Sensor Domain of Histidine Kinase KinB of *Pseudomonas aeruginosa*

A HELIX-SWAPPED DIMER*  

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Kemin Tan1‡,‡, Gekleng Chhor‡, T. Andrew Binkowski‡, Robert P. Jedrzejczak1, Magdalena Makowska-Grzyska1†, and Andrzej Joachimiak1,‡,‡

From the 1Midwest Center for Structural Genomics and 2 Structural Biology Center, Biosciences, Argonne National Laboratory, Argonne, Illinois 60439 and the 4Center for Structural Genomics of Infectious Diseases, University of Chicago, Chicago, Illinois 60637

**Background:** The sensor domain (SD) of histidine kinase (HK) KinB (KinB-SD) receives signals from the environment and induces a transduction cascade.

**Results:** Structures of the KinB-SD were obtained in ligand-free, phosphate-bound, and mutant forms.

**Conclusion:** The unique helix-swapped KinB-SD structure forms a ligand-binding cavity on the dimer interface.

**Significance:** KinB-SD studies provide insights into the signal transduction and identity of potential signaling molecules.

The overproduction of polysaccharide alginate is responsible for the formation of mucus in the lungs of cystic fibrosis patients. Histidine kinase KinB of the KinB-AlgB two-component system in *Pseudomonas aeruginosa* acts as a negative regulator of alginate biosynthesis. The modular architecture of KinB is similar to other histidine kinases. However, its periplasmic signal sensor domain is unique and is found only in the *Pseudomonas* genus. Here, we present the first crystal structures of the KinB sensor domain. The domain is a dimer in solution, and in the crystal it shows an atypical dimer of a helix-swapped four-helix bundle. A positively charged cavity is formed on the dimer interface and involves several strictly conserved residues, including Arg-60. A phosphate anion is bound asymmetrically in one of the structures. In silico docking identified several monophosphorylated sugars, including β-D-fructose 6-phosphate and β-D-mannose 6-phosphate, a precursor and an intermediate of alginate synthesis, respectively, as potential KinB ligands. Ligand binding was confirmed experimentally. Conformational transition from a symmetric to an asymmetric structure and decreasing dimer stability caused by ligand binding may be a part of the signal transduction mechanism of the KinB-AlgB two-component system.

Overproduction of the exopolysaccharide alginate by *Pseudomonas aeruginosa* in bacterial pulmonary infections leads to the buildup of mucus in the lungs of patients with cystic fibrosis (1). Alginate biosynthesis is tightly controlled by a set of genes (2–4). One of these is AlgB, which was shown to serve as a negative regulator of alginate production (5). AlgB is a two-component response regulator of the NtrC family (6). The cognate sensor kinase of AlgB is KinB (GI:15600677), which is a histidine kinase (HK) (7).

Similar to many HKs, KinB, at its N terminus, has a periplasmic sensor domain (KinB-SD) (~120 amino acids) flanked by two transmembrane helices that anchor it to the membrane (7). The sensor domain (SD) is unique to the *Pseudomonas* genus and has no significant sequence similarity with any other known proteins. Within the different strains of *P. aeruginosa*, the sequence identities are larger than 85%. The sequence identity with other members of the *Pseudomonas* genus is between 28 and 35%. After the second transmembrane helix, the cytoplasmic portion of KinB is a regular HK that is composed of several domains as follows: (i) a histidine kinases, adenyl cyclases, methyl-accepting proteins, and phosphatases (HAMP) domain; (ii) Per-Arnt-Sim (PAS) domain, and (iii) a His-kinase-A (phosphoacceptor) core containing a specific histidine-containing dimerization subdomain and a kinase subdomain. In alginate regulation, it is believed that the KinB-AlgB (HK-RR) pair works similarly to other two-component systems (TCSs) in signal relay from the periplasm to cytoplasm, such as the PhoQ-PhoP and PmrB-PmrA pairs among others found in *P. aeruginosa* (8). A typical TCS signaling mechanism involves the periplasmic KinB-SD receiving an environmental signal. Binding a signal molecule causes a structural change in the SD that is transmitted across the membrane resulting in the auto-phosphorylation of the specific histidine in the dimerization subdomain of the HK core. The phosphate is then transferred to a specific aspartate of the response regulator, AlgB. Subsequently, AlgB mediates AlgW-dependent MucA proteolysis leading to the reduction of alginate biosynthesis (5). Recent studies have significantly expanded the function of KinB. It was

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‡ To whom correspondence should be addressed. E-mail: andrzejj@anl.gov.

‡‡ The abbreviations used are: HK, histidine kinase; SD, sensor domain; TCS, two-component system; Man-6-P, β-D-mannose 6-phosphate; Fru-6-P, β-D-fructose 6-phosphate; SdhEt, seleno-methionine; r.m.s.d., root mean square deviation; BLI, biolayer interferometry; DSF, differential scanning calorimetry; DLS, dynamic light scattering; PDB, Protein Data Bank; PAS, Per-Arnt-Sim; SAD, single-wavelength anomalous diffraction; LS, least squares.

§‡ The atomic coordinates and structure factors of the KinB-SD in ligand-free, phosphate-bound, and mutant forms were deposited in the Protein Data Bank (PDB) (http://www.pdb.org/) (3KKB, 3L34, 4LLC, and 4LLC1).
shown that in addition to alginate synthesis down-regulation, KinB seems to control a large number of genes coding for virulence factors that are important to the development of acute pneumonia and causes a switch between chronic and acute infection (9, 10). KinB also appears to be essential in acute infections in zebrafish embryos and other virulence-associated phenotypes, such as quorum sensing, biofilm formation, and motility (11). These phenotypes, however, are independent of KinB’s kinase activity and its response regulator AlgB, indicating an atypical, noncanonical TCS signaling mechanism (10). Moreover, one report suggested that in the regulation of virulence, KinB functions as a pho-phosphate dephosphorylate AlgB instead of an AlgB kinase (12). It is not clear whether these KinB functions involve signaling molecule(s). All of these data added complexity to the regulatory activities of KinB and the signal transduction pathways. It is believed that a part of the intricacy may involve possible diverse responses of SDs to different signaling molecules. Despite these recent discoveries, the KinB-SD and its signaling molecule(s) have remained uncharacterized. Understanding the KinB signal transduction mechanism and identifying the signaling molecules may be important for developing cystic fibrosis treatments.

