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Signal Transduction and Phosphoryl Transfer by a FixL Hybrid Kinase with Low Oxygen Affinity: Importance of the Vicinal PAS Domain and Receiver Aspartate

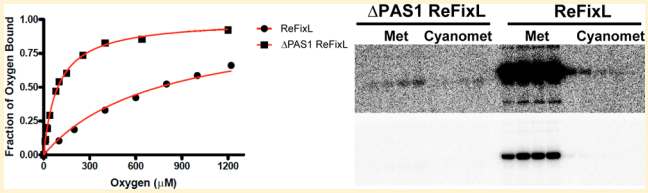
Eduardo H. S. Sousa,*[,‡] Jason R. Tuckerman,[†] Ana C. S. Gondim,[†] Gonzalo Gonzalez,[†] and Marie-Alda Gilles-Gonzalez*[,†]

[†]Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9038, United States

[‡]Department of Organic and Inorganic Chemistry, Federal University of Ceara, Center for Sciences, CEP 60455-760 Fortaleza-Ceara, Brazil

Supporting Information

ABSTRACT: FixL is a prototype for heme-based sensors, multidomain proteins that typically couple a histidine protein kinase activity to a heme-binding domain for sensing of diatomic gases such as oxygen, carbon monoxide, and nitric oxide. Despite the relatively well-developed understanding of FixL, the importance of some of its domains has been unclear. To explore the impact of domain−domain interactions on oxygen sensing and signal transduction, we characterized and investigated *Rhizobium etli* hybrid sensor *Re*FixL. In *Re*FixL, the core heme-containing PAS domain and kinase region is preceded by an N-terminal PAS domain of unknown function and followed by a C-terminal receiver domain. The latter resembles a target substrate domain that usually occurs independently of the kinase and contains a phosphorylatable aspartate residue. We isolated the full-length *Re*FixL as a soluble holoprotein with a single heme *b* cofactor. Despite a low affinity for oxygen (K_d for O_2 of 738 μM), the kinase activity was completely switched off by O_2 at concentrations well below the K_d. A deletion of the first PAS domain strongly increased the oxygen affinity but essentially prohibited autophosphorylation, although the truncated protein was competent to accept phosphoryl groups in trans. These studies provide new insights into histidyl−aspartyl phosphoryl transfers in two-component systems and suggest that the control of ligand affinity and signal transduction by PAS domains can be direct or indirect.



Hemeproteins are well-known for their roles in catalysis as well as for ligand transport and storage in many organisms. A third role as dedicated sensors was identified more recently, and novel physiological roles have been ascribed to these sensors in bacteria, archae, insects, and mammals.[1,2] There are at least seven families of heme-based sensors based on the folds of the heme-binding regions. These include PAS, HNOB, globin, CooA, GAF, LBD, and SCHIC.[2−5] Upon sensing O_2, CO, or NO, heme-based sensors trigger a response that can involve changes in catalysis or macromolecular interactions (protein−protein or protein−nucleic acid), because of the impressive modularity and versatility of coupled functional domains.

The prototype for a heme-based sensor is the FixL protein, whose discovery was a hallmark in defining this young field.[6,7] FixL possesses a kinase activity specific for the FixJ protein, its partner in a two-component regulatory system. Such systems are widespread in bacteria and are utilized for sensing and signaling of environmental cues leading to physiological adaptations.[8−10] *Sinorhizobium meliloti* FixL (*Rm*FixL) was the first sensor shown to be reversibly inhibited by O_2. Anaerobically, *Rm*FixL is fully activated to phosphorylate its protein substrate, FixJ. Phosphorylated FixJ is a transcription factor that binds to specific DNA sequences to induce a cascade of hypoxia-specific gene expression. The *nif* genes encoding the nitrogenase subunits and *fix* genes for respiration at low O_2 concentrations are expressed as an end point to this cascade. However, oxygen-sensing two-component systems are not limited to FixL and FixJ or to Rhizobia. In *Mycobacterium tuberculosis*, for example, adaptations to dormancy are in part governed by an O_2-sensing system comprised of a transcription factor called DevR and two sensor kinases, DosT and DevS.[10] DevR has a domain organization similar to that of FixJ; in these proteins, an N-terminal receiver domain with the aspartate for phosphorylation (around residue 54) precedes a C-terminal helix−turn−helix DNA-binding region. DosT and DevS resemble FixLs, with a major exception being binding of heme in a GAF domain instead of a PAS domain.[3]

Interesting molecular details have emerged from enzymatic studies of a naturally soluble full-length *Bradyrhizobium*

