Function of the N-terminal region of the phosphate-sensing histidine kinase, SphS, in *Synechocystis* sp. PCC 6803

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In *Synechocystis* sp. PCC 6803 the histidine kinase SphS (*sll0337*) is involved in transcriptional activation of the phosphate (P_i)-acquisition system which includes alkaline phosphatase (AP). The N-terminal region of SphS contains both a hydrophobic region and a Per-Arnt-Sim (PAS) domain. The C-terminal region has a highly conserved transmitter domain. Immunological localization studies on heterologously expressed SphS in *Escherichia coli* indicate that the hydrophobic region is important for membrane localization. In order to evaluate the function of the N-terminal region of SphS, deletion mutants under the control of the native promoter were analysed for *in vivo* AP activity. Deletion of the N-terminal hydrophobic region resulted in loss of AP activity under both P_i -deficient and P_i -sufficient conditions. Substitution of the hydrophobic region of SphS with that from the Ni^{2+} -sensing histidine kinase, NrsS, resulted in the same induction characteristics as SphS. Deletion of the PAS domain resulted in the constitutive induction of AP activity regardless of P_i availability. To characterize the PAS domain in more in detail, four amino acid residues conserved in the PAS domain were substituted with Ala. Among the mutants R121A constitutively expressed AP activity, suggesting that R121 is important for the function of the PAS domain. Our observations indicated that the presence of a transmembrane helix in the N-terminal region of SphS is critical for activity and that the PAS domain is involved in perception of P_i availability.

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INTRODUCTION

Phosphorus is an essential element for living organisms and is present in cells predominantly as phosphate. Inorganic phosphate (P_i) is incorporated into many cellular components, including nucleotides, nucleic acids, phospholipids and a large variety of metabolic intermediates. It is also important in the covalent modification of proteins via phosphorylation, which is an essential component of many signal transduction and regulatory pathways. Despite its importance phosphorus is one of the least available nutrients in the environment (Schweitzer & Simon, 1995; Tyrrell, 1999). The responses brought about by P_i deficiency in *Escherichia coli* have been extensively studied. A two-component signal transduction system is required for regulation of the expression of the *pho* regulon

(Makino *et al.*, 1989), which encodes proteins for acquisition, storage and utilization of P_i .

Two-component signal transduction systems consist of two proteins, a histidine kinase and a response regulator (Stock *et al.*, 1989). Typically histidine kinases are membrane-bound sensory proteins. Once a specific stimulus is perceived by the kinase, a conserved histidine residue in the protein is autophosphorylated and the phosphate group is transferred to a cognate response regulator. The activity of the response regulator is determined by phosphorylation. Since many response regulators affect transcription, this provides a way in which the two-component system can modulate physiological responses to intra- and extracellular changes. The regulatory pathway is further complicated by the discovery of a third component modulating the activity of specific two-component systems (Chen *et al.*, 2008; Salinas *et al.*, 2007). The P_i -responsive two-component system is present in a wide variety of bacteria. In *E. coli* it consists of the histidine kinase PhoR, and the response regulator PhoB (Makino *et al.*, 1989). Disruption of *phoB* results in complete loss of response to P_i deficiency, consistent with the two-component system being essential for induction of the *pho* regulon. A third component, PhoU, is also

Abbreviations: AP, alkaline phosphatase; PAS, Per-Arnt-Sim; TM, transmembrane.

A supplementary table, listing primers used in this study, and five supplementary figures, showing construction of the modified genes and plasmids used in this study and amino acid alignment of hydrophobic regions from SphS and NrsS, are available with the online version of this paper.

involved in regulation of the *pho* regulon. Inactivation of *phoU* constitutively activates expression of the *pho* regulon via PhoR–PhoB regardless of P_i availability (Nakata *et al.*, 1984). In *E. coli*, PhoU thus functions as a negative regulator of the two-component system under P_i -sufficient conditions. Under P_i -deficient conditions the inhibitory effect of PhoU is overcome and the PhoR–PhoB sensory relay activity is restored, resulting in expression of the *pho* regulon.