The HK SDs of TCSs have been investigated previously (13–15). These domains show significant sequence diversity, and it is believed that they can recognize a wide variety of chemical signals. Upon binding a ligand, the information is transmitted into the cell, initiating a kinase response. The majority of previously characterized SDs adopt two main types of structural scaffolds, the PAS-like and four-helix bundle domains. A PAS-like (16) or “PDC” fold (17) is represented by the structures of PhoQ (17, 18), DcuS (19), and Cta (16). The double PAS-like domain has also been observed in LuxQ (20), DctB (21, 22), and in recently reported KinD (23). PAS-like SDs are believed to serve as ligand recognition units in extracytoplasmic signal transduction proteins (24). A four-helix bundle SD is represented by NarX (25), and a double four-helix bundle SD is represented by TorS (26). This domain is believed to be a ubiquitous sensor module for HKs, and it contributes conserved residues to the dimerization interface (27). In both PAS-like and four-helix bundle SDs, a conformational change in the dimer upon signaling molecule binding is recognized as a necessary and key step in the signal transduction from the periplasmic to the cytoplasmic portion of TCSs. Other structural scaffolds for HK have also been observed. Hendrickson and co-workers (28) reported a functionally uncharacterized SD (HK29r) of HK encoded by the Geobacter sulfurreducens GSU2755 gene. HK29r shows a periplasmic solute-binding protein fold described as a “Venus flytrap” (28). A double periplasmic solute-binding protein sensor domain has also been identified and described in the sensor kinase BvgS of the TCS BvgAS (29), which regulates the virulence of Bordetella pertussis in whooping cough (30). Recently, crystal structures of a SD consisting of two seven-blade ß-propellers and an Ig-like fold have also been reported from a Bacteroides thetaiotaomicron hybrid TCS, which contains all the domains of a typical HK in a single polypeptide (31). The discovery of a periplasmic solute-binding protein, a seven-blade ß-propeller, and an Ig-like SD expands the structural modules used for signaling in TCSs and shows a more diverse set of signaling molecule receptors associated with HKs.

Some SDs are found across many species and super-kings, but some are unique to a specific genus. Pseudomonas KinB-SDs belong to the latter group and show a unique sequence. A secondary structure prediction suggests an all-helical structure placing it in a four-helix bundle subgroup. However, the conserved residues found in four-helix bundle SDs are not present (27). Initially, KinB was aligned and compared with a SD of HK PhoR from Bacillus subtilis, with which it shares 31% amino acid sequence identity and 59% similarity (7). A recently reported PhoR SD structure revealed an ß/ß PAS-like fold (24).

Here, we present high resolution crystal structures of the KinB-SD in a ligand-free form and a complex with a bound phosphate. These structures reveal an all-helical four-helix bundle structure, consistent with secondary structure predictions, forming an atypical helix-swapped dimer. Interestingly, KinB sequence similarity to PhoR suggests that PAS-like SDs may be evolutionarily related. Several site-directed mutations were introduced to confirm helix swapping, and structures of R60L and R60E mutants were determined. Inspired by the unique cavity formed on the interface between two monomers where the phosphate group was found, in silico ligand screening was performed. Among the top scoring ligands were several monophosphorylated sugars, including ß-D-fructose 6-phosphate (Fru-6-P) and ß-D-mannose 6-phosphate (Man-6-P), a precursor and an intermediate of alginate synthesis, respectively (32). Several assays were performed to evaluate ligand binding and the potential impact on KinB and its structure and function. The combination of the structural, mutagenesis, computational, and biophysical data provides clues to potential signaling molecule(s) of the KinB and a possible signal transduction mechanism.

**EXPERIMENTAL PROCEDURES**

**Protein Cloning, Expression, and Purification**—A portion of the P. aeruginosa PAO1 kinB gene (Gln-42 to Ala-168) corresponding to the periplasmic SD was cloned into the pMC6G19 vector. The gene was overexpressed in Escherichia coli BL21. The cells were grown at 37 °C in enriched M9 medium containing seleno-methionine (SeMet) under conditions known to inhibit methionine biosynthesis (33). The SeMet-labeled KinB-SD was purified from the E. coli cells using nickel-affinity chromatography (34). The protein was concentrated using an Amicon Ultra 3K centrifugal filter device (Millipore Inc.) in 20 mM HEPES, pH 8, 250 mM NaCl, and 2 mM dithiothreitol (DTT). The protein showed a final yield of about 40 mg/liter culture. Site-directed mutations of R60A, R60L, and R60E were created by the PIPE cloning method (35). The plasmid mentioned above was used as the template; primers were added to a final concentration of 0.4 µM, and mutagenesis was performed in a final volume of 50 µl using PfuUltra Hotstart PCR Master Mix (Stratagene). The procedure from the subsequent transformation of PCR products to mutant expression and purification was the same as described above for wild-type KinB-SD. Plasmids of mutated proteins were sequenced to confirm the presence of mutations.
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The native avi-tagged KinB-SD utilized in a biosensor interferometry (BLI) study using an Octet RED system (FortéBIO) was created by cloning the same wild-type kinB-SD gene into the pMCSG62 vector, which contains an avi tag (36). The sequence confirmation, plasmid transformation, protein expression, and purification of the avi-tagged KinB-SD were the same as described for the native KinB-SD with the following exceptions: the cells were grown in LB containing 40 mM K₂HPO₄, and the protein was purified and concentrated in buffer containing 20 mM Tris, pH 8, 50 mM NaCl, and 2 mM DTT.

