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Backbone chemical shift assignments for the sensor domain of the *Burkholderia pseudomallei* histidine kinase RisS – “missing” resonances at the dimer interface

Garry W. Buchko^{a,b,*}, Thomas E. Edwards^{a,c}, Stephen N. Hewitt^{a,d}, Isabelle Q.H. Phan^{a,e}, Wesley C. Van Voorhis^{a,d}, Samuel I. Miller^{d,f}, and Peter J. Mylera^{e,g}

^aSeattle Structural Genomics Center for Infectious Disease, Pacific Northwest National Laboratory, Richland, WA 99352, USA

^bBiological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA

^cBeryllium, Bainbridge Island, WA 98110, USA

^dDepartment of Medicine, University of Washington, Seattle, WA 98195-7185, USA

^eSeattle BioMed, Seattle, WA 98109-5219, USA

^fDepartment of Microbiology & Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA

^gDepartment of Biomedical Informatics and Medical Education & Department of Global Health, University of Washington, Seattle, WA 98195, USA

Abstract

Using a deuterated sample, all the observable backbone ¹H^N, ¹⁵N, ¹³C^α, and ¹³C^γ chemical shifts for the dimeric, periplasmic sensor domain of the *Burkholderia pseudomallei* histidine kinase RisS were assigned. Approximately one-fifth of the amide resonances are “missing” in the ¹H-¹⁵N HSQC spectrum and map primarily onto α-helices at the dimer interface observed in a crystal structure suggesting this region either undergoes intermediate timescale motion (μs – ms) and/or is heterogeneous.

Keywords

signal transduction; infectious diseases; two-component system; chemical shift perturbations; melioidosis

Biological content

Bacteria use a sophisticated, two-component, signal transduction mechanism to control specific gene expression that enables adaptation to physical and environmental changes (Matsushita et al. 2002). The system is composed of a histidine kinase and a response regulator. The histidine kinase component is a multifunctional, transmembrane protein that

*Correspondence: garry.buchko@pnnl.gov.

usually functions as a dimer on the cell's surface. The signal transduction cascade begins with the exterior sensor domain responding to environmental stimuli and triggering the autophosphorylation of a conserved histidine residue in the interior part of the protein. In turn, the phosphorylated histidine kinase transfers the phosphate group to an aspartic acid residue in the partner response regulator protein. The phosphorylated response regulator protein is then able to positively or negatively affect expression of the targeted gene. Because signal transduction occurs via a different mechanism in eukaryotes and some signal transduction events may play a role in virulence (Bonner et al. 2004), histidine kinases are an attractive target for new antibacterial agents (Schreiber et al. 2009). Among the infectious bacteria in need of new treatment strategies is *Burkholderia pseudomallei*, an aerobic, Gram-negative, soil-dwelling bacterium responsible for melioidosis (Wiersinga et al. 2006). This infectious disease is a serious health problem in Southeast Asia and Northern Australia and the US Centers for Disease Control and Prevention are concerned about the potential use of this organism for biological terrorism. Analysis of the *B. pseudomallei* genome has identified over 20 genes that may be histidine kinases. An X-ray crystal structure for the periplasmic sensor domain of the histidine kinase RisS has been solved (PDB ID 3LR5) although the external stimulus for RisS has not yet been identified. To assist identification of the external stimulus specific for RisS and the structural changes this stimulus may induce to initiate the signal cascade, the backbone amide resonances for the periplasmic sensor domain of RisS (residues 30 – 151: sRisS) have been determined in solution using a triple-labeled (^2H , ^{13}C , ^{15}N) sample. These assignments, together with the crystal structure, will facilitate chemical shift perturbation studies (Prost et al., 2007) and may enable the identification of ligand binding surfaces on the protein.

Materials and methods

Cloning, expression, and purification

The predicted sensor domain (residues 30 – 151) of the *RisS* gene (BURPS1710b_2508) was amplified by PCR using the genomic DNA of *Burkholderia pseudomallei* strain 1710b and the oligonucleotide primers 5'-GGGTCCTGGTTCGATGAGCTTCCGAGTGATCGAGC-3' (forward) and 5'-CTTGTTTCGTGCTGTTTATTAGCCGGTGACGGTATCGAGCTG-3' (reverse) (Invitrogen, Carlsbad, CA) containing LIC primers (underlined). This amplified region of the *RisS* gene was then inserted into the NruI/PmeI digested expression vector AVA0421 by ligation-independent cloning that provided a 21-residue tag (MAHHHHHHMGTLEAQTQGPGS-) at the N-terminal of the expressed protein (Choi et al. 2011). The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3)-R3-pRARE2 cells (gift from SGC-Toronto, Toronto, ON) using a heat shock method. Uniformly 2H -, ^{15}N -, ^{13}C -labeled sRisS was obtained (37°C) using minimal medium (Miller) containing D_2O , NH_4Cl (1 mg/mL) and D-glucose (2.0 mg/mL) supplemented with NaCl (50 $\mu\text{g/mL}$), MgSO_4 (120 $\mu\text{g/mL}$), CaCl_2 (11 $\mu\text{g/mL}$), Fe_2Cl_3 (10 ng/mL) and the antibiotics chloramphenicol (34 $\mu\text{g/mL}$) and ampicillin (150 $\mu\text{g/mL}$). Culture growth was initiated by the inoculation of 20 mL of minimal media (D_2O) containing unlabeled NH_4Cl and D-glucose with 1 mL of frozen glycerol stock of the transformed cells. After an OD_{600} reading of ~ 0.6 was reached, these cells were gently spun down, supernatant removed, and

