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Structural characterization and modeling of the *Borrelia burgdorferi* hybrid histidine kinase Hk1 periplasmic sensor: a system for sensing small molecules associated with tick feeding

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Abstract

Two-component signal transduction systems are the primary mechanisms by which bacteria perceive and respond to changes in their environment. The Hk1/Rrp1 two-component system (TCS) in *B. burgdorferi* consists of a hybrid histidine kinase and a response regulator with diguanlylate cyclase activity, respectively. Phosphorylated Rrp1 catalyzes the synthesis of c-di-GMP, a second messenger associated with bacterial life-style control networks. Spirochetes lacking either Hk1 or Rrp1 are virulent in mice but destroyed within feeding ticks. Activation of Hk1 by exogenous stimuli represents the seminal event for c-di-GMP signaling. We reasoned that structural characterization of Hk1's sensor would provide insights into the mechanism underlying signal transduction and aid in the identification of activating ligands. The Hk1 sensor is composed of three ligand-binding domains (D1-3), each with homology to periplasmic solute-binding proteins (PBPs) typically associated with ABC transporters. Herein, we determined the structure

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for D1, the most N-terminal PBP domain. As expected, D1 displays a bilobed Venus Fly Trap-fold. Similar to the prototypical sensor PBPs HK29S from *Geobacter sulfurreducens* and VFT2 from *Bordetella pertussis*, apo-D1 adopts a closed conformation. Using complementary approaches, including SAXS, we established that D1 forms a dimer in solution. The D1 structure enabled us to model the D2 and D3 domains. Differences in the ligand-binding pockets suggest that each PBP recognizes a different ligand. The ability of Hk1 to recognize multiple stimuli provides spirochetes with a means of distinguishing between the acquisition and transmission blood meals and generate a graded output response that is reflective of the perceived environmental threats.

Introduction

Two-component signal transduction systems (TCS) are a principal molecular mechanism by which bacteria sense and adapt to perturbations in their surroundings (Capra and Laub, 2012; Gao and Stock, 2009; Mascher et al., 2006). Prototypical TCSs are composed of a sensory histidine kinase (HK) and a response regulator (RR) (Gao and Stock, 2009). The vast majority of HKs, contain a highly variable periplasmic (or, with Gram-positive bacteria, extracellular) sensor and a well-conserved cytoplasmic histidine kinase core that is comprised of (i) an N-terminal HisKA domain involved in histidine phosphorylation/dimerization (DHP) and (ii) a C-terminal catalytic and ATP-binding (CA) domain (Gao and Stock, 2009; Krell et al., 2010; Mascher et al., 2006). Although less common, HKs also can be entirely cytoplasmic. The RR component contains a readily identifiable receiver (REC) and a variable effector domain (*i.e.*, DNA binding, enzymatic activity) (Galperin, 2010; Gao and Stock, 2009). Activation of bacterial HKs most often begins with a ligand-induced conformational change within the sensor which, when transmitted across the cytoplasmic membrane *via* one or more flanking transmembrane (TM) domains, stimulates autophosphorylation of a conserved His residue within the histidine kinase core (Cheung and Hendrickson, 2010; Gao and Stock, 2009; Krell et al., 2010; Lowe et al., 2012b). The high-energy phosphoryl group is then transferred to an Asp residue within the corresponding RR REC domain (Galperin, 2010; Gao and Stock, 2009). Hybrid sensory HKs contain both histidine kinase core and REC domains, enabling them to function as “stand alone” systems or as part of more elaborate signal transduction networks (Gao and Stock, 2009; Krell et al., 2010). While tens of thousands of HK sensor domains have been identified in bacterial genomes, only a handful of activating molecules have been defined (Gao and Stock, 2009; Mascher et al., 2006; Ulrich and Zhulin, 2010).

Periplasmic-sensing HKs represent the largest group of membrane-bound sensory HKs and, in general, fall into one of four structural classes (Cheung and Hendrickson, 2010; Mascher et al., 2006): (i) PhoQ/CitA/DcuS (PDC)-type sensor domains adopt a “PAS-like” $\alpha+\beta$ fold and respond to a variety of environmental stimuli, including divalent cations (Ca^{2+} and Mg^{2+}), antimicrobial peptides, and citrate and C4-dicarboxylates (*i.e.*, succinate, fumarate, and malate) (Cheung and Hendrickson, 2010; Gao and Stock, 2009; Krell et al., 2010; Mascher et al., 2006). (ii) NarX- and TorS-type sensors consist of single or double four-helix bundles (Baraquet et al., 2006; Cheung and Hendrickson, 2009; Moore and Hendrickson, 2009) and respond to nitrite/nitrate and trimethylamine-N-oxide, respectively

(Jourlin et al., 1996; Lee et al., 1999). (iii) Reg-prop-type sensors display a β -propeller fold and appear to respond primarily to sugars (Lowe et al., 2012a; Zhang et al., 2014). (iv) PBP-type sensor domains which display strong structural similarity to high-affinity type 2 periplasmic binding proteins (PBPs) for ABC transporters (Berntsson et al., 2010; Mascher et al., 2006; Tam and Saier, 1993). First described in 2009 by Cheung *et al.* (Cheung et al., 2009), sensor PBPs, represented by HK29_S from *Geobacter sulfurreducens* (Cheung et al., 2009) and VFT1 and VFT2 from *Bordetella pertussis* (Dupré et al., 2015; Herrou et al., 2010), are thought to function by the Venus Fly Trap (VFT) mechanism most often associated with ABC transporters (Berntsson et al., 2010; Mao et al., 1982; Quijcho and Ledvina, 1996). With ABC transporter PBPs, occupancy of the ligand binding pocket induces a transition from an open to a closed conformation, allowing the liganded-PBP to interact with its cognate membrane-bound permease (Berntsson et al., 2010; Mao et al., 1982; Quijcho and Ledvina, 1996). In the case of sensor PBPs, the open-to-closed conformational change induced by ligand binding is translated across the cytoplasmic membrane *via* a piston-like or rotational motion within the TM domains, which in turn, activates (or antagonizes) the kinase core (Cheung and Hendrickson, 2010; Lowe et al., 2012b; Mascher et al., 2006; Moore and Hendrickson, 2009; Neiditch et al., 2006; Sevvana et al., 2008).

