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ORIGINAL PAPER

The hybrid histidine kinase Slr1759 of the cyanobacterium *Synechocystis* sp. PCC 6803 contains FAD at its PAS domain

Klaus-Peter Michel · Ann-Kristin Schröder · Maike Zimmermann · Sonja Brandt · Elfriede K. Pistorius · Nicole Frankenberg-Dinkel · Dorothee Staiger

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Abstract The cyanobacterium *Synechocystis* sp. PCC 6803 harbours 47 histidine kinases (Hiks). Among these are hybrid histidine kinases with one or two response regulator domains as well as numerous Hiks with several sensory domains. One example is the hybrid histidine kinase Slr1759 (Hik14) that has two PAS domains arranged in tandem linked to a predicted GAF domain. Here, we show that a Slr1759 derivative recombinantly expressed in *Escherichia coli* has a flavin cofactor. Using truncated Slr1759 variants, it is shown that the flavin associates with the first PAS domain. The cofactor reconstitutes the activity of D-amino acid oxidase apoprotein from pig kidney, indicating that the flavin derivative is FAD. Furthermore, the Slr1759 histidine kinase domain indeed undergoes autophosphorylation in vitro. The phosphorylated product of a recombinant Slr1759 derivative is sensitive to acids, pointing to a histidine residue as the phosphate-accepting group.

Keywords Hybrid histidine kinase · FAD · PAS domain · *Synechocystis*

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K.-P. Michel · A.-K. Schröder · M. Zimmermann · E. K. Pistorius · D. Staiger (✉)
Department of Molecular Cell Physiology, University of Bielefeld, Universitätsstraße 25, 33615 Bielefeld, Germany
e-mail: dorothee.staiger@uni-bielefeld.de

S. Brandt · N. Frankenberg-Dinkel
Physiology of Microorganisms, Ruhr-University Bochum, Bochum, Germany

Introduction

Histidine kinases play a central role in the perception and processing of environmental signals in bacteria, cyanobacteria, fungi and plants, and ultimately impact gene expression and processes like phototaxis, chemotaxis, and virulence in bacteria (Chang and Stewart 1998; West and Stock 2001). In the so-called two-component systems, sensor histidine kinases combine with cognate response regulators. Signal-dependent autophosphorylation of a conserved histidine residue within the histidine protein kinase domain is followed by phosphoryl transfer to a conserved aspartate residue within the response regulator. As a result, the response regulator interacts with downstream signaling components or directly acts on promoters of target genes (Mascher et al. 2006; Gao et al. 2007).

The cyanobacterium *Synechocystis* sp. PCC 6803 harbours 47 genes encoding predicted Hiks many of which are implicated in stress reactions (Mizuno et al. 1996; Murata and Suzuki 2006) including responses to cold stress (Suzuki et al. 2001), high salt (Marin et al. 2003), high osmolarity (Mikami et al. 2002), heavy metals (Lopez-Maury et al. 2002) or low phosphate (Hirani et al. 2001), as well as in manganese homeostasis (Ogawa et al. 2002; Yamaguchi et al. 2002) or phototaxis (Shin et al. 2008). We have previously characterized the *slr1759* mutant constructed in the Murata laboratory (Suzuki et al. 2000; Nodop et al. 2006). It is insertionally inactivated in *slr1759* encoding the hybrid histidine kinase Hik14 and also does not express *slr1760* encoding a response regulator that is part of an operon with *slr1759* (Nodop et al. 2006). Thus, Slr1759 and Slr1760 presumably represent a two-component system encoded by linked genes.

In cultures that were inoculated at low cell density and aerated with 2% CO_2, growth of the Hik14 mutant was

strongly delayed compared to WT. Under these conditions, the pH drops and the equilibrium between CO_2 and HCO_3^- is shifted towards CO_2. Thus, the mutant presumably is less able to cope with acidification of the medium and the more energy-demanding metabolisation of CO_2. Further characterization of the mutant pointed to an enhanced respiratory activity and a slightly reduced photosynthetic activity, implicating Slr1759 in the coordination of carbon assimilation, pH homeostasis, respiration and photosynthesis (Nodop et al. 2006).

Slr1759 is a multidomain hybrid histidine kinase (Fig. 1). Based on its transmembrane segments and a CHASE (cyclases/histidine kinases associated sensing extracellular) domain, an extracellular sensory domain, Slr1759 is predicted to be membrane-anchored. Indeed, upon subcellular fractionation of *Synechocystis* sp. PCC 6803 cells Slr1759 was found associated with the cytoplasmic membrane consistent with a potential involvement in pH sensing (Nodop et al. 2006).