In general, histidine kinase proteins consist of two portions: a signal-input domain located at the N terminus and a transmitter domain located at the C terminus (Mascher *et al.*, 2006). The former perceives the specific stimulus and modulates the activities of autophosphorylation and phospho-transfer to the cognate response regulator located in the latter domain. Analysis of the N-terminal region in sensory histidine kinases is important in order to understand the mechanism of signal perception. In *E. coli*, the N-terminal region of PhoR possesses two transmembrane (TM) helices and a Per-Arnt-Sim (PAS) domain (Scholten & Tommassen, 1993). Deletion of the TM region and/or the PAS domain from PhoR results in constitutive activation of the transmitter domain irrespective of P_i availability. This suggests that both domains are involved in repression of kinase activity under P_i -sufficient conditions (Scholten & Tommassen, 1993; Yamada *et al.*, 1990). In *Bacillus subtilis* the PhoR protein also contains two TM helices and a PAS domain. However, it has a large periplasmic loop between the TM helices which is absent from PhoR in *E. coli*. In *B. subtilis* deletion of the two TM helices and the periplasmic loop from the PhoR protein does not affect induction of the *pho* regulon, suggesting that the cytosolic transmitter domain senses P_i availability and induces expression of the *pho* regulon (Shi & Hulet, 1999). The molecular mechanism for sensing the availability of P_i and regulating it via a histidine kinase thus clearly differs between organisms and the role of specific protein domains warrants further investigation.

Histidine kinases and response regulators are expressed at a very low level and this is likely to be an important factor in downstream gene regulation. Overaccumulation of histidine kinases and response regulators in cells can cause excess signal transduction between cognate partners or between non-cognate partners. (Ehira & Ohmori, 2006; Krall & Reed, 2000). This phenomenon has been utilized to screen for novel histidine kinase activities. For example overexpression of the histidine kinase SasA from the cyanobacterium *Synechococcus* sp. PCC 7942, which functions as a regulator of circadian rhythm, functionally complements mutations of EnvZ and PhoR in *E. coli* (Nagaya *et al.*, 1993), which function as osmosensors and phosphosensors respectively. Investigation into the specific function of histidine kinases and the role of component domains should thus be conducted under conditions of gene expression equivalent to those of the native histidine kinases in wild-type cells in order to prevent erroneous conclusions. Previous analyses of the signal input domains

of PhoR in *E. coli* and in *B. subtilis* have utilized multicopy plasmids with strong promoters to express the kinases and it would be advisable to avoid this.

In order to express genetically modified kinases at equivalent levels to the native protein *in vivo*, the coding sequence of the native gene in the chromosome could be replaced with genes encoding the target proteins via homologous recombination and driven from the original promoter. Since the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) readily undergoes double homologous recombination between transformed DNA fragments and chromosomal DNA (Williams, 1988), we decided to exploit this ability to study the function of subdomains of the P_i -sensing histidine kinases in this organism utilizing the original promoter. Additionally since most of the genes for histidine kinases in *Synechocystis* are transcribed in a monocistronic manner there is no need to take the expression levels of the genes downstream from the histidine kinase into account as they are not cotranscribed (Murata & Suzuki, 2006). These characteristics of *Synechocystis* greatly simplify studies of genetically modified histidine kinases.

The genome of *Synechocystis* encodes the histidine kinase SphS (Slr0337) and the response regulator SphR (Slr0081) and a negative regulator SphU (Slr0741), which are structural and functional orthologues of PhoR, PhoB and PhoU respectively (Aiba *et al.*, 1993; Hirani *et al.*, 2001; Juntarajumnong *et al.*, 2007a; Suzuki *et al.*, 2004). In *Synechocystis* these genes are located on the genome separately and should be transcribed independently. The SphS–SphR pathway regulates the expression of at least 12 genes, including *phoA*, which encodes a periplasmic alkaline phosphatase (AP) (Suzuki *et al.*, 2004). Induction of AP activity is a highly conserved response to P_i deficiency in many bacteria and has been used as an indicator of the activity of the SphS–SphR pathway in *Synechocystis* (Juntarajumnong *et al.*, 2007a). Burut-Archana *et al.* (2009) have demonstrated that deletion of a portion of the N-terminal sequence of SphS results in constitutive induction of AP activity, indicating that this region is essential to respond to P_i fluctuations.

In this report we describe an expression system in *Synechocystis* cells for modified forms of SphS utilizing the native promoter of the *sphS* gene, so eliminating any artefacts due to overproduction. We have expressed a series of SphS derivatives and assessed the signal-sensing ability of the modified SphS by measuring cellular AP activity. The possible functions of the hydrophobic region and the PAS-like domain of SphS are discussed.

METHODS

Bacterial strains and culture conditions. A glucose-tolerant strain of *Synechocystis* (Williams, 1988) was used as the wild-type strain. The wild-type strain and its derivatives were grown at 34 °C in BG11

medium (Stanier *et al.*, 1971) buffered with 20 mM HEPES–NaOH (pH 7.5) under continuous illumination provided by incandescent lamps at 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Phosphate-free BG11 medium was prepared by substitution of K_2HPO_4 with KCl as described previously (Suzuki *et al.*, 2004). Mutants of SphS were grown in the presence of 25 μg kanamycin ml^{-1} or 25 μg spectinomycin ml^{-1} , depending on the inserted antibiotic resistance gene. Liquid cultures were continuously bubbled with 1.0 % (v/v) CO_2 -containing air. *E. coli* strains JM109 (TaKaRa Bio) and BL21(DE3) (Merck KGaA) were used as hosts for genetic manipulation and protein expression respectively, and were grown in LB medium with appropriate antibiotics at 37 °C (Miller, 1972).