Received: July 24, 2012
Revised: December 18, 2012
Published: January 2, 2013

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japonicum FixL (*Bj*FixL) and a *Rm*FixL truncation lacking a transmembrane N-terminal region.[1,2,11,12] An important advance was the demonstration that the reaction of a FixL–FixJ complex with ATP is more responsive to O_2 and more efficient in its overall anaerobic rate and extent than the sequential phosphorylations of FixL with ATP and FixJ with phospho-FixL.[13] These data suggested that an efficient signaling response might depend on intimate interactions of FixL with FixJ. Along these lines, a remarkable hysteretic effect of O_2 concentration on the FixJ phosphorylation rate suggested that FixL maintains a memory of O_2 binding. This memory is thought to be due to a slow recovery of the kinase from inactivation, probably because of a structural reorganization involving domain–domain interactions.[14] The memory effect allows a surprisingly stringent regulation of FixL by O_2 even at concentrations of this ligand that do not permit full saturation.[14]

Clearly, to convey the status of the FixL iron center to the kinase, the regulatory heme-PAS domain must interact with the enzymatic region. One aspect of FixL signaling that has been minimally explored to date is the influence of additional domains, apart from the heme and kinase domains, on regulation. At least two additional domains are known to occur in FixLs. The first is a non-heme-containing N-terminal PAS domain that usually precedes the heme-PAS region; *Bj*FixL is an example of this. In addition, a C-terminal region resembling a FixJ receiver domain can occur in some FixL proteins that have been denoted hybrids. Similar organizations have been noted for other sensory systems. Possibly, the extra PAS and receiver domains compete for binding to regions of the kinase, with important consequences for the enzymatic activity, as has been proposed.[1]

Here we examine for the hybrid *Rhizobium etli* FixL (*Re*FixL) protein the impact of domain–domain interactions on ligand binding and regulation. *Re*FixL contains not only the non-heme-binding N-terminal PAS domain but also a C-terminal FixJ-like receiver domain. O_2 regulation of *Re*FixL has been investigated in vitro, but until now, its biochemistry had not been explored.

MATERIALS AND METHODS

The full-length wild-type *R. etli fixL* gene was amplified by a polymerase chain reaction that appended NdeI and HindIII sites to overlap the 5′ and 3′ ends of the gene, respectively. The DNA that served as a template was kindly provided by M. Soberon and colleagues.[15] For expression, the full-length *fixL* gene was placed after a tac promoter in a pUC19-derived *Escherichia coli* expression vector that conferred ampicillin resistance. The *D573N RefixL* mutant was generated with the QuikChange site-directed mutagenesis kit (Stratagene). The Δ*PAS1 RefixL* truncation (without the first PAS domain) was designed by amplifying the region from codon 135 to the end of the *fixL* gene and cloning it as described above. The cloned DNAs were verified by sequencing (McDermott Center for Human Growth & Development, University of Texas Southwestern Medical Center).

Gene Expression and Protein Purification. A 4 L culture of *E. coli* strain TG1 harboring the wild-type *RefixL*, Δ*PAS1 RefixL*, or *D573N RefixL* plasmid was grown overnight in a Bioflow 3000 fermentor at 37 °C, 200–500 rpm, and 20% atmospheric O_2. When the culture reached an OD_{600} of ∼0.5, expression of the recombinant protein was induced with 1 mM IPTG. The cells were harvested 4–6 h later and lysed by sonication, and the lysate was cleared by centrifugation at 70000 rpm (Ti 70 rotor, Beckman). Purifications of *Re*FixL, ΔPAS1 *Re*FixL, and D573N *Re*FixL were conducted by a strategy similar to the previously reported purifications of *B. japonicum* and *S. meliloti* FixLs, with minor changes.[16] Protein expression and purification were monitored from the UV–vis absorption spectra and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The protein concentration was measured by Bradford assays (Bio-Rad), with bovine serum albumin as the standard. The resulting protein was >90% pure as evaluated by SDS–PAGE, analytical gel filtration, and the UV–vis spectra. Small aliquots were fast-frozen in liquid nitrogen for single use upon being thawed and stored at −80 °C.

