

NMR assignments for the *Sinorhizobium meliloti* response regulator Sma0114

Sarah R. Sheftic · Preston P. Garcia ·
Victoria L. Robinson · Daniel J. Gage ·
Andrei T. Alexandrescu

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Abstract Response regulators are terminal ends of bacterial two-component systems that undergo extensive structural reorganization in response to phosphoryl transfer from their cognate histidine kinases. The response regulator encoded by the gene *sma0114* of *Sinorhizobium meliloti* is a part of a unique class of two-component systems that employ HWE histidine kinases. The distinct features of Sma0114 include a PFx₂FATGY motif that houses the conserved threonine in the “Y–T coupling” conformational switch which mediates output response through downstream protein–protein interactions, and the replacement of the conserved phenylalanine/tyrosine in Y–T coupling by a leucine. Here we present ¹H, ¹⁵N, and ¹³C NMR assignments for Sma0114. We identify the secondary structure of the protein based on TALOS chemical shift analysis, ³J_{HNH_α coupling constants and hydrogen–deuterium exchange. The secondary structure determined by NMR is in good agreement with that predicted from the sequence. Both methods suggest that Sma0114 differs from standard CheY-like folds by missing the fourth α-helix. Our initial NMR characterization of Sma0114 paves the way to a full investigation of the structure and dynamics of this response regulator.}

Keywords Two-component systems · Response regulator · HWE histidine kinase · Rossmann-fold · CheY motif

Biological context

Bacteria, being single cell organisms in direct contact with their surroundings, have evolved sophisticated signaling pathways to detect and respond to changes in environmental conditions. The two-component systems are amongst the most ubiquitous signal transduction mechanisms used by bacteria (Stock et al. 2000). Two-component systems consist of a sensor histidine kinase that autophosphorylates in response to an external stimulus, and a response regulator that catalyzes the transfer of a phosphate from the histidine kinase to a conserved aspartate residue in its “receiver domain” (Laub and Goulian 2007; Stock et al. 2000). Phosphorylation results in a conformational transition of the response regulator, which mediates its interactions with downstream molecules that affect gene expression and physiology (Laub and Goulian 2007; Stock et al. 2000). The subject of the present study is a two-component system recently identified in a screen for genes that regulate catabolite repression and carbon metabolism in the nitrogen-fixing symbiont *Sinorhizobium meliloti*. The two-component system is comprised of the histidine kinase Sma0113 and the response regulator Sma0114.

Receiver domains of response regulators are typically about 110 amino acids long and are structurally and functionally analogous to members of the Ras family of small eukaryotic GTPases (Stock et al. 1991). We thus expect the 120 amino acid (13.5 kDa) Sma0114 protein to have a CheY-like structure, belonging to the α/β Rossmann-fold superfamily. Regulatory regions of receiver domains

S. R. Sheftic · V. L. Robinson · D. J. Gage (✉) ·
A. T. Alexandrescu (✉)
Department of Molecular and Cell Biology,
University of Connecticut, 91 N. Eagleville Rd.,
Storrs, CT 06269-3125, USA
e-mail: daniel.gage@uconn.edu

A. T. Alexandrescu
e-mail: andrei@uconn.edu

P. P. Garcia
Natural Sciences Department, Castleton State College,
Castleton, VT 05735, USA

are characterized by a set of conserved residues clustered in an active site at the C-terminal edge of the β -sheet. An aspartate (D60 in Sma0114) serves as the site of phosphorylation, a pair of acidic residues are typically involved in magnesium binding (E15 and D16), and a lysine (K105) interacts through electrostatic interactions with the phosphate. Two additional conserved amino acids a hydroxylic residue (Ser/Thr) and an aromatic residue (Phe/Tyr) play direct roles in the activation mechanism. Transfer of a phosphoryl group to the aspartate, prompts reorientation of the Ser/Thr to hydrogen bond with the phosphate, creating a space that allows the Phe or Tyr residue to rotate inward. This conformational rearrangement called “Y–T coupling” transforms the overall character of the α 4- β 5- α 5 face of the protein. The conformational transition in turn governs the specific interactions that mediate the output response (Laub and Goulian 2007; Stock and Guhaniyogi 2006; Stock et al. 2000), which in the case of Sma0114 are probably mediated through protein–protein interactions.