Immediately downstream of the CHASE domain Slr1759 contains two PAS (PER/ARNT/SIM) domains with adjacent PAC (PAS-associated) domains. PAS domains are sensors that monitor changes in light, redox potential, oxygen or small molecules in the cytosol (Taylor and Zhulin 1999). Downstream of the PAS/PAC domains, Slr1759 harbours a GAF (cyclic guanosine monophosphate associated factor) domain that is present in cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, formate hydrogen lyase, and the phytochrome photoreceptors (Martinez et al. 2002).

The C-terminal half comprises a histidine kinase domain (HisKA) with the conserved histidine residue as predicted primary phosphoacceptor and the ATP-binding HATPaseC (histidine kinase-, DNA gyrase B-, phytochrome like-ATPase) domain followed by two putative response regulator (RR) domains and a histidine phosphotransfer (Hpt) domain. The two RR might act as alternative acceptor sites, allowing input from multiple phosphate donors that are triggered by different stimuli.

Here, we demonstrate that Slr1759 is able to autophosphorylate in vitro and has a flavin cofactor attached to its first PAS domain. Furthermore, we show that this flavin is FAD as it reconstitutes the activity of a D-amino acid oxidase apoprotein. Slr1759 is the first cyanobacterial histidine kinase for which an association with FAD has been shown experimentally.

Materials and methods

Bacterial strains and DNA manipulation

E. coli strains DH10B and BL21 (DE3) (Novagen, Madison, WI) were cultivated in LBG (LB + 1 g/L glucose) medium. Cloning was performed according to standard procedures (Sambrook et al. 1989). Plasmids, strains, and oligonucleotide primers for PCR amplification with *Pwo* polymerase (Roche Applied Science, Munich, Germany) are listed in Table 1.

Expression and purification of Trx-His_6- and GST-His_6-tagged truncated *Slr1759* variants

Recombinant proteins were expressed with the pET vector system (Novagen, Madison, WI). The fusion proteins carry either an N-terminal thioredoxin domain (Trx) or N-terminal glutathione S-transferase (GST)-tag for enhanced solubility, and one or two His_6-tags (Table 2). PCR-derived DNA fragments of *slr1759* encoding single or multiple domains of Hik14 were cloned into pET vectors. The integrity of the resulting plasmid constructs was confirmed by sequencing. BL21 (DE3) strains harbouring the different variants were grown in LBG medium supplemented with 150 µg/mL Ap to an OD_{600} of 0.5–1.0. Protein expression was induced with 1 mM isopropyl-β-thiogalactoside (IPTG), and growth was continued for an additional 20 h at 15°C to minimize the formation of insoluble protein. After harvesting and adding the protease inhibitors PMSF

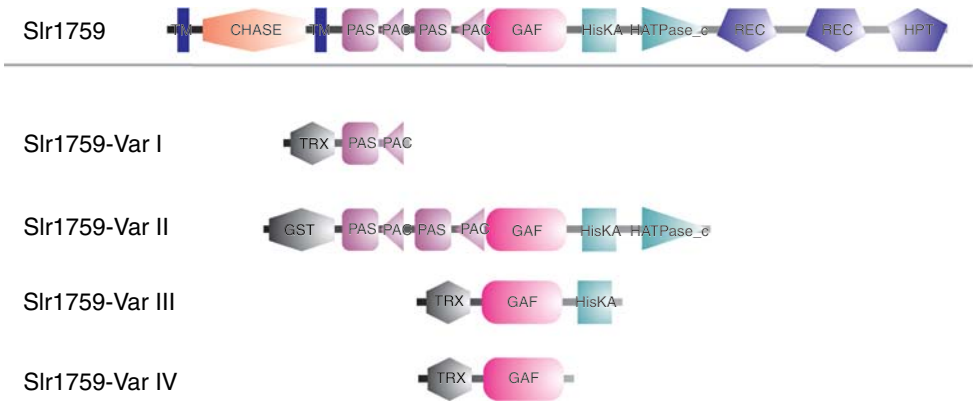

Fig. 1 Constructs for expression of recombinant Hik14 protein variants. Predicted domain structure of Slr1759 based on InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) (*top*) and truncated Slr1759 derivates recombinantly expressed in *E. coli*