transferred to 100 mL of minimal media containing $^{15}\text{NH}_4\text{Cl}$ and $\text{D}-[^2\text{H}_7; ^{13}\text{C}_6]\text{glucose}$. When this culture reached an OD_{600} reading of ~ 0.6 the culture was poured into 650 mL of labeled minimal media. When this culture reached an OD_{600} reading of ~ 0.6 it was transferred to a 25°C incubator and protein expression induced with isopropyl β -D-1-thiogalactopyranoside ($0.026\text{ }\mu\text{g/mL}$). Approximately 12 hours later the cells were harvested by mild centrifugation and frozen at -80°C . After thawing the frozen pellet, sRisS was purified using two chromatography steps: immobilized metal ion affinity (20 mL Ni-Agarose 6 FastFlow column (GE Healthcare, Piscataway, NJ)) and Superdex75 gel-filtration (HiLoad 26/60 column) (Buchko et al. 2013). After the first chromatography step, the fraction containing most of the sRisS ($\sim 40\text{ mL}$) was exchanged into 3C protease cleavage buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.6) by overnight dialysis (4 L) at 278 K. The protein was concentrated to $\sim 20\text{ mg/mL}$ (Amico Centriprep-10) and the N-terminal polyhistidine tag removed by overnight incubation with 3C protease ($1\text{ }\mu\text{g}$ per $50\text{ }\mu\text{g}$ target protein). The second chromatography step, in addition to removing minor impurities and the polyhistidine tag, exchanged sRisS into the buffer used for the NMR studies: 100 mM NaCl, 20 mM Tris-HCl, 1.0 mM dithiothreitol, pH 7.1. Note that after removal of the polyhistidine tag the protein contained five non-native amino acids at the N-terminus, GPGSM-, numbered G1 through M5 here. The first native residue, labeled S6 here, is residue S30 in the native protein.

Nuclear magnetic resonance spectroscopy

All the NMR data was collected at 303 K on a triple-labeled (^2H , ^{13}C , ^{15}N) sample ($\sim 0.7\text{ mM}$) using four-channel Varian Inova-600, and -750 spectrometers equipped with triple resonance probes and pulse field gradients. Assignment of the ^1H , ^{13}C , and ^{15}N chemical shifts of the backbone resonances were made from a standard two-dimensional ^1H - ^{15}N HSQC experiment and three-dimensional HNCACB, HNCA, HNCACO, HNCO, HNN, and ^{15}N -edited NOESY experiments using the Varian Biopack suite of pulse programs. Deuterium decoupling and a recycle delay of 1.5 ms were used in all the experiments with the HNCACB experiment optimized for the detection of β -carbons using a tauCC value of 7 ms. A mixing time of 100 ms was used in the ^{15}N -edited NOESY experiment. An overall rotational correlation time (τ_c) for sRisS was estimated from backbone amide ^{15}N $T_1/T_{1\rho}$ ratios (Buchko et al. 2008). All NMR data was processed using Felix2007 (MSI, San Diego, CA) software and analyzed with the program Sparky (v3.115). Indirect methods (DSS = 0 ppm) were used to reference the ^1H , ^{13}C , and ^{15}N chemical shifts that were deposited into the Biological Magnetic Resonance Bank database (www.bmrb.wisc.edu) under the accession number BMRB-25278.