Size Exclusion Chromatography—Size exclusion chromatography of the wild-type KinB-SD was performed on a Superdex 200 10/300GL column using an ÄKTA FPLC (GE Healthcare). The column was pre-equilibrated with the crystallization buffer (20 mM HEPES, pH 8, 250 mM NaCl, 2 mM DTT) and calibrated with premixed protein standards, which included ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and blue dextran (13.7 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and blue dextran (2,000 kDa). The chromatography was carried out at room temperature with a flow rate of 0.3 ml/min using a 100-µl protein sample at a concentration of 47.5 mg/ml. The subsequent size exclusion chromatography of wild-type KinB and point mutants (R60A, R60L, and R60E) was performed on an SRT SEC-150 column (Sepax Technologies) connected to a Dionex HPLC equipped with a temperature-controlled autosampler housing two 96-well plate sample racks, a GP50 gradient pump, and a PDA-100 photodiode array detector (Thermo Scientific). The chromatography runs were carried out at room temperature with a flow rate of 1 ml/min. Calibration of the SRT SEC-150 column was performed with protein standards as described above.

Protein Crystallization—The proteins were screened for crystallization conditions with the help of a Mosquito nanoliter liquid handler (TTP LabTech) using the sitting drop vapor diffusion technique in 96-well CrystalQuick plates (Greiner). For each condition, 0.4 µl of protein (at 47.5 mg/ml) and 0.4 µl of crystallization formulation were mixed; the mixture was equilibrated against 140 µl of the crystallization solution in each reservoir well. The crystallization screens used were Index (Hampton Research), ANL-1 (Qiagen), and ANL-2 (Qiagen) at two temperatures, 4 and 24 °C. Diffraction quality crystals appeared under multiple conditions including the following: 1) 1.6 M sodium dihydrogen phosphate, 0.1 M phosphate-citrate, pH 4.2, and 0.4 M di-potassium hydrogen phosphate at 24 °C; 2) 0.1 M ammonium acetate, 0.1 M sodium acetate, pH 5.6, 30% PEG 4000, and 15% glycerol at 24 °C. Prior to data collection, the crystals were treated with cryoprotectants and cryocooled directly in liquid nitrogen. Co-crystallization trials with mono-phosphorylated sugars, including Fru-6-P and Man-6-P, were performed under crystallization condition 1 described above and supplemented with 10% of the sugars.

X-ray Diffraction and Structure Determination—A single-wavelength anomalous diffraction (SAD) data set was collected near the selenium absorption peak (12.66 keV) at 100 K from each type of KinB-SD crystal. All data were obtained at the 19-ID beamline of the Structural Biology Center at the Advanced Photon Source at Argonne National Laboratory using the program SBCcollect (37). The intensities of each data set were integrated and scaled with the HKL3000 program suite (Table 1) (38). For the phosphate-bound KinB-SD crystal containing SeMet-labeled protein, two molecules with four SeMet residues were expected in one asymmetric unit. Heavy atom sites were located using the program SHELXD (39), and four selenium sites were found and used for phasing with the program MLPHARE (40). After density modification (40), a partial model comprised of 180 residues in two chains (71% of a dimer) with 121 side chains was built in three cycles of Arp/wArp (41) model building. All of the above programs are integrated within the program suite HKL3000 (38). The model was completed manually using the program COOT (42). The final model, a helix-swapped all-helical dimer of the KinB-SD, was refined using the program Phenix.refine (Table 1) (43). The structure of the ligand-free crystal was determined using the molecular replacement method (44). The search template used was the KinB-SD swapped dimer with the connecting loops truncated. Four copies of the swapped dimers were located in the asymmetric units of the crystal. After several cycles of refinement, the connecting loops between the helices were rebuilt using $F_o - F_c$ difference electron density maps. Final refinement was carried out with the program Phenix.refine (Table 1) (43). The structure of mutant R60L was determined using the same molecular replacement approach as described for the ligand-free KinB-SD (Table 1). The structure of mutant R60E was determined using the same SeMet SAD phasing approach as described for the structure of SeMet-labeled, phosphate-bound KinB-SD (Table 1).

Differential Scanning Fluorimetry Assay—A differential scanning fluorimetry (DSF) assay was carried out using an iCycler thermal cycler (Bio-Rad) controlled by the iQ5 optical system software (Bio-Rad). The assay was performed in a 96-well PCR plate. The fluorescent dye used was SYPRO Orange (Invitrogen), which interacts with the hydrophobic portion of a protein to greatly enhance its fluorescence emission. In each well, 4 µl of 50× dye was mixed with 36 µl of protein at a concentration of 20 µM. The sample plate was sealed and centrifuged for 1 min at 1000 rpm and then placed in the thermal cycler. After 2 min, the temperature was increased gradually with a heating rate of 1 °C/min. The fluorescence intensity from each well versus temperature (melt curve) was measured, and a melting temperature ($T_m$) was calculated from the maximum value of the negative first derivative of the melt curve. The data were analyzed using GraphPad Prism Version 5.00 for Mac OS X (GraphPad Software).

