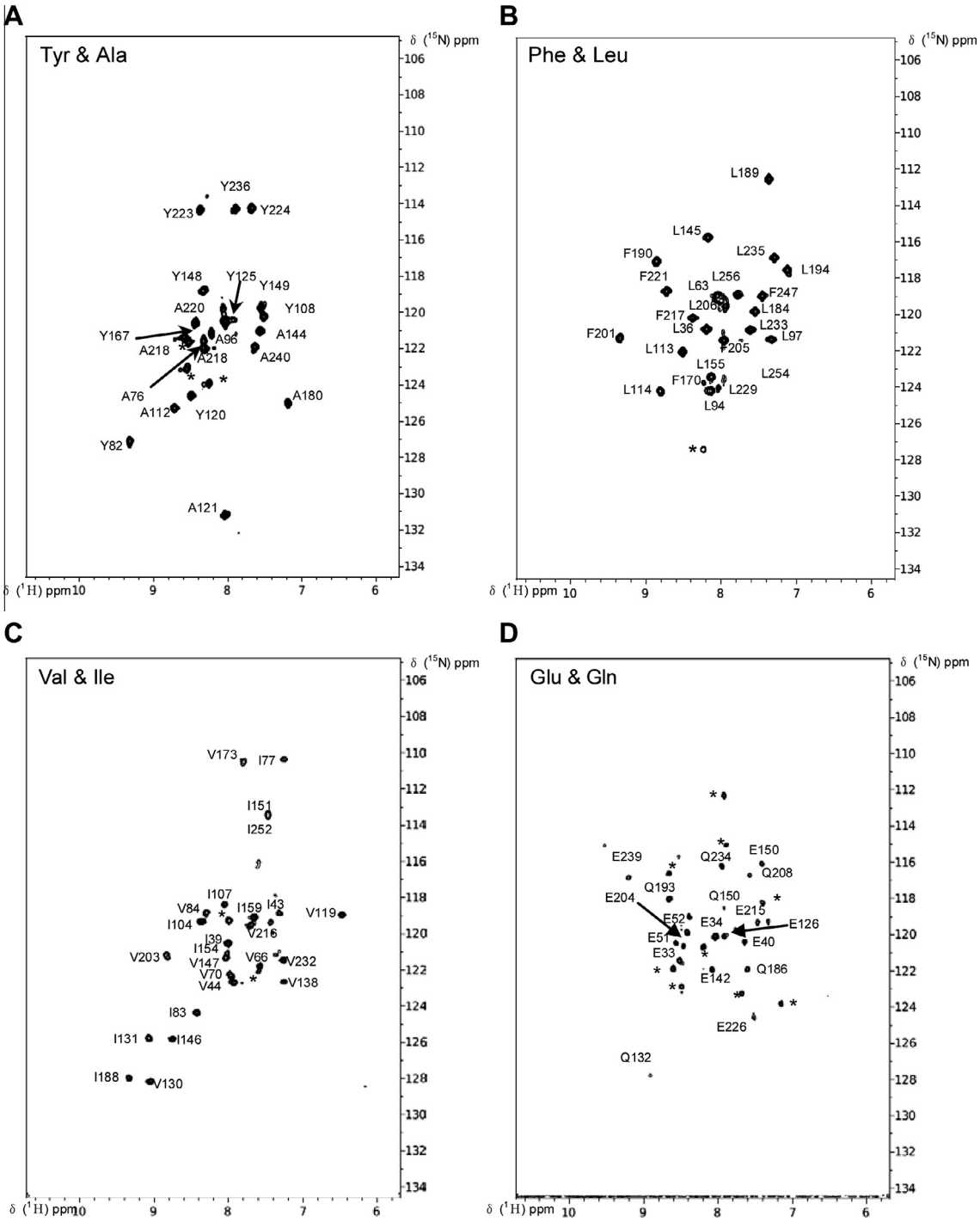

Fig. 2. 1H–15N TROSY spectra of amino acid-selectively 15N labeled N_ALF$_{233}$ samples. (A) ^{15}N Tyr and ^{15}N Ala labeled, (B) ^{15}N Phe and ^{15}N Leu labeled, (C) ^{15}N Val and ^{15}N Ile labeled, and (D) ^{15}N Glu and ^{15}N Gln labeled. Asterisks indicate cross-peaks, which do not correspond to the desired 15N-labeled amino acids.