Borrelia burgdorferi, the Lyme disease spirochete, is maintained in nature via an obligate enzootic cycle involving *Ixodes* ticks and mammalian reservoir hosts (Pal and Fikrig, 2010; Radolf et al., 2012; Steere et al., 2004). The transmission, survival and pathogenic potential of *B. burgdorferi* depend on the bacterium's ability to modulate its transcriptome as it transits between its arthropod vector and mammalian reservoir hosts (Groshong and Blevins, 2014; Iyer et al., 2015; Pal and Fikrig, 2010; Radolf et al., 2012; Skare et al., 2010). Remarkably, differential gene expression in *B. burgdorferi* is orchestrated by just two Two-Component Systems (TCS) (Fraser et al., 1997). One, Hk2/Rp2, is composed of an entirely cytoplasmic HK with a PAS-like sensor and a cytoplasmic response regulator, respectively (Galperin, 2010; Xu et al., 2010; Yang et al., 2003). Although the contribution of Hk2 to phosphorylation of Rp2 remains uncertain (Xu et al., 2010), Rp2-P acts in concert with the DNA-binding Fur ortholog BosR to promote RpoN-dependent transcription of *rpoS* at the onset of the nymphal blood meal (Hyde et al., 2009; Katona et al., 2004; Ouyang et al., 2008; Ouyang et al., 2015; Ouyang et al., 2012; Radolf et al., 2012; Samuels, 2011; Smith et al., 2007; Yang et al., 2003). RpoS, in turn, controls the reciprocal expression of genes involved in tick transmission and mammalian infection (Caimano et al., 2007; Dunham-Ems et al., 2012; Fisher et al., 2005; Groshong and Blevins, 2014; Hubner et al., 2001; Ouyang et al., 2012; Xu et al., 2012). *B. burgdorferi*'s other TCS, Hk1/Rp1, consists of a membrane-bound hybrid sensory HK and a cytoplasmic RR with diguanlylate cyclase activity, respectively (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). Upon phosphorylation by Hk1, Rp1 catalyzes the synthesis of c-di-GMP (Caimano et al., 2015; Kostick et al., 2011; Ryjenkov et al., 2005), a second messenger associated with a wide range of bacterial life-style control networks, including the transition from planktonic to sessile states during biofilm formation (Povolotsky and Hengge, 2012; Romling et al., 2013). *B. burgdorferi* lacking either Hk1 or Rp1 are virulent in mice by needle-inoculation but are destroyed within the midguts of feeding ticks during acquisition and transmission

(Caimano et al., 2015; Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). These phenotypic data, combined with results from transcriptomics analyses of Rrp1-deficient organisms grown *in vitro* (Caimano et al., 2015; He et al., 2011; Rogers et al., 2009), suggest that the Hk1/Rrp1 TCS activated in response to exogenous ligands encountered during tick feeding and functions to promote utilization of alternate substrates for energy generation and biosynthesis of intermediary metabolites and/or protective remodeling of the spirochete cell envelope.

Phosphorylation of Rrp1 by Hk1 represents the seminal event for c-di-GMP signaling by *B. burgdorferi* (Caimano et al., 2015; Caimano et al., 2011; He et al., 2011; Kostick et al., 2011; Rogers et al., 2009; Ryjenkov et al., 2005). The periplasmic sensor for Hk1 is composed of three sensor PBP domains (D1, D2 and D3; Fig. 1A), all of which could be involved in mediating signal transduction. We reasoned that a detailed structural analysis of this sensor would provide valuable insight into the molecular mechanisms underlying Hk1-mediated signal transduction and aid in the identification of its activating ligand(s). Toward this end, we determined the crystal structure for D1, the most N-terminal PBP. As expected, D1 displays the bilobed VFT-fold that is characteristic of both ABC transporter and sensor PBPs. Similar to the HK29_S and VFT2, the prototypes for sensor PBPs (Cheung et al., 2009; Herrou et al., 2010), D1 adopts a closed conformation in the absence of ligand. Using complementary approaches (*e.g.*, size-exclusion chromatography and small angle X-ray scattering [SAXS]), we established that D1 forms a stable homodimer in solution. Using D1 as a guide, we modeled Hk1's other two sensor PBPs, D2 and D3. Despite their overall structural similarity, non-synonymous differences in the residues lining the putative ligand binding pockets for D1, D2 and D3 suggest that each recognizes a different ligand. The ability of Hk1 to perceive multiple exogenous stimuli provides Lyme disease spirochetes with a facile means of distinguishing the acquisition and transmission blood meals and calibrating c-di-GMP-dependent gene expression according to the perceived environmental threats.

Materials and Methods

Cloning, overexpression and purification of recombinant D1

The Hk1 (BB0420) domain D1 was amplified from *B. burgdorferi* strain 5A4 NP1 genomic using oligonucleotide primers D1-5'-NheI (5'-TAGCTAGCAACGTCAATTTATTTCTAAGGAT-3') and D1-3'-XhoI (5'-GTGCTCGAGCTTAGCATTGCTATCTAAAAAAGA-3'). The resulting amplicon was cloned into *NheI/XhoI*-digested pET28a (Novagen, San Diego, CA) for overexpression in *Escherichia coli* Overexpress™ C41 (DE3) cells (Lucigen, Middleton, WI). A hexahistidine affinity tag was fused in-frame at the Hk1 N-terminus to facilitate purification by metal affinity chromatography. The nucleotide sequence of the resulting construct, pMC2748, was confirmed by sequencing (Genewiz, Inc., South Plainfield, NJ). For overexpression of recombinant protein, an overnight culture of MC2748 was used to inoculate 4-L of LB-Miller broth containing kanamycin (50 µg ml⁻¹). Cultures were incubated at 37°C with shaking at 200 rpm. At A₆₀₀ = ~0.6, expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (final concentration, 0.5 mM), followed by further growth for 3-4