Absorption Spectra, Autoxidation, and Ligand Binding Measurements. Unless otherwise specified, all of these measurements were taken in 50 mM Tris (pH 8.0) at 25 °C. Anaerobic samples were prepared inside a glovebag (COY laboratories) by treating concentrated FixL with a stoichiometric amount of dithionite and immediately removing the dithionite with a gel exclusion column (G25 Sephadex resin). For autoxidation experiments, these samples were diluted 100-fold in air-saturated buffer at 25 °C, and their absorption spectra were monitored with a Cary 4000 UV–vis spectrophotometer (Varian). Laser-flash photolysis and stopped-flow measurements were conducted with an LKS.60 laser kinetic spectrometer fitted with a PiStar stopped-flow drive unit (Applied Photophysics, Leatherhead, U.K.). For sample excitation, the LKS.60 spectrometer was coupled to a Quantel Brilliant B Nd:YAG laser with second harmonic generation. Ligand binding kinetics were followed at a wavelength of maximal difference between the starting and final species. Each association rate constant was calculated from a linear plot of k_{obs} versus ligand concentration, including at least four ligand concentrations. Association rates for CO (100–960 μM) with the deoxy state were measured by laser-flash photolysis. The association rates for O_2 (0.10–1.2 mM) with the deoxy state and for imidazole (0.5–8.0 mM) and cyanide (2–5 mM) with the met state were measured by stopped-flow methods. The dissociation rate constant for O_2 was estimated from the intercept of a linear plot of k_{obs} versus O_2 concentration obtained from stopped-flow measurements of the association rates. The equilibrium dissociation constants (K_d values) for binding of the proteins to O_2 and CO were measured directly by titrating the deoxy state of *Re*FixL (2–3 μM) with 25–1220 μM O_2 or 1–960 μM CO at 25 °C as previously described.[17] The K_d values for binding were determined from saturation changes calculated by multiple-linear regression analysis of whole spectra and fit to a quadratic single-binding equation using Prism version 5.0.

Phosphorylation Assays. An initial investigation of the effect of divalent cations on *Re*FixL phosphorylation led us to adopt a divalent metal mixture of 50 μM Mn^{2+} and 1 mM Mg^{2+} for the experiments, as was previously done for the *Bj*FixL–*Bj*FixJ complex.[14] Experiments conducted with deoxy, NO-bound, or CO-bound *Re*FixL were conducted inside an anaerobic glovebag, while aerobic and oxidized forms of *Re*FixL (met, cyanomet, and imidazolomet) were prepared outside of this bag. The experiment conducted in air also included DTT to prevent any potential oxidation, which was checked before and after each experiment. Unless otherwise specified, reactions were conducted in phosphorylation buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 5% (v/v) ethylene glycol]

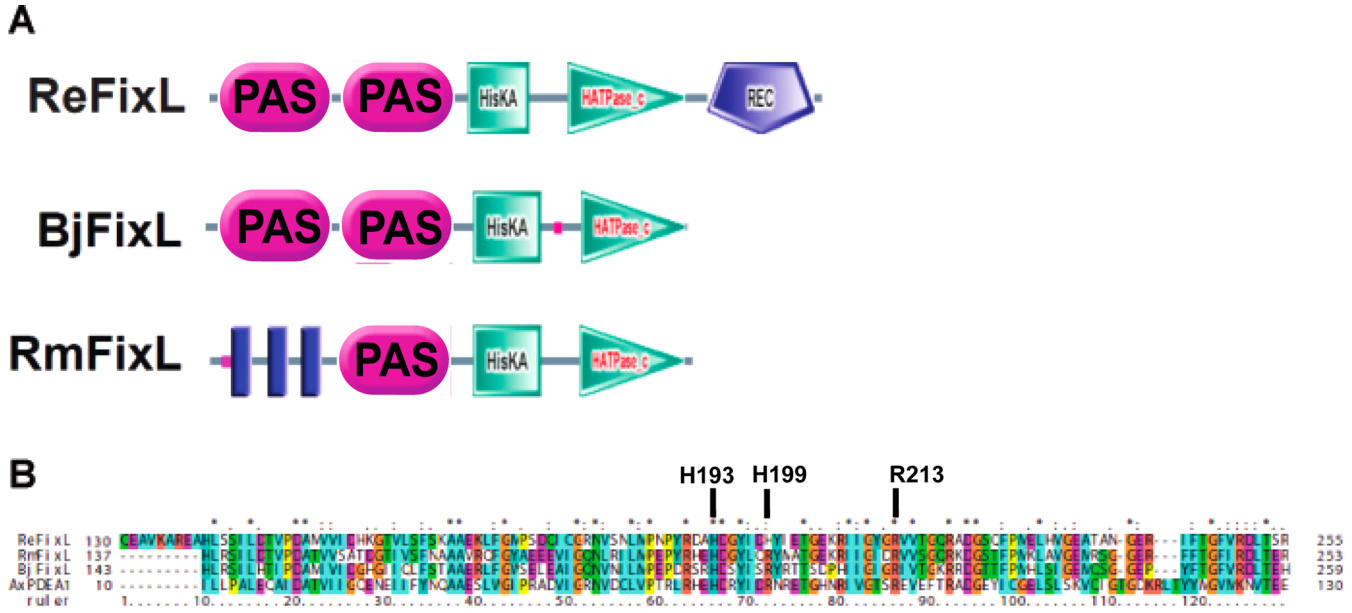


Figure 1. FixL domain organization and conservation of heme-binding PAS domains in heme-based sensors. The analysis of domain organization is based on SMART (A). The alignment of heme-binding PAS domains indicates (top bars) the proximal histidine residue and two arginines involved in signaling transduction and oxygen binding (B).

