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Addition at the Molecular Level: Signal Integration in Designed Per–ARNT–Sim Receptor Proteins

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Survival of organisms in dynamic environments requires accurate perception and integration of signals. At the molecular level, signal detection is mediated by signal receptor proteins that largely are of modular composition. Sensor modules, such as the widespread Per–ARNT–Sim (PAS) domains, detect signals and, in response, regulate the biological activity of effector modules. Here, we exploit the modularity of signal receptors to design and engineer synthetic receptors that comprise two PAS sensor domains responsive to different signals, and we use these signals to control the activity of a histidine kinase effector. Designed two-input PAS receptors detected oxygen and blue light in a positive cooperative manner. The extent of the response to the signals was dictated by domain topology: the dominant regulatory effect was exerted by the PAS domain proximal to the effector domain. The presence of one sensor domain modulated the signal response function of the other. Sequence and structural data on natural receptors with tandem PAS domains show that these are predominantly linked by short amphipathic α-helices. Signals from multiple sensor domains could be integrated and propagated to the effector domain as torques. Our results inform the rational design of receptors that integrate multiple signals to modulate cellular behavior.

Keywords: Per–ARNT–Sim; photoreceptor; protein design; signal integration; signal transduction

Introduction

Excitability, the ability to detect and react to environmental stimuli, is a basic property of life. Living organisms must sense diverse chemical and physical input signals such as light, temperature, electric field, or the concentration of nutrients or toxins, and respond with appropriate output behavior. Multiple signals must be processed and integrated, which may be achieved at the molecular, cellular, or tissue level.[1] At the molecular level, signal detection is mediated by so-called signal receptor proteins. A hallmark of many signal receptors is their modular composition, which largely separates input (or sensor) and output (or effector) functionalities into distinct structural entities (i.e., proteins or protein domains).[1,2] Signal-dependent interactions between sensors and effectors modulate the biological activity of the receptor.

Per–ARNT–Sim (PAS) domains represent a widespread and versatile class of signal sensor and protein interaction domains.[3,4] As a family, PAS domains detect a wide range of physical and chemical signals and regulate the activities of diverse effector proteins. Despite this diversity, PAS domains share similar tertiary structures and certain signaling principles and mechanisms, and at least some PAS domains are functionally interchangeable.[4] For example, we replaced the oxygen-sensing PAS B domain of the histidine kinase FixL from *Bradyrhizobium japonicum* with a blue-light-sensitive PAS domain of the light–oxygen–voltage (LOV) subfamily[5] from the *Bacillus subtilis* photoreceptor YtvA. The resulting chimeric protein YF1 largely retained the catalytic efficiency of FixL, yet was regulated by blue light instead of oxygen.[6] We thus successfully reprogrammed the signal specificity of the protein. The modularity of natural signaling proteins provides a powerful means for producing novel protein switches by recombination of sensor and effector domains.[7]

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Abbreviations used: PAS, Per–ARNT–Sim; LOV, light–oxygen–voltage; FMN, flavin mononucleotide.

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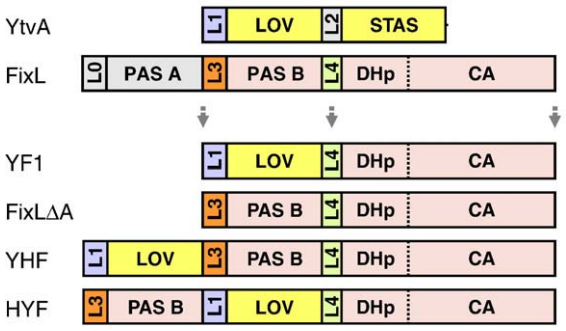

Fig. 1. Domain architectures of the parent PAS receptor proteins YtvA and FixL and the synthetic PAS receptor proteins derived from them: YF1, FixLΔA, YHF, and HYF. Linkers between protein domains are denoted L0–L4. YHF is equivalent to LOV-FixLΔA, and HYF is equivalent to PAS-B-YF1.

Many proteins contain multiple PAS domains or combine PAS domains with other signaling domains,[8] but the role of multiple PAS domains is poorly understood. How do several signaling domains interact? Do they detect multiple signals? If so, how are these signals integrated? What determines their gating properties? We address these questions by synthesis. We engineered variants of FixL comprising both its oxygen-sensitive PAS B domain and the blue-light-sensitive LOV domain of YtvA, and examined how these two-input PAS receptor proteins responded to the signals oxygen and blue light. Our findings provide the basis for the rational design of synthetic protein switches that respond to multiple signals.