Table 1 Oligonucleotides used to PCR-amplify partial sequences of *slr1759* from *Synechocystis* sp. PCC 6803 genomic DNA for subcloning into pET vectors

Introduced or native restriction enzyme sites are underlined

Primer	Protein	Amplified product (bp)	DNA sequence 5′ → 3′
slr1759-F1 *slr1759*-R7a	Slr1759-Var I	386	TAGCCATGGAAAAAACCGCCCT AAACCGGCCGTCACTTTCGC
slr1759-F12 *slr1759*-R12	Slr1759-Var II	2,178	ACCACTAGTCAGGAATTAGCCGTG ATCCTCGAGAATCCGCCGGT
slr1759-F19 *slr1759*-R19	Slr1759-Var IV	661	ATCATGGCCAGGAATTCACC GTCTGGATATCACTGACCGT

Table 2 Characteristics of truncated Slr1759 variants expressed in *E. coli* BL21 (DE3)

Protein	Tag	Amino acids	MM (kDa)	Domains	Solubility/colour
Slr1759-Var I	Trx-His_6	335–455	32.7	1.PAS-PAC	Soluble/yellow
Slr1759-Var II	GST-His_6	329–1,048	108.7	1.PAS-HATPase_c	Soluble/yellow
Slr1759-Var III	Trx-His_6	581–1,041	71.6	GAF-KinA	Soluble/colourless
Slr1759-Var IV	Trx-His_6	567–781	43.9	GAF	Soluble/colourless

(1 mM), benzamidine (1 mM) and benzonase (0.5 U/mL, Roche), cells were passed twice through a prechilled French press (SML Aminco, Urbana, Il) at 137.9 MPa. The cell-free extract was clarified by centrifugation for 30 min at 12,000*g*. This cleared lysate was subjected to affinity purification either on nickel-nitrilotriacetic acid resin (Ni-NTA; Qiagen, Hilden, Germany) according to the manufacture's instructions. Slr1759-Var II was purified either via its His_6-tag or via its GST-tag on glutathione sepharose resin (GE Healthcare, Munich, Germany).

[γ-^{32}P] ATP autophosphorylation assay

Slr1759 autophosphorylation assays were done as previously described (McCleary and Zusman 1990; Nishiwaki et al. 2000). Purified recombinant protein was added to the standard phosphorylation buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM dithiothreitol, and 0.15 mM γ-^{32}P ATP). Reactions were stopped by adding SDS sample buffer and loaded onto a 10% SDS polyacrylamide gel (acrylamide:bisacrylamide 121:1). Gels were dried and scanned on a Typhoon 8000 PhosphorImager (GE Healthcare).

Chemical stability measurements were performed as described in McCleary and Zusman (1990). Following electroblotting of the gel-separated proteins onto a PVDF membrane, the membrane was incubated in 1 M HCl, 2 M NaOH, 0.8 M NH_2OH pH 6.8 or 50 mM Tris-HCl pH 7.5, respectively.

Determination of FAD

The identification and quantification of FAD was done by reconstitution of D-amino acid oxidase (D-Aox) activity. D-Aox apoprotein was prepared from commercially available pig kidney D-Aox (Sigma, Munich, Germany). In order to remove its cofactors, 10 mg lyophilized protein (30 U) was dissolved in 1 mL 10 mM sodium phosphate buffer pH 7. Subsequently, 1 mL H_2O, 0.7 mL saturated ammonium sulphate solution and 0.8 mL 0.1 M HCl were added. After gentle mixing, the assay was centrifuged for 10 min at 25,000*g*. The FAD-containing supernatant was discarded and the resulting protein pellet was resuspended in 1 mL 10 mM sodium phosphate buffer pH 7. The washing step was repeated once or twice.

To extract the cofactor of Slr1759-Var I (Table 2), 0.2 mL of the affinity-purified protein was mixed with 20 µL 1 M H_2SO_4 and incubated for 30 min on ice. After centrifugation for 10 min at 20,000*g*, the FAD-containing supernatant was removed and neutralized with 20 µL 2 M NaOH. This supernatant was used for all subsequent reconstitution assays in a Clark-type O_2 electrode. The reaction mixture contained 0.5 mL 0.2 M Tricine-NaOH pH 8, 50 µL of D-Aox apoprotein, and the extract from the recombinant protein in a total volume of 3 mL. Once the mixture showed constant oxygen content, the assay was started by the addition of 0.3 mL 0.1 M D-alanine. A calibration curve was produced using 1–50 µM FAD.

