Role of the PAS Sensor Domains in the *Bacillus subtilis* Sporulation Kinase KinA

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Histidine kinases (HKs) are the most ubiquitous molecular sensors used by bacteria. They work in concert with a cognate response regulator (RR) to sense and respond to a plethora of environmental stimuli, including changes in pH, light, temperature, cellular energy levels, redox state, and the presence of toxins and food (1, 2). Some HKs are essential for bacterial viability due to their role in essential cellular processes, while others are important for mediating antibiotic resistance and virulence; this has led to the idea that some HKs might be good antimicrobial targets (2–5).

HKs function by autophosphorylating on a conserved histidine residue and then transferring the resultant high-energy phosphate to a conserved aspartate residue on the RR (6, 7). The RR is usually (but not always) a transcription factor that displays altered or enhanced affinity for its cognate DNA recognition elements upon phosphorylation (1). HKs are modular, homodimeric proteins. The cytoplasmic C-terminal domain of the protein is known bioinformatically as the HisKA domain. It is always involved in dimerization, autophosphorylation, and phosphate transfer and is made up of a four-helix bundle (the dimerization and histidine phosphotransfer [DHp] domain) that carries the phosphorylatable histidine and a C-terminal catalytic domain (often termed “Cat”), which binds ATP (8–10). HisKA is preceded by an N-terminal “sensor” module that varies in length and domain complexity between different HKs (11). Most HKs are membrane bound, and the body of the sensor module is typically separated from the catalytic domain by the membrane and the membrane-spanning regions of the protein. There are several HKs, however, that are entirely cytoplasmic and others that are membrane bound with both their N-terminal sensor and C-terminal catalytic modules in the cytoplasm.

The most common cytoplasmic signaling domains are PAS domains (12, 13). These domains are found in combination with a great variety of other signaling domains in both plant and animal proteins, but in bacteria, they are almost exclusively associated with HKs. PAS domains often mediate protein-protein interactions, and this function in turn is often modulated via ligand binding to the PAS domain (14–16). PAS domains have been shown to bind a diverse array of ligands, including heme, flavins, 4-hydroxycinnamic acid, carboxylic acids, and divalent metal ions (17).

Sporulation of *Bacillus subtilis* is a major developmental step that occurs upon nutrient starvation. Whether or not the cell commits to sporulation is determined by the level of phosphorylated Spo0A, a master transcription regulator (18, 19), which in turn is governed by a complex phosphorelay (20) initiated primarily by autophosphorylation of KinA, a cytoplasmic HK. One way in which the phosphorelay is controlled is through regulation of KinA activity via a number of antikinases; these proteins include Sda and KipI, both of which block KinA autophosphorylation (21–26). There is also a causal link between the cellular level of KinA and the bacterium’s sporulation status (27).

KinA is an unusual HK in that, as well as being non-membrane bound, its N-terminal sensor module is comprised of three tandem PAS domains, termed PAS$_A$, PAS$_B$, and PAS$_C$ (13, 28). It was suggested that the sensor module of KinA detects a sporulation-specific signal that regulates the activity of the autokinase (AK) domain. Although this hypothesis cannot be discounted as a mechanism for fine-tuning of KinA function (29), it was recently shown that the sensor module is not essential for KinA activity, as...
it can be substituted with a chimeric construct that supports both
KinA multimer formation and host cell sporulation (30). This
suggests that the N-terminal region of KinA does not have to rec-
ognize a sporulation signal in order to activate KinA and that it
instead plays a largely structural role by enhancing KinA dimeriza-
tion, which then allows autophosphorylation (31). In support of
this, the KinA catalytic domain by itself does not drive sporula-
tion, but it will allow sporulation when tagged with sections of the
N-terminal sensor module that support multimer formation (32).
Although an order of affinity for the putative PAS-PAS ho-
modimer interactions in the KinA sensor has been proposed (32),
some questions remain about how the N-terminal domain holds the
catalytic domain of KinA in a functional conformation.

In this study, we examined the KinA PAS domains from a
structural perspective in an attempt to better define their struc-
tural and functional roles as well as the overall architecture of
KinA. We have defined the minimal autonomously folding unit of
each PAS domain, determined their oligomeric state, and exam-
ined their contribution to KinA autokinase activity. Taken to-
gether, the results allow us (i) to clarify aspects of the sensor struc-
ture that have previously been unclear and (ii) to propose a model
for how the KinA sensor module holds the autokinase module in a
functional conformation.

MATERIALS AND METHODS

Plasmid construction, bacterial strains, and growth media. Standard
procedures were used for DNA manipulation (33). Plasmids expressing
wild-type KinA (residues 1 to 606) and the His-autokinase (AK) module
(residues 383 to 606) were gifts from K. Cunningham (22). The strains
and plasmids used in this study are described in Table 1. Escherichia coli
DH5α (34) and E. coli BL21 (DE3) (35) were used for cloning experi-
ments and protein overproduction, respectively. E. coli strains were grown in
Luria-Bertani medium and transformed by heat shock (33) with selection
on plates supplemented with 100 μg/ml ampicillin (Amp) or 25
μg/ml kanamycin (Kan).