3. Results and discussion

The recombinant polypeptide N_ALF$_{233}$ corresponding to the N-terminal domain of Anthrax Lethal Factor was successfully expressed as a GST fusion protein in *E. coli* and isolated from the soluble fraction of the cell lysate while it was also partly detected as insoluble in inclusion bodies (Fig. 1A). Using M9 minimal growth media N_ALF$_{233}$ was obtained at a yield of ~8 mg/l. After GST affinity chromatography and on-column digestion of the fusion protein by thrombin, N-ALF$_{233}$ was further purified by size exclusion chromatography, which suggested that it is monomeric. The purity of the final product was verified by SDS PAGE analysis (Fig. 1A). Samples suitable for NMR spectroscopy were prepared by concentrating the protein up to 0.3 mM. Higher concentrations lead to precipitation.

The first ^{1}H–^{15}N TROSY spectrum of a ^{15}N-labeled N_ALF$_{233}$ sample showed a large chemical shift dispersion indicative of a folded polypeptide exhibiting a tertiary structure. The protein was stable and no signs of proteolytic degradation were observed in the spectra even after 7 days. However, as expected for a mainly helical polypeptide (according to the LF crystal structure (PDB entry 1J7N) [7]; UniProt entry code (P15917)) with MW ≈ 27 kDa

there was heavy overlap of peaks in the central region of the spectrum. Therefore, various labeling techniques were applied to achieve a nearly complete backbone assignment of N_ALF$_{233}$.

First, a perdeuterated ^{15}N/^{13}C-N_ALF$_{233}$ sample was obtained which gave a significantly improved TROSY spectrum (Fig. 1B) displaying almost all of the theoretically expected 227 backbone amide cross peaks (six of the 233 residues are prolines). The spectra of this sample allowed the backbone assignment of 160 out of 233 residues (69%). To confirm and extend this assignment, amino-acid selective ^{15}N-labeling was applied. More specifically, using the auxotrophic strain *E. coli* DL39 we successfully incorporated (*i*) both ^{15}N-Phe and ^{15}N-Leu (Fig. 2A), (*ii*) both ^{15}N-Tyr and ^{15}N-Ala (Fig. 2B), as well as (*iii*) both ^{15}N-Ile and ^{15}N-Val (Fig. 2C) in ^{14}N-N_ALF$_{233}$ samples without observing any significant cross-labeling. To this end, the concentrations of the ^{14}N amino acids that have to be added to the M9 minimal medium (see Section 2) were meticulously screened for effective suppression of scrambling due to the metabolism of the two ^{15}N amino acids. In all three cases the yield of N_ALF$_{233}$ was comparable to the yield obtained with the prototrophic strain *E. coli* BL21(DE3). In the TROSY spectrum of the ^{15}N-Phe/^{15}N-Leu sample, 25 out of 31 expected peaks were observed (Fig. 2A), while the spectrum of ^{15}N-Tyr/^{15}N-Ala N_ALF$_{233}$ (Fig. 2B) contained 26 out of 28 expected peaks. In the case of ^{15}N-Ile/^{15}N-Val N_ALF$_{233}$ (Fig. 2C) all 34 peaks expected were observed. In each case, the two simultaneously labeled amino acids could be safely identified and assigned based on different ^{13}C$^{\alpha}$ and/or ^{13}C$^{\beta}$ chemical shift values. Overall, the dual labeling strategy leads to 18 new assignments of backbone amides.

It is well-known that it is extremely difficult to achieve amino acid selective labeling of glutamate due to its prominent role in bacterial metabolism [17]. Past efforts in our lab have demonstrated that the addition of ^{15}N-Glu to unlabeled M9 medium leads to an HSQC spectrum very similar to that of a uniformly ^{15}N labeled sample because of extensive scrambling of the label. While efforts to reduce scrambling have been reported to the literature, the addition of all the amino acids along with labeled source of nitrogen was necessary[18], while we managed to highly reduce cross-labeling in a simpler, cost effective way by using auxotroph *E. coli* strain and adding only a few amino acids. Cross-labeling could be suppressed to a very high degree by two modifications to our protocol for the simultaneous ^{15}N labeling of two amino acids that are based on previous observations of those amino acids exhibiting severe cross-labeling when ^{15}N-Glu had been added to the culture: (i) the quantities of Leu and Asn were increased, and (ii) Arg, Ser and Thr [19] which are not necessary for the growth of the auxotrophic strain were added to the medium along with an excess of ^{15}N-Glu. The obtained TROSY spectrum (Fig. 2D) showed a significant improvement. While N_ALF$_{233}$ contains 30 glutamate residues, 28 peaks were observed. However, cross-labeling was not completely eliminated, mainly due to the conversion of ^{15}N-Glu to ^{15}N-Gln (Fig. 2D). Moreover, several of the peaks show in the 3D spectra ^{13}Cα and/or ^{13}Cβ chemical shift values that are non-typical for Glu- or Gln-residues. This scrambling of the ^{15}N label was found to be non-systematic, as the peaks that do not correspond to Glu/Gln residues were assigned to different residue types.