Circular Dichroism Spectroscopy Assay—Circular dichroism (CD) spectroscopy was performed on a J-810 CD spectropolarimeter (Jasco). The cell path length was 1 mm, and the sensor domain protein concentration was 0.1 mg/ml with a volume of 300 µl. Each CD spectrum acquired represents an average of three scans collected from 200 to 260 nm with a step size of 0.1 nm at a rate of 50 nm/min and a bandwidth of 1 nm. For the thermal stability experiment, one spectrum was measured every 5° from 25 to 90 °C. In each step, the temperature was ramped up slowly and stabilized for 3 min before data acquisition. The baseline of each spectrum was corrected by subtracting the spectrum of a buffer blank obtained at 25 °C. The results were converted to per residue molar absorption units, $\Delta e$ (M⁻¹ cm⁻¹).
The data were analyzed using the program GraphPad Prism mentioned earlier.

**Dynamic Light Scattering Assay**—Dynamic light scattering (DLS) was performed using DynaPro Plate Reader Plus (Wyatt Technologies, Inc.) in a 384-microwell plate format. The experiment was to measure the hydrodynamic radius of the KinB-SD and its evolution during a heating ramp in the presence and absence of small ligands. An increase of the hydrodynamic radius of a protein with a temperature is an indication of protein thermal denaturation and subsequent aggregation (45). A shift of the temperature in the presence of a potential ligand suggests an influence of the ligand on the thermal stability/unfolding/aggregation of the protein. Typically, the molecular hydrodynamic radius does not necessarily reach a plateau during heating, and the measurement of the hydrodynamic radius of the protein tends to fluctuate after a protein starts to unfold and aggregate; a melting temperature cannot be simply defined based on a given fitting model, such as the calculated peak position of the first derivation of the melting curve using a Gaussian distribution model. Therefore, the onset of the hydrodynamic radius expansion was defined as a characteristic temperature ($T_{onset}$) when the calculated first derivative reaches 1, which approximately means that the hydrodynamic radius doubles its size per degree Kelvin at the temperature. The radius usually rapidly becomes larger after this point. Before the calculation of the first derivative values, melting curves were subjected to a rapid basis runs were conducted, one in which the bound phosphate group, a list of potential signaling molecules was obtained identified that adhere to the geometric and biochemical constraints of the pocket.

Reducing the set by filtering for a phosphate group, a list of potential signaling molecules was obtained (Table 2). The molecules were docked using the software FRED (OpenEye Scientific). During docking, all conformations of the molecules were iterated to identify a consensus pose. Two independent runs were conducted, one in which the bound phos-

| TABLE 1 |
| --- |
| **Data collection and crystallographic statistics** |

|                | PO$_2^-$-bound | Ligand-free | R60L mutant | R60E mutant |
|----------------|----------------|-------------|-------------|-------------|
| **Data collection** |                |             |             |             |
| Space group     | $P_3\overline{1}$ | $P_3\overline{1}$ | $P_1$       | $P_3\overline{1}$ |
| Unit cell       | $a = b = 75.36$, $c = 75.02$ Å | $a = 61.51$, $b = 103.08$, $c = 69.47$ Å, $\beta = 90.01^\circ$ | $a = 45.23$, $b = 47.80$, $c = 49.45$ Å | $a = b = 84.65$, $c = 73.9$ Å |
| Molecular mass (residue) | 14,448 Da (130$^a$) | 14,448 Da (130$^a$) | 14,448 Da (130$^a$) | 14,448 Da (130$^a$) |
| Mol (ÅU)        | 2              | 4           | 2           | 2           |
| SeMet (ÅU)      | 4              | 16          | 8           | 4           |
| Wavelength      | 0.9794 Å (peak) | 0.9793 Å (peak) | 0.9793 Å (peak) | 0.9793 Å (peak) |
| Resolution      | 37.8 to 1.88 Å | 41.4 to 1.70 Å | 34.0 to 1.68 Å | 36.7 to 2.00 Å |
| No. of unique reflections | 20,202$^g$ | 93,984$^d$ | 4,068$^d$ | 2,012$^d$ |
| Redundancy      | 5.4 (3.8)$^g$ | 4.5 (3.8)$^d$ | 5.0 (5.0)$^d$ | 7.5 (7.6)$^d$ |
| Completeness    | 98.8% (86.3%)$^g$ | 99.5% (94.7%)$^d$ | 95.6% (95.9%)$^d$ | 99.7% (100.0%)$^d$ |
| $R_{merge}$     | 9.7% (54.1%)$^g$ | 6.8% (73.5%)$^d$ | 6.5% (51.8%)$^d$ | 7.6% (55.1%)$^d$ |
| $I/|I_0|$       | 23.8 (2.3)$^g$ | 26.1 (1.5)$^d$ | 41.0 (4.0)$^d$ | 43.8 (3.1)$^d$ |
| Solvent content | 42.2% | 35.5% | 28.7% | 53.6% |
| **Phasing**     |                |             |             |             |
| $R_{calcd}$     | 87%            | MR$^f$      | MR$^f$      | 83%         |
| Figure of merit | 20.4%$^a$ | 15.2%$^b$ | 15.2%$^b$ | 15.2%$^b$ |

**Refinement**

|                |                |             |             |             |
| Resolution      | 37.8 to 1.88 Å | 41.4 to 1.70 Å | 34.0 to 1.68 Å | 36.7 to 2.00 Å |
| Reflections (work/test) | 18,538/982 | 87,857/4875 | 40,952/2054 | 20,191/1026 |
| $R_{work}$      | 16.3/2.00%    | 18.2/2.55%  | 19.6/2.42%  | 19.5/2.29%  |
| r.m.s.d. from ideal geometry, Bond length/angle | 0.006 Å/0.876$^e$ | 0.006 Å/0.964$^e$ | 0.006 Å/0.924$^e$ | 0.007 Å/0.961$^e$ |
| No. of atoms (Protein/HETATM) | 1974/223 | 7501/731 | 3700/282 | 1868/142 |
| Ramachandran plot statistic | | | | |
| Residues in most favored regions | 98.7% | 96.9% | 97.1% | 94.5% |
| in additional allowed regions | 1.3% | 3.1% | 2.9% | 5.5% |
| in generously allowed regions | 0% | 0% | 0% | 0% |
| in disallowed region | 0% | 0% | 0% | 0% |
| Solvent content | 42.8% | 35.5% | 28.7% | 53.6% |
| PDB deposit    | 3KKB         | 3L34        | 4LLE        | 4LLC        |