DNA for the truncation constructs PASa, PASb, PASc, PASbc-AK, and
PASa-AK (where the subscript indicates which PAS domain is in-
cluded) was obtained by PCR amplification using B. subtilis chromosomal
DNA as the template. PCR products were purified, digested by using
EcoRI and BamHI, and ligated into either pGEX-2T (GE Healthcare) to
generate N-terminally glutathione S-transferase (GST)-tagged KinA con-
structs or pET28a (Novagen) to generate N-terminally His6-tagged con-
structs. KinA expression is isopropyl-thiogalactopyranoside (IPTG)
inducible in both plasmids. Each plasmid was sequenced to verify the
insert sequence.

Protein production and purification. 15N-labeled PAS domains were
expressed and purified according to a method that we described previ-
ously for Sda (24). Unlabeled proteins were produced by using Luria
broth and then purified in the same manner as the labeled proteins.
Briefly, E. coli BL21(DE3) cells containing expression plasmids (listed in
Table 1) were grown at 37°C, and the temperature was then shifted to 18°C
once the culture optical density at 600 nm (OD600) reached 0.4. Protein
expression was then induced with 100 μM IPTG at an OD600 of 1.0. Cells
were harvested by centrifugation at 22 h postinduction.

His6-tagged proteins (wild-type [WT] KinA and the AK construct)
were purified by resuspending the cell pellet in equilibration buffer (250
mM NaCl, 25 mM Tris, 10 mM imidazole, 5 mM β-mercaptoethanol [pH
7.5]) and then lysing the cells by using a cell disruptor (Constant Systems
TS series benchtop) operating at a constant pressure of 25 kpsi. The
cell lysate was centrifuged at 30,882.8 × g for 45 min at 4°C to remove insol-
uble debris, and the supernatant was then loaded onto Ni-nitrilotriacetic
acid (NTA) resin (Qiagen), which was then washed with 10 column vol-
umes of equilibration buffer. Proteins were liberated from the His6 tag
on-column cleavage with thrombin (150 U per liter of culture); cleavages
were performed in equilibration buffer without imidazole. Removal of the
His6 tag leaves a vestigial tripeptide sequence (GSH) at the N terminus of
the protein. Cleaved proteins were eluted from the column with equili-
bration buffer.

For constructs harboring N-terminal GST tags (i.e., PASbc-AK and
PASa-AK), cell lysates were obtained as described above, and the super-
natant from the centrifugation step was then loaded onto glutathione
affinity beads (Sigma). The column was first washed with lysis buffer (pH
8.0) and then lysing the cells by using a cell disruptor (Constant Systems
TS series benchtop) operating at a constant pressure of 25 kpsi. The
cell lysate was centrifuged at 30,882.8 × g for 45 min at 4°C to remove insol-
uble debris, and the supernatant was then loaded onto Ni-nitrilotriacetic
acid (NTA) resin (Qiagen), which was then washed with 10 column vol-
umes of equilibration buffer. Proteins were liberated from the His6 tag
by on-column cleavage with thrombin (150 U per liter of culture); cleavages
were performed in equilibration buffer without imidazole. Removal of the
His6 tag leaves a vestigial tripeptide sequence (GSH) at the N terminus of
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natant from the centrifugation step was then loaded onto glutathione
affinity beads (Sigma). The column was first washed with lysis buffer (12
mM NaF, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], 0.5
mM phenylmethylsulfonyl fluoride [PMSF] [pH 7.2]) to remove nonspe-
cifically bound proteins, and the column was then equilibrated with
thrombin cleavage buffer (150 mM NaCl, 50 mM NaF, [pH 7.5]). Proteins
were liberated from the GST tag by on-column cleavage with thrombin
(150 U per liter of culture). Removal of the GST tag leaves a vestigial

TABLE 1 Plasmids and strains used in this study

<table>
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<tr>
<th>Plasmid or strain</th>
<th>Description or genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>WT KinA (PASbc-AK)</td>
<td>IPTG-regulated promoter, pET28a origin; Kan²; His₆-KinA wild type (residues 1–606); thrombin cleavage site</td>
<td>22 (His₆-KinA1–606)</td>
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<tr>
<td>PASa1-138, PASa1-117, PASb1-117</td>
<td>IPTG-regulated promoter, pGEX-2T origin; Amp³; GST-PASa (residues 1–138, 1–117, or 1–117); thrombin cleavage site</td>
<td>This study</td>
</tr>
<tr>
<td>PASb145–264, PASb145–257, PASb136–255</td>
<td>IPTG-regulated promoter, pGEX-2T origin; Amp³; GST-PASb (residues 145–264, 145–257, or 136–255); thrombin cleavage site</td>
<td>This study</td>
</tr>
<tr>
<td>PASc</td>
<td>IPTG-regulated promoter, pGEX-2T origin; Amp³; GST-PASc (residues 269–382); thrombin cleavage site</td>
<td>This study</td>
</tr>
<tr>
<td>PASbc-AK</td>
<td>IPTG-regulated promoter, pGEX-2T origin; Amp³; GST-PASbc-autokinase (residues 136–606); thrombin cleavage site</td>
<td>This study</td>
</tr>
<tr>
<td>PASa-AK</td>
<td>IPTG-regulated promoter, pGEX-2T origin; Amp³; GST-PASa-autokinase (residues 145–606); thrombin cleavage site</td>
<td>This study</td>
</tr>
<tr>
<td>AK</td>
<td>IPTG-regulated promoter, pET28a origin; Kan²; His₆-autokinase (residues 383–606); thrombin cleavage site</td>
<td>22 (His₆-KinA383–606)</td>
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</tbody>
</table>

Plasmids

<table>
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<tr>
<th>Strains</th>
<th>Description or genotype</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>E. coli BL21</td>
<td>F⁻ ompT hsdSB(r– m–) gal dcm (DE3)</td>
<td>35</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>F’ Δ(argF-lacZ)U169 phoA gblv44 Δ(lacZ)M15 gyaA96 recA1 relA1 endA1 thi-1 hsdRI7</td>
<td>34</td>
</tr>
</tbody>
</table>
dipeptide sequence (GS) at the N terminus of the protein. Cleaved pro-
teins were eluted with lysis buffer and collected in the eluate.