h. Cells were harvested by centrifugation at $6000 \times g$ for 30 min at 4°C and the resulting pellets were suspended in buffer containing 20 ml of 50 mM Tris (pH 7.5), 10% glycerol, 100 µg of lysozyme (Sigma-Aldrich, St. Louis, MO) and 100 µl of protease inhibitor mixture (Sigma-Aldrich) and lysed by sonication. The sonicated lysate was centrifuged at $20,000 \times g$ for 30 min at 4°C followed by filtration through a 0.22 µm filter before loading onto a 5-ml nickel-nitrilotriacetic acid (Ni-NTA)(Qiagen, Valencia, CA) column equilibrated with Buffer A (50 mM Tris [pH 7.5], 100 mM NaCl, and 10 mM imidazole). The column was washed once with 25-mls of Buffer A, followed by successive washes with Buffer A containing 20 mM and 40 mM imidazole. Recombinant D1 was cleaved off the Ni-NTA column by digestion with thrombin (100 units) for 3–4 h at 22°C. Cleaved D1 was loaded onto a SP-Sepharose (Bio-Rad, Hercules, CA) cation-exchange column, which was washed extensively with 25 mM Tris (pH 7.5) and 10% glycerol. D1 was eluted with a gradient of 0–1.0 M NaCl. Fractions containing D1 were pooled and subjected to size-exclusion chromatography over a Superdex 200 10/300 Tricorn column (GE Healthcare Life Sciences, Pittsburgh, PA) equilibrated with 50 mM Tris (pH 7.5) and 100 mM NaCl. Selenomethionine (SeMet)-substituted D1-His was purified as described above using from *E. coli* grown in M9 minimal medium supplemented with a mixture of 100 mg each of lysine, phenylalanine and threonine, 50 mg of isoleucine, leucine and valine, and 60 mg of SeMet. Far-UV circular dichroism spectroscopy was performed as previously described (Luthra et al., 2011) to ensure that the purified recombinant D1 protein remained properly folded.

Analytical size exclusion chromatography

Size-exclusion chromatography was carried out on a Superdex 200 10/300 Tricorn column (GE Healthcare Life Sciences, Pittsburgh, PA) on a BioLogic DuoFlow FPLC workstation (Bio-Rad) using the following calibration standards: Thyroglobulin (Stokes radius [Rs] 8.5 nm; 670 Da), Ferritin (Rs 6.1 nm; 440 Da), Aldolase (Rs 4.8 nm; 158 Da), Conalbumin (Rs 3.6 nm; 75 kDa), Chymotrypsinogen (Rs 2 nm; 25 kDa) and Ribonuclease A (Rs 1.6 nm; 13.7 kDa). The column was equilibrated and run in desired buffer at 25°C. The relative elution volume was determined using Prism Graph (Graph Pad, LaJolla, CA) from regression analysis of the $\log_{10}$ values for protein standards as a function of the available partition coefficient (K_{av}) using the equation: $K_{av} = V_e - V_0/V_g - V_0$, where V_e is the elution volume, V_0 is the void volume determined by elution of Blue Dextran (2000 kDa) and V_g is the geometric column volume. To assess the stability of the D1 dimer, protein samples (1 mg/ml) incubated with 3 M urea at room temperature for 1 hour before being applied to a Superdex 75 16/60 column pre-equilibrated with buffer containing 50 mM Tris-HCl (pH 7.0), 0.1 M NaCl, 3 M urea and 1 mM TCEP. Protein was eluted at room temperature in the same buffer.

Crystallization, data collection and processing

Crystallization of the recombinant Hk1 SBP domain D1 (D1) was carried out at 22°C using the sitting-drop vapor-diffusion method. D1 samples were concentrated to 1.7 mg/ml in 50 mM Tris (pH 7.5), 50 mM NaCl and 1 mM Tris (2-carboxyethyl) phosphine (TCEP). Initial crystallization was performed using conditions derived by the high-throughput screening (HTS) laboratory at the Hauptman-Woodward Medical Research Institute (Luft et al., 2011).

Diffraction quality crystals were obtained for the native D1 protein in conditions containing 0.1 M Tris (pH 8.0), 0.1 M $\text{Mg}(\text{NO}_3)_2$ and 20% PEG 20,000. Crystallization drops were set up by adding 2 μl of protein solution to 2 μl of mother liquor. Streak-seeding was used to improve crystal quality and reproducibility. Before flash cooling in liquid N_2 , crystals were cryoprotected by transferring through a 3:1 mixture of Paratone-N (Hampton Research) and Paraffin oil (Hampton Research). SeMet-substituted protein was concentrated to 1.7 mg/ml in 50 mM HEPES (pH 7.0) and 50 mM NaCl and crystallized by streak seeding as described above for the native crystals in conditions containing 0.1 M Tris-HCl (pH 8.0), 0.1 M $\text{Mg}(\text{NO}_3)_2$ and 18% PEG 20,000. Crystals of SeMet-substituted D1 were cryo-cooled using a method similar to the native crystals. All data sets were collected remotely at 100 K on beamline 17-ID-B of IMCA-CAT at the Advanced Photon Source (APS) at Argonne National Laboratory (Argonne, Illinois) using a Dectris Pilatus 6M Pixel Array. Diffraction data were measured at absorption, inflection and remote wavelengths for multiwavelength anomalous dispersion (MAD) phasing and subsequently processed with DENZO and SCALEPACK in HKL-2000 (Otowski and Minor, 1997). Details of the data collection statistics are summarized in Table 1.