Linear tetrapyrrole binding

Truncated Slr1759 variants harbouring the GAF domain were tested for the autocatalytic attachment of the linear tetrapyrroles biliverdin IXα (BV) and phycocyanobilin (PCB). In order to obtain holoproteins, *E. coli* BL21 (DE3) cells were cotransformed with the plasmids Slr1759-Var II, Var III or Var IV and pAT-BV encoding heme oxygenase 1 from *Synechocystis sp.* PCC 6803 (for production of BV) or

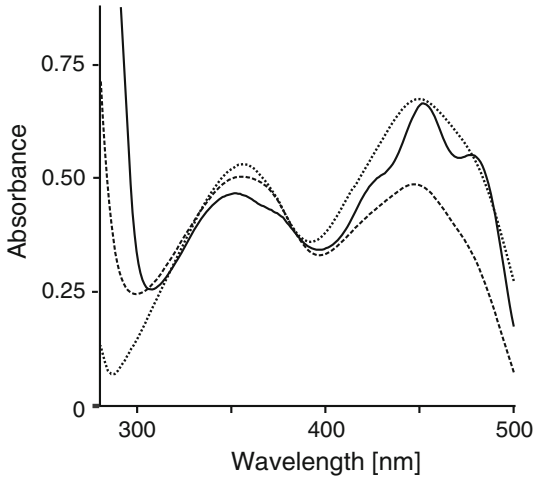

Fig. 2 Absorption spectra of fractions obtained during purification of Var I. Native protein of Slr1759-Var I after affinity purification (*continuous line*), cofactor extracted from affinity-purified Slr1759-Var I protein (*dashed line*). For comparison, absorption of 10 µM FAD-solution is shown (*dotted line*)

pAT103 (for production of PCB) under control of an IPTG-inducible P_{lac} promoter as described before (Gambetta and Lagarias 2001; Tasler et al. 2005). For coexpression, the medium contained 50 µg/mL Ap and 25 µg/mL Km. Protein synthesis was induced using 500 µM IPTG to provide sufficient amounts of recombinant Slr1759 variants and chromophore. During production and purification of holoproteins all steps were carried out under green safelight or in the dark.

Results and discussion

Recombinant Slr1759 harbours a flavin cofactor in its first PAS domain

To characterize the predicted domains of Slr1759, truncated recombinant proteins were constructed (Fig. 1; Tables 1, 2) and expressed in *E. coli*.

Slr1759 variants either harbouring the two PAS domains or extending from the first PAS domain to the HATPase domain, to the GAF domain, or to the response regulator domains fused to the thioredoxin-tag were recovered in an insoluble form. The membrane pellets obtained from the corresponding *E. coli* cells showed a prominent yellow colour (not shown), pointing to the presence of a flavin cofactor.

When the Trx-tag was exchanged by a GST-tag, the first PAS domain by itself could be recovered in a soluble form (Slr1759-Var I). The cleared lysate again showed a yellow colour, suggesting that a flavin cofactor is associated with the PAS domain. The absorption spectrum displayed bands around 375 and 450 nm which were very similar to the flavin reference (Fig. 2). The 450-nm band exhibits a fine structure that indicates binding of this flavin chromophore to the protein (Kleiner et al. 1999).

Table 3 FAD content of recombinantly expressed Slr1759-Var I purified on Ni^{2+}-NTA

Sample	FAD content		Protein content		FAD: protein
	pmol/mL	µM	mg/mL	µM	
Var I-E1	42.96	12.37	1.54	47[a]	0.26:1
Var I-E2	27.24	39.23	11.15	340	0.11:1
Var I-E3	40.06	52.38	7.15	220	0.24:1

Quantification of FAD was done by reconstitution of D-Aox apoprotein activity (see "Materials and methods"). The Slr1759-Var I protein was recovered in three consecutive fractions E1–E3 from the Ni^{2+}-NTA column

[a] The flavin content of Slr1759-Var I was also determined from the absorbtion spectrum on the basis of the molar extinction coefficient for flavin. The value was 49 µM. This implies that besides FAD, there is no FMN in the sample

The flavin cofactor is FAD

The identity of the flavin cofactor (riboflavin, FMN or FAD) was determined using reconstitution of D-Aox activity that depends on FAD (Rao et al. 1967; Casalin et al. 1991). Apoenzyme from pig kidney D-Aox was incubated with an acidic extract of affinity-purified Slr1759-Var I, and the O_2 consumption during oxidative deamination of D-alanine was monitored. Since the extract of Slr1759-Var I reconstituted D-Aox activity of the apoprotein, we conclude that the first PAS domain of Slr1759 contains FAD. The FAD content of affinity-purified Slr1759-Var I protein corresponded to 0.11–0.26 µmol cofactor per 1 µmol protein (Table 3). The FAD content could not be increased by supplementing the cultures with riboflavin. Therefore, the low ratio of FAD to PAS domain presumably is due to loss of FAD during the purification. In fact, some FAD was found in the flow-through of the affinity column that retained the protein.