For autophosphorylation assays, all proteins were first desalted into
phosphorylation buffer [25 mM Tris, 250 mM NaCl, 0.5 mM EDTA, 1
mM tris-(2-carboxyethyl)phosphine (TCEP), 20 mM MgCl2 (pH 7.5)] by
using a HighPrep 26/10 desalting column (GE Healthcare). Protein purity
was analyzed by using fast protein liquid chromatography (FPLC)
and SDS-PAGE.

Size exclusion chromatography (SEC) and all subsequent experiments
for PASa and PASb were performed by using buffer A (20 mM Tris, 200
mM NaCl, 1 mM EDTA, 1 mM TCEP, 0.1% NaN3 [pH 7.5]), while buffer
B (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 10 mM TCEP, 0.1% NaN3
[pH 7.5]) was used for PASc. Proteins were desalted into these buffers as
required. The solubility of PASc was improved at lower NaCl concentra-
tions, and the higher concentration of reducing agent in buffer B was
necessary to stop PASc from forming a disulfide-bonded dimer. Nuclear
magnetic resonance (NMR) experiments were performed with buffer C
(20 mM Tris, 150 mM NaCl, 1 mM TCEP [pH 6.85]).

**Multiangle laser light scattering.** Multiangle laser light scattering
(MALLS) was performed as described previously (24, 36), using a
miniDAWN Tristar laser light scattering photometer and an Optilab DSP
interferometric refractometer (both from Wyatt Technology). SEC was
performed with buffer B by using a Superdex 75 HR 10/30 column (Phar-
macia Biotech). All samples were injected in a final volume of 200 µl to
avoid volume-related retention time artifacts. Estimates of the weight-
average molecular mass (Mw) were determined by using Debye fitting;
reported errors are the standard deviations (SD) of the Mw estimate.

**NMR spectroscopy.** Two-dimensional (2D) 1H-15N heteronuclear
single-quantum coherence (HSQC) spectra were acquired at a tempera-
ture of 298 K on Varian Inova 500- and 600-MHz spectrometers using
15N-labeled PAS domains (50 to 150 µM for PASa, 100 to 550 µM for
PASb, and 20 to 50 µM for PASc), using buffers A and B. The HSQC
spectra of PASc in buffer A and buffer B were identical at the concentra-
tions of protein used for these experiments, but PASc solubility was
greater at 100 mM NaCl (buffer B). Data were processed with NMRPipe
(37), and spectra were analyzed by using XEASY (38).

**Analytical ultracentrifugation.** Analysis of PAS domains using ana-
lytical ultracentrifugation (AUC) was carried out by using buffer A
with either 1 mM TCEP (PASa and PASb) or 10 mM TCEP (PASc). Additional
AUC experiments with PASc were carried out by using buffer B. The following molecular masses (M) and partial specific volumes (v) of the
PAS domain constructs were calculated at 20°C from the amino acid com-
position using SEDNTERP (39): PASa, M = 13,749, v = 0.7424 ml/g; PASb,
M = 13,265, v = 0.7351 ml/g; PASc, M = 13,796, v = 0.7432 ml/g. AUC
experiments were conducted by using a Beckman XL-1 ultracentri-
fuge using the interference optical system. Sedimentation velocity (SV)
experiments were performed by using aluminum-Epon double-sector
synthetic boundary centerpieces at 20°C. Initially, the sedimentation pro-
files were analyzed by using the time-derivative g(r) method with
DCDT+ (40) and the c(s) method with Sedit (41). Data sets at multiple
loading concentrations were also globally fitted to single ideal species
(PAS) or monomer-dimer association models (PAS2) by using both Sed-
phat (42) and Sedanal (43). Weight-average sedimentation coefficients
were obtained by integration of the main peak from the c(s) distributions,
and the resulting Sinc isotherms were fit to a monomer-dimer association
model using IGOR Pro. Sedimentation equilibrium (SE) measurements
to correspond to PASa

RESULTS

Defining the N- and C-terminal boundaries for each PAS do-
main. Previous studies of the primary structure of the 382-residue
KinA sensor module indicated that it contained three PAS do-
 mains (13). Although the structure of most PAS domains con-
forms to the canonical PAS fold, the minimum autonomous fold-
in g unit of a PAS domain often cannot be determined from
sequence analyses alone. The domain boundaries predicted by
TIGRFAM for PASa, PASb, and PASc are residues 1 to 123, 139 to
262, and 263 to 387, respectively, but the PROSITE, SMART, and
Pfam databases all make different predictions about the N- and
C-terminal boundaries of the PAS domains. Thus, our initial sub-
cloning of these domains was guided by additional information,
including known three-dimensional (3D) structures of PAS do-
main and the likely position of interdomain linker regions based on
V8 proteolysis of KinA (45).