Crystal structure determination, refinement and validation

SeMet-substituted D1 crystals diffracted to 3.0 Å and exhibited different cell dimensions compared to native crystals. The SeMet-substituted D1 domain construct contained 233 residues including four SeMet residues per monomer with a total of 4 monomers. Only two out of a possible 16 SeMet peaks were identified in the asymmetric unit by PHENIX (ver. 1.8.4-1496) (Adams et al., 2010). Therefore, only one Se anomalous signal was used per 466 residues in the structural determination. As a result, the initial electron density maps were not of sufficient quality to manually or automatically build a model. The electron density for one copy of D1 within the asymmetric unit was cut out using the programs Mapmask and Maprot from the CCP4 suite of programs (Collaborative Computational Project, 1994) and subsequently used as a search model for molecular replacement for both the SeMet and native D1 datasets using PHASER (Storoni et al., 2004). Successive cycles of map cutting, MR, and then NCS averaging were performed in order to improve phases. The quality of the map was manually inspected after each round of refinement to ensure that the map averaging was successful and to redefine the map boundaries in an attempt to identify the region corresponding to a single molecule. Once the maps were of a sufficient quality to clearly identify three of the molecules in the asymmetric unit, six-fold, multi-crystal averaging was performed with DMMulti from CCP4 to improve map quality and ultimately transfer phases from the low-resolution Se-Met data to the high-resolution native data set. After molecular replacement, NCS averaging was applied to improve the quality of the maps. Utilizing this improved map, an initial model of the high-resolution native data was built automatically using the AutoBuild program in PHENIX (Adams et al., 2010) with a NCS matrix enforced. Initial model building revealed the presence of the fourth molecule in the asymmetric unit. With new the four-fold NCS matrix enforced, AutoBuild was able to place approximately 80% of the total number of residues automatically. Iterative rounds of model building and restrained refinement were performed using Coot (Emsley et al., 2010) and PHENIX (ver. 1.8.4-1496) (Adams et al., 2010), respectively. NCS restraints were used during initial stages of refinement, but were released for later stages. The final refinement

protocol included the addition of riding hydrogens, individual B-factor, optimized X-ray/stereochemistry and X-ray/ADP weights, and TLS refinement. Amino acid residues for D1 are numbered according to the full-length Hk1 protein. All crystallographic figures were generated in PyMOL (<http://www.pymol.org/>). The stereochemistry and quality of the model was validated using MolProbity (Adams et al., 2010). Dimer interaction analysis was aided by the use of PDBSum (<http://www.ebi.ac.uk/pdbsum>) (de Beer et al., 2014). Data processing and refinement statistics are summarized in Table 1. Coordinates and structure factors have been deposited in the PDB (PDB ID 5BWJ).

Small angle X-ray scattering (SAXS) data acquisition and analysis

SAXS and WAXS data were collected simultaneously at beamline X9 at the National Synchrotron Light Source (NSLS, Upton, NY) at 10°C by two overlapping detectors, a Mar 165 CCD SAXS detector 3.4 m from the sample, and a custom built Photonic Science CCD WAXS detector. The two-dimensional scattering images collected on the CCD detectors were circularly averaged using software developed at the beamline to yield one-dimensional scattering profiles as a function of momentum transfer q ($q = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle and λ is the wavelength). The X-ray wavelength was 0.855 Å and the angular range collected was $0.008 < q < 1.0060$. The sample cell contained a glass capillary sealed across the evacuated chamber. Protein samples and matching buffer solutions were flowed through the capillary during exposure to reduce radiation damage. For data collection, 90 µl of the protein sample at 1, 3 and 6 mg/ml or matching buffer (50 mM Tris [pH 7.5], 100 mM NaCl) was exposed for 90 s, subdivided into three 30 s exposures of 30 µl. The raw scattering data were scaled and buffer was subtracted using pyXS developed at X9 beamline at Brookhaven National Laboratory (Allaire and Yang, 2011). Individual scattering curves were visually inspected for radiation damage and aggregation prior to merging. Scattering curves for the three concentrations were scaled and merged in *PRIMUS* (Konarev et al., 2003) using predominantly the low Q data for the low concentrations, the high Q range data for the highest concentrations, and the mid-range intensities for the second highest concentration. Radii of gyration (R_g) and $I(0)$ values, extrapolated from the Guinier region of the Guinier plot, were computed using *PRIMUS* (Konarev et al., 2003). $P(r)$ functions were calculated using the program *GNOM* (Svergun, 1992). Theoretical scattering curves were computed from the D1 crystal structure and compared to experimental scattering curves using the program FoXS module (Schneidman-Duhovny et al., 2010) built in UCSF chimera (Pettersen et al., 2004). The relative molecular weight was determined from the SAXS data using *AUTOPOROD* (Petoukhov et al., 2012).

Bioinformatics, homology modeling and multiple sequence alignment

Conserved domains within Hk1 were identified using CD-Search (Marchler-Bauer and Bryant, 2004). Cellular location and topology for Hk1 was predicted using SignalP v 3.0 (Bendtsen et al., 2004), TMPred (Hofmann and Stoffel, 1993) and TOPCONS (Tsirigos et al., 2015). Dimeric interfaces between PBP domains were determined using PDBSum (<http://www.ebi.ac.uk/pdbsum>) (de Beer et al., 2014; Laskowski et al., 1997). Homology models of Hk1 D2 and D3 domains were generated by comparative modeling (Marti-Renom et al., 2000) approaches using MODELLER 9.13 (<http://salilab.org/modeller>) (Sali and Blundell, 1993). Multiple sequence alignments and graphical displays were generated using ClustalW

(<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and ESPript (Gouet et al., 1999), respectively. The BLOSUM scoring matrix option was used for all alignments (Henikoff and Henikoff, 1992). Secondary structure prediction for all three sensor PBPs were generated using PsiPred (Buchan et al., 2013; McGuffin et al., 2000). Opening angles were calculated as previously described (Herrou et al., 2010) and are based on the angle formed between the vectors defined by α carbons of three residues (one on the lip of Lobe 1, one in the hinge region, and one on the lip of Lobe 2). Except where noted, opening angles obtained herein were equivalent or highly similar to those previously reported for the same structure (Herrou et al., 2010).

Results

The Hk1 periplasmic sensor is comprised of three tandem sensor PBP domains

Previously we reported that the periplasmic sensor for Hk1 contained two tandem domains (D1 [residues 23-249] and D2 [residues 255-461]), both of which share sequence similarity to the Cluster 3 family of bacterial type 2 periplasmic binding proteins (PBPs) (Caimano et al., 2011; Tam and Saier, 1993). Despite their low overall sequence similarity (Fig. 1B), type 2 PBPs display a high degree of structural similarity (Cheung et al., 2009; Herrou et al., 2010; Tam and Saier, 1993). Reanalysis of the Hk1 full length periplasmic sensor (residues 23-717) by CD-Search (Marchler-Bauer and Bryant, 2004) revealed a third PBP domain, D3 (residues 471-684; E value=1.29e-17). As expected, D1-3 are predicted by PsiPred to have similar secondary structures (Supplementary Fig. 1). A search of the Pfam database identified ~400 known or putative HKs containing 1-2 sensor PBP domains. However, only a handful of putative HKs contain 3 sensor PBPs; all of these appear to be with hybrid HKs (*i.e.*, contain both histidine kinase and REC domains). Interestingly, with the exception of Hk1 and its orthologs in related *Borrelia* species (*i.e.*, relapsing fever spirochetes), almost all of the hybrid HKs containing 3 or more PBPs are found in marine bacteria (data not shown).