Sma0114 has a number of important differences from previously characterized response regulators. Response regulators associated with the HWE family of histidine kinases (Karniol and Vierstra 2004) either because the two components are linked on the same polypeptide chain or encoded in the same operon like the Sma0113/114 pair, often have a conserved motif at the C-terminal end of strand β 4. Response regulators containing this PFx FATGY, or “FAT GUY” motif have not been structurally characterized. While Sma0114 has the conserved threonine (T86) involved in the classic Y–T coupling mechanism of activation (Stock and Guhaniyogi 2006), the conserved (Phe/Tyr) residue on strand β 5 is replaced by a leucine (L102). To better understand the unique features of Sma0114 including the non-conserved Y–T coupling mechanism, the role of the PFx FATGY motif, and activation by its cognate HWE kinase, we have undertaken an NMR characterization of the structure and dynamics of the protein.

Experimental procedures

Protein expression and purification

The *sma0114* gene was amplified from *S. meliloti* strain Rm1021, cloned into the pET28(+) plasmid, and transformed into *Escherichia coli* strain BL21(DE3)pLysS. Cells were grown in 1 l of M9 medium containing 0.5 g $^{15}\text{N-NH}_4\text{Cl}$ and/or 3 g of ^{13}C -glucose supplemented with kanamycin to a final concentration of 25 $\mu\text{g/ml}$. Following incubation at 30°C to mid-log phase, the recombinant His-tagged Sma0114 protein was induced by addition of IPTG to a final concentration of 0.5 mM. Cells were harvested 18 h after induction and lysed by sonication in affinity

column binding buffer (100 mM Tris–HCl, 0.5 M NaCl, pH 7.4) containing lysozyme and 1 mM PMSF. The cell debris was sedimented and the His₆-Sma0114 fusion protein in the supernatant was purified by nickel affinity chromatography on a 1 ml HisTrap column. Following elution, fractions containing His₆-Sma0114 were pooled and cleaved overnight at 23°C with 10 U/ml of bovine thrombin. PMSF was subsequently added to inactivate thrombin, and the Sma0114 component was further purified by gel filtration on a Superdex S75 column. The fractions eluted from this column were run over a 1 ml HisTrap column to remove any uncut His₆-Sma0114. The remaining flow-through containing Sma0114 at >95% purity by SDS–PAGE, was dialyzed extensively against the buffer used for NMR: 50 mM sodium phosphate, pH 6.0. Following dialysis, samples were concentrated for NMR using 10,000 molecular weight cut-off centrifugal filter units.

The Sma0114 construct used for these studies contains the extra three N-terminal residues GSH from the thrombin cleavage site used to purify the protein. These residues are included in our numbering scheme for the protein, which runs from residues G1 to V123 with only residues M4–V123 part of the wild type Sma0114 amino acid sequence. We did not detect NMR signals from the amide protons of the extra residues G1–H3, suggesting they are disordered.

Nuclear magnetic resonance spectroscopy

Sma0114 samples for NMR contained 500 μM protein in 50 mM sodium phosphate buffer pH 6.0, with 1 mM DTT. Samples of 250 μl volumes were taken up Shigemi microcells. Spectra were recorded on a Varian INOVA 600 MHz spectrometer equipped with a cryogenic probe. The temperature for all experiments was 37°C.

NMR assignments were obtained using 2D and 3D experiments (Cavanagh et al. 2006) implemented in the Varian Protein Pack. Starting from the 2D ^1H - ^{15}N HSQC spectrum, backbone ^1H , ^{15}N and ^{13}C resonances were assigned using two redundant sequential walk pathways. The first used 3D HNCACB, HNCA and HN(CO)CA data to establish $C\alpha$ and $C\beta$ connections across peptide bonds. The second pathway used HNCO and HN(CA)CO experiments to connect C' carbons across peptide bonds. The backbone assignments for Sma0114 are summarized in Fig. 1. Side chain assignments were obtained from 3D HCCH-TOCSY, H(CCO)NH, C(CO)NH, HNHA, HNHB, ^1H - ^{15}N TOCSY-HSQC, and ^1H - ^{13}C HSQC-NOESY data. Stereo-specific assignments for methylene protons were based on short mixing time (25 ms) 2D NOESY, 3D ^1H - ^{15}N NOESY-HSQC and HNHB experiments as described (Case et al. 1994). Aromatic ^1H and ^{13}C resonances were obtained from 2D ^1H - ^{13}C HSQC, DFQ-COSY