With all these labeling efforts about 85% of the backbone amide resonances (^{1}H^{N} and ^{15}N) of N_ALF$_{233}$ could be assigned. In order to extend this assignment further, a partially deuterated ^{15}N/^{13}C sample was prepared according to protocols from literature [20], replacing M9 minimal medium with LB medium (50% D_2O) in the first culture and finally inducing protein expression in M9 minimal medium containing 70% D_2O. It was estimated that 45% of the non-exchangeable protons were replaced with deuterium. This partial replacement of non-exchangeable protons with ^{2}H allowed the acquisition of two selective CBCA(CO)NH spectra [15] which provide correlations of a backbone amide group with the side chain of the preceding residue when the latter lacks either an aliphatic γC atom or a γCO atom, thus facilitating considerably the identification of Asn, Asp, Cys, Ser, Ala and Gly residues as predecessors in the sequence.

Taking into account all this information, we obtained an almost complete assignment of the backbone amides (96%) and extended the overall assignment of ^{1}H^{N}, ^{15}N, ^{13}C$^{\alpha}$, ^{13}C$^{\beta}$ and ^{13}C$'$ chemical shifts to ~85%. These chemical shifts were analyzed with TALOS+ (http://spin.niddk.nih.gov/bax/software/TALOS/; [21]) which predicts the backbone dihedral angles Φ and Ψ and thus is able to identify the secondary structural elements of the polypeptide. According to TALOS+, 53.2% of the residues in N_ALF$_{233}$ have a helical conformation (Fig. 3). This number is in good agreement with the 59.7% helical content of the N-terminal domain of LF in the crystal structure of the full-length protein (PDB entry 1J7N) [7]. More specifically, the backbone chemical shifts of N_ALF$_{233}$ identify 18 helices that coincide well with the helices in the crystal structure of the full-length protein (Fig. 3). The 6 short β-strands

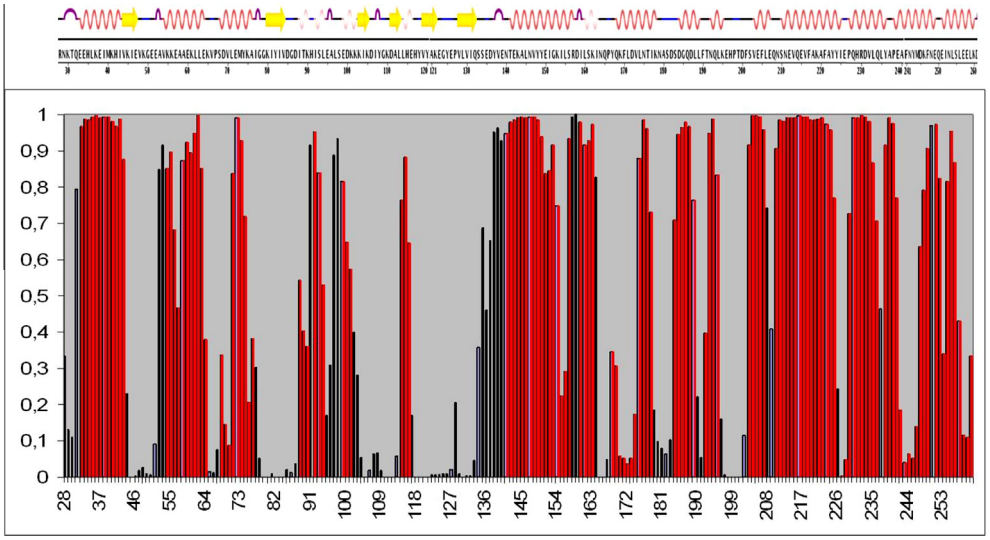

Fig. 3. Secondary structure analysis of N_ALF$_{233}$. The propensity of each residue to exhibit a helical conformation as calculated by TALOS+ is compared with its secondary structure in the crystal structure of full-length ALF protein (cartoon in the top row). The bars in red correspond to those residues that have been shown to actually form α-helixes in the crystal structure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the N-terminal domain of crystallized LF were not found by TALOS+, which is most likely due to incomplete assignment of the residues involved in these strands.

In summary, we report the expression in *E. coli* and purification of a recombinant 233-residue protein corresponding to the N-terminal domain of Anthrax Lethal Factor (N_ALF_{233}). The protein is a stable, well-folded monomer in solution and was structurally analyzed by NMR spectroscopy. Due to the relatively high MW of ~27 kDa the sequence specific assignment could be completed only by means of various labeling techniques including partial and complete deuteration and amino-acid selective ^{15}N-labeling, which were carried out by modifying some commonly used protocols. This allowed the application of a dual labeling approach, which is clearly more time- and cost-effective. Finally, TALOS+ identified the mainly helical secondary structure of N_ALF_{233} that is very similar to that of N-ALF in the crystal structure of full-length LF.

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