The parent protein YtvA comprises an N-terminal LOV photosensor domain and a C-terminal STAS effector domain[9] (Fig. 1), and elicits the general stress response in *B. subtilis* upon blue-light absorption.[10] In the second parent FixL, two tandem PAS domains, denoted PAS A and PAS B, are linked N-terminally to a histidine kinase domain comprising the dimerization/phosphoacceptor subdomain DHp and the catalytic subdomain CA (Fig. 1). FixL forms part of the FixL/FixJ two-component system that regulates nitrogen metabolism in *B. japonicum* in response to oxygen levels.[11,12] The PAS A domain does not bind any known cofactor, and its specific biological role is uncertain. The PAS B domain is a sensor domain: it binds a heme (iron protoporphyrin IX) cofactor that can exist in reduced (ferrous; Fe^{II}) and oxidized (ferric; Fe^{III}) states.[13] Under anaerobic conditions, ferrous deoxy FixL is active as a histidine kinase. Using ATP as substrate, deoxy FixL first undergoes autophosphorylation at a histidine residue in the dimerization/phosphoacceptor subdomain and then transfers the phosphate group to an aspartate residue in its cognate noncovalently bound response regulator, FixJ.[11] Phospho-FixJ stimulates the transcription of genes involved in nitrogen metabolism. Binding of molecular oxygen to the ferrous heme produces oxy FixL with greatly reduced kinase activity. Oxidation to the ferric form generates met FixL with kinase activity identical with that in the ferrous deoxy state.[14] Binding of cyanide or imidazole to the met state yields cyanomet FixL or imidazolemet FixL, respectively, both of which are largely devoid of kinase activity.

Results

Engineering two-input PAS receptors

Fusion proteins comprising two PAS sensor domains and a histidine kinase domain were generated by modular recombination of domains from the two parent proteins *B. subtilis* YtvA and *B. japonicum* FixL (Fig. 1). Previously, we constructed the fusion protein YF1 by replacing both the PAS A domain and the PAS B domain of FixL with the single LOV domain of YtvA (Fig. 1).[6] The signal specificity of histidine kinase activity was thus reprogrammed from the diatomic ligands oxygen and cyanide to blue light. Both *in vitro* and *in vivo*, YF1 displayed histidine kinase activity in the dark; upon blue-light absorption, net kinase activity was eliminated, and YF1 acted as a light-activated phosphatase.[6]

Here, we investigate the role and interplay of multiple PAS sensor domains by protein design of histidine kinases that comprise two sensor domains. We generated the chimeric protein YHF in which we replace the PAS A domain of FixL with the LOV

Table 1. Description and activity of histidine kinases

Protein	Description of fusion	Turnover [mol FixJ (mol kinase)$^{-1}$ h^{-1}][a]			
		Dark	Light	Cyanide	Light and cyanide
YF1[b]	YtvA (1–127)–FixL (258–505)	56.4±2.8	*	—	—
FixL[b]	FixL (1–505)	80.2±3.6	—	0.086±0.014	—
FixLΔA	FixL (131–505)	2.1±0.2	—	0.33±0.04	—
YHF	YtvA (1–127)–FixL (130–505)	123±12	38.9±5.5	2.1±0.3	*
YHFΔ138–139	YtvA (1–127)–FixL (130–137)–FixL (140–505)	*	*	*	*
YHFΔ136–139	YtvA (1–127)–FixL (130–135)–FixL (140–505)	*	*	*	*
YHFΔ137–140	YtvA (1–127)–FixL (130–136)–FixL (141–505)	*	—	—	—
YHFΔ138–141	YtvA (1–127)–FixL (130–137)–FixL (142–505)	*	—	—	—
HYF	FixL (133–261)–YtvA (5–127)–FixL (258–505)	3.8±0.7	0.81±0.08	2.0±0.2	*

[a] Activities of histidine kinases in FixJ phosphorylation assays. (*) No detectable activity; (—) corresponding data not determined.
[b] Data taken from Möglich *et al.*[6]

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domain of YtvA (Fig. 1, Table 1). YHF thus comprises both the light-sensitive LOV domain of YtvA and the heme-binding oxygen-sensitive PAS B domain of FixL. A characteristic DID sequence motif at the C terminus of FixL PAS A, which is related to the DIT consensus motif found in other PAS domains,[4,6] precisely defines the C-terminal domain boundary of the PAS A domain and facilitates the design of YHF even in the absence of explicit structural information.

UV/Vis absorption spectroscopy confirmed that YHF incorporated its two cofactors, flavin mononucleotide (FMN) and heme, and interacted with its separate signals (light or diatomic ligands) similarly to its two parent proteins (Fig. S1a). Upon blue-light absorption, YHF reversibly underwent the characteristic LOV photocycle,[5] which is accompanied by spectral changes indicative of the formation of a thioether bond between a conserved cysteine residue in the LOV domain and atom C(4a) of the FMN cofactor.[15] Light-induced spectral changes were closely similar to those observed for YF1[6] and the parent YtvA.[9] Absorption spectroscopy also confirmed that, similar to the other parent FixL, YHF can adopt both ferrous and ferric states. Upon binding of cyanide or imidazole, met YHF displayed spectral changes that correspond to those for met FixL (Fig. S1a). As indicated by difference absorption spectra, the LOV photoreaction was unaffected by the presence of cyanide (Fig. S1b) and, *vice versa*, cyanide binding induced the same spectral changes under both dark and light conditions (Fig. S1c).