**PASa.** We acquired 2D 1H-13C-15N HSQC spectra of several PASa
constructs in order to assess folding and aggregation status
(46). The longest construct (PASa

1–117) yielded an HSQC spectrum with overall very good chemical shift dispersion. However, there were many intense peaks with random-coil chemical shift values (i.e., 1H chemical shifts of 7.8 to 8.6 ppm), suggestive of unstructured termini and possibly other disordered regions. Moreover, these longer constructs were unstable during purification and were naturally proteolyzed into a smaller fragment that was revealed from N-terminal sequencing and mass spectrometry to correspond to PASa

1–117. V8 proteolysis of PASa

1–138 also yielded a single fragment that corresponded to PASa

1–117 (data not shown). The peaks in 2D HSQC spectra of both PASa

1–117 and a shorter construct, PASa

1–112, had excellent chemical shift dispersion with very few intense peaks in the random coil region, indicating that each of these proteins folds au-
tonomously into a well-ordered tertiary structure (data not
shown). In each case, however, the spectra contained approxi-
mately 25 fewer peaks than expected, even though SDS-PAGE gels
indicated that the proteins had not been proteolyzed. The HSQC
spectrum did not change significantly, and no additional peaks
were detected with changes in protein concentration, pH, or tem-
perature or the addition of potential ligands (ATP, ADP, and GTP
at several concentrations). Similar NMR results were obtained
independently in another laboratory for an almost identical
PASa

10–117 construct (47).

An HSQC spectrum with a defined number of missing peaks is
usually indicative of a protein that is undergoing interconversion
between multiple states, such as monomer-dimer exchange, which
generally makes the protein unsuitable for structure deter-
mination using NMR methods. Nevertheless, the NMR results
indicate that residues 1 to 117 (and residues 11 to 117) of the KinA
sensor module form a stable, autonomously folded domain, as

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indicate that residues 1 to 117 (and residues 11 to 117) of the KinA
sensor module form a stable, autonomously folded domain, as
reported previously by Lee and coworkers for PASA\textsubscript{10–117} (47). Moreover, in contrast to the results obtained previously by Wang \textit{et al.} (45), but consistent with the previously determined crystal structure of PASA\textsubscript{10–117} (47), we find that this protein domain is not monomeric at concentrations above 10M (see below).

**PASB.** We initially subcloned a PASB construct that encompassed residues 145 to 264. A 2D HSQC spectrum of this construct showed a larger-than-expected variation in peak intensity, suggestive of aggregation and/or chemical exchange-induced line broadening (46). We next produced a construct with trimmed N- and C-terminal boundaries (PASB\textsubscript{154–257}), which corresponds to a literature prediction for PASB (28). This fragment was unstable during purification and yielded an HSQC spectrum with very little chemical shift dispersion that is typical of an unfolded protein. However, a 9-residue shift of the initial PASB domain boundaries produced a fragment (PASB\textsubscript{136–255}) that yielded a significantly improved HSQC spectrum with excellent chemical shift dispersion (Fig. 1A), indicating that this fragment autonomously folds to give a well-ordered tertiary structure. However, the peak line widths obtained for this construct were broader than those obtained for PAS\textsubscript{C} (see below) (Fig. 1B); this might indicate that PASB is a dimer or undergoing a chemical exchange process that leads to NMR line broadening.

**PAS\textsubscript{C}.** The HSQC spectrum of PAS\textsubscript{C}\textsubscript{269–382} displayed excellent resolution (i.e., well-dispersed peaks), narrow line widths (i.e., no evidence of aggregation or other line-broadening phenomena), and minimal peak overlap (Fig. 1B). This indicates that PAS\textsubscript{C}\textsubscript{269–382} is an autonomously folded monomer with very few, if any, disordered regions and is likely to be suitable for NMR structure determination.

**Figure 1A** shows the PAS domain boundaries as defined by the NMR analyses reported here. It is significant, as discussed further below, that these boundaries (particularly the N terminus of PAS\textsubscript{B}) differ from those used in two previous studies where KinA domain deletion studies were performed (31, 45). A third study (32) used boundaries more similar to those which we have defined. Correct assignment of the PAS domain boundaries is critical for examining the properties of each domain.

**Self-association properties of the isolated PAS domains.** We used MALLS and analytical ultracentrifugation to determine the self-association properties of the purified PAS domains. For SV studies, protein samples were prepared at multiple concentrations, and data were initially analyzed to define the homogeneity of the preparations, to assess the association state(s), and to test for concentration-dependent self-association. The SV data were examined by using the time-derivative (\(c/\ln c\)) method, a model-independent transformation of the data that enables determination of an apparent sedimentation coefficient distribution function, \(g(s^*)\) (48). For PAS\textsubscript{B}, which does not undergo reversible self-association (see below), the data were examined by using the \(c(s)\) method, which models the data as a sum of noninteracting Lamm equation solutions to remove diffusional broadening (41).

