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Chemical shift assignments and secondary structure prediction of the phosphorelay protein VanU from *Vibrio anguillarum*

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Abstract

Vibrio anguillarum is a biofilm forming Gram-negative bacterium that survives prolonged periods in seawater and causes vibriosis in marine life. A quorum-sensing signal transduction pathway initiates biofilm formation in response to environmental stresses. The phosphotransferase protein VanU is the focal point of the quorum-sensing pathway and facilitates the regulation between independent phosphorelay systems that activate or repress biofilm formation. Here we report the 1H, 13C, and 15N backbone and side chain resonance assignments and secondary structure prediction for VanU from *V. anguillarum*.

Keywords

VanU; NMR; Vibrio anguillarum; Phosphorelay

Biological context

Vibrio anguillarum is a Gram-negative marine pathogen causing the disease known as vibriosis/septicaemia in a variety of salmonid fish. It is known to have a particularly high mortality rate (Frans et al. 2011), with antibiotic resistant strains emerging. Like many other vibrios, *V. anguillarum* contains a sophisticated quorum-sensing pathway ensuring its survival under the harsh conditions of the sea. This pathway eventually results in the formation of biofilms to help ensure bacterial survivability (Frans et al. 2011). *V. anguillarum*'s quorum-sensing pathway is comprised of six main components (Croxatto et al. 2004; Weber et al. 2011). VanN, VanP/Q, and CqsS are hybrid histidine kinases (containing both histidine kinase and response regulator domains), all of which are able to phosphorylate the phosphotransferase protein VanU. After becoming phosphorylated, VanU transfers the phosphoryl group to the response regulator VanO. VanO is a σ-54 dependent response regulator that once phosphorylated (in conjunction with the alternative σ-54 factor RpoN) induces expression of four small regulatory RNA molecules termed Qrr1-4. In turn, Qrr1-4 (in conjunction with RNA chaperon Hfq) destabilize *vanT* mRNA and represses the expression of the quorum-sensing transcriptional regulator VanT. The functional impact is inhibition of protease activity, pigment, serine production and biofilm formation (Croxatto et al. 2004; Weber et al. 2008). The phosphotransferase protein VanU is the convergence point

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in this quorum-sensing pathway. The independent quorum-sensing histidine kinase responses are channeled through this single protein (Weber et al. 2011). VanU is of significance as this convergence point protein regulates Vibrio's ability to survive long periods of time under harsh marine conditions through the development of biofilms. Consequently, structural studies of VanU are important for characterizing the mechanisms of biofilm formation in *V. anguillarum* and their outbreaks in marine life.

VanU serves a dual purpose as a phosphoreceiver and phosphodonor in the quorum-sensing pathway in V. *anguillarum. vanU* mutants have been shown to shorten the half-lives of *vanT* mRNA leading to a decreased VanT expression and thus biofilm formation (Weber et al. 2011). Due to VanU's critical role in the quorum sensing pathway and biofilm formation, VanU is of significant structural interest. VanU is a 12.5 kDa protein hypothesized to exist as a functional monomer. An up-down-up-down four-helix bundle core with the purported site of histidine phosphorylation located at a position on the second helix is predicted from secondary structure predictions and homologous structures (Ulrich et al. 2005). As a first step toward solving the NMR solution structure of VanU (112 residues, 12.5 kDa), we report nearly complete sequence specific backbone and side chain chemical shift assignments of VanU from *V. anguillarum*.

Methods and experiments

VanU (residues 1–112) from *V. anguillarum* was cloned into expression vector pGEX-4T2 (GE Health Care) with a Thrombin cleavable N-terminal GST fusion tag and transformed into BL21(DE3) cells (VWR) for expression. To uniformly label VanU with $^{13}C/^{15}N$, cells were grown in 1 L of M9 medium supplemented with $^{15}NH_4Cl$ and/or ^{13}C-glucose (CIL and Sigma Aldrich) at 33°C. At OD_{600} of~0.7 the culture was induced with 1 mM IPTG and allowed to grow for 8 hours. The cells were harvested by centrifugation at 7,000 rpm for 15 min. Cell pellets were suspended in lysis buffer (PBS, 1 mM DTT, 1 mM EDTA and 0.02% sodium azide and sonicated 20 times, for two minute pulses with 5 minutes rest in between. The resulting cell lysate was clarified by centrifugation at 13,500 rpm and the resulting supernatant was loaded on to GST-Bind resin (Novagen). The GST tag was removed from the purified protein by incubation with Thrombin (calbiochem). Samples for NMR experiments were buffer exchanged into NMR buffer (25mM Tris-HCl, 100mM KCl,, 1 mM DTT, pH 6.5 and 0.02% sodium azide, in 10 and 100% D_2O) at pH 6.0 and concentrated to 500 μM – 1 mM.