Sedimentation equilibrium centrifugation at YHF protein concentrations of between 2 and 7 μM revealed the presence of a single species with an apparent molecular mass of 121.2±9.4 kDa. Based on the expected dimer molecular mass of 115.8 kDa, we conclude that YHF, like FixL and YF1, predominantly exists as a dimer in solution.[6]

Heterotropic cooperativity between signals in YHF

We determined the catalytic activity of YHF in FixJ phosphorylation experiments under multiple turnover conditions, as described for YF1.[6,14] Briefly, fusion kinases were equilibrated at 23 °C in the presence of an excess of FixJ. Reactions were initiated by addition of an excess of [γ-^{32}P]ATP, aliquots were taken at different times and separated by gel electrophoresis, and the extent of phospho-FixJ formation was quantitated by phosphorimaging. The initial reaction velocity under these conditions was taken as a measure of net kinase catalytic activity.[6] In the absence of signals (i.e., in the met state in the dark), YHF phosphorylated FixJ with an initial reaction velocity of 123±12 mol FixJ (mol kinase)$^{-1}$ h^{-1} (Fig. 2)‡. Upon the introduction of saturating amounts of either light or oxygen as signal, activity was reduced by 2-fold to 3-fold. The activity of met YHF in the light was 38.9±5.5 h^{-1}, and the activity of oxy YHF in the dark was 54.5±7.6 h^{-1}. In the presence of both light and oxygen, YHF activity was reduced by 280-fold to 0.44±0.05 h^{-1}. Together, the signals led to a greater than multiplicative reduction of activity compared with the reduction in activity observed for light and oxygen separately. Thus, the presence of both signals light and oxygen had a positive heterotropic cooperative effect on YHF activity.[16] Heterotropic cooperativity was not restricted to light and oxygen, but was also observed in the met state (Fig. 3). In the dark, addition of cyanide to form cyanomet YHF reduced the activity of met YHF by 60-fold to 2.1±0.3 h^{-1}; in the light, the activity was too low to be measurable (<0.04 h^{-1}), corresponding to at least a 1000-fold reduction of activity in comparison to met YHF in the dark. Similarly, addition of imidazole reduced the activity of met YHF by 2.9-fold to 42.6±4.9 h^{-1} in the dark and by 110-fold to 1.1±0.1 h^{-1} in the light.

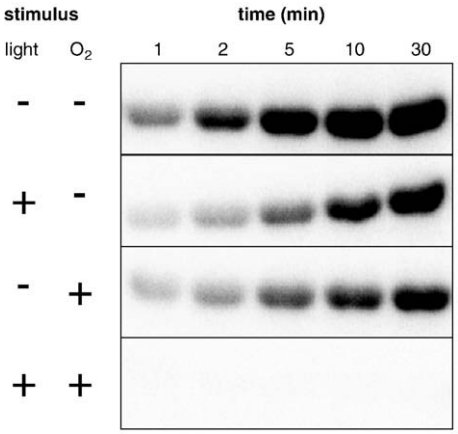

Fig. 2. YHF kinase activity is regulated by blue light and molecular oxygen in a positive cooperative manner. Phosphate incorporation into FixJ over time was detected by autoradiography using [γ-^{32}P]ATP. In the presence of either light or oxygen, the activity of YHF is moderately reduced; introduction of both signals strongly reduces activity.

Under identical reaction conditions, the net kinase activity of YF1 was 56.4±2.8 h^{-1} in the dark (Fig. 3).[6] Light absorption led to a more than 1000-fold reduction. For the parent FixL, the activity was 80.2±3.6 h^{-1} in the met state and 0.086±0.014 h^{-1} in the cyanomet state.[6] Complete removal of the PAS A domain from FixL to yield met FixLΔA (Fig. 1) led to a reduction of kinase activity to 2.1±0.2 h^{-1}. Addition of cyanide further reduced activity to 0.33±0.02 h^{-1}.

Signal response in YHF

We next studied the effect of signals on YHF activity upon partial saturation with the signals. Cyanide could not be used as a signal in these experiments due to its long equilibration times and

‡ In the following, we drop the two mole terms and report kinase activities in units of h^{-1}.

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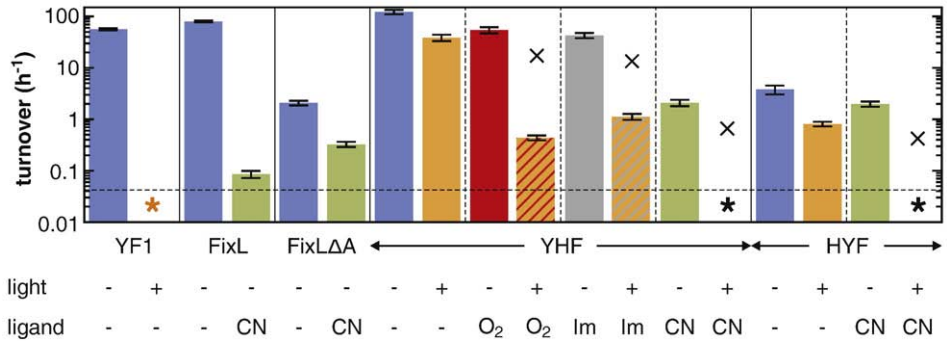

Fig. 3. Kinase activity and signal-dependent regulation of histidine kinase variants. Assay conditions were as indicated in the table below the figure and by different colors. Blue bars denote net kinase activities in the dark; orange bars denote activities in the light; red bars denote activities in the presence of oxygen; gray bars denote activities in the presence of 200 mM imidazole; and green bars denote activities in the presence of 10 mM cyanide. Asterisks indicate activities below the detectable limit of 0.04 h^{-1} (broken line). Crosses (X) indicate expected activities in the presence of the two signals if the signals acted noncooperatively.

high affinity ($K_d \sim 1$ μM) for the PAS B domain;[17] thus, imidazole was used. First, we conducted activity assays in the dark at varying imidazole concentrations (Fig. 4a). As determined by absorption spectroscopy, imidazole binding by met YHF was best described by a single-site isotherm with dissociation constant $K_d = 31.7 \pm 3.9$ mM. No homotropic cooperativity between the two imidazole binding sites within dimeric YHF was observed. The effect of imidazole on YHF activity could similarly be fitted by a single-site isotherm with the same dissociation constant $K_d = 27.5 \pm 6.8$ mM. Thus, binding of imidazole to YHF was linearly correlated to reduction in kinase activity.