Results and discussion

While it is well established that full length histidine kinases function as dimers *in vivo*, the sensor domain by itself is often monomeric in solution (Zhang and Hendrickson, 2010). The sensor domain of RisS is non typical as all the data suggests that sRisS is a dimer in solution. After removal of the N-terminal tag the sRisS construct has a molecular weight of 14.5 kDa. However, its behavior in size exclusion chromatography and NMR spectroscopy experiments were characteristic of an $\sim 30\text{ kDa}$ protein. First, at room temperature ($\sim 295\text{ K}$)

using a flow rate of 1.0 mL/min, sRisS eluted off a 16/60 Superdex75 size exclusion column with a retention time of 74 minutes, a value characteristic of an ~30 kDa protein on this column (data not shown). Second, the estimated isotropic overall rotational correlation time (τ_c) for sRisS, as inferred from ^{15}N spin relaxation times, was 15.8 ± 0.5 ns at 293 K and 15.1 ± 0.5 ns at 313 K, values consistent with an ~30 kDa protein. Third, the ^1H - ^{15}N HSQC spectrum of sRisS was not especially well resolved at 293 K and featured broad cross peak line shapes (data not shown) typical of a large protein. The spectral properties of the ^1H - ^{15}N HSQC spectrum were improved by collecting the backbone assignment data at 303 K, a temperature at which circular dichroism spectroscopy showed no significant changes in global structure relative to 293 K (data not shown). Fourth, there was little magnetization transfer to the β -carbons in the HNCACB experiment using a double-labeled (^{13}C , ^{15}N) sample at temperatures as high as 313 K, a feature characteristic of a large protein. Magnetization transfer to the β -carbons was obtained using a triple-labeled (^2H , ^{13}C , ^{15}N) sample at 303 K. These solution observations were corroborated by a crystal structure for sRisS (3LR5) showing a homo-dimer with a favorable buried surface area of 2910 $\text{\AA}^3$.

The ^1H - ^{15}N HSQC spectrum for sRisS is illustrated in Figure 1 and features wide chemical shift dispersion of the amide resonances in both dimensions that is characteristic of a structured protein (Yee et al., 2002). All the backbone and two tryptophan amide resonances detectable in the spectrum were assigned as labeled in Figure 1. For three of the residues near the N-terminus (S6, I10, and E11) a duplicate weak resonance was also identified suggesting a second minor orientation for this unstructured tail (no inter-residue NOEs for these amides were observed in 3D ^{15}N -edited NOESY data). In Figure 2A these assigned amide resonances are highlighted (blue) on the primary sequence of the protein. It is evident that amide cross peaks are “missing” for 25 residues (red). While a couple of these “missing” amide cross peaks may exist underneath assigned residues (with carbon chemical shift overlap in the backbone 3D experiments as well), the clustering of missing amide resonances in two regions near the N-terminus suggests that most of these “missing” residues are truly absent. Various strategies were unsuccessfully employed to observe more of the sRisS resonances in the ^1H - ^{15}N HSQC spectrum (Yee et al., 2002). Increasing the temperature from 303 to 318 K produced sharper spectra but no additional amide cross peaks while lowering the temperature from 303 to 273 K resulted in a loss of resolution and the “merging” of resonances due to line broadening. Little change was observed in the spectra by varying the NaCl concentration between 50 – 300 mM and no change observed by adding EDTA to a final concentration of 5 mM. The protein contains no cysteine residues and therefore it was not surprising that the presence or absence of DTT had no effect on the number of observed amide resonances. The use of TROSY to collect an ^1H - ^{15}N HSQC spectrum improved the resolution but did not reveal additional resonances. Attempts to increase the concentration of the protein above ~ 0.7 mM resulted in protein precipitation and the ^1H - ^{15}N HSQC spectrum following a 10-fold decrease in the protein concentration showed no chemical shift perturbations. Lowering the pH of the solution below 5.5 resulted in precipitation of the protein (an event that was reversible). Hence, the missing amide resonances appear to be a genuine property of the sRisS dimer.

Missing amide cross peaks in ^1H - ^{15}N HSQC spectra of proteins typically identify regions undergoing motion or chemical exchange in the intermediate timescale (ms to μs) (Buchko

et al., 2004; Ådén et al., 2011) and the literature contains many examples where this feature has provided information regarding the biophysical properties of proteins. Amide cross peaks disappear from the ^1H - ^{15}N HSQC spectrum of the intrinsically disordered anti-sigma factor, FlgM, upon binding to its target, σ^{28} , due to conformational exchange between a disordered and more ordered state (Daughdrill et al., 1997). The early events of ionic strength induced self-assembly of amelogenin into nanospheres were followed by the pattern of disappearing amide resonances at the protein-protein interfaces as the salt concentration was increased (Buchko et al., 2010). The low pH ^1H - ^{15}N HSQC spectrum of α -lactalbumin in the molten globular state contains few cross peaks due to extreme line broadening from millisecond conformational fluctuations due to local side chain disorder (Redfield et al., 1999). *Arabidopsis thaliana* peroxiredoxin Q remains monomeric and fully structured in going from the oxidized to the reduced state, yet this transition results in the disappearance of ~ 50% of the resonances in the ^1H - ^{15}N HSQC spectrum due to internal intermediate motion in the protein (Ådén et al., 2011). Many of the missing amide resonances in the ^1H - ^{15}N HSQC spectrum of the *E. coli* DNA repair protein formamidopyrimidine-DNA glycosylase map onto the linker region and the interface between the N- and C-terminal domains suggesting internal intermediate motion between these domains (Buchko et al., 2005).