Genetic modifications of the SphS gene in *Synechocystis*. To replace the entire ORF of the *sphS* gene with a kanamycin-resistance cassette, DNA fragments upstream and downstream of the ORF of *sphS* were amplified by PCR with upstream primers, 0337uF and 0337uR, and downstream primers, 0337dF and 0337dR, using genomic DNA of *Synechocystis* as a template. Primer sequences used in this study are shown in Supplementary Table S1, available with the online version of this paper. The upstream fragment was cloned between the *NsiI* and *NdeI* sites of pGEM-T easy vector (Promega). This was followed by cloning of the downstream fragment between the *SalI* and *SacII* sites of the plasmid containing the upstream fragment to obtain pSK01. All plasmids constructed in this study and their descriptions are shown in Table 1. A kanamycin-resistance cassette, containing *NdeI* and *SalI* sites at each end, was amplified by PCR using kanF and kanR primers and EZ-Tn5 (Epicentre) as template. The amplified DNA fragment was digested with *NdeI* and *SalI* and inserted into pSK01, which had been cleaved with the same enzymes, to construct pSK02. To obtain the *sphS*-deleted strain, wild-type cells of *Synechocystis* were transformed with pSK02 as described by Williams (1988).

In order to replace the kanamycin-resistance cassette with modified *sphS* genes, a DNA fragment containing the sequence of the transmitter domain of SphS was amplified by PCR using kinF1 and 0337R primers and cloned between the *HindIII* and *SalI* sites of pSK02 to obtain pSK03. A spectinomycin-resistance gene cassette, containing *SalI* sites at both ends, was amplified by PCR using speF and speR primers and the *aadA* gene in pAM1146 (Tsinoremas *et al.*, 1994) as template. This fragment was cloned into the *SalI* site of pSK03 to obtain pSK04.

The full-length *sphS* gene was amplified by PCR using 0337F and 0337R primers to construct the complemented strain (SphSC). The SphS construct lacking a hydrophobic region at the N terminus (*sphS* ΔHy) was obtained by PCR using primers ΔHyF and 0337R. To construct the PAS-domain-deleted SphS (*sphS* ΔPAS), the partial *sphS* sequences were amplified by PCR using primer pairs 0337F plus PASuR, and PASdF plus 0337R. The two fragments were mixed and further amplified by PCR using 0337F and 0337R primers, generating a 0.95 kbp DNA fragment. The PCR products were cloned into pGEM-T easy vector and the DNA sequences were confirmed. Each of the DNA fragments cloned into pGEM-T easy vector was excised from the vector with *NdeI* and *HindIII* and cloned between the *NdeI* and *HindIII* sites of pSK04 to obtain pSK05, pSK06 and pSK07 for strains SphSC, SphS ΔHy and SphS ΔPAS , respectively (see Supplementary Fig. S1).

To construct strain SphSAN, expressing the signal-input-domain-deleted SphS, the DNA fragment corresponding to the transmitter domain of SphS was amplified by PCR using kinF2 and 0337R primers. The fragment was cloned between the *NdeI* and *SalI* sites of pSK01, and the spectinomycin-resistance cassette was inserted into the plasmid as described above to construct pSK08. ΔSphS cells were transformed with the plasmids constructed above and spectinomycin-resistant cells were selected.