Second, we measured the activity of YHF after partial photobleaching, the extent of which was monitored by absorption spectroscopy (Fig. 4b). Upon illumination with low-intensity blue light, the fraction of YHF in the dark state decayed in a first-order reaction with time constant $\tau = 109 \pm 27$ s. The kinase activity of YHF also decayed in a first-order reaction with the same time constant $\tau = 138 \pm 31$ s. As for imidazole binding, the extent of signal saturation (here light absorption) was linearly correlated to reduction in kinase activity.

Third, we measured the dark recovery of the absorption spectra and the kinase activity of YHF after complete photobleaching (Fig. 4c). Similar to YF1,[6] the absorption spectra of YHF fully recovered from photobleaching in a biphasic manner. Approximately 70% of the amplitude was regained with time constant $\tau_1 = 6720 \pm 20$ s, and the remaining 30% was regained in a very slow phase ($\tau_2 > 25{,}000$ s). The kinase activity of photobleached YHF samples fully recovered in a first-order reaction with $\tau = 7900 \pm 2500$ s, corresponding to the time constant of the main phase of the absorption data.

Interestingly, YF1 kinase activity recovered after photobleaching in a sigmoid fashion, implying homotropic cooperativity between subunits within the dimeric YF1 protein.[6] In contrast, no such homotropic cooperativity is observed for YHF. For both signals imidazole and light, the effect on YHF kinase activity is directly proportional to the degree of saturation with the signal. Thus, homotropic interactions between signals in YHF are noncooperative, but heterotropic interactions (e.g., between imidazole and light) are cooperative.

Signal response is governed by domain topology

We investigated whether the sequential order of sensor domains affects the activity and regulation of two-input PAS proteins by engineering the protein HYF in which the order of PAS sensor domains is reversed in comparison to YHF (Fig. 1). HYF thus links the PAS B domain of FixL to the LOV domain of YtvA and the histidine kinase domain of FixL. As for YHF, HYF also incorporated its cofactors heme and FMN, interacted with its signals light and cyanide, and, as shown by sedimentation equilibrium centrifugation, formed a dimer in solution with a molecular mass of 115.8±13.8 kDa.

In the dark, the kinase activity of met HYF was 3.8±0.7 h^{-1}, a value substantially lower than that of met YHF (123±12 h^{-1}) (Fig. 3). Cyanide binding to met HYF resulted in a 1.9-fold reduction of activity to 2.0±0.2 h^{-1}; blue-light absorption led to a 4.7-fold reduction of activity to 0.81±0.08 h^{-1}. As for YHF, heterotropic interactions between light and cyanide exerted a positive cooperative effect on HYF; the combination of both signals lowered activity by more than 90-fold to below the detection threshold of 0.04 h^{-1}. However, the relative magnitude of the response to the signals light and cyanide was reversed in HYF in comparison to YHF (Fig. 3). In YHF, the effect of cyanide on kinase activity much exceeded that of light; in HYF, the effect of light modestly exceeded that of cyanide. Thus, in both YHF and HYF, the PAS domain proximal in sequence to the histidine kinase domain exerted a stronger effect on catalytic activity than the distal PAS domain.

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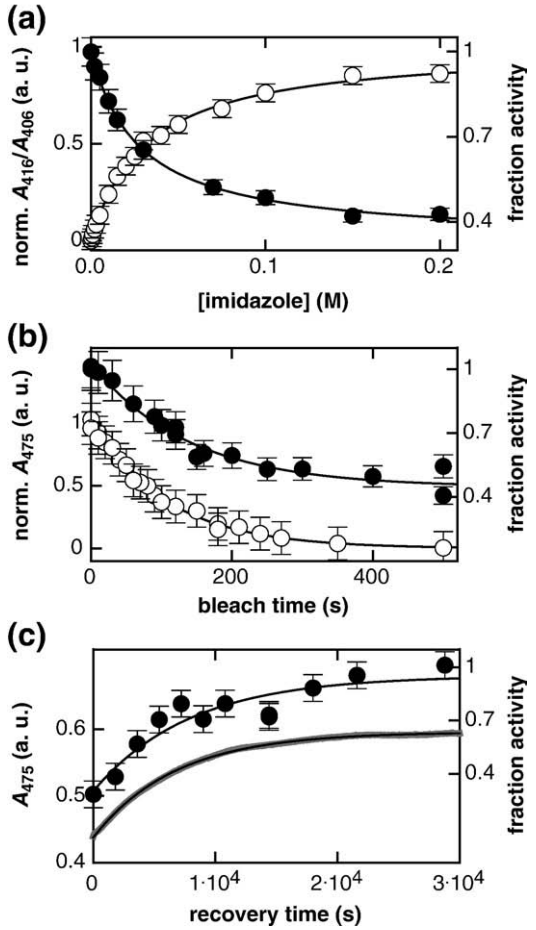