In Figure 2A the elements of secondary structure observed in the crystal structure of sRisS (PDB ID 3LR5) determined under crystallization conditions near neutral pH are shown above the primary amino acid sequence of the protein. The vast majority of the missing residues are located in the first two α -helices and first β -strand near the N-terminal. Amide protons in α -helices and β -strands usually exchange with water slowly, therefore, unless these regions are disordered in solution and adopt canonical structure only in the crystal lattice, it is unlikely that the missing resonances are due to proton chemical exchange with water on the millisecond timescale, and hence, must be due to other reasons. In Figure 2B the crystal structure of the sRisS dimer is shown and the residues with missing amide cross peaks in the sRisS ^1H - ^{15}N HSQC spectrum mapped onto the structure in red. The dimer interface consists of the first two α -helices near the N-terminal of sRisS; elements of secondary structure containing the majority of missing amide resonances. Intermolecular dynamics due to dimerization on the millisecond timescale is likely not responsible for the missing amide resonances of residues on the dimer interface because no chemical shift perturbations or additional resonances were observed in the ^1H - ^{15}N HSQC spectrum of sRisS upon diluting the sample 10-fold. Consequently, intramolecular dimer dynamics are likely responsible for most of the missing amide resonances and one possible explanation is the chemical shifts have broadened out beyond detection due to the presence of many different dimer interfaces (heterogeneous interface) (Buchko et al., 2008), and/or millisecond fluctuations (conformational exchange) of the side chains at the interface (Redfield et al., 1999).

The observations at the dimer interface of sRisS may be due to truncation of the protein. Full length RisS contains 445 residues and only 122 residues of the predicted sensor domain were used in the sRisS construct. The intrinsic affinity for the sensor domain of histidine kinases to dimerize is inherently weak (Zhang and Hendrickson, 2011) and may be further

destabilized by the absence of the entire transmembrane domain along with a lipid bilayer to hold it in place. On the other hand, the intermediate timescale motion or heterogeneous interactions at the dimer interface of sRisS may serve a role in signal transduction, providing plasticity for ligand/stimulus recognition. Upon interaction with ligand/stimulus that is specific for RisS the timescale motion or heterogeneity at the dimer interface may change. Either of these effects may disrupt an interaction network and initiate the transduction of the signal through the membrane leading to the eventual regulation of the targeted gene (Prost et al. 2007). To test this hypothesis it is first necessary to identify the external stimulus specific for RisS and the chemical shift assignments for the sensor domain of RisS presented here will facilitate chemical shift perturbation studies to identify this stimulus. The identification of the stimulus and the structural/dynamic changes the stimulus induces will potentially accelerate efforts towards the development of a new generation of intervention strategies to treat and control melioidosis (Stacy et al. 2011).

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Fig. 1.

The ^1H - ^{15}N HSQC spectrum for sRisS (~0.7 mM) collected at a proton resonance frequency of 750 MHz, 303 K, in 100 mM NaCl, 20 mM Tris, 1 mM DTT, pH 7.1. Size exclusion chromatography and the estimated correlation time indicate that the 127-residue construct is a dimer in solution. The assigned amide cross peaks are labeled in blue, tryptophan side chain amides identified with an “r”, the side chain resonances are identified with a dashed red horizontal line, and cross peaks below the contour threshold are marked with an “x”. The three purple circled cross peak are related to S6, I10, and E11. A tryptophan side chain amide, W114r, (10.83 and 130.7 ppm), and the amide resonances for A93 (9.93 and 129.3 ppm) and V96 (6.23 and 120.8 ppm) are not shown.

Fig. 2.

A) Primary amino acid sequence for the sensor domain of sRisS highlighting, in red, residues for which amide cross peaks were not observed in the ^1H - ^{15}N HSQC spectrum. Prolines and residues assigned to amide cross peaks are colored blue. The first five residues are non-native and remain after removal of the poly-histidine tag. Above the sequence are the elements of secondary structure observed in the crystal structure of sRisS obtained at neutral pH (PDB ID 3LR5): α -helices = gold, 3_{10} -helix = magenta, β -strands = cyan. B) Mapping of the missing amide resonances in the ^1H - ^{15}N HSQC spectrum of sRisS (red) onto a cartoon representation of the sRisS crystal structure (PDB ID 3LR5).