**PAS\textsubscript{A} weakly self-associates.** MALLS analysis of PAS\textsubscript{A}\textsubscript{1–117} (13.7 kDa) yielded a peak with a calculated \(M_w\) of 21.0 ± 0.8 kDa when the protein was loaded onto an SEC column at a concentration of 18 \(\mu\)M (0.25 mg/ml) (data not shown). Doubling or halv-
FIG 2  AUC and MALLS analysis of the N-terminal PAS domains of KinA. (A) Sedimentation velocity analysis of PASA. Shown are normalized g(s*) distributions obtained for PASA at concentrations of 0.1 mg/ml (solid), 0.6 mg/ml (dashes), and 2.0 mg/ml (dots). Conditions were as follows: rotor speed of 55,000 rpm, temperature of 20°C, and interference optics. The g(s*) distributions and weight-average sedimentation coefficients were calculated by using DCDT+ (32). (B) Sedimentation equilibrium analysis of PASA. Data were collected at five concentrations (0.05, 0.1, 0.2, 0.4, and 0.8 mg/ml) and three rotor speeds (28,000, 36,000, and 44,000 rpm) at 20°C using interference optics. The data are shown as points (for clarity, only every third point is shown), and the best fits are shown as lines; the inset shows the residuals. The data were globally analyzed with a monomer-dimer model using HeteroAnalysis (44) to give a best-fit Kd value 11.0 μM with an RMS deviation of 0.016 fringes. (C) PASB and PASC were fractionated on a Superdex 75 size exclusion column, and weight-average molecular masses (Mw) obtained by integration of the sedimentation coefficient distributions obtained for PASA at concentrations of 0.2 mg/ml (solid), 0.6 mg/ml (dashes), and 2.0 mg/ml (dots). Conditions were as follows: rotor speed of 60,000 rpm, temperature of 20°C, and interference optics. The sedimentation coefficient distribution, c(s), and estimates of molecular masses were obtained by using SEDFIT (53). (D) Sedimentation velocity analysis of PASB. Shown are the c(s) concentration distributions obtained for PASA at concentrations of 0.1 mg/ml (solid), 0.6 mg/ml (dashes), and 2.0 mg/ml (dots). Conditions were as follows: rotor speed of 55,000 rpm, temperature of 20°C, and interference optics. The g(s*) distributions and weight-average sedimentation coefficients were calculated by using DCDT+ (32).

We further characterized the self-association of PASA using SV. Figure 2A shows the g(s*) sedimentation coefficient distributions for three concentrations of PASA ranging from 0.1 to 2.0 mg/ml (7.2 to 144 μM). The peak of the distribution shifts to the right with increasing PASA concentrations, as do the weight-average sedimentation coefficients (s_w) obtained by integration of the peaks, which increase from 1.87 S (0.1 mg/ml) to 2.14 S (2.0 mg/ml). The increase in s with increasing PASA concentrations indicates that the protein exists in a reversible mass action equilibrium that is rapid on the time scale of the sedimentation experiment. The SV data were globally analyzed by using a reversible monomer-dimer model with s_{monomer} = s_{dimer} and the dimer dissociation constant (K_d) as fitting parameters. Given the small range of PASA concentrations examined, it was necessary to fix the sedimentation coefficients to constrain the fit. Using estimates of s_{monomer} of 1.5 S and s_{dimer} of 2.3 S, as reported previously for PASA (47), global analysis provided an estimated K_d of 10 to 20 μM. This value is consistent with our findings from the MALLS analysis.

To further refine the K_d measurement, we rigorously characterized the behavior of PASA in solution using SE over a concentration range of 0.05 to 1.6 mg/ml (3.6 to 115 μM) at three rotor speeds (Fig. 2B). The data were globally analyzed by using a simple monomer-dimer model with HeteroAnalysis. A good fit was obtained, yielding a K_d of 11.0 μM (2-standard-deviation confidence interval of 9.2 to 12.9) with a root mean square (RMS) deviation of 0.016 fringes. Allowing the stoichiometry (N) to float gave a best-fit value of an N of 1.99. The fit was not further improved by allowing for thermodynamic nonideality or an incompetent monomer or dimer.

Thus, taken together, the MALLS, SV, and SE measurements convincingly demonstrate that PASA_1–117 exists in a weak monomer-dimer equilibrium with a K_d of ~10 μM. Thus, at the concentrations used for NMR analysis (30 to 150 μM), we would expect PASA_1–117 to be primarily dimeric. The absence of peaks in
the HSQC spectrum of PASA$^{1-117}$ is, however, suggestive of line broadening due to chemical exchange that is intermediate on the NMR time scale (46). What is this chemical exchange process if it is not interconversion between monomer and dimer? The answer is provided by the crystal structure of PAS, $^{11-117}$, which reveals that PASA is capable of forming two structurally distinct dimers in which the relative orientation of the two monomeric subunits is very different (47). Thus, we conclude that the absence of peaks in the HSQC spectrum of PASA$^{1-117}$ is reflective of exchange between structurally distinct dimers rather than exchange between monomer and dimer.

We do not believe that the self-association of PASA and/or the exchange between structurally distinct dimers is physiologically relevant. The cellular concentration of KinA is very low; previous studies indicated that it varies between 0.2 μM during vegetative growth and 1.8 μM during sporulation (49). Although we cannot rule out an enhancement of dimerization affinity due to the proximity of PASA monomers in the context of full-length KinA, the current data suggest that PASA will be primarily monomeric even at the elevated KinA concentrations present during sporulation.