Fig. 4. Regulation of YHF activity by the signals imidazole and light. In (a)–(c), UV/Vis absorption data are shown as open symbols (○) or gray lines, kinase activity data are shown as closed symbols (●), and fits of the experimental data are shown as black lines. (a) Imidazole binds to YHF in a noncooperative fashion with dissociation constant $K_d = 31.7 \pm 3.9$ mM, as determined by absorption spectroscopy. The decrease in YHF activity upon addition of imidazole can be fitted by an isotherm with $K_d = 27.5 \pm 6.8$ mM. (b) Upon partial photobleaching, the fraction of the YHF LOV domain in the dark state decays in a first-order reaction with time constant $\tau = 109 \pm 27$ s, as determined by absorption spectroscopy. Under these conditions, YHF activity decays in a first-order reaction with time constant $\tau = 138 \pm 31$ s. (c) After saturating photobleaching, the fraction of the YHF LOV domain in the dark state recovers with a time constant $\tau_1 = 6720 \pm 20$ s, followed by a slower phase. YHF activity fully recovers in a first-order reaction with time constant $\tau = 7900 \pm 2500$ s.

PAS–PAS linkers largely adopt an α-helical conformation

Our previous work on YF1 showed that the length of the linker between its LOV domain and its histidine kinase domain crucially affects catalytic activity and the extent and nature of its regulation by light.[6] To explore the properties of PAS–PAS linkers, we analyzed the domain architecture and sequences of a large group of natural proteins that contain more than one PAS domain. A survey of the Pfam database[8] revealed that multiple PAS domains within one protein are common and that some proteins contain up to 15 annotated PAS domains. We identified 5002 pairs of tandem PAS domains and aligned the sequences of their intervening linkers (Fig. 5a). As in our previous analyses of PAS–histidine kinases[6] and PAS–GGDEF proteins,[4] many PAS domains within the current data set showed a conserved DIT sequence motif at their C terminus. However, the DIT motif was conserved to a lesser extent than in PAS-histidine kinases, and a sizable fraction of PAS domains displayed different sequence motifs. As observed for the linkers between PAS domains and several effector domains,[4,6] the PAS–PAS linkers between tandem PAS domains were short and showed an amphipathic sequence signature with a heptad periodicity of hydrophobic residues. Strikingly, 48% shared the same discrete length of 28 residues (Fig. 5b); FixL, YHF, and HYF all fall into this most populated class. Minor populations of linker length occurred at regular intervals of three or four residues shorter or longer (Fig. 5b; Fig. S2). These data argue for PAS–PAS linkers of well-defined structure and largely rule out unstructured linkers. The amphipathic sequence signature and the constrained length distribution (Fig. 5) suggest a predominantly α-helical conformation of PAS–PAS linkers. The multiple PAS–PAS linkers in proteins with more than two tandem PAS domains show closely similar properties; no clear correlation was found between the lengths and the sequences of individual PAS–PAS linkers within the same protein. Since natural PAS proteins are usually at least dimeric,[4] amphipathic linker helices could associate with each other or with other helices to form helical bundles or coiled coils, as we proposed for YF1.[6]

Deletion or insertion of two residues within an α-helical or coiled-coil PAS–PAS linker would drastically change the relative orientation of tandem PAS domains and thus be expected to have detrimental effects on signal transmission and regulation of protein activity. We tested this prediction experimentally and found that deletion of two residues from the PAS–PAS linker of YHF to form YHFΔ138–139 indeed completely abolished kinase activity under all conditions (Table 1). Similarly, removal of four consecutive residues from different locations within the linker (YHFΔ136–139, YHFΔ138–141, and YHFΔ137–140) resulted in a complete loss of kinase activity (Table 1). All linker deletion variants incorporated their cofactors FMN and heme as evidenced by absorption spectroscopy (data not shown), indicating that their sensor domains are correctly folded. Interestingly, the distribution of linker lengths (Fig. 5b) shows that PAS–PAS linkers in different proteins frequently differ by three or four residues (e.g., 24 and 28 residues). While these results clearly confirm that the integrity of the PAS–PAS linker is important for function, more research is needed to fully understand its properties.