$^a$ Data include a three-residue N-terminal tag, SNA.
$^b$ Data include Bijvoet pairs.
$^c$ The last resolution bin was 1.88–1.91 Å.
$^d$ The last resolution bin was 1.70–1.73 Å.
$^e$ The last resolution bin was 1.68–1.71 Å.
$^f$ The last resolution bin was 2.00–2.03 Å.
$^g$ Molecular replacement (MR) method was used (44).
$^h$ This number was obtained after SAD phasing before density modification (40).

| TABLE 2 |
| --- |
| **Results of docking to KinB-SD for potential signaling molecules** |

| Compound | Mannose 6-phosphate | Glucose 1-phosphate | Fructose 6-phosphate | Mannose 1-phosphate | Glucose 1-phosphate |
|----------|---------------------|---------------------|---------------------|---------------------|---------------------|
| FRED Chemgauss 4 Score | −10.96 | −9.84 | −9.78 | −9.49 | −8.45 |
helix was used as a constraint to pose the molecules and one where the molecules were able to explore all possible conformational spaces. Both iterations provided the same final pose. The Chemgauss 4 scores for each molecule are shown in Table 2.

Ligand Binding Using Biolayer Interferometry—BLI was used to measure the interaction of the KinB-SD with the top two ligands predicted by in silico analysis and involved in alginate synthesis. The KinB-SD with an avi tag was purified as described earlier. Assays were performed in 96-well black microplates (Fisher) at 30 °C on an Octet RED instrument (FortéBIO). The KinB-SD was loaded onto Super Strepavidin biosensors (FortéBIO) at a concentration of 2 mg/ml in phosphate-buffered saline (PBS) buffer. The reference Super Strepavidin biosensors were blocked with EZ-link biocytin (Thermo Scientific) at 10 μg/ml in PBS buffer. For comparison, both sugars and their phosphorylated derivatives were tested. Each sugar was titrated from 0.23 to 30 mM in a buffer containing 20 mM Tris, pH 8.0, 50 mM NaCl, and 2 mM DTT. Triplicate samples were prepared for each sugar dilution. Assays were run first using the protein biosensors, followed by the reference biosensors using the same protocol. The reference data were then subtracted from the protein data to account for nonspecific binding. No binding to the KinB-SD was observed for fucose and manose. To better estimate binding affinity for the phosphorylated sugars, an expanded concentration range was tested for Man-6-P (0.23–266 mM) and Fru-6-P (0.23–658 mM) with the highest values being near the solubility limit for the examined sugar. To determine apparent equilibrium dissociation constants (K_D), the response signal at equilibrium was plotted against sugar concentration. Steady-state analysis of the data were performed using GraphPad Prism.

RESULTS AND DISCUSSION

KinB-SD Forms a Helix-swapped Dimer—The recombinant KinB-SD crystallized under several conditions. We have determined high resolution structures of the ligand-free and phosphate-bound forms (Table 1). In the ligand-free structure, there are eight monomers in one asymmetric unit. The phosphate group-bound structure was determined using a crystal obtained from a crystallization solution containing 2.1 M phosphate; there are two monomers in one asymmetric unit. The ligand-bound form is more structurally ordered and is used below to describe details of the KinB-SD.

In the phosphate-bound structure, two all-helical KinB-SDs form an α4 swapped dimer with an approximate 2-fold symmetry (Fig. 1, A and B). Monomer A includes residues from Gln-42 to Ala-164 and monomer B from Gln-42 to His-166. In both monomers, the N-terminal SNA sequence (cloning artifact) is visible in the electron density, and it extends the N-terminal α1 helix. Each monomer is composed of four helices with its fourth helix (α4) exchanged in position with the α4 helix of its partner, creating an eight-stranded α-helical bundle. The two hinge loops between the α3 and α4 and α3′ and α4′ helices, involved in the α4 swapping, are well ordered, and their electron densities are unambiguous in the high resolution structure. However, the hinge loop conformation is very different in the two monomers (Figs. 1, B and C, and 24). In monomer A, this loop is short (Ala-127 to Asn-132), but in monomer B it is four res-
idues longer (Thr-121 to Ala-130). The longer loop in monomer B is a result of unwinding of the C terminus of the /H9251 helix.

The two hinge loops show distinct conformations. Particularly, the Met-129 residue in monomer A points inward and is completely buried. The same residue in monomer B is fully solvent-exposed (Fig. 1C). When the two monomers are superimposed (using C atoms) by least squares (LS) fitting, the resulting root mean square deviation (r.m.s.d.) value is 1.71 Å, and the hinge loops and their proximity show the largest differences.

In a typical four-helix bundle, the /H9251 helix would make the majority of contact with the /H9251 and /H9251 helices of the same chain and in its dimeric form it would interact with two helices, /H9251 and /H9251, of the second chain (Fig. 1D). In the KinB-SD swapped four-helix bundle dimer, the /H9251 helix interacts only with the /H9251 helix.
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helix of the same chain and with three helices, α3’, α1’, and α4’, of the opposite chain. Therefore, because of swapping, the dimer interface now involves three helices (α1, α3, and α4) from each monomer instead of two (α1 and α4) (Fig. 1B). The α4-swapping nearly doubles the surface of the dimer interface as compared with the unwrapped dimer (Table 3). The total buried surface-accessible area of the α4-swapped dimer is 4714 Å² with a complexation significance score of 1 (Table 3) (47). The size exclusion chromatography data (Fig. 2B) also indicate that in solution the wild-type KinB-SD exists as a dimer.