All NMR experiments were performed at 298 K on a Varian Inova 600 MHz and Bruker Avance 700 MHz, both equipped with cryoprobes. Backbone chemical shifts were assigned in a sequential manner from [^{15}N–^{1}H] HSQC, HNCO, HN(CA)CO, CBCA(CO)NH, HNCACB, and a CC(CO)NH experiments. Sidechain proton chemical shifts were assigned using HBHA(CO)NH, (H)CC(CO)NH, ^{15}N-TOCSY (50 and 100 ms), and an HCCH-TOCSY experiments. Aromatic assignments were made from a ^{13}C-HSQC, (HB)CB(CGCD)HD, and (HB)CB(CGCDCE)HE. Data was processed using NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson and Blevins 1994). Phi/Psi dihedral angles and resulting secondary structure prediction was calculated using the program TALOS+ (Shen et al. 2009).

Assignments and data deposition

Complete backbone amide assignments have been obtained for all non-proline residues 2–114 as shown in the 2D [^{1}H–^{15}N] HSQC spectrum in Fig. 1. Near complete backbone resonances for Cα (99%), Cβ (100%), C′ (99%), Hα (99%) have been assigned along with 98% of all side chain atoms (excluding aromatic side chains). VanU consists of 112 residues

(12.5 kDa) potentially forming a 4-helix bundle. A simple sequence BLAST search on PDB.org shows that VanU shares significant sequence homology with LuxU from *V. harveyi* (Ulrich et al. 2005). By structural similarity to LuxU from *V. harveyi*, the histidine required for phosphotransferase for VanU is residue 56 (Ulrich et al. 2005). Secondary structure prediction by the program TALOS+ indicates that VanU does indeed conform to a phosphotransferase like structure (four-helix bundle) as shown in Fig. 2 (Shen et al. 2009). The secondary structure prediction is quite similar to the structure of LuxU from *V. harveyi* (Ulrich et al. 2005). The chemical shift assignments have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the accession number 19006.

Acknowledgments

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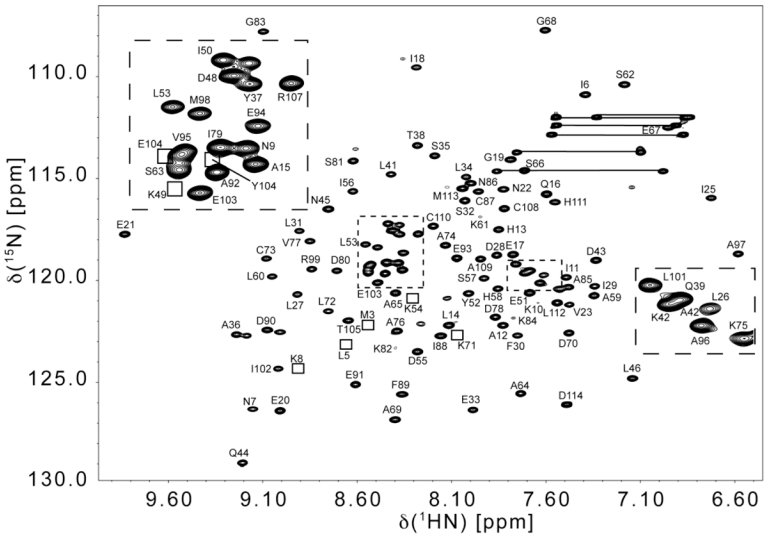

Figure 1.

2D [^{1}H-^{15}N] HSQC spectrum of 750 μM VanU protein from *Vibrio anguillarum* in 25mM Tris-HCl, 100mM KCl,, 1 mM DTT, pH 6.5 and 0.02% NaN_3 at 600 MHz (Varian Inova spectrometer).