Overall structure of Hk1 sensor domain D1

To better understand how exogenous stimuli are perceived by Hk1, we overexpressed and affinity-purified D1, the most N-terminal PBP domain, from *Escherichia coli* for structural characterization. The D1 crystal structure, determined by multiple-wavelength anomalous dispersion (MAD) phasing, was refined at 2.05 Å. Details of the data collection parameters and refinement statistics are summarized in Table 1. Crystals of D1 grew in the monoclinic space group P2₁, with four molecules in the asymmetric unit arranged as a dimer of dimers (Chains A/B and C/D; Fig. 2A). Using Chain A as a reference, the root mean square deviation (RMSD) values for the B, C and D chains were 1.1 Å, 1.0 Å and 1.0 Å, respectively (also see Supplemental Table 1). In the final refined MAD structure (data not shown), it was revealed that ten of the SeMet residues were completely disordered or had disordered side chains. In the final native structure, only Met70 was consistently ordered in all chains. Met233 was present in 3 of the 4 chains (A, C and D) and Met235 was only ordered in Chain D. The high degree of disorder of the MSE residues complicated the data analysis and reduced the overall anomalous signal. The structure was ultimately solved by using the MAD dataset maps as a molecular replacement model by cutting out the density

and using molecular replacement and NCS averaging over several cycles (see Materials and Methods).

The Hk1 D1 monomer adopts the bilobed Venus Fly Trap (VFT) architecture that is common to ABC transporter and HK sensor PBPs (Cheung et al., 2009; Dupré et al., 2015; Fukami-Kobayashi et al., 1999; Fulyani et al., 2013; Herrou et al., 2010). The VFT-fold is characterized as having two mixed α/β globular domains (Lobes 1 and 2), each comprised of a 5-stranded β -sheet enclosed in 3-4 α -helices, connected by a two β -strand hinge region (Fig. 2B). Using the D1 structure as a query, we searched for D1 structural homologs within the RSCB Protein Data Bank (PDB) (Berman et al., 2000) using the DALI server (Holm and Rosenström, 2010). As expected, HK29_S (PDB id: 3H7M) and VFT2 (PDB id: 3MPL), the prototypes for bacterial sensor PBPs and numerous amino acid ABC transporter PBPs, were among the highest scoring matches (Supplemental Table 2).

The putative ligand binding pocket (LBP) for D1, located in the cleft between Lobes 1 and 2 (Fig. 2B and C), contained only ordered water molecules (Table 1). All three subdomains (Lobe 1, Lobe 2 and the β strand hinge region) contribute amino acid side chains potentially involved in ligand binding (Lobe 1: Asp36, Phe38, Thr92, Asn209; Hinge: Ile139, Thr142, Ser162; Lobe 2: Tyr144, Asp180, Thr183). While the residues lining the LBP are predominantly acidic (Asp36, Asp180) or proton donors (Thr92, Thr142, Ser162, Tyr144, Thr183), the cavity also includes both polar (Asn209) and hydrophobic (Phe38) side chains. Many of the side chains facing the interior of the cavity participate in a water-mediated hydrogen-bonding network. We saw little to no amino acid similarity between residues forming the putative LBPs for D1 and the ABC transporter PBPs ArtJ, GlnBP and HisJ (data not shown), strongly suggesting that preferred ligand(s) for D1 differs from those of its structural homologs. On the other hand, we did see a higher degree of similarity between the D1 and VFT2 LBPs, including conservation of a phenylalanine residue (Phe38 and Phe317 in D1 and VFT2, respectively) involved in ligand binding by VFT2 (Herrou et al., 2010), although, overall, the D1 LBP is much more negatively charged (Supplemental Fig. 2).

Apo-D1 adopts a closed conformation

As per the Venus Fly Trap model, ABC transporter PBPs undergo a substantial conformational change upon ligand binding, which enables the liganded protein to interact with its cognate membrane-bound permease (Felder et al., 1999; Mao et al., 1982; Quirocho and Ledvina, 1996). Based on DALI search results, D1 most closely resembled the closed-liganded forms of ABC transporter PBPs (Supplemental Table 2). Of note, the two previously characterized sensor PBPs, HK29_S and VFT2, also crystallized in a closed conformation in the absence of ligand (Cheung et al., 2009; Herrou et al., 2010). We next determined the opening angles for D1 along with a diverse panel of 10 ABC transporter and 2 HK sensor PBPs (Supplemental Fig. 3 and Supplemental Table 2). The value of the opening angle for D1 (22.3°) was the smallest of the 12 PBPs examined, strongly suggesting that this domain is in a closed conformation when unliganded. However, opening angles for unliganded transporter PBPs can vary substantially depending on the orientation of the domain (Wilkinson and Verschuere, 2003). Thus, it is possible that the opening angle for

D1 in the crystal structure may not be representative of the native domain within the full length membrane-bound sensor.

Hk1 D1 forms a stable dimer

Although HKs typically function as homodimers *in vivo*, periplasmic sensor domains expressed without their corresponding TM and/or cytoplasmic domains are usually monomeric or dimerize only at very high concentration (Cheung and Hendrickson, 2010). Analysis of the D1 crystal structure using PDBsum (Laskowski et al., 1997) and PISA (Xu et al., 2008) indicated that the dimeric conformation is the most stable form; the free energies of dissociation (G^{diss}) for the dimeric interfaces between Chains A/B and C/D, estimated based on the crystal structure, are 8.3 and 9.0 kcal/mol, respectively. Using PDBsum, we identified the residues responsible for D1 dimer formation (Supplemental Fig. 4). The dimeric interfaces between Chains A/B and C/D are stabilized by numerous hydrogens bonds and van der Waals interactions mainly contributed by helix $\alpha 5$ (residues 181-187) (Supplemental Figs. 4 and 5A). The putative LBPs for each domain are oriented on opposite sides of the dimer and tilted by $\sim 45^\circ$ relative to each other (Supplemental Fig. 5A). Using complementary approaches, we confirmed that D1 also forms a dimer in solution: (i) By analytical size-exclusion chromatography (SEC), D1 eluted at 14.4 mls with an estimated Stokes radius (R_s) of ~ 3.35 nm (Supplemental Fig. 5B and C), which is consistent with that of a globular protein having a molecular mass of ~ 60 kDa, twice that of the monomer (27.2 kDa). (ii) The SEC elution profile of D1 did not change when the protein was purified under denaturing conditions (*i.e.*, 3 M urea) (Supplemental Fig. 5D). (iii) As described below, the *ab initio* envelope model for D1, determined by SAXS, most closely resembled that of the Chain A/B dimer. The ratio between the Stokes radius and radius of gyration (R_s/R_g) for D1 determined by SAXS (see below and Table 2) was ~ 0.81 , which is consistent with D1 being a homogenous sphere (Rizos et al., 2006).