A Cavity within the Dimer Interface—The KinB-SD dimer interface shows several distinctive features. In the eight-helix bundle dimer, four helices (α1, α4, α1’, and α4’) are longer and protrude away. Two N and two C termini are located in close proximity (12–16 Å apart) on the same side (Fig. 1A bottom). Two N and two C termini are located in close proximity (12–16 Å apart) on the same side (Fig. 1A bottom) of the dimer. In the full-length protein, these helices are presumed to extend into the membrane, and therefore this region is proximal to the membrane. On the opposite side of the dimer (Fig. 1A, top), the hinge loop-containing region is most distal from the membrane. In the dimer α-helical bundle, there are eight discrete layers of side chain interactions. Six layers of the side chain packing involve all eight helices, and two layers involve only the four longest helices (α1, α4, α1’, and α4’). These interactions involve intra- and inter-monomers. As expected, the majority of interactions are hydrophobic or comprise hydrogen bond networks by highly conserved residues and solvent molecules.

The fifth layer of the KinB-SD shows quite a unique interaction, and it includes strictly conserved Arg-60 residues from helix α1/α1’ on the dimer interface. The planar guanidinium groups of the Arg-60/Arg-60’ side chains from the two opposing monomers are stacked in an almost perfectly parallel fashion with their C5–C5 interatomic distance of 3.68 Å (Fig. 3, A and B). Stacking of guanidinium moieties of arginine pairs has been reported previously and is quite common in proteins (48). For example, a similar guanidinium-guanidinium ion pair (Arg-3/Arg-89) is present in tryptophan synthase from Salmonella typhimurium. This interaction is stabilized by an aspartic acid carboxylate (PDB entry 1KFC) (49). However, this pair as well as many other arginines reported previously are exposed toward the solvent region instead of being buried in the core of the protein (48). Solvent molecules surrounding such an ion pair are believed to provide additional stabilization to the close face-to-face approach of two arginines (50). In the KinB-SD, the energetically unfavorable packing of two positively charged Arg-60 residues at the dimer interface seems mitigated by the local environment. Multiple hydrogen bonds, both direct and water-mediated, between these two arginines and the glutamine residues (including Gln-147/Gln-147’ from the fifth layer, and Gln-57 and Gln-157, from both monomers that form the fourth layer of interaction just below the arginines (not shown in Fig. 3, A and B) may help reduce the repelling force between the two guanidinium groups. Nevertheless, this site may be well suited to accept a negatively charged ion.

Above the positively charged fifth layer is the sixth layer that features strictly conserved Gln/64 and Arg-143 and conserved Asp/Glu/Asn-65 (Fig. 2A). In this layer, the side chains of the two Arg-143 residues pull away from the center and point outward, forming salt bridges with the Asp-65 residues (Fig. 3, A and B). The Arg-143 residues are also contacting hydrophobic residues in the seventh layer. If the side chains of these arginines would assume conformation similar to the two Arg-60 residues, the guanidinium groups of Arg-143 residues would have approached each other and positioned exactly above the two Arg-60 guanidinium groups in the fifth layer described earlier, forming a highly unusual cluster of four guanidinium groups at the center of the dimer interface. It appears that Asp-65 serves as a guide for Arg-143 side chain conformation and forces it to bend away and consequently create a cavity on the dimer interface of a volume of 1009.6 Å³ in the middle of the sixth layer of the helical bundle (Fig. 3, B and C). The cavity spans across an approximate 2-fold axis that relates the A and B monomers. The core of the seventh layer includes Leu-67 and Phe-139, which seemingly provide a hydrophobic cap for the cavity centered in the sixth layer. Most of the residues that are critical in forming the cavity, including Arg-60, Gly-64, Phe-139, and Arg-143, are completely conserved in KinB HKs within the Pseudomonas genus (Fig. 2A). This is the only cavity in the KinB-SD that can accommodate a 200–300-Da ligand, and we propose that the cavity serves as the signal molecule-binding site.

Asymmetric Binding of a Phosphate in Cavity—In the cavity, a phosphate group is positioned asymmetrically (Fig. 3). It
forms two strong hydrogen bonds with Arg-60 (2.8 and 3.2 Å), one weak interaction with Arg-60’ (3.6 Å), a strong hydrogen bond with main chain carbonyl of Arg-143 (2.8 Å), and a water-mediated hydrogen bond (2.6 Å) that is held by the guanidinium group of Arg-143 and the amino group Gln-147 side chain. There are also several hydrogen bonds to water molecules (not shown in figures) that bridge the anionic group with other surrounding protein atoms. The phosphate group is shifted toward monomer B but makes the strongest interaction with Arg-60 of monomer A. The position of the phosphate group inside the ligand-binding site and the hinge loops are the two most asymmetric elements of the KinB-SD dimer.

**Nearly Symmetric Ligand-Free KinB-SD**—In the ligand-free structure, eight monomers form four similar helix-swapped dimers in one asymmetric unit, which are packed in an alternative up-and-down fashion. In the cavity of each dimer, only water molecules are found. When compared with the phosphate-bound SD, the ligand-free structure has higher atomic displacement parameters (B-factors), although its crystal has lower solvent content and a higher diffraction limit. This suggests higher plasticity of the ligand-free KinB-SD dimer (Table 1) and, as often observed, the binding of a phosphate orders the structure.