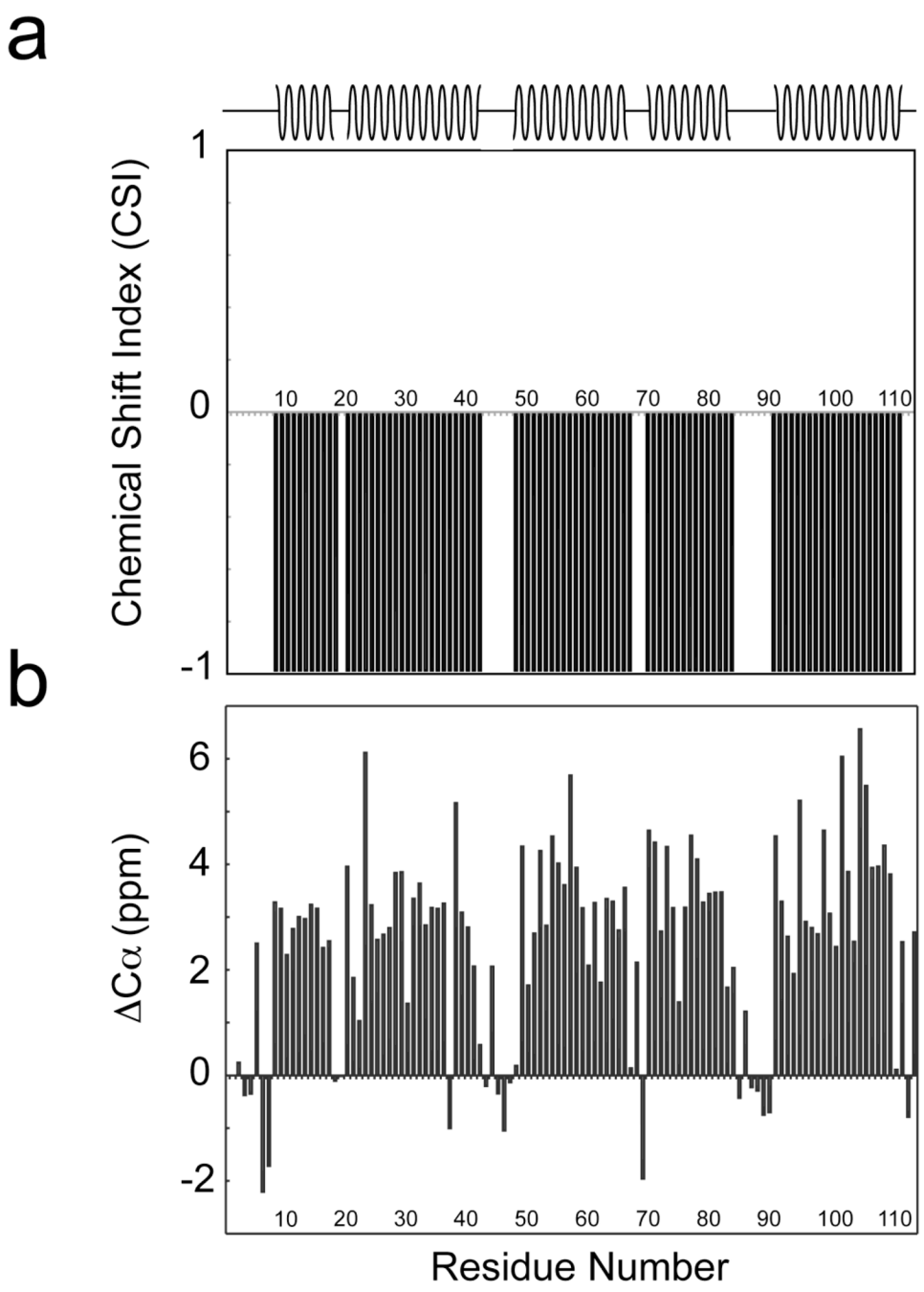

Figure 2.

a, Chemical shift index of C-terminal VanU as determined from TALOS+ using backbone Cα, Cβ, Hα, and C′ atoms. Prediction with areas of positive values indicate extended sheet regions and negative values indicate helical secondary structure. b, Difference of Cα chemical shifts from random coil values predicting secondary structure. Based on the deviations, areas of negative values indicate extended sheet regions and positive values indicate helical secondary structure