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NMR Assignments of the N-terminal Signaling Domain of the TonB-dependent Outer Membrane Transducer PupB

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Abstract

Outer membrane TonB-dependent transducers (TBDTs) actively transport ferric siderophore complexes from the extracellular environment into Gram-negative bacteria. They also participate in a cell-surface signaling regulatory pathway that results in upregulation of the transducer itself, in response to iron-deplete conditions. The TBDT PupB transports ferric pseudobactin, and signals through its N-terminal signaling domain (NTSD), while the TBDT homolog PupA is signaling-inactive. Here, we report the NMR chemical shift assignments of the PupB-NTSD. This information will provide the basis for structural characterization of the PupB-NTSD to further explore its signaling properties.

Biological context

Gram-negative bacteria maintain iron homeostasis via transcriptional activation of iron transport proteins under iron-limited conditions to uptake extracellular ferric complexes into the cell across the outer and inner membranes (Hantke, 1981). This active transport process relies upon the TonB:ExbB:ExbD inner membrane complex to activate the outer membrane transporters; hence, their designation as TonB-dependent (Kadner & McElhaney, 1978). A subset of these transporters have been characterized as “transducers,” as in addition to mediating the active uptake of ferric molecules, they activate a cell surface signaling (CSS) pathway that results in their own transcriptional up-regulation (Koebnik, 2005). Signaling by this pathway is dependent upon interaction of an N-terminal signaling domain (NTSD) of the transducer with the periplasmic domain of an inner membrane sigma regulator (Harle *et al.*, 1995). In the Pup signaling and transport pathway in *Pseudomonas capeferrum* (formerly *Pseudomonas putida* WCS358), two outer membrane proteins, PupB and PupA, are signaling-competent or incompetent, respectively, although both have similar transport functions (Koster *et al.*, 1994). In the context of full-length PupA, replacement of the PupA-NTSD with the PupB-NTSD maintains transport but also restores signaling-competence; conversely, replacement of the PupB-NTSD with the PupA-NTSD in full-length PupB

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retains transport activity but renders the protein signaling-incompetent (Bitter *et al.*, 1991, Koster *et al.*, 1993, Koster *et al.*, 1994).

Previously, NMR spectroscopy was used to determine the solution structures of the PupA-NTSD and the *E. coli* ortholog FecA-NTSD (Garcia-Herrero & Vogel, 2005, Ferguson *et al.*, 2007). Additionally, the structure of the full-length *Pseudomonas aeruginosa* transducer, FpvA, including its NTSD, was determined by X-ray crystallography (Cobessi *et al.*, 2005, Brillet *et al.*, 2007, Wirth *et al.*, 2007, Greenwald *et al.*, 2009). All known structures of NTSDs possess similar secondary and tertiary structural elements. Therefore, the PupB-NTSD was proposed to contain the conserved $\beta\alpha\beta$ - $\beta\alpha\beta\beta$ motif, despite sharing only 33% sequence identity and 39% sequence similarity with the PupA-NTSD. Thus, the differences in signaling activity between the two Pup NTSDs must relate to divergence in primary sequence.

To characterize the structure of the NTSDs, as well as correlate structural differences between the PupA and PupB-NTSDs to variations in functionality, we have obtained NMR chemical shift assignments of the PupB-NTSD.

Methods and experiments

The NTSD of PupB from *Pseudomonas caeferrum*, amino acids 49-128, was synthesized and codon optimized (GenScript) into pUC57. The PupB-NTSD fragment was excised using *Bam*HI and *Xho*I exonucleases and subcloned into a modified pGEX vector, which included a TEV protease recognition site (ENLYFQG) between the glutathione S-transferase (GST) tag and the PupB-NTSD sequence.

E. coli BL21(DE3)pLysS cells, transformed with the pGEX-PupB-NTSD vector, were used for protein expression. Cells were grown in LB medium with 100 μ g/mL ampicillin to an OD₆₀₀ = 0.6-0.8, as measured using an Implen Nano-Photometer Classic (Implen, Inc.). Protein expression was induced by addition of 0.5 mM IPTG, followed by overnight expression at 20°C. Cells were harvested by centrifugation at 4000 *g* and cell pellets stored at -80°C until use. Uniform isotope labeling was obtained by expressing protein as described above, except that cells were grown in M9 minimal medium containing 3 g/L [U-¹³C₆]-D-glucose and 1 g/L ¹⁵NH₄Cl (Cambridge Isotope Laboratories, Inc.).

Cell pellets were suspended in lysis buffer (25 mM Tris pH 7.8, 150 mM NaCl, 2 mM DTT) and disrupted in a Nano DeBEE homogenizer (BEE International) at 15,000 psi. Cellular debris was separated by centrifugation at 20,000 *g* for 45 min. Clarified lysate was applied to Glutathione Sepharose 4 Fast Flow resin (GE Lifesciences) and washed with lysis buffer. Recombinant TEV protease was applied to the column in a 1:10 TEV protease:fusion protein mass ratio, then incubated for 16 h at 4 °C to remove the GST tag. Cleaved PupB-NTSD was washed off the column with lysis buffer. The NTSD was further purified by gel filtration on a Superdex 200 column (GE Lifesciences) in lysis buffer without DTT, then concentrated to 15-20 mg/mL (1.8-2.5 mM). Mass spectrometry analysis was used to verify that this purification protocol yielded the expected 82-residue product comprising of 80 residues corresponding the NTSD, preceded by 2 N-terminal residues, Gly and Ser, which

were artifacts of expression construct design. Concentrated protein was flash-cooled in LN₂ and stored at -80 °C. Typical protein yields were 7 mg of PupB NTSD per L of culture in LB media and 4 mg/L from expression in M9 minimal media. The purity of the collected protein was estimated to be >95% by SDS-PAGE stained with Coomassie Blue. Protein concentration was determined by measuring absorbance at 280 nm ($\epsilon_{280} = 2980 \text{ M}^{-1} \text{ cm}^{-1}$); the theoretical molecular weight of WT PupB-NTSD is 8101 g/mol.