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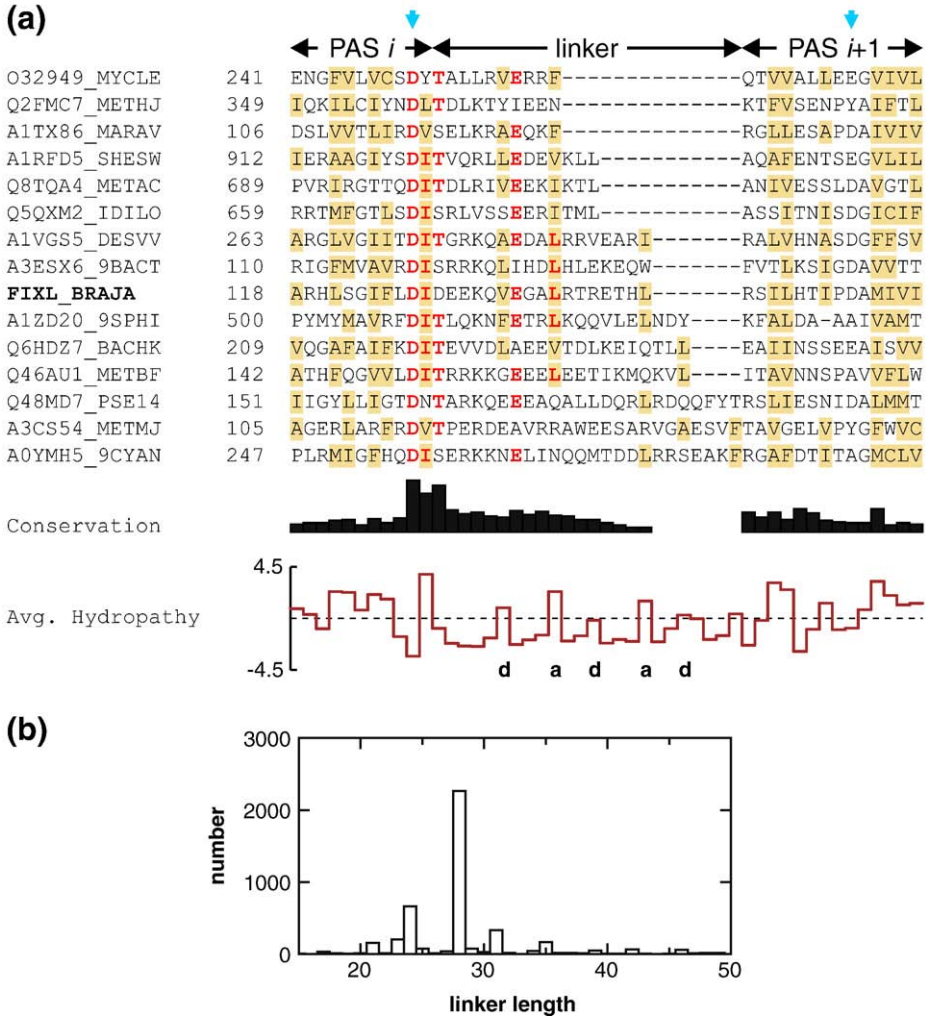

Fig. 5. Tandem PAS domains are connected by α-helical linkers. (a) Multiple sequence alignment of the PAS–PAS linkers between 5002 tandem PAS domains. A representative subset of 15 sequences is shown and labeled with their UniProt[18] identifiers; *B. japonicum* FixL (FIXL_BRAJA) is highlighted in boldface. Residues conserved in more than half of all 5002 sequences are shown in bold red; brown shading denotes columns with more than 50% hydrophobic residues. On average, PAS–PAS linkers display an amphipathic sequence signature with a heptad periodicity of hydropathy. Hydrophobic positions are labeled **a** and **d** according to standard nomenclature.[19] (b) Distribution of linker lengths in tandem PAS domains as calculated by the number of residues between the blue arrows indicated in (a). About 48% of all sequences display a discrete linker length of 28 residues. Minor populations of linkers are longer or shorter by multiples of three or four residues.

Discussion

The presence of predominantly short and largely α-helical PAS–PAS linkers constrains the relative orientations that tandem PAS domains can assume. Together with the limited but suggestive structural data on tandem PAS proteins, which we review below, we develop a structural and mechanistic model for signal integration in multi-input PAS receptors.

Structure of multi-input PAS receptors

High-resolution structures of tandem PAS domains are available for the prokaryotic proteins DctB from *Sinorhizobium meliloti*[20] (Protein Data Bank entry 3E4P), LuxQ from *Vibrio harveyi*[21] (2HJ9), MmoS from *Methylococcus capsulatus*[22] (3EWK), and Mcp_N from *Vibrio cholerae* (3C8C) (Fig. 6a; Fig. S3). In agreement with our sequence analysis, their PAS–PAS linkers consistently form short α-helices. Interestingly, in all tandem PAS structures, the PAS A and PAS B domains are linearly oriented in a head-to-tail fashion; the C termini of the PAS A and PAS B domains face approximately the same direction (Fig. S4). The structural interface between the cores of PAS A and PAS B is relatively small and, on average, buries 560±80 Å^2 of solvent-accessible surface area. Crucially, this spatial arrangement leaves the outer surface of the canonical five-stranded antiparallel β-sheet of the PAS core domains largely exposed and thus available to undergo signal-dependent intramolecular and intermolecular interactions that form a key feature of signal transduction by PAS proteins.[4]

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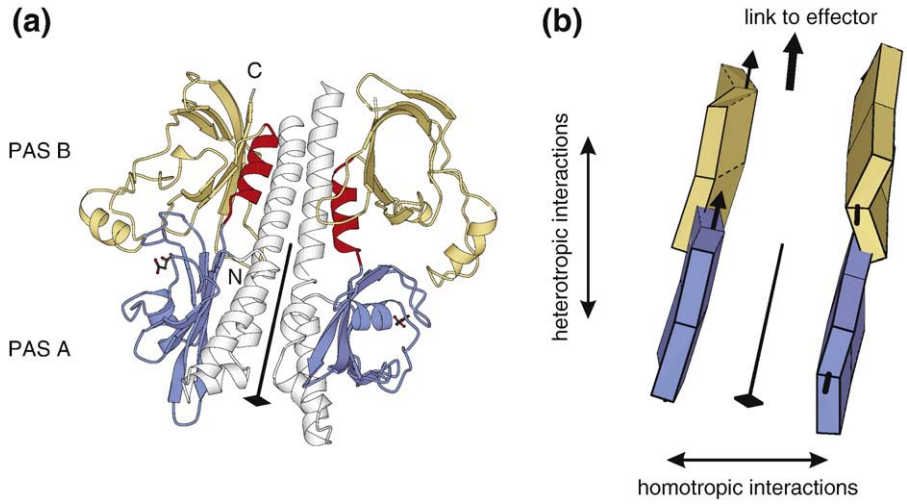