Envelope structure of D1 determined by SAXS

We next employed small angle X-ray scattering (SAXS) to define the oligomeric state and envelope structure of at three different sample concentrations (1, 3 and 6 mg/ml) (Fig. 3A). Radii of gyration (R_g) and $I(0)$ values, extrapolated from the Guinier region of the Guinier plot (Supplemental Fig. 6) using *PRIMUS* (Konarev et al., 2003), were highly similar at all three concentrations (Table 2), indicating that D1 is monodisperse in solution. Molecular mass values computed at all three sample concentrations are consistent with that of a dimer. The merged experimental scattering curve for D1 showed excellent agreement with the theoretical scattering curves for the crystal structure dimers ($\chi^2 = 3.02$ and 2.62 for Chains A/B and C/D, respectively) calculated with FoXS implemented in Chimera (<http://www.cgl.ucsf.edu/chimera>) (Fig. 3B). In contrast, the theoretical scattering curve for the Chain A monomer correlated poorly ($\chi^2 = 9.92$) with the experimental curve (Fig. 3B). The distance distribution functions $P(r)$, derived using an indirect Fourier transformation of the scattering curve, as implemented in the program *GNOM* (Svergun, 1992), showed a unimodal peak at ~ 32 Å, which is consistent with D1 having an inflexible globular shape. The maximum distance (D_{max}) for D1 (~ 76 Å) (Fig. 3C) was similar to the theoretical D_{max} (70 Å) calculated from the crystal structure dimer.

Ab initio shape reconstructions from the SAXS data were performed using DAMMIN (Svergun, 1999) and DAMAVER (Volkov and Svergun, 2003) using ten independent models with (P2) and without enforcing symmetry (P1) (Fig. 3D). The mean normalized spatial discrepancy (NSD) values for the P1 and P2 consensus models were similar (0.518 ± 0.04 and 0.545 ± 0.04 , respectively); the NSD value comparing the P1 and P2 consensus models was 0.45. Thus, both symmetries consistently yielded stable reconstructions of similar shapes. The molecular mass estimated from the excluded volume of the DAMMIF models (57.4 kDa) agrees with the expected mass for the D1 dimer (54.4 kDa) based on amino acid sequence. Using COLORES implemented within SITUS, we performed rigid body docking of monomeric and dimeric D1 into the *ab initio* envelope model. As shown in Fig. 3D, the SAXS-reconstructed envelope most closely resembled that of the Chain A/B dimer (NSD = 2.9); the NSD value for the monomer was 5.5.

Homology modeling of Hk1 D2 and D3 PBP domains

Using the D1 structure as a template, we generated homology models for D2 and D3, Hk1's two other sensor PBPs (Fig. 1A). RMSD values between D1 Chain A and the homology models for D2 and D3 were calculated by the secondary structure matching (SSM) method implemented in Coot (Emsley and Cowtan, 2004; Emsley et al., 2010). As expected, the structures for D2 and D3 overlapped very closely with D1 (1.2 Å and 0.96 Å, respectively) (Fig. 4A). Single amino acid changes in the LBPs for sensor and ABC transporter PBPs have been shown to dramatically affect ligand specificity and/or binding affinity (Bjorkman and Mowbray, 1998; Fulyani et al., 2013; Herrou et al., 2010). We next compared the putative LBPs for all three Hk1 sensor PBPs; as shown in Fig. 4B and Table 3, we saw greater overall sequence conservation between D1 and D3. Most notably, substitution of a positively charged residue (Arg407) in D2 for conserved negatively charged residues in D1 and D3 (Asp180 and Asp636, respectively) presumably alters the electrostatic environment of the corresponding LBP cavities substantially (Fig. 4C). Likewise, non-synonymous substitution of Asp36 in D1 for Asn259 and Lys497 in D2 and D3, respectively, would be expected to affect ligand binding. Based on these analyses, we conjecture that Hk1's three sensor PBPs recognize different ligand(s).

Discussion

Bacterial HK sensor PBPs are thought to function by analogy to the Venus Fly Trap mechanism first described for ABC transporters (Cheung et al., 2009; Herrou et al., 2010; Mascher et al., 2006). According to this model, ligand binding by sensor PBPs induces a conformational change within flanking TM domain(s) that is required for activation (or antagonism) of the cytoplasmic kinase core domain (Cheung and Hendrickson, 2010; Lowe et al., 2012b; Mascher et al., 2006; Moore and Hendrickson, 2009; Neiditch et al., 2006; Sevvana et al., 2008). Recently, Dupré *et al.* (Dupré et al., 2015) provided compelling evidence supporting this model; using a mutagenesis approach, they demonstrated that ligand binding by the *B. pertussis* BvgS VFT1 and/or VFT2 sensor domains had a negative effect on kinase activity, presumably by inducing a conformational change within the adjacent transmembrane domain. Structural differences between Hk1 and BvgS suggest that the molecular mechanism underlying signal transduction by these HKs differs: (i) Signal

transduction by BvgS is mediated primarily by inter-domain interactions between VFT1 and VFT2 (Dupré et al., 2015). The presence of a third PBP in the Hk1 sensor substantially increases the number and complexity of intermolecular interactions that could be involved in regulating kinase activity, and, ultimately, synthesis of c-di-GMP by Rrp1. (ii) In contrast to BvgS, Hk1's periplasmic sensor is predicted to be tethered to the cytoplasmic membrane by both N- and C-terminal TM domains. Being constrained at both ends, presumably, decreases the overall flexibility of the full-length sensor and/or help maintain one or more of the PBPs in a more open conformation. (iii) Unlike VFT1 and VFT2 (Dupré et al., 2015; Herrou et al., 2010), D1 forms a dimer in solution; dimerization of D1 in the native membrane-bound Hk1 homodimer could provide an important “control point” for kinase activation by limiting movement of the N-terminal TM domain. Collectively, the above features could confer on Hk1 the ability to sense and respond to the variety of small molecules that *B. burgdorferi* encounters during its enzootic cycle and generate a graded output response that is reflective of the concentration and nature of the ligands bound.