Fig. 1 ^1H - ^{15}N HSQC spectrum of Sma0114. Backbone assignments are indicated with pink labels. Peaks that could only be seen at contours lower than shown (M4, E17) are denoted by squares. Side chains amide resonances are indicated with blue dashed lines and labels, with the superscript SC. Data were collected at pH 6.0 and a temperature of 37°C

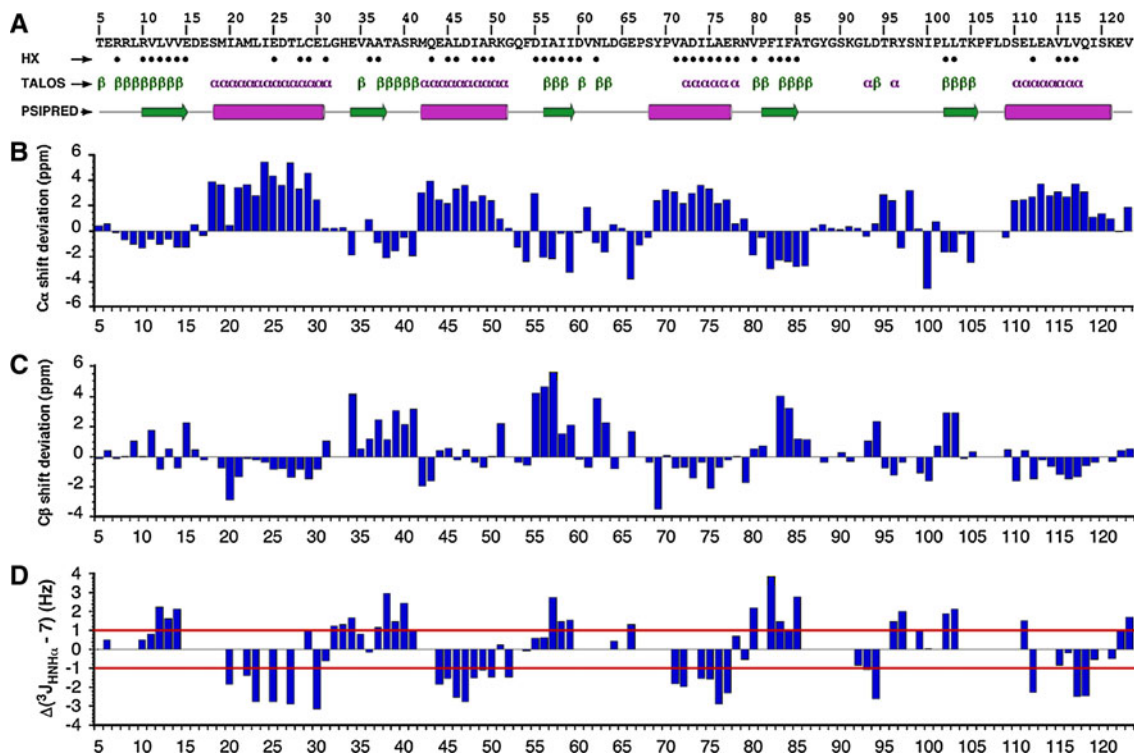
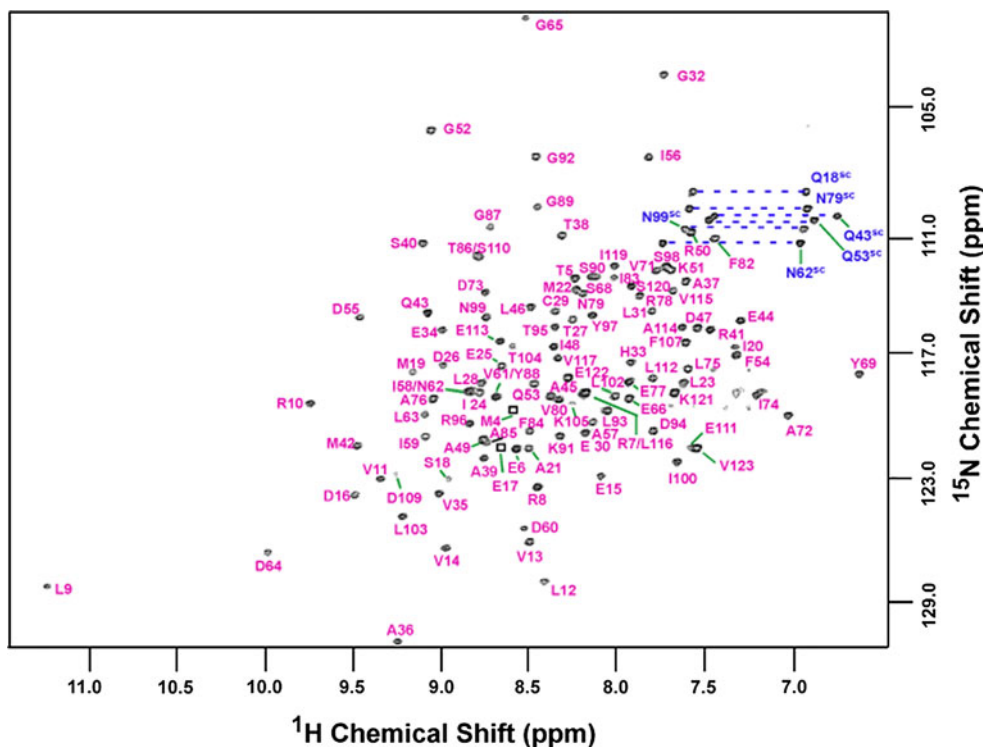