Fig. 6. Models for the structure and signal transduction mechanism of tandem PAS signal receptors. (a) Three-dimensional structure of the dimeric tandem PAS domains of *S. meliloti* DctB (3E4P). Within each molecule, the PAS A domain is shown in blue, the PAS B domain is shown in yellow, and the PAS–PAS linker is shown in red. A dyad symmetry axis is shown as a black line. (b) Homotropic interactions occur between like PAS domains across the dyad axis, and heterotropic interactions occur between tandem PAS domains along the dyad axis. Signals from multiple sensor domains could be integrated as torque and propagated along the central dyad symmetry axis to the C-terminal effector domain.

The outer surfaces of the PAS β-sheets form extensive contacts with long α-helices N-terminal to PAS A. Interactions between the cores of the PAS A and PAS B domains and the PAS–PAS linker helix are almost exclusively confined to the PAS-B domain. All of the above tandem PAS proteins dimerize within the crystal lattice[20,21] or in solution.[22] All form parallel dimers (Fig. S3a–c), as was also suggested for several related multi-PAS proteins.[23,24] Dimerization is mediated by a central helical bundle or spine that comprises the long α-helices N-terminal to PAS A and spans the (crystallographic or approximate) dyad symmetry axis of the molecule. Homotropic interactions between the two PAS A domains (and the two PAS B domains) are indirect and mediated by the long N-terminal α-helices§. These tandem PAS structures derive from transmembrane chemoreceptors, but FixL, YHF, and HYF are water soluble. The precise geometry of the central spine may differ between PAS proteins. In particular, FixL, YHF, and HYF all lack similar N-terminal α-helices. Homodimerization of individual PAS domains could be mediated through direct interactions between the outer surfaces of the PAS β-sheets, which is frequently observed in the structures of isolated PAS domains,[4] among them those of FixL PAS B[25] and YtvA LOV.[26]

A similar spatial configuration in which multiple sensor or sensor-related domains are arranged along a central helical spine is also found in the structures of bacteriophytochrome red-light sensors.[27–29] Related α-helices, often with amphipathic sequence, also connect various forms of sensor, effector, and other domains in widely diverse proteins.[8,30] So-called signaling helices thus serve as widespread linkers in signaling proteins.[8,31]

In contrast to the prokaryotic tandem PAS proteins, the eukaryotic clock protein Per from *Drosophila melanogaster* (1WA9)[32] and its *Mus musculus* homolog Per2 (3GDI)[33] connect tandem PAS domains by linkers that are largely devoid of secondary structure (Fig. S3e and f), although they display head-to-tail orientations of tandem PAS domains similar to those in prokaryotic proteins (Fig. S3a–c). In other eukaryotic proteins, PAS–PAS linkers are very long (e.g., the linker between the LOV1 domain and the LOV2 domain of plant phototropins comprises more than 150 residues).[34] Prokaryotic and eukaryotic multi-PAS proteins evidently differ in their architecture and signaling mechanism.

Signal integration by multi-input PAS receptors

Introduction of signals induced small changes in quaternary structure within the dimeric sensor domain for both the isolated LOV domain from YtvA and the PAS B domain from FixL.[25,26] Data on YF1 and variants suggested that such light-induced conformational and dynamic changes are propagated as torque from the LOV sensor domain through a coiled-coil linker to the histidine kinase effector domain: the signal acts as a rotary switch.[6] Since YF1, YHF, HYF, and FixL all form dimers and share a common histidine kinase effector domain, we propose that a similar model for signal transduction

§ A very recent article by Zhang and Hendrickson reports several crystal structures of tandem PAS domains that overall adopt conformations closely similar to the structures shown in Fig. S3a–c.[45]

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also holds for the tandem PAS proteins and provides a general basis for signal integration. As discussed above, multiple PAS (and possibly other) sensor domains are linked by short α-helices and arranged in an essentially linear fashion around the dyad symmetry axis of a homodimeric protein (Fig. 6a; Fig. S3). Heterotropic interactions between tandem PAS domains occur along the central spine; homotropic interactions occur in a direction perpendicular to and across the spine (Fig. 6b). The linear spatial arrangement of multiple sensor domains is consistent with addition of torques along axes roughly parallel with the dyad axis and, thereby, integration of rotary signals. Torque originating in the distal PAS domains could propagate to the proximal PAS domains, where it may be further amplified or reduced by torque originating in the proximal PAS domains. An integrated torque signal is transmitted to the C-terminal effector domain, where it regulates biological function (here, histidine kinase activity).[6] Although our model is consistent with the present functional and structural data, we are aware that both are limited in extent and that further experimental verification is required.