NMR spectroscopy

500 μL of 50 – 100 μM U-¹⁵N-labeled PupB SD, or 500 μM – 1 mM U-¹⁵N/¹³C-labeled PupB-NTSD dialyzed into 50 mM Na phosphate pH 6.0, 100 mM NaCl overnight at 4 °C was utilized for data collection and chemical shift assignment (NTSD = 82 residues). Prior to data collection, D₂O was added to a final concentration of 10% v/v. NMR data were recorded at 25 °C on Agilent DD2 600 and 800 MHz spectrometers equipped with triple-resonance cryogenic probes. The following double and triple resonance experiments were performed: ¹H-¹⁵N HSQC, ¹H-¹³C HSQC (all carbon), ¹H-¹³C HSQC (aliphatic carbon only), HNCO, HNCACB, CBCA(CO)NH, C(CO)NH, H(CCO)NH, HCCH TOCSY, HNHA, HBCB(CGCD)HD, HBCB(CGCDCE)HE, 2D ¹H-¹H NOESY, ¹H-¹⁵N HSQC-NOESY, and ¹H-¹³C HSQC-NOESY. NMR data were processed with NMRPipe (Delaglio *et al.*, 1995) and displayed with NMRViewJ.

Assignments and data deposition

Backbone chemical shift assignments were initially performed via semi-automation with RunAbout in NMRViewJ (Johnson & Blevins, 1994, Johnson, 2004), then transferred for continued manual assignment in CCPNmr (Vranken *et al.*, 2005). Sequential assignment of backbone chemical shifts were accomplished with through-bond experiments: 3D HNCO, HNHA, HNCACB, and CBCA(CO)NH (Bax & Ikura, 1991, Constantine *et al.*, 1993). Side chain resonances were assigned from CBCA(CO)NH, H(CCO)NH, C(CO)NH, HCCH TOCSY, HBCB(CGCD)HD, and HBCB(CGCDCE)HE spectra (Cavanagh *et al.*, 2007). Aromatic side chain assignments were obtained from HBCB(CGCD)HD, HBCB(CGCDCE)HE, and 2D ¹H-¹H NOESY experiments. Backbone chemical shifts were analyzed with TALOS+ to predict secondary structure elements (Shen *et al.*, 2009).

All backbone atoms were assigned, with the exception of the 4 proline residues which lack backbone amide hydrogens. Overall, 97.3% of all hydrogens, 89.6% of all carbons, and 85.0% of all nitrogens were assigned. The remaining unassigned atoms are primarily Asp C_T, Arg guanidine groups, and some Tyr or Phe sidechain protons.

CheckShift (<http://www.checkshift.services.came.sbg.ac.at>) was utilized to assess the quality of chemical shift assignments, by comparison of the target chemical shifts to the reference, DSS, set to 0 ppm (Ginzinger *et al.*, 2007, Ginzinger *et al.*, 2009). CheckShift output includes the target shifts with reference correction. Proposed chemical shift offset values were -0.02, +0.02, -0.29, +0.35, and 0 for N, C, C _{β} , C _{α} , and H, respectively, indicating that referencing was consistent and accurate, with respect to the reference chemical shifts.

Analysis of backbone chemical shifts by TALOS+ indicated that the PupB-NTSD possesses the $\beta\alpha\beta$ - $\beta\alpha\beta\beta$ motif that has been observed in other NTSDs (Fig. 2A and Figure 2B).

Structure calculations, as well as detailed investigation of structural and biochemical differences of the PupB-NTSD compared to the PupA-NTSD, are ongoing.

The ^1H , ^{15}N , and ^{13}C chemical shift assignments have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 27141.

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Fig. 1. ^1H - ^{15}N HSQC spectrum of the PupB-NTSD. The spectrum was recorded on a 600 MHz Agilent DD2 spectrometer at 25 °C. Backbone resonance assignments are indicated using single letter codes. Signals boxed in red are likely folded arginine side chain ϵ -NH resonances from the three arginine residues encoded in the PupB-NTSD. Horizontal lines indicate glutamine and asparagine side chain NH_2 resonances. NTSD numbering begins at residue 49, pertaining to full-length PupB, including the signal peptide. Residue S48 derives from the expression construct.

Fig. 2.

Primary sequence alignment of the PupB- and PupA-NTSDs (A). Identical residues are shaded in black and similar residues in grey. The blue arrows and red cylinders indicate consensus β -strands and α -helices, respectively. Comparison of secondary structure elements from the PupA-NTSD NMR structure (PDB 2A02) with the probabilities for secondary structural elements for the PupB-NTSD as indicated by TALOS+ (B). Consensus β -strands and α -helices in (A) were determined from this analysis.