PASB is a stable dimer. Previous analyses indicated that the N-terminal region of KinA self-associates (31). Although PASA has the capacity to dimerize, the $K_d$ for self-association is 10-fold higher than the physiological concentration range measured for KinA, and previous studies have shown that this self-association occurs in a nonspecific manner (47). This indicates that another region of the sensor module must be involved in KinA dimerization. Our NMR data indicated that this region is most likely PASB.

We initially used MALLS to analyze PASB$^{136-255}$ (13.8 kDa) at a very high concentration (950 μM; 13 mg/ml). We obtained a small peak with a very large $M_w$ and a much more heavily populated peak with a calculated $M_w$ of 26.6 ± 0.3 kDa (Fig. 2C), which is very close to the predicted size of a PASB$^{136-255}$ dimer (27.6 kDa). This suggests that although PASB$^{136-255}$ has a very slight tendency to aggregate, it is primarily a stable dimer in solution.

We used SV to further characterize the self-association of PASB$^{136-255}$. Figure 2D shows the $s^*$ sedimentation coefficient distributions obtained for PASB over a concentration range of 0.1 to 2.0 mg/ml (7.25 to 218 μM). A major feature is observed at an $s=\sim 2.3$ S, along with a minor component at an $s=\sim 3.3$ S. An additional feature at an $s=0.5$ S is likely associated with a mismatch of buffer components between the sample and reference solutions. The position of the main peak does not shift, and there is no evidence of an increasing relative contribution of the $s=3.3$ S feature with increasing concentrations. This indicates that the peak at 2.3 S corresponds to a stable, nonequilibrating species. The higher level of S material presumably is associated with a nonequilibrating aggregate.

To define the nature of the $s=2.3$ S species, the data at all three concentrations were globally analyzed by using a model that includes two discrete species and a continuous distribution to account for the higher-S aggregates. A good fit was obtained with RMS deviations of 0.0073 fringes and best-fit parameters for the major species of an $s_{1w}=2.41$ S and an $M=27.4$ kDa. The measured molecular weight is close to the predicted size of a dimer of PASB$^{136-255}$. Thus, over the concentration range examined, the PASB sample is composed of two species: an aggregate that comprises <5% of the total and a stable dimer. Based on the absence of any dissociation at the lowest concentration, we estimate that the $K_d$ is <10 nM. Since this $K_d$ value is 200- to 180-fold lower than the measured cellular concentrations of KinA (0.2 μM to 1.8 μM) (49), we conclude that PASB mediates dimerization of KinA at normal intracellular concentrations.

PASC is predominantly monomeric. A previous SEC analysis of a PASC construct indicated that it dimerizes under nonreducing conditions (45). In our hands, PASC underwent a concentration-independent disulfide-mediated dimerization in the absence of a reducing agent, and consequently, our experiments were performed in the presence of the strong reducing agent TCEP (10 mM). MALLS analysis of PASC yielded a peak with an estimated $M_w$ of 13.3 ± 0.8 kDa, consistent with a monomer (13.27 kDa) (Fig. 2C). There was also a small amount of a larger species with an elution time consistent with a dimer. The peak was too small to accurately estimate the $M_w$. Thus, using MALLS, we could not tell if this larger species was a residual disulfide-bonded dimer or a noncovalent dimer, so we performed AUC for clarification.

Figure 2E shows the g($s^*$) sedimentation coefficient distributions for PASC$^{169-382}$ at concentrations ranging from 0.12 to 3.0 mg/ml (9 to 226 μM) in buffer containing 200 mM NaCl. The distributions shifted slightly to higher $s$ values at the highest concentration, and the weight-average sedimentation coefficients increased from 1.45 to 1.69 S over this concentration range, indicating that PASC undergoes a rapid, reversible self-association. However, the weight-average sedimentation coefficients are close to the predicted monomer value of ~1.5 S, indicating that this self-association is weak. This PASC self-association cannot be accurately quantified by using AUC under conditions where the oligomer is significantly populated due to complications from hydrodynamic and thermodynamic nonideality at high protein concentrations. However, if we assume that PASC associates to form dimers, extrapolation of our lower concentration data yields a $K_d$ estimate of 200 to 500 μM.

Our finding that PASC is more soluble at lower salt concentrations led us to repeat this experiment in buffer containing 100 mM NaCl, with concentrations ranging from 0.1 to 2.0 mg/ml (7.5 to 150 μM). At the lower salt concentration, the g($s^*$) distributions also shifted slightly to higher $s$ values with increasing concentrations, and the weight-average sedimentation coefficients increased from 1.45 to 1.52 S. At 100 mM NaCl, the self-association of PASC appeared even weaker than that at 200 mM NaCl, with a $K_d$ of >500 μM. Thus, regardless of the salt concentration, the $K_d$ values measured for PASC dimerization are >100-fold higher than the cellular concentration of KinA (49), and hence, self-association of PASC is unlikely to be physiologically relevant.

The PAS domains do not heteroassociate. We used NMR chemical shift mapping (46) to examine the ability of the PAS domains to associate with one another. In these experiments, we first acquired an HSQC spectrum of a $^{13}$N-labeled PAS domain (PASA, PASB, or PASC). After this, we mixed the labeled protein sample with an unlabeled, nonself, domain preparation, and a second spectrum was acquired. All experiments were conducted under conditions where both partner proteins were soluble and folded (as indicated by the HSQC spectra). In these types of experiments, peaks are obtained for only the labeled protein. Differences in peak positions (i.e., chemical shifts) between samples with and without additional proteins are indicative of an interaction between the two proteins (46).