Our experimental results indicate that the regulatory roles of sensor domains strongly depend on their protein context. This is most clearly seen for the two-input receptors YHF and HYF, which comprise the same PAS sensor domains yet link them in different sequential orders (Fig. 1). Both proteins exhibit heterotropic cooperativity: blue light and diatomic ligands affected catalytic activity in a positive cooperative manner. However, YHF and HYF differed in the extent of the effect of the two signals. The signal sensed by the PAS domain proximal in sequence to the effector domain exerted the stronger effect on activity than the signal sensed by the distal PAS domain. Another example is provided by the comparison of YF1 and HYF (Fig. 1). While blue-light absorption completely abolished kinase activity in YF1, it only moderately reduced the activity of HYF (Fig. 3). Thus, the additional N-terminal PAS B domain in HYF strongly attenuates the regulatory effect of the LOV domain. Deletion of the PAS A domain of FixL led to a 40-fold reduction of activity in FixLΔA and attenuated the regulatory effect of cyanide (Fig. 3). N-terminal addition of a distal LOV domain to FixLΔA to yield YHF restored robust kinase activity and increased the regulatory effect of cyanide. A qualitative general conclusion is that the signaling properties of isolated domains or truncated proteins differ considerably from those of intact signaling proteins.[35] This is also exemplified by plant phototropins whose two LOV domains, LOV1 and LOV2, both detect blue light yet mediate different functional roles.[36] Whereas LOV2 is proximal to a kinase effector domain and exerts the dominant regulatory effect on its activity, the distal LOV1 domain fine-tunes the response to light.[36] Moreover, the photochemical properties of LOV1 and LOV2 vary depending on the protein construct in which they are embedded.[34]

While many details remain unclear, several not mutually exclusive roles for multiple PAS domains begin to emerge. First, as shown here, multiple PAS domains within a single receptor can detect multiple signals. A natural example of a dual-input PAS receptor is provided by *Rhodospirillum centenum* Ppr, which comprises both red-light-detecting and blue-light-detecting sensor modules.[37] Cellular two-component systems are usually well insulated from each other to minimize cross-talk and thus only permit signal integration at the transcriptional level or further downstream.[38] However, integration of signals at the receptor level, instead of at the transcriptional level, could be of advantage because it occurs very rapidly and ensures spatial and temporal coincidence of the signals. Second, PAS domains, even if they do not themselves act as sensor domains, can modulate the signal response function of other (PAS) sensor domains, as demonstrated here and in several other systems (e.g., *Lee et al.*,[23] Slavny *et al.*,[24] Christie,[34] and Kyndt *et al.*[37]). Combinatorial mixing of sensor domains throughout evolution may thus have created novel receptors with graded and optimized signal response functions. Third, (multiple) PAS domains frequently mediate oligomerization and signal-dependent protein interactions.[3,4] For example, signal detection was proposed to involve transient dissociation of individual PAS domains within the multi-PAS proteins *S. meliloti* DctB,[20,39] *B. subtilis* KinA,[23] and *Azotobacter vinelandii* NifL.[24] Most of these functions do not require a PAS domain to be an explicit sensor domain, which concurs with the observation that many PAS domains apparently do not directly detect a signal.[4] Different classes of signaling domains may share certain mechanistic principles,[2,4,40] and these findings could thus be of broader relevance.

Modular recombination of protein domains has proven to be a powerful design strategy for synthetic signal receptors. In particular, the recent design of artificial photoreceptors[6,35,41–43] greatly expands the repertoire of optogenetics.[44] Synthetic photoreceptors can be genetically encoded and employed *in vivo* to control the properties of cells and, indeed, the behavior of whole organisms by light.[6,42,43] As we have demonstrated, several signals can be processed by multi-input PAS receptors and integrated at the protein level. Synthetic signal receptors with defined gating logic provide an additional layer of control and specificity and could become versatile tools for studies on living systems.

Materials and Methods

A more detailed description of Materials and Methods is provided in Supplementary Material.

Molecular biology and protein purification

Fusion constructs were generated by overlap extension PCR and site-directed mutagenesis, as described previously.[6] Proteins were purified by metal-ion affinity

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and ion-exchange chromatography in accordance with Möglich *et al.*[6] Purified proteins were reconstituted with their cofactors heme and FMN. Protein concentrations were determined by absorption spectroscopy using an extinction coefficient of 1.26×10^5 M^{-1} cm^{-1} for the heme cofactor at 398 nm and an extinction coefficient of 1.25×10^4 M^{-1} cm^{-1} for FMN at 450 nm.[6]

Phosphorylation assays

Assays were conducted in reaction buffer [50 mM Tris–HCl (pH 8.0), 50 mM KCl, 100 μM $MnCl_2$, and 5% (vol/vol) ethylene glycol] at 23±1 °C, as described previously.[6,14] Measurements on YHF, HYF, and variants were routinely conducted in their oxidized met state. The cyanomet and imidazolemet forms were obtained by addition of 10 mM KCN or 200 mM imidazole, respectively. The oxy forms of the fusion kinases were produced by reduction of the heme cofactor to its ferrous state with 10 mM dithiothreitol in the presence of oxygen. The oxidation and ligation states of the heme cofactor were evaluated by absorption spectroscopy. Kinase experiments were conducted either in the dark or under illumination with a fiber optics lamp, as described previously.[6] In photobleaching and photorecovery experiments, kinase activities were normalized to the activity of a reference sample incubated in the dark.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.05.019

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