In each of the four ligand-free swapped dimers, two monomers are more similar to each other and therefore the dimer is more symmetric (pseudo 2-fold) than that in the phosphate-bound dimer. The orientation of Met-129, which points inward in monomer A and outward in monomer B, seems to be the only major difference between the two monomers (Fig. 4A). The r.m.s.d. values of the pairwise structural alignment (LS fitting, Cα atoms) of the monomers between the four A-chain monomers (6 pairs), between the four B-chain monomers (6 pairs), and between the A- and B-chains (16 pairs) are 0.67 ± 0.22, 0.35 ± 0.05, and 0.74 ± 0.17 Å, respectively. None of the individual r.m.s.d. values are larger than 1 Å. This is in contrast to the r.m.s.d. value (r.m.s.d. 1.71 Å) from the LS alignment between the chain A and B monomers in the phosphate-bound structure.

The comparison of the phosphate-bound dimer structure with the four ligand-free dimers shows conformational changes induced by phosphate binding (Fig. 4, B and C). In addition to the large conformational change of the monomer B hinge loop (α3-α4 loop), the phosphate-bound dimer also shows an outward movement of its α2-α3 turn (Fig. 4, B and C). Most interestingly, in monomer A, the N-terminal portion of α1 and the C-terminal region of the α4 helices shift away from the dimer interface (Fig. 4, B and C). As indicated earlier, this region is proximal to the membrane, and this shift when propagated across the membrane may change the relative orientation of the HK cytoplasmic domains.

**Structures and Dimerization of R60A, R60L, and R60E Point Mutants**—Arginine 60 is one of the highly conserved residues of KinB (Fig. 2A); it forms a unique symmetrically stacked guanidinium interaction buried on the dimer interface and binds the phosphate anion. To test the structural and functional roles of Arg-60, three point mutations, R60A, R60L, and R60E, were produced and characterized. These mutants, when compared with the wild type, shift the oligomeric state of the KinB-SD in solution from a dimer to a monomer (Fig. 5A). In addition, diffraction quality crystals of the R60L and R60E mutants were obtained, and their structures were determined. In solution, R60L is a mixture of monomer and dimer in a ratio of ~2:1.
dimer, arginine 60 is buried at the dimer interface, whereas in the R60E mutant the glutamate side chain is solvent-exposed. There are two R60E monomers in one asymmetric unit, which are very similar except for the α3−α4 hinge loop region (Fig. 5C) and can be superimposed with an r.m.s.d. of 0.62 Å (Ca atoms). The structures of both the R60L and R60E mutants show B-factors even higher than the ligand-free wild-type protein (Table 1), underscoring the higher flexibility of the ligand-free structure.

Structural Homologues—A Dali structural homology search (51) using a structure of the helix-swapped monomer form (monomer A) results in no significant hits. The closest match is a three-helix bundle of a spectrin repeat of plectin that aligns with the α1, α2, and α3 helices of monomer A (PDB entry 2ODV, Z-score 8.5, r.m.s.d. value of 2.0 Å, and length of alignment of 87 amino acids with a sequence identity of 7%). The low structural homology of the KinB-SD to protein structures underscores the novelty of the helix-swapped four-helix bundle dimer. In contrast, using the R60E mutant monomer structure as a search template yields a large number of hits. This is not surprising because a four-helix bundle exists as a common structural motif in many protein structures, either as part of a core structure or at protein-protein interfaces (52). Three helix bundle SDs with known structures, TAR (53), NarX (25), and TorS (26), are all among the top hits. For example, the SD of E. coli HK NarX has a Z score of 11.5 (PDB entry 3EZB, r.m.s.d. value of 2.5 Å, and length of alignment of 107 amino acids with a sequence identity of 11%).

Binding of a Phosphate Group in Solution—We carried out DSF and CD assays (54) to investigate the binding of a phosphate in solution to the KinB-SD. The protein unfolding temperature (Tm) was measured using DSF at different phosphate concentrations. The Tm gradually shifts to a higher temperature from 47 to 54 °C as the phosphate concentration increases from 0.1 to 1 M (Fig. 6, A and B). A similar stabilization effect was observed in the pH range of 4.2 to 7.5. However, it appears that the affinity for the phosphate anion is low. CD spectra were also recorded to measure the thermal denaturation of the KinB-SD (Fig. 6C). These spectra, based on the measurement of the helical content of the SD, correlate helix unfolding to the thermal denaturation of this all-helical protein. The first derivatives of the unfolding curves of the KinB-SD fits a single Gaussian distribution (Fig. 6, D and E), suggesting that the unfolding of the SD dimer is largely a one-step event. We also observed an increased Tm at a high phosphate concentration in good agreement with the results of the DSF assay. It suggests that the phosphate ligand bound in the KinB-SD stabilizes the structure and may be a part of a signaling molecule.

Potential Identity and Structural Impact of Signaling Molecule(s)—The nature of the KinB signaling molecule(s) remains unknown, although the KinB-AlgB TCS has drawn noteworthy interests. In silico ligand screening was performed to provide a rationale for the exploration of potential ligands based on the cavity properties of the KinB-SD dimer. Surprisingly, the top hits of the docking experiment are monophosphate sugars (Table 2 and Fig. 7), including the precursor and the intermediate of alginate biosynthesis (55), which the KinB-AlgB TCS negatively regulates. These ligands show orientations
with a nearly identical overlap of the phosphate moiety position in the cavity. To test these results experimentally, binding of precursor Fru-6-P and intermediate Man-6-P of alginate biosynthesis was examined using DSF, CD, and BLI assays. In contrast with the phosphate binding experiments, no significant Tm shift for the KinB-SD could be detected in the presence of these monophosphate sugars using DSF and CD assays (Fig. 6).