Hk1's sensor PBPs share sequence and structural similarity to the substrate-binding proteins for ABC transporters of polar amino acids (Berntsson et al., 2010; Cheung and Hendrickson, 2010; Cheung et al., 2009; Mascher et al., 2006; Tam and Saier, 1993). The fact that Hk1 is active when *B. burgdorferi* is cultivated in BSK-II medium (Caimano et al., 2015; Caimano et al., 2011; He et al., 2011; Kostick et al., 2011; Rogers et al., 2009), a complex, semi-defined growth medium contains abundant free amino acids (provided by the medium component CMRL 1066), bovine serum albumin and rabbit serum (Barbour, 1984), strongly suggests that one or more of Hk1's sensor PBPs also could recognize amino acids and/or small peptides. Non-synonymous substitutions in the ligand binding pockets suggest, however, that their preferred ligands for D1-3 differ from their transporter orthologs. In an effort to identify the ligands that activate Hk1 *in vitro*, we incubated recombinant D1, which we showed herein is purified from *E. coli* in an unliganded state, with complete BSK-II medium and individual medium components. Using liquid chromatography-mass spectrometry, however, we were unable to detect a media-derived ligand bound to the recombinant protein (data not shown). Likewise, using a thermal shift assay, a technique often applied to ligand-PBP interactions (Giuliani et al., 2008; Pantoliano et al., 2001), we did not detect any binding interactions between recombinant D1 and a panel of potential ligands (i.e., BSK-II, medium components and free amino acids) (data not shown). We envision at least three possible explanations for these results. First, the preferred ligand(s) for D1 may not have been among those tested. Second, activation of Hk1 by BSK-II *in vitro* may be mediated by D2 and/or D3. Third, the LBP for recombinant D1 may be inaccessible given this domain's closed conformation. Distinguishing between these possibilities will require structural and/or biochemical characterization of D2 and D3 and, conceivably, presentation of all three sensor PBP domains within a more native, preferably membrane-bound, configuration.

Activation of the Hk1/Rrp1 TCS is required for *B. burgdorferi* to survive both the acquisition and transmission blood meals (Caimano et al., 2015; Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). During larval acquisition, chemotactic signals emanating from the bite site induce spirochetes to migrate from the skin of the reservoir host into the

tick midgut where they colonize the epithelial cell surfaces (Pal and Fikrig, 2010; Tilly et al., 2008; Zung et al., 1989). During transmission, *B. burgdorferi*, revived from a state of nutrient-deprivation by the influx of the nymphal blood meal, undergo exponential replication and traverse the midgut epithelium *en route* to a new host (de Silva and Fikrig, 1995; Dunham-Ems et al., 2009; Ohnishi et al., 2001; Pal and Fikrig, 2010; Ribeiro et al., 1987). Structural analysis and modeling of D1-3 suggest that the Hk1 sensor has the requisite versatility to distinguish between these two feeding states. These data also raise a key question -- What types of ligands might be used by *B. burgdorferi* to differentiate between acquisition and transmission? Spirochetes within mammalian tissues are exposed continuously to free amino acids within interstitial fluid, thus it seems unlikely that these molecules would be solely responsible for activating Hk1. The Hk1 sensor, most likely, has the ability to recognize host- and/or tick-derived ligands that are unique to the feeding process. One provocative possibility is that one or more of Hk1's PBPs bind neurotransmitters, which are derived from amino acids and would be released by the host in response to tissue injury (i.e., formation of the feeding lesion) (Askenase et al., 1995; Dai et al., 2010; Theoharides et al., 2012). Tick saliva also contains a plethora of bioactive molecules, including dopamine and dopamine sulfate (Koci et al., 2014; Simo et al., 2012; Simo et al., 2011; Yalcin et al., 2010). Interestingly, using qRT-PCR, we recently reported significantly greater induction of c-di-GMP-dependent genes by *B. burgdorferi* in feeding nymphs compared to larvae (Caimano et al., 2015). These data suggest that the Hk1 calibrates the synthesis of c-di-GMP by Rrp1 according to the level of protection required within different tick life stages. The structural data presented herein make it possible to devise a mutagenesis-based strategy to: (i) identify residues involved in ligand binding by each PBP; (ii) determine when during the enzootic cycle each domain is required; and (iii) establish whether these sensor domains act independently or cooperatively to promote Hk1-mediated signal transduction.

Supplementary Material

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Figure 1. The *B. burgdorferi* Hk1 periplasmic sensor is comprised of three domains, each with sequence similarity to HK sensor PBPs and ABC transporter substrate-binding proteins

A. Schematic representation of sensor domains of *B. burgdorferi* Hk1. Based on bioinformatics, Hk1 is predicted to be anchored to the inner membrane by an uncleaved signal sequence (residues 6-26; TM1) and an internal transmembrane domain (residues 716-737; TM2). Conserved domains were identified using CD-Search (Marchler-Bauer and Bryant, 2004). **B.** Multiple sequence alignment of Hk1 sensor domains D1, D2 and D3 and prototypical sensor PBPs and ABC transporter substrate-binding proteins performed using a BLOSUM matrix. Conserved residues (60%) are highlighted in red and outlined in blue. The α -helices and β -strands are marked with coils and arrows, respectively, and are based on the secondary structure of HK29s from *Geobacter sulfurreducens* (PDB id: 3H7M). BvgS_VFT1 and BvgS_VFT2, periplasmic sensor domains from *Bordatella pertussis* BvgS (GenBank accession AAA22970); EvgS_D1 and EvgS_D2, periplasmic sensor domains from *Escherichia coli* EvgS (GenBank accession BAA03108); ArtJ, Arginine-, Histidine-, Lysine-binding protein ArtJ from *Geobacillus stearothermophilus* (GenBank accession KFX35677); GlnBP, GlnP Substrate Binding Domain 2 (sbd2) from *Lactococcus lactis* (GenBank accession WP_003132767); HisJ, ABC transporter substrate-binding protein HisJ from *E. coli* (GenBank accession AAA85769).