We observed no changes in chemical shifts or signal intensity for any of the combinations of PAS domains that we tried (15N-PASA with PASB or PASC, 15N-PASB with PASA or PASC), and
Although we cannot rule out a role for (i) proximity due to tethering or (ii) accessory factors in driving PAS domain interactions in intact KinA, our results suggest that there is no heterodomain interactions between PASA, PASB, and PASC.

Role of the PAS domains in KinA autophosphorylation. In order to examine the influence of each PAS domain on KinA autokinase activity, we constructed a series of truncated KinA proteins in which each of the three PAS domains was removed stepwise, as shown in Fig. 3B. The NMR data were used to determine the domain boundaries for each construct. SDS-PAGE analysis revealed purity greater than 90% for all constructs (Fig. 3A), and the apparent molecular masses were close to the predicted values (WT/H11005 69.4 kDa; PASBC-AK/H11005 53.7 kDa; PASC-AK/H11005 52.7 kDa; AK/H11005 25.1 kDa).

Autophosphorylation of full-length WT KinA was measured via the incorporation of radioactivity into KinA upon incubation with [γ-32P]ATP. Samples were electrophoresed by using SDS-PAGE, and phosphorylated KinA was then visualized by using autoradiography (Fig. 4A). Autophosphorylation of WT KinA reached a maximum after 90 min of incubation under the chosen experimental conditions (data not shown), and a comparison of the extent of autophosphorylation of WT KinA and the various truncation constructs over this time period is shown in Fig. 4A.

The intensities of phosphorylated protein on the autoradiogram were quantified by using the Quantity One program, and mean values are shown in Fig. 4B. The autokinase activity of WT KinA was set at 100%. Deletion of PAS A had little effect on autokinase activity, as the PASBC-AK construct showed only a small decrease in autophosphorylation (92% relative to that of WT KinA). As mentioned above, we initially subcloned a PAS B domain that started at residue 145 (since domain boundary predictions by several bioinformatics programs were inconclusive). However, our NMR data revealed that a fully folded and stable PASB domain requires residues 136 to 145. Consequently, we conclude that the PASBC-AK construct comprising residues 145 to 606 contains an incomplete PAS B domain that is likely partially unfolded and highly flexible at the N terminus, and hence, we have renamed this construct PASC-AK. Interestingly, the autophosphorylation ability of this protein is almost abolished; it shows only 2.5% activity compared to that of WT KinA (Fig. 4B), indicating that a fully folded PASB domain is required for kinase activity. These in vitro data are consistent with previously reported in vivo observations that (i) the PASC domain alone does not support sporulation and (ii) in experimental situations, the PAS B domain is required for sporulation (32). Since it appears that PASB is critical for autophosphorylation of KinA, it was not surprising that the AK con-
ylation. NMR and AUC experiments indicated that PASB is the individual PAS domains, (ii) carefully define their functional approaches in order to (i) examine the structural features of the boundaries in various protein constructs.

The existing data is difficult due to the use of different PAS domain main of KinA in a functional conformation, but interpretation of module influences and regulates KinA. Some information exists numerous efforts, much remains to be learned about how the sensor boundaries in various protein constructs.

We used a combination of biochemical and biophysical approaches in order to (i) examine the structural features of the individual PAS domains, (ii) carefully define their functional boundaries, and (iii) elucidate their roles in KinA autophosphorylation. NMR and AUC experiments indicated that PAS$_B$ is the only PAS domain in the KinA sensor module that can form a stable dimer at physiologically relevant concentrations. We demonstrated that, in the context of native KinA, the autokinase activity of the protein is critically dependent on PAS$_B$ but that PAS$_A$ is dispensable. We suggest that the primary role of PAS$_A$ is structural: it links PAS$_B$ and the AK domain in the correct orientation to allow both ends of the monomer to dimerize effectively.

The data presented here are to some extent consistent with results reported previously by Wang et al. (45), who showed that the sensor module of KinA is critical for kinase activity, with deletion of all three PAS domains abolishing autophosphorylation. In contrast to our findings, however, Wang and coworkers observed that deletion of PAS$_A$ caused a >90% reduction in autophosphorylation activity; they concluded that PAS$_A$ is essential for efficient autokinase activity. Similarly, Lee and colleagues (47) concluded that deletion of PAS$_B$ significantly diminished KinA activity, as measured indirectly via a green fluorescent protein (GFP) reporter assay of sporulation pathway activation. These contrary observations may be due to the different domain boundaries chosen to construct truncated KinA proteins: the “PAS$_A$ deletion” construct used by Wang et al. (45) lacks residues 136 to 143 of the PAS$_B$ domain that we have defined and shown here to be critical for KinA autophosphorylation, while the construct used by Lee et al. (47) lacks PAS$_B$ residues 136 to 151. Our results are consistent with recent in vivo functional studies showing that KinA with a deletion of residues 1 to 136 (which deletes PAS$_A$ completely but leaves the PAS$_B$ domain intact) induced sporulation at wild-type levels irrespective of nutrient availability (31).