Table 1. Plasmids used in this study

Plasmid	Description	Reference or source
pGEM-T easy	Cloning vector, Ap ^r	Promega
pAM1146	Source of Sp ^r cassette, Sp ^r	Tsinoremas <i>et al.</i> (1994)
pTrc99a	Source of P _{trc} and <i>lacI^q</i> , Ap ^r	Amann & Brosius (1985)
pET28a	Expression vector, Km ^r	Merck KGaA
pSK01	P _{sphS} , Ap ^r	This study
pSK02	P _{sphS} , <i>sphS</i> [−] , Ap ^r Km ^r	This study
pSK03	P _{sphS} , Ap ^r Km ^r	This study
pSK04	P _{sphS} , Ap ^r Km ^r Sp ^r	This study
pSK05	P _{sphS} - <i>sphS</i> ⁺ , Ap ^r Sp ^r	This study
pSK06	P _{sphS} - <i>sphS</i> ΔHy ⁺ , Ap ^r Sp ^r	This study
pSK07	P _{sphS} - <i>sphS</i> ΔPAS ⁺ , Ap ^r Sp ^r	This study
pSK08	P _{sphS} - <i>sphS</i> ΔN ⁺ , Ap ^r Sp ^r	This study
pSK09	P _{sphS} -[<i>nrsS</i> (N)- <i>sphS</i> ΔHy] ⁺ , Ap ^r Sp ^r	This study
pSK10	P _{sphS} -[<i>nrsS</i> (N)- <i>sphS</i> ΔN] ⁺ , Ap ^r Sp ^r	This study
pSK11	P _{sphS} - <i>sphS</i> (P80A) ⁺ , Ap ^r Sp ^r	This study
pSK12	P _{sphS} - <i>sphS</i> (N96A) ⁺ , Ap ^r Sp ^r	This study
pSK13	P _{sphS} - <i>sphS</i> (R121A) ⁺ , Ap ^r Sp ^r	This study
pSK14	P _{sphS} - <i>sphS</i> (E124A) ⁺ , Ap ^r Sp ^r	This study
pSK15	P _{sphS} - <i>sphS</i> (H207A) ⁺ , Ap ^r Sp ^r	This study
pSK16	P _{trc} - <i>sphS</i> (H207A) ⁺ , Ap ^r Sp ^r	This study
pSK17	P _{trc} - <i>sphS</i> ΔHy (H207A) ⁺ , Ap ^r Sp ^r	This study
pSK18	P _{trc} -[<i>nrsS</i> (N)- <i>sphS</i> (H207A) ΔHy] ⁺ , Ap ^r Sp ^r	This study

Construction of fusion-protein-expressing strain. To express a fusion protein of the N-terminal part of the nickel-sensing histidine kinase from *Synechocystis*, NrsS (Lopez-Maury *et al.*, 2002), and SphS, a DNA fragment corresponding to the N-terminal region (amino acid residues 1–222) of *nrsS* was amplified by PCR using primer pair *nrsSF* and *nrsSR*. The resultant fragment was cloned into the *NdeI* site of pSK06 or pSK08 to construct pSK09 and pSK10 (see Supplementary Fig. S2). The direction of the inserted fragment was confirmed by PCR using *nrsSF* and 0337R primers. Δ SphS cells were transformed with pSK09 or pSK10 to yield strains expressing NrsS(N)-SphSAHy and NrsS(N)-SphS Δ N, respectively.

Construction of strains expressing point-mutated SphS. In order to substitute the conserved amino acid residues in the PAS domain of SphS with Ala, the *sphS* gene containing the targeted substitution was synthesized by a fusion PCR strategy (Wang *et al.*, 2002). This resulted in point-mutated *sphS* fragments (see Supplementary Fig. S3). The fragments were then digested with *NdeI* and *XhoI* and cloned between the *NdeI* and *XhoI* sites of pSK04. Δ SphS cells were transformed with the plasmids, resulting in strains expressing SphS(P80A), SphS(N96A), SphS(R121A), SphS(E124A), and SphS(H207A).

Measurement of AP activity. Wild-type and mutant strains of *Synechocystis* grown in BG11 medium for 16 h were harvested by centrifugation at 25 °C at 3000 g for 5 min and the cells were washed twice with phosphate-free BG11 medium. The cells were subsequently inoculated into BG11 or phosphate-free BG11 medium at OD₇₃₀ 0.2–0.4 and further incubated for 24 h. The activity of AP in the intact cells was measured by the rate of degradation of *p*-nitrophenyl phosphate (Aiba *et al.*, 1993).

Preparation of recombinant SphS protein and raising an antibody. The coding region of the *sphS* gene in pSK05 was cleaved by *NdeI* and *SalI* and cloned into pET28a (Merck KGaA) digested with both enzymes. The resulting plasmid, pET-SphS, was then introduced into *E. coli* BL21(DE3) cells (Supplementary Fig. S3).

E. coli transformants were grown at 37 °C in LB medium supplemented with 25 µg kanamycin ml^{−1}. The recombinant protein was induced in exponentially growing cells by 1 mM IPTG. After 4 h incubation, cells were harvested by centrifugation, washed with buffer A [50 mM HEPES/NaOH (pH 7.5), 100 mM NaCl], and suspended in buffer A. The cells were then disrupted by sonication at 4 °C using a UD-201 sonicator (TOMY). The cell extract was centrifuged at 20 000 g for 10 min and the inclusion body containing recombinant protein was collected. This was washed twice with buffer B [50 mM HEPES/NaOH (pH 7.5), 100 mM NaCl, 5 mM EDTA, 0.05 % (w/v) sodium deoxycholate, 0.5 mg lysozyme ml^{−1}] at room temperature for 30 min. The inclusion body was suspended in buffer C [50 mM HEPES/NaOH (pH 7.5), 100 mM NaCl, 8 M urea]. Insoluble material was removed by centrifugation and the supernatant containing His-tagged SphS was loaded onto a HiTrap chelating column (GE Healthcare Bio-Science) and washed with buffer C. Bound proteins were eluted with buffer C containing 250 mM imidazole.