Figure 2. Refined crystal structure of *B. burgdorferi* Hk1 sensor domain D1

A. Ribbon drawing of the Hk1 D1 asymmetrical unit (dimer of dimers; Chains A/B and C/D). Chain A, green; Chain B, cyan; Chain C, magenta; and Chain D, yellow. **B.** Ribbon drawing depicting Lobe 1 [residues 27-109, 208-234] and Lobe 2 [residues 110-207] of Chain A. β strands and helices are indicated in magenta and cyan, respectively. The dashed line between β strands 7 and 8 indicates an unstructured loop. The putative ligand binding pocket (LBP) positioned between two lobes is indicated by a dashed circle. **C.** Side chains of residues predicted to form the D1 LBP are shown in gray. Amino acid residue numbering is based on full length native Hk1.

Figure 3. SAXS analysis of domain

A. Experimental scattering curves for D1 collected at 1, 3 and 6 mg/ml. **B.** Experimental curve for D1 (black) represents the merged scattering data collected at all three protein concentrations. Theoretical scattering curves were computed using the crystal structure coordinates for the Chain A monomer (green) and Chain A/B (red) and C/D (blue) dimers. **C.** $P(r)$ function plot of D1 calculated from averaged experimental curves in panel A using *GNOM* (Svergun, 1992). **D.** *Ab initio* reconstruction of the D1 molecular envelope calculated using DAMMIF and DAMAVER (Franke and Svergun, 2009) overlaid with the crystal structure Chain A/B dimer. Helix $\alpha 5$ in each monomer is shown in red.

Figure 4. Homology modeling of Hk1 sensor domains D2 and D3

A. Homology model of D2 (yellow) and D3 (cyan) overlaid with D1 (green). **B.** Multiple sequence alignment of all three Hk1 sensor PBPs was generated using Clustal W (Thompson et al., 1994) and ESPript (Gouet et al., 1999). Residues that are identical or similar in all three PBPs are highlighted and outlined in black, respectively. Residues predicted to form the LBPs for each domain are outlined in red. Cartoon depictions of α helices (loops) and β strands (arrows) in the D1 crystal structure are shown above the corresponding residues. **C.** Predicted ligand binding pockets for Hk1 D1 (green), D2 (yellow) and D3 (cyan) sensor PBP domains. Amino acid numbering is based on full length native Hk1.

Table 1

Data collection and refinement statistics

	Native	Inflection	Peak	Remote
Data Collection				
Wavelength (Å)	0.97910	0.97935	0.97910	
Resolution range (Å)	37.66 – 2.05	34.42 – 3.00	34.42 – 3.05	
Space group	P 1 2 ₁ 1	P 1 2 ₁ 1	P 1 2 ₁ 1	P 1 2 ₁ 1
Unit cell	58.30, 62.85, 111.81, 90, 101.32, 90	67.08, 63.27, 107.10, 90, 106.89, 90	66.89, 63.17, 106.58, 90, 106.58, 90	67.08, 63.15, 106.98, 90, 106.77, 90
R _{sym}	0.063 (0.554) ¹	0.064 (0.368)	0.083 (0.662)	0.051 (0.353)
Completeness (%)	99.55 (95.0)	91.70 (91.4)	100 (100)	99.3 (92.1)
Mean I/σ(I)	8.82 (1.29)	13.9 (3.87)	14.63 (4.54)	14.30 (4.21)
Redundancy	3.3	2.4	13.3	2.5
Refinement Statistics				
R (work/free)	0.1945/0.2206			
No. of Unique Reflections	49534 (4772)			
Number of Residues	796			
Molecules / ASU	4			
Number of Atoms:				
Non-hydrogen	6644			
Protein	6354			
Ligands	33			
Water	257			
Wilson B-factor (Å ²)	38.64			
RMS (bonds)	0.003			
RMS (angles)	0.650			
Ramachandran favored (%)	96.0			
Ramachandram outliers (%)	0			
Clashscore	0.32			
Overall Molprobity Score	0.94			
PDB Deposit	5BWJ			

Parentheses denote statistics for the outer shell.

RMS, root mean square deviations from ideal geometry of bonded angles.

Table 2

Summary of SAXS analysis.

Protein	Conc. ^a	Rg(Å) ^{b,c}	I(0) ^c	I(0)/Conc (μM)	MM ^d (Daltons)
1	18 μM	26.05 ± 0.78	23.1 ± 0.08	1.28	65,641
3	54 μM	27.74 ± 1.1	57.6 ± 0.28	1.06	55,829
6	108 μM	29.68 ± 0.6	79.1 ± 0.28	0.75	59,795
Merged data ^e		27.70 ± 1.03	56.6 ± 1.09		60,422

^aProtein concentration determined using Nanodrop. Molarity is based on the dimeric conformation

^bThe radius of gyration (*R_g*) is the root mean square distance from the center of mass to each electron.

^cDetermined by Guinier approximation in *PRIMUS* (Konarev et al., 2003).

^dMolecular masses (MM) are based on Porod volumes calculated using *AUTOPOROD* (Petoukhov et al., 2012).

^eValues represented the merged normalized data for all three protein concentrations.

Table 3Residues lining the LBPs for each PBP.¹

D1	D2	D3
Val35	Tyr258	Val496
Asp36	Asn259	Lys497
Phe38	Tyr261	Trp499
Thr92	Gly316	Thr554
Ile139	Phe363	Ile595
Thr142	Ile366	Phe598
Tyr144	Leu368	Tyr600
Ser162	Tyr386	Phe618
Asp180	Arg407	Asp636
Thr183	Ser410	Thr639
Asn209	Asn427	Thr657

¹Residues are based on the full length Hk1.