The amount of FAD in the recombinant protein determined by the D-Aox apoprotein assay corresponded to the FAD amount calculated from the absorption spectra using the molar extinction coefficient of FAD ($\varepsilon = 11{,}300$ M^{-1} cm^{-1} at 450 nm). This implies that besides FAD no FMN is present in Slr1759-Var I.

When the second PAS domain was expressed in *E. coli*, FAD was found in the crude lysate but was lost upon further purification, suggesting that the second PAS domain may harbour a loosely bound FAD (not shown).

PAS domains that harbour FAD have been found in the redox sensing proteins *Azotobacter vinelandii* NifL and *E. coli* Aer; the latter senses O_2 indirectly as changes in the electron transport system. Although not enough PAS domains have been characterized in sufficient detail to

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   ***    * * *  *    **  ** *      * *       *  *      * * *   **      *    * * * *   *    **      **
ISITDLKANILYANRAFRTITGYGSEEVLGKNESILSNGTTPRLVYQALWGRLAQKKPWSGVLVNRRKDKTLYLAELTVAPVLNEAGETIYYLG-MHRDTS  NifL
VAITDTEGVITYVNDKFVEVSGYSREELIGNTHRLVSSGYHSPEFFQQFWQTIRAGKVWHGQINNRAKAGNTYWVDSTVVPFLDDNGNPYQYLAIRFEITS  Slr1759
MSTTDLQSYITHANDTFVQVSGYTLQELQGQPHNMVRHPDMPKAAFADMWFTLKKGEPWSGIVKNRRKNGDHYWVRANAVPMVRE-GKISGYMSIRTRATD  Aer
   **    **  ** ** ****  ** *  *  *         *   * *   *   * *   ** **  * **    **     *    *   *   *
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Fig. 3 Alignment of the first PAS domain of Slr1759 upon the FAD-binding PAS domains of *Azotobacter vinelandii* NifL and *E. coli* Aer. The alignment was performed using the ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). *Asterisks* indicate residues identical between Slr1759 and NifL (*top*) and between Slr1759 and Aer (*bottom*), respectively. Conserved Asn, Trp and Tyr residues implicated in FAD binding (Key et al. 2007; Taylor 2007) are in *bold*

correlate cofactor binding with amino acid residues (Hefti et al. 2004), we note that several residues that based on the NifL crystal structure contribute to FAD binding are also found in the first PAS domain of Slr1759 (Key et al. 2007; Taylor 2007) (Fig. 3). Overall, it shares 31% identity with the N-terminal PAS domain of NifL and 35% identity with the Aer PAS domain. In analogy to these, Slr1759 may be involved in sensing the energy status or changes in the redox poise within the cell via FAD.

Autophosphorylation of Slr1759

To investigate whether Slr1759 indeed autophosphorylates at the predicted histidine kinase-like domain, purified Slr1759-Var II, which comprises the predicted histidine kinase domain linked to the two PAS/PAC domains was incubated with [γ-^{32}P]-ATP. Phoshorylation of the protein was detected a few minutes after the start of the reaction and increased up to 45 min (Fig. 4a). To determine the optimal temperature range of the enzyme, the phosphorylation assay was performed for 30 min at temperatures ranging from 0 to 60°C (Fig. 4b). The highest level of autophosphorylated protein was obtained at 25°C. The amount of phosphorylated Slr1759 increased with rising concentration of [γ-^{32}P]-ATP substrate with a maximal activity obtained at 0.6 mM (Fig. 4c). The addition of increasing amounts of unlabeled ATP decreased the yield of ^{32}P labeled protein (Fig. 4d).

To obtain information on the phospho-accepting amino acid, chemical stability of the phosphorylated protein was tested. The phosphoester bond was stable under basic conditions but labile under acidic conditions, pointing to histidine as phospho-accepting amino acid (Fig. 4e).