Eluted proteins were subjected to 12.5 % SDS-PAGE and the gels were stained as described by Ortiz *et al.* (1992). The band corresponding to the recombinant protein was excised and the gel slice crushed and suspended in electrophoresis buffer (Laemmli, 1970). Protein was eluted from the gel by shaking for 12 h at room temperature, filtered with a 5 µm centrifugal filter device (Ultrafree-MC, Millipore) and the purified recombinant SphS was used to immunize a rabbit to obtain an antibody against SphS.

Localization of SphS in *E. coli*. To express SphS, SphSAHy and NrsS(N)-SphSAHy in *E. coli*, we constructed pSK16, 17 and 18 which

include the entire sequence for SphS or modified SphS substituting the phosphorylatable His residue with Ala under control of the strong artificial *trc* promoter (see Supplementary Fig. S4). Mutation of the His residues in SphS derivatives was included to inactivate the kinase activity, so minimizing possible effects of overproduction of the kinases on the growth of *E. coli* cells. To exchange the H207 of SphSAHy with alanine, the DNA fragment between the *XbaI* and *XhoI* sites of pSK06 was exchanged with that of pSK15, producing plasmid pSK06a. In order to introduce the *trc* promoter into pSK15 and pSK06a, a DNA fragment including the *trc* promoter was amplified by PCR using *trcF* and *trcR* primers and pTrc99a (Amann & Brosius, 1985) as template. After digesting the amplified DNA fragment with *NdeI*, it was cloned into the *NdeI* site of pSK15 and pSK06a; the resulting plasmids were named pSK16 and pSK17, respectively. The direction of the inserted *trc* promoter was confirmed by PCR. To construct plasmid pSK18 the *trc* promoter sequence was amplified by PCR using *trcF2* and *trcR* primers and pTrc99a DNA as the template. The amplified DNA was digested with *NsiI* and *NdeI*, and inserted between the *NsiI* and *NdeI* sites of pSK17. The amplified *nrsS* DNA fragment as described above was digested with *NdeI* and introduced into the *NdeI* site of the resulting plasmid.

Plasmids pSK16, pSK17 and pSK18, and a control plasmid, pSK05, were introduced into *E. coli* JM109 and the transformants were cultured in LB medium at 37 °C. Cells in the exponential growth phase were collected by centrifugation, resuspended in two different buffers systems: buffer D [50 mM Tris/HCl (pH 8.0), 50 mM NaCl] or buffer E [buffer D containing 100 mM Na₂CO₃ (pH 11.5)] at 4 °C and disrupted by sonication. Clear cell lysates obtained after removal of cell debris by centrifugation at 20 000 g for 10 min were then ultracentrifuged at 160 000 g, for 1 h at 4 °C. The supernatant was withdrawn and termed the cytosolic protein fraction. The precipitates were washed by suspending in the same buffer used for sonication and recovering by ultracentrifugation under the same conditions. In order to distinguish between membrane-bound proteins and insoluble matter which was not removed by the initial centrifugation, the final pellet was resuspended in buffer F [50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 % (v/v) Tween 20] and insoluble matter was removed by centrifugation. The detergent-soluble fraction was designated the membrane proteins.

For Western blot analysis, proteins separated by SDS-PAGE were electro-transferred to PVDF membrane. Rabbit anti-SphS serum and alkaline-phosphatase-conjugated anti-rabbit IgG (Bio-Rad) were used as the primary and secondary antibodies. The primary antibody was used at a 1:1,000 dilution with TBS [Tris-buffered saline; 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl] containing 1 % (w/v) skim milk and the secondary antibody was used at a 1:20 000 with TBS. Detection was carried out using the amplified Alkaline Phosphatase GAR kit according to manufacturer's instructions (Bio-Rad). A sample (10 µg protein) from each fraction was subjected to 10 % SDS-PAGE. Protein concentrations were determined by the Bio-Rad Protein Assay kit, with BSA as standard.