Symmetric model of the sensor domain. Our findings allow us to propose a model for the dimeric KinA sensor module in which the two monomers align in a symmetric fashion (Fig. 5), with PAS$_B$ comprising the core of the dimeric structure. This configuration is consistent with the HSQC spectrum obtained for PAS$_B$, which has the requisite number of backbone peaks for a

**DISCUSSION**

Phosphorylation of KinA (and, to a lesser extent, KinB, KinC, and Kind [50]) is the initial step required to activate the multicomponent phosphorelay that controls sporulation in *B. subtilis*. The regulation of KinA is therefore critical to ensure that bacteria switch to the irreversible stages of sporulation only under severely nutrient-deprived conditions. The N-terminal sensor module (or another protein sequence that supports KinA multimerization) is essential for the activity of WT KinA (Fig. 4B). To detect autophosphorylation of this construct, very high protein concentrations had to be used (52 μM) (Fig. 4A). The observations reported here are consistent with previously reported in vivo data showing that the AK domain alone is not able to induce sporulation in *B. subtilis* (32).

**FIG 5 Models for possible conformations of the KinA sensor dimer.** The DHp and PAS$_A$ domain structures are shown as Richardson representations, with monomers indicated by color (blue and green) and the inclusion of a prime. Structure coordinates were taken from data reported under Protein Data Bank (PDB) accession numbers 3DGE (DHp from *Thermotoga maritima* [?]) and 2VLG (PAS$_A$ from *B. subtilis* [47]). The dimeric structure is consistent with data for native, nontagged KinA reported previously by Lee et al. (47). (A) Sensor arrangement with PAS$_B$ in a parallel dimer configuration. R1, R2, and R3 designate three side chains so that the viewer can orient the structure and examine the symmetry of the dimer. (B) Sensor arrangement with PAS$_B$ in the antiparallel dimer configuration (top view, looking down on the DHp four-helix bundle). In this arrangement, the PAS$_B$ domains lies “flat” across the end of the DHp domain. In the two configurations shown here, each monomer in the dimer experiences the same twisting forces. B and C, PAS$_B$ and PAS$_C$, respectively; α1, N terminus of DHp helix 1; black line, peptide backbone of the protein; cat, catalytic ATP-binding domain. Two-headed arrows indicate the movements that may lead to HK activation and deactivation. These are (i) 26° helical rotations of α2 against α1, as shown previously for EnvZ by Ferris et al. (52), and (ii) “helix cracking” α2 proposed previously for KinA by Dago et al. (51).
protein of the size of PAS\(_d\) but displays line-broadening characteristic of a dimer. The peak count indicates that only a single chemical state exists for each of the backbone amide protons, suggesting that PAS\(_d\) exists as a symmetric dimer. An asymmetric configuration of the dimer would expose each residue to two different environments, and thus, each \(^{1}H-^{15}N\) pair would generate two HSQC peaks, which is not what we observed. It should be noted that a symmetric dimer can be achieved in two ways (Fig. 5), via a parallel or antiparallel alignment. In the parallel arrangement (Fig. 5A), the PAS\(_d\) dimer would most likely protrude vertically from the end surface of the DHp domain. In the antiparallel arrangement (Fig. 5B), the PAS\(_d\) dimer can lie flat across the end surface of the four helices of the DHp domain. Our data do not allow us to discriminate between these two possible conformations.

An interesting prediction of the sensor domain model is the position of PAS\(_c\) and its role in linking PAS\(_d\) with the AK module. Presumably, the relative orientation of PAS\(_d\) and PAS\(_c\) must be important, and homology searches reinforce this point. There are currently 31 records in UniProt for KinA homologues with all three PAS domains in the N-terminal region (search conducted on 16 January 2013). Figure S1 in the supplemental material shows an alignment of a subset of these proteins, one from each representative species from the BLAST results. This alignment reveals an extraordinary level of conservation of the entire region from the beginning of PAS\(_d\) to the phosphorylatable His405 in the DHp domain of KinA. In this region of KinA, PAS\(_d\) is actually the least conserved region (72% similarity across all homologues); in contrast, PAS\(_c\) is 83% conserved, and quite remarkably, the PAS\(_{dc}\) linker is 100% conserved. In contrast, the PAS\(_{dc}\) linker is very divergent between homologues (only 5.5% conservation), while PAS\(_d\) itself also shows more moderate conservation (67%). It is interesting to note that residues 351 to 513 are 100% conserved between homologues: this region neatly spans the C-terminal region of PAS\(_c\), the beginning of helix 1 in the KinA DHp domain, and the crucial His405 residue.

Clearly, this region of KinA is functionally important, but how does the sensor module regulate KinA activity and thereby control sporulation? It has been suggested that KinA might be regulated by starvation-associated intracellular ligands that, upon recognition by the sensor module, would activate autophosphorylation of KinA. This idea is controversial. Unstarved vegetative cells can sporulate in the absence of a nitrogen source, suggesting that PASB exists as a symmetric dimer. An asymmetric arrangement tethers the N-terminal regions of the DHp helix 1 pair near one another, but it does not necessarily affect the orientation or location of helix 2. It is possible that PAS\(_d\) and PAS\(_c\) can flex and that, in doing so, they interact variably with helix 1, helix 2, or the Cat domain. This may provide a mechanism for KinA regulation. In contrast, PAS\(_d\) is a tight dimer and is unlikely to be a modulator of KinA activity; instead, it helps maintain KinA in a conformation where it is able to function as a dimer and auto-phosphorylate.

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