Fig. 2 Secondary structure of Sma0114. **a** Amino acid sequence of Sma0114, amide protons protected from exchange after 2 h in D_2O at pH 6.0 and 37°C, TALOS predictions of secondary structure based on HN, N, Hz, C α , C β and C' chemical shifts (Cornilescu et al. 1999), and secondary structure predicted using the PSIPRED server (Bryson et al. 2005). **b** C α chemical shifts minus random coil values (Wishart

et al. 1995a). Positive deviations correspond to α -helix, negative to β -sheet. **c** C β chemical shifts minus random coil values (Wishart et al. 1995a). Negative deviations correspond to α -helix, positive to β -sheet. **d** $^3J_{\text{HNH}\alpha}$ couplings obtained from a 3D HNHA experiment, presented as $^3J_{\text{HNH}\alpha} - 7$ Hz. Positive and negative differences correspond to β -sheet and α -helix secondary structure, respectively

and TOCSY data. 2D NOESY experiments (150 ms mixing time) using a sample in D₂O were used to connect aromatic resonances with previously assigned aliphatic resonances. Side chain amide resonances were linked through intra-residue NOEs to previously assigned aliphatic protons using 3D ¹H-¹⁵N NOESY-HSQC data. DSS was used as an internal reference for ¹H resonances, and ¹³C and ¹⁵N resonances were referenced indirectly (Wishart et al. 1995b). Spectra were processed and analyzed using FELIX-NMR and iNMR.

The limits of secondary structure elements were determined using ³J_{HNH α} -coupling constants (Vuister and Bax 1993), TALOS chemical shift analysis (Cornilescu et al. 1999) and hydrogen–deuterium exchange data. These data are summarized in Fig. 2. The secondary structure established by NMR agrees with the secondary structure predicted from the sequence using the PSIPRED server (Bryson et al. 2005). Both methods indicate that helix α 4 of the prototypical CheY-fold, is absent in Sma0114 (corresponding to residues 90–99). The α 4- β 5- α 5 face of response regulators is critical in transmitting the kinase phosphorylation signal to downstream binding partners (Gao and Stock 2010; Stock et al. 2000), so the absence of helix α 4 in Sma114 may signify a distinct mechanism of coupling between HWE kinases and their cognate response regulators.

Assignments and data deposition

¹HN, ¹⁵N, ¹³C', C α , C β , H α and H β resonances were assigned for 94% of the amino acids in Sma0114. In total, we obtained assignments for 84% of all carbons (90% of aliphatic carbons and 65% of aromatic carbons), 84% of all protons (84% aliphatic and 85% of aromatic protons) and 75% of all nitrogens. Five of 118 non-proline residues were not observed in the ¹H-¹⁵N HSQC spectrum: F107 and L108, as well as residues G1-H3 which are not part of the wild type sequence but were introduced as part of a thrombin cleavage site used in purification of Sma0114. Chemical shift assignments are deposited in the Biological

Magnetic Resonance Bank (<http://www.bmrb.wisc.edu>) under accession code BMRB-16905.

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