RESULTS AND DISCUSSION

Construction of domain-deleted SphS expressing strains

Juntarajumnong *et al.* (2007b) have reported that the translation start site of the *sphS* gene is located at the ATG 141 bp upstream from the GTG originally assigned as the translation initiation codon (Kaneko *et al.*, 1996, <http://bacteria.kazusa.or.jp/cyanobase/>). The newly annotated SphS consists of 430 amino acid residues and contains a

hydrophobic stretch in the N-terminal region, a transmitter domain in the C-terminal region, and a putative PAS domain between them (Fig. 1). This is a structure which many orthologues of SphS have. To investigate molecular mechanisms of signal perception by SphS, we decided to express the domain-deleted SphS from the original promoter of the *sphS* gene and examine its responsiveness *in vivo* using the AP reporter protein activity. This strategy ensures that the sensor molecule is maintained at the same level as in the wild-type. To express genetically modified SphS in cells of *Synechocystis* from the native promoter of the *sphS* gene, the entire ORF of the *sphS* was substituted with a kanamycin-resistance gene cassette (Fig. 1a). Segregation of the *sphS*-deleted chromosome was confirmed by PCR (data not shown) and the strain, named Δ SphS, was utilized as a host for secondary transformations introducing modified SphS. The kanamycin-resistance gene of Δ SphS was replaced with the modified *sphS* genes containing a spectinomycin-resistance gene cassette which was inserted immediately downstream of the termination codon of *sphS*. (Supplementary Fig. S1). The strains Sph Δ N, Sph Δ Hy and Sph Δ PAS contained deletions in the entire N-terminal region (amino acid residues 2–196) excluding the transmitter domain, the hydrophobic region (amino acid residues 2–50), and the putative PAS domain (amino acid residues 69–177) respectively. The intact *sphS* gene was also introduced to obtain a complemented strain, SphSC. Replacement of all the Δ SphS chromosomal copies with the inserted genes was confirmed by PCR and sensitivity to kanamycin (data not shown). All the spectinomycin-resistant strains grew well in P_i -sufficient conditions (data not shown).

Effects of the domain deletions of SphS on the expression of AP activity

Since the expression of the gene for AP is solely regulated by the SphS–SphR pathway in *Synechocystis* (Suzuki *et al.*, 2004), we assayed AP activity to evaluate functionality of the SphS derivatives (Fig. 1b). The AP activity in cells of Δ SphS was negligible under both P_i -sufficient and -deficient conditions. AP activity in response to P_i availability in the SphSC strain was similar to that observed in the wild-type. This indicates that the SphSC protein was functioning normally regardless of the base substitutions introduced into the upstream and the downstream coding regions to insert the gene constructs into *Nde*I and *Sal*I sites; these substitutions did not alter the amino acid sequence. Similarly, insertion of a spectinomycin-resistance gene cassette downstream of the coding region did not cause any alterations to the normal function of the gene. We next analysed the AP activities in response to P_i availability in cells of the domain-deleted strains. Sph Δ N and Sph Δ Hy did not exhibit any AP activity at any level of P_i availability. This suggests that the hydrophobic domain in the N-terminal region of SphS is essential for sensing P_i availability and/or activating kinase activity *in vivo*. Cells of Sph Δ PAS constitutively expressed AP activity regardless of P_i availability, as has been reported for cells of the *sphU*-deleted strain (Juntarajumnong *et al.*, 2007a), suggesting that the PAS domain might function to suppress SphS activity under P_i -sufficient conditions, possibly together with SphU. The construct containing the hydrophobic domain minus the PAS domain (Sph Δ PAS) exhibited constitutive kinase activity. However, the construct minus the hydrophobic domain but containing the PAS domain

Fig. 1. Effects of deletion of domains in SphS on the expression of AP activity. (a) Domain architectures of native and mutated SphS. The domains were depicted with the Pfam program (Finn *et al.*, 2008). Numbers indicate the positions of amino acid residues from the translation start site of the native SphS. Dashed lines represent the regions deleted from the native protein. PAS, Hik and black rectangles indicate PAS domain, transmitter domain of the histidine kinase and the hydrophobic region, respectively. (b) AP activities in each strain grown under P_i -sufficient (P_i +, white bars) or P_i -deficient (P_i -, shaded bars) conditions for 24 h. The assays were carried out three times with cultures grown independently; means $\pm$ SEM are plotted.

(Sph Δ Hy) was inactive. This indicates that the hydrophobic domain is essential for activation of the transmitter domain.

Signal perception/kinase activation only requires a hydrophobic region and is not sequence-specific

To investigate the dependence of the specific amino acid sequence of the SphS hydrophobic domain on kinase activation and sensing we decided to completely replace this region with the amino acid sequence from the Ni^{2+} -sensing histidine kinase NrsS (Lopez-Maury *et al.*, 2002). These two hydrophobic regions have very little amino acid sequence similarity (see Supplementary Fig. S5). We constructed strains that expressed a fusion protein with the N-terminal region of NrsS and the truncated forms of SphS to obtain NrsS(N)-Sph Δ N and NrsS(N)-Sph Δ Hy (Fig. 2a). NrsS upregulates the *nrsBACD* operon coding for an ABC transporter involved in Ni^{2+} tolerance in the presence of Ni^{2+} in *Synechocystis*. NrsS has two membrane-spanning helices at the N-terminal region and is predicted to be located in the plasma membrane (Lopez-Maury *et al.*, 2002). If the hydrophobic domain of SphS is essential in sensing changes in P_i availability, replacement with the hydrophobic region from NrsS may result in loss of P_i sensing.