However, we were able to estimate binding affinity of Man-6-P and Fru-6-P toward KinB using BLI and an Octet RED system (Fig. 7B). For Man-6-P, the estimated equilibrium dissociation constant, $K_D$, was 25 ± 3 mM. It was more difficult to measure the $K_D$ value for Fru-6-P due to solubility limits and instrument limitations, but it was estimated to be ~250 mM. Importantly, the nonphosphorylated analogues (fructose and mannose) were also tested and did not show any binding to the KinB-SD. These results when combined with the phosphate stabilization effect on the KinB-SD suggest that the phosphate group plays an important role in the binding of these monophosphate sugars to the KinB-SD, although overall binding affinities are weak. The apparent $K_D$ value for Fru-6-P is so low that it may be nonspecific. Interestingly, DLS data show that the binding of monophosphate sugars, which induces some unfavorable conformational changes and thus decreases the structural stability of the KinB-SD dimer. We attempted to determine structures of KinB-SD complexes with phosphorylated sugars, but thus far co-crystallization with these ligands did not produce crystals containing a bound monophosphate sugar.

The impact of signal molecule binding on the structure and stability of the SD has been rarely studied (23). To produce a conformational change, the binding of a signal molecule may induce the formation of an energetically favorable SD dimer or stabilize a weak SD dimer. However, binding of a signal molecule may disrupt an SD dimer or lower its stability. Both protein thermal stabilization and destabilization by bound ligands have been observed and examined (56). The binding of monophosphate sugars to the KinB-SD may be an example of the latter case, as observed in our DLS experiments. The binding may induce a conformational change in the KinB-SD dimer, reducing its stability; this change is then propagated through the membrane.

A closer inspection of the KinB-SD structure (Fig. 3) and the docking model (Fig. 7A) reveals the extensive potential for binding of a phosphate anion-containing sugar molecule that can form hydrogen bonds and hydrophobic interaction in addition to the ionic ones observed for the phosphate anion-Arg-60 (Figs. 2 and 7). However, the lack of hydrogen bonds from the
hydroxyl groups of monophosphate sugars to the protein apparently limits the strength of their binding to the KinB-SD. The actual binding modes of monophosphate sugars may be somewhat different from the ones predicted in *in silico* studies (Fig. 7A), in which ligands were docked to a rigid KinB-SD dimer. The KinB-SD dimer with a bound monophosphate sugar within the dimer interface may adopt a slightly more open and less energetically favorable conformation.

Several experiments were performed to characterize the effect of binding of monophosphate sugars on the structure and stability of the KinB-SD. Both DSF and CD measurements showed an increase in KinB-SD stability in the presence of phosphate (Fig. 6) but showed no effect in the presence of sugar monophosphates. In contrast, only DLS showed a destabilization of the KinB-SD in the presence of Man-6-P and Fru-6-P, respectively. C, DLS experiments of KinB and KinB in the presence of Fru-6-P or Man-6-P in low salt conditions (50 mM NaCl, 20 mM Tris, pH 8). The values of $T_{\text{onset}}$ of each melting curve is an average of three independent runs. Under high salt conditions (250 mM NaCl, 20 mM HEPES, pH 8), none of the melting curves reached plateaus. The values of $T_{\text{onset}}$ are 46.3 °C (KinB), 43.2 °C (KinB + Fru-6-P (F6P)), and 41.2 °C (KinB + Man-6-P (M6P)), respectively (data not shown).

**Implication to Signaling Transduction Mechanism**—The binding of a signal molecule and dimerization of the HK-SD are regarded as critical steps for signal transduction across the membrane from the periplasm to the cytoplasm. However, these issues continue to remain controversial, because in many cases the nature of a signal molecule and dimerization state of the SD are difficult to prove. For example, PAS-like sensor domains are monomeric or weakly dimeric in solution (19). Some even appear to be monomers in crystals that are grown from highly concentrated protein solutions (22, 28). The known four-helix bundle SD, NarX (25), forms dimers in the crystal, but no oligomeric states in solutions have been reported. The KinB-SD, existing as a dimer in both structure and solution, seems to form a stable homodimer, presumably benefiting from the helix swapping. In HKs it was shown that transmembrane helices, cytoplasmic HAMP, PAS, and His-Kinase-A domains all form homodimers (14). Therefore, the two periplasmic SDs of an HK dimer are very likely physically close to each other in the periplasm, regardless of whether or not a dimer is formed.

Several mechanisms have been proposed on how a SD dimer transmits a signal across the membrane (13–15). These mechanisms generally propose a small structural change induced by ligand binding that is propagated through the membrane. The conformational changes include a ligand binding-induced asymmetry in the SD dimer (57). In this study, we found that the...
KinB-SD forms a stable dimer in solution and in the crystal. A cavity is formed at the dimer interface that is large enough to accommodate a small ligand, and it contains a buried arginine pair that may be well suited to accept a negatively charged anion. In the absence of any ligand, the KinB-SD dimer appears to be a flexible and symmetric structure, but it becomes more rigid and somewhat asymmetric upon the binding of a phosphate anion. The binding of the phosphate occurs in the middle of the molecule, but it seems to cause a tilt of the KinB membrane-proximal α1 N- and α4 C-terminal helices that may be a part of the signaling across the membrane. Conformational changes may be caused by asymmetric binding of a ligand that is negatively charged and binds to positively charged Arg-60 pairs inside the dimer. Although the binding of a phosphate group to the KinB-SD is of low affinity, it is suggestive that the signaling molecule may include a phosphate group such as monophosphate sugars, which have been predicted in silico and experimentally evaluated in this study. Although these monophosphate sugars seem to fit well into the profile of a potential signaling role, the monophosphate sugars and their signal transduction mechanism are of high priority to be addressed. Consideration of the roles of the monophosphate sugars and their signal transduction mechanism is of high priority to be addressed. Considering the complexity of TCSs, we cannot exclude possibilities of other signaling molecule(s) interacting with the KinB-SD.

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