The GAF domain of Hik14 does not bind linear tetrapyrrole

GAF domains can bind linear tetrapyrroles via a thioether bond of a conserved cysteine (Martinez et al. 2002). In order to investigate whether the GAF domain of Slr1759 is able to bind a linear tetrapyrrole cofactor, Slr1759-Var III harbouring the GAF and the histidine kinase domain and Slr1759-Var IV harbouring only the GAF domain were expressed in pigment-producing *E. coli* cells and subjected to a spectrophotometric analysis. Neither Slr1759-Var III

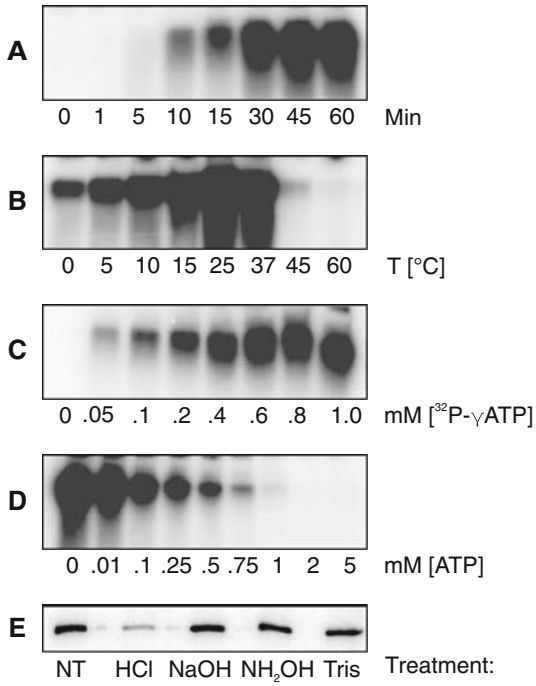

Fig. 4 Autophosphorylation activity of Slr1759. Recombinant Slr1759-Var II was incubated with [γ-^{32}P] ATP under the conditions indicated. **a** Time course: aliquots were withdrawn at the time points indicated. **b** Temperature dependence of histidine kinase activity. Incubation was performed for 30 min at the temperatures indicated. **c** Dependence of phosphorylation activity on [γ-^{32}P] ATP concentration. **d** Competition by unlabelled ATP. Increasing concentrations of unlabelled ATP were added to the assay mixture containing 0.15 mM [γ-^{32}P] ATP. **e** Chemical stability of the phospho-modified amino acid residue. The gel-separated phosphorylation products were transferred onto a PVDF membrane and subjected to treatment with the chemicals as indicated. *NT* no treatment. To resolve the phosphorylated product on polyacrylamide gels, it was necessary to use a low degree of cross-linking. The phosphorylated product merely entered standard gels, perhaps due to the formation of higher molecular weight adducts upon phosphorylation

nor Slr1759-Var IV showed a change in absorbance when coexpressed with the pigment biosynthesis genes (data not shown). Therefore, it seems unlikely that the GAF domain of Slr1759 binds linear tetrapyrrole(s). In line with this, no change in the red–far-red difference spectrum could be recorded in crude lysate or in solutions containing the partially-purified protein, not even when supplementary biliverdin or phycocyanobilin were added (data not shown). In a phylogenetic tree of all GAF domains of *Synechocystis*,

Slr1759 clusters together with Hik4 (Sll1228) and Hik26 (Slr0484) which have not yet been investigated for tetrapyrrole binding (Ikeuchi and Ishizuka 2008).

Conclusion

Proteins with complex domain structures linking multiple sensory domains are prevalent in cyanobacterial genomes, presumably reflecting the need to mediate their numerous responses to a suite of environmental cues in the respective habitats. The multiple sensory domains of Slr1759 may enable this hybrid histidine kinase to perceive different input stimuli via its cognate cofactors.

Interestingly, a correlation between the number of PAS domains and the number of proteins participating in electron transport reactions has been noted in bacterial genomes (Zhulin and Taylor 1998). *Synechocystis* sp. PCC 6803 with its photosynthetic and respiratory electron transport chains has 17 PAS-containing proteins with a total of 47 PAS domains, while the heterotrophic *Helicobacter pylori* with its simple electron transport chain has no PAS domain proteins at all (Zhulin and Taylor 1998). Our findings that Slr1759 has an FAD attached to its first PAS domain may point to an involvement of this hybrid histidine kinase in redox perception related to the coordination of photosynthetic and respiratory activity.

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