AP activity in cells expressing NrsS(N)-Sph Δ Hy responded to P_i availability to the same extent as those expressing SphSC. However, whilst the SphSC-expressing strain did not have any AP activity under P_i -sufficient conditions, detectable activity was present in the NrsS(N)-Sph Δ Hy-expressing strain. We also looked at AP activity in the cells of the strain expressing NrsS(N)-Sph Δ N, which has the PAS domain deleted, and as for Sph Δ PAS, the activity was constitutive (Figs 1 and 2).

These findings suggest that the hydrophobic region of SphS might not be essential for signal perception and/or kinase activation. It also indicates that the presence of any hydrophobic region may be sufficient for induction of AP activity by SphS.

The N-terminal region of SphS is essential for membrane localization

To investigate the region of SphS that may be required for membrane localization, and hence probably signal perception, we designed a series of constructs which were truncated/deleted in specific domains. We also generated an antibody that was capable of recognizing all of the domains in the protein. Following expression and fractionation into membrane and soluble fractions it should be possible to determine the location of the protein immunologically.

The antibody could specifically detect the recombinant SphS via Western blotting at a level of 5 ng (data not shown). At first, we attempted to detect SphS protein in wild-type cells. Cells in the exponential growth phase were collected and disrupted by sonication. After removing the unbroken cells and cell debris, the cell lysate was separated into cytoplasmic, plasma membrane and thylakoid membrane fractions by discontinuous sucrose-density-gradient centrifugation (Murata & Omata, 1988). When 10 μ g of each sample was subjected to Western blotting analysis, no protein was detected (data not shown). This indicates that the amount of SphS *in vivo* is below the detection limit; this is perhaps not surprising, as sensor proteins are liable to be of very low abundance.

We next attempted to overexpress SphS in *Synechocystis*. The Δ SphS strain was transformed with plasmid pSK16 or pSK17, which contain the *sphS* and *sph Δ Hy* gene,

Fig. 2. Fusions of truncated SphS constructs with the TM region of NrsS. (a) Domain architectures of the fusion proteins; (b) AP activities under P_i -sufficient (P_i +, white bars) or P_i -deficient (P_i -, shaded bars) conditions. The assays were carried out three times with cultures grown independently; means $\pm$ SEM are plotted.

respectively, under the control of P_{trc} a strong bacterial artificial promoter (Supplementary Fig. S4). To regulate expression levels of the introduced gene, the *lacI^q* gene was concomitantly integrated into the chromosome via homologous recombination (Supplementary Fig. S4). To minimize unexpected effects of the overexpression of the sensor proteins, the phosphorylatable His residues of both proteins were substituted with an Ala residue. After transformation, spectinomycin-resistant colonies of both transformants were obtained. However, we could not cultivate the cells in liquid BG11 medium containing 25 μ g spectinomycin ml^{-1} even without addition of IPTG. The increased level of SphS in these cells may be causing deleterious effects on cellular function. The precise mechanism for this is unclear.

Since elevated expression of SphS, and its derivatives, in *Synechocystis* was deleterious we decided to investigate expression in a heterologous system. SphS, SphS Δ Hy and NrsS(N)-SphS Δ Hy were successfully expressed in *E. coli* cells and fractionated for immunological determination of subcellular localization. We used two different pH treatments for preparation of the soluble and membrane fractions, pH 8.0 and pH 11.5. The latter pH was employed to overcome any problems which might be associated with proteins being trapped inside membrane vesicles. Treatment of membranes at pH 11.5 is a well-documented method of linearizing closed membrane vesicles (Griff *et al.*, 1992). If a positive result was obtained on pH 8.0 isolated membranes and a negative one with pH 11.5 membranes this would indicate that the result was not due to membrane insertion but rather to trapping of the protein inside a closed vesicle. True membrane insertion could only be verified if the protein was detected in both pH 8.0 and pH 11.5 preparations. All the membrane preparations isolated at pH 8.0 gave a positive signal (Fig. 3). SphS Δ Hy could be solubilized after the pH 11.5 treatment, indicating that it is not membrane inserted. SphS and NrsS(N)-SphS Δ Hy, which contain the

hydrophobic domain, remained membrane bound after pH 11.5 treatment, consistent with the hydrophobic domain being essential for membrane localization.

Identification of critical amino acid residues in the PAS domain

Our results above indicate that the PAS domain may be involved in the regulation of kinase activity depending on P_i availability (Figs 1, 2, and 4). To identify amino acid residues required for PAS domain function, we aligned PAS domains from several orthologues of SphS, including PhoR from *E. coli* (Fig. 4a) and identified four absolutely conserved hydrophilic residues that could be critically important. It is noteworthy that the PAS domains of cyanobacterial SphS proteins have lower identity with that of PhoR, but the function is still conserved. Thus the conserved amino acid residues might have an important role for the function of the domain. The four conserved residues P80, N96, R121 and E124, and autophosphorylatable His residue, H207, in SphS, were separately substituted with Ala and introduced into Δ SphS *Synechocystis* cells. Point mutations of P80A and E124A did not influence the induction of AP activity, suggesting that these residues are not critical for activity of the PAS domain in SphS (Fig. 4b). Mutations of N96A and H207A resulted in loss of induction of AP activity regardless of P_i availability. N96 might be important for the activation of the transmitter domain since substitution resulted in complete inactivation. Mutation of R121A caused constitutive expression of the AP activity even under P_i -sufficient conditions, suggesting that R121 may have an important function in sensing by the PAS domain.

In *E. coli*, PhoR lacking its TM region constitutively expresses the AP gene, suggesting that this region is required for negative regulation of PhoR activity. In SphS of *Synechocystis*, deletion of part of the hydrophobic region (amino acid residues 8–15) also constitutively induces AP

Fig. 3. Western blot analysis of SphS and modified SphS proteins expressed in cells of *E. coli*. Cells of *E. coli* expressing the native and modified SphS proteins were disrupted at pH 8.0 and at pH 11.5. The crude extracts were further separated by ultracentrifugation into cytosolic (C) and membrane (M) fractions. A 10 μ g sample of each fraction was subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue (a) or electroblotted on PVDF membranes and detected with an anti-SphS polyclonal antibody (b). These results were reproducible; representative results are presented. Numbers indicate molecular mass (kDa).

Fig. 4. Effects of point mutations in the PAS domain on the expression of AP activity. (a) Alignment of amino acid sequences of the PAS domain of SphS orthologues. tel, *Thermosynechococcus elongatus* BP-1 (NP_681715); cya, *Synechococcus* sp. JA-3-3Ab (YP_475742); syn, *Synechocystis* sp. PCC 6803 (NP_442021); mar, *Microcystis aeruginosa* NIES-843 (YP_001660279); ana, *Anabaena* sp. PCC 7120 (NP_488542); amr, *Acaryochloris marina* MBIC11017 (YP_001519542); syf, *Synechococcus elongatus* PCC 7942 (YP_400028); eco, *Escherichia coli* K-12 MG1655 (NP_414934). Asterisks indicate the amino acid residues substituted with Ala. Numbers indicate positions of amino acid residues. The amino acid alignment was created by CLUSTAL W (Thompson *et al.*, 1994). (b) AP activity in the cells grown under P_i-sufficient (P_i +, white bars) or P_i-deficient (P_i -, grey bars) conditions. The assays were carried out three times with cultures grown independently; means $\pm$ SEM are plotted.

expression (Burut-Archanai *et al.*, 2009). Our results indicate that deletion of the entire hydrophobic region of SphS was unable to activate reporter AP activity regardless of

P_i availability. Moreover, a protein in which the hydrophobic region of SphS was replaced by that from NrsS retained P_i responsiveness, suggesting that the hydrophobic region does not play a role in sensing the P_i signal but rather is important in localizing the sensory protein to a specific location on the membrane. There are several examples of histidine kinases in bacteria being located at the poles of cells (Hallez *et al.*, 2007). The hydrophobic region of SphS might be important to localize it to a specific location. In order to detect SphS expressed *in vivo* in *Synechocystis* new techniques will have to be developed.

In *E. coli*, it has been suggested that the activity of PhoR is repressed through formation of a hetero complex with PhoU and the high-affinity P_i transporter (Wanner, 1993). No direct evidence of such protein interaction has been demonstrated (Baek *et al.*, 2007). In this study we found that recombinant SphS expressed in *E. coli* was localized to the membrane via the hydrophobic domain. This suggested that a similar situation may occur in *Synechocystis* but we were not able to demonstrate it because of the low amount of SphS in this cyanobacterium. The presence of this domain is required for activation of kinase activity. The repressive function of the PAS domain under P_i-sufficient conditions was similar to that of the SphU protein. It may be that the SphU protein interacts with SphS via the PAS domain. This could be tested by looking for direct protein-protein interactions. The mutants which we have generated affecting the function of the PAS domain, such as R121A and N96A, could be very useful materials to demonstrate the interaction between SphS and SphU genetically.

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