containing 1 mM $MgCl_2$, 50 μM $MnCl_2$, and 5 μM protein (*Re*FixL, ΔPAS1 *Re*FixL, or D573N *Re*FixL) and started via the introduction of 0.5 mM ATP (unlabeled ATP from Roche and [γ-^{32}P]ATP from Amersham Biosciences, with a specific activity of 0.21 Ci/mmol) at 23 °C. After each specified time course, aliquots (10 μL) of the reaction mixture were removed and mixed with one-third volume of stop buffer [0.50 M Tris-HCl (pH 6.8), 40 mM EDTA, 4.0% (w/v) sodium dodecyl sulfate, 0.20 M NaCl, 50% (v/v) glycerol, and 2.0% (v/v) β-mercaptoethanol] and placed on ice. For every reaction course, the status of the heme iron was verified from the 250−700 nm absorption spectra before and after the assay. The reaction products were electrophoresed on 11% (w/v) polyacrylamide gels, and the gels were dried in air. Thin-layer chromatography (TLC) was additionally used to monitor the ATP and P_i in the reaction mixtures. For TLC, reaction samples with stop buffer (1 μL) were spotted onto a polyethyleneimine-cellulose plate, ~1 cm apart, and 2 cm from the bottom of the plate. Controls with only the radiolabeled ATP were included on every plate. The plates were air-dried and developed in a sealed TLC container with a 1 cm layer of 0.75 M NaH_2PO_4 (pH 3.5). The phosphorylated proteins in the dried gels and ATP and P_i on the TLC plates were quantified with a phosphorimager (Bio-Rad Personal Molecular Imager FX).

The reversibility of the O_2 inhibition was investigated via addition of dithionite or DTT. An inhibited phosphorylation was initially conducted in air-saturated buffer as described above, and after 9 min, 500 μM dithionite or 10 mM DTT was added to the samples in an anaerobic chamber. The reaction products were mixed with stop buffer 11, 24, and 35 min after the addition of DTT or 0.5, 1.5, 3.0, 6.0, 18, and 48 min after the dithionite treatment.

Assays of the intermolecular phosphoryl transfer, the reverse reaction with ADP, and the extent of chemical dephosphorylation were conducted with prephosphorylated *Re*FixL or D573N *Re*FixL that had been cleared of ATP and metals on a P-6 Biospin column (Bio-Rad, Hercules, CA). For the intermolecular phosphoryl transfer, phospho-*Re*FixL or phospho-D573N *Re*FixL was mixed with a 2-fold excess of ΔPAS1 *Re*FixL in phosphorylation buffer with 1 mM $MgCl_2$ and 50 μM $MnCl_2$, under aerobic conditions, and the reactions were halted with stop buffer. For the reverse reaction with ADP, phospho-*Re*FixL or phospho-D573N *Re*FixL was mixed with phosphorylation buffer containing 5 mM $MgCl_2$ and 1 mM ADP; as controls, the same reactions were conducted without ADP or with 5 mM EDTA. For the chemical dephosphorylations, phospho-*Re*FixL or phospho-D573N *Re*FixL was mixed with denaturing buffer A, B, or C [buffer A consisted of 2.0 mM EDTA and 2.0 M guanidinium chloride (pH 8.0), buffer B consisted of buffer A and 0.20 M NaOH, and buffer C consisted of buffer A and 0.20 M HCl]; the reactions were allowed to proceed and then halted via the addition of stop buffer and immediately cooled on ice. Samples were electrophoresed and run on TLC plates as described above.

RESULTS AND DISCUSSION

Domain Organization and Quaternary Structure. *Re*FixL is a hybrid sensor kinase that strongly resembles *Bj*FixL, with residues 1−490 of the two proteins being 49.5% similar and 33% identical. By contrast to *Bj*FixL, which contains 505 residues, *Re*FixL contains 641 residues because of an additional C-terminal receiver domain, as assigned by Pfam (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) (Figure 1). A Clustal X alignment of the *Re*FixL primary sequence with proven O_2-sensing PAS domain proteins such as FixLs, *Ax*PDEA1, and *Ec*DosP identified the PAS2 domain of *Re*FixL as being the likely heme-binding region. This domain features several canonical heme-PAS residues, including the expected proximal histidine (H193), and two basic side chains (H199 and R213) known to influence O_2 affinity and signal transduction in other FixLs.[16,18] Likewise, Clustal X alignments of the *Re*FixL sequence with FixJ proteins identified the D573 residue of *Re*FixL as a candidate phosphoryl acceptor. We set out to study wild-type *Re*FixL

dx.doi.org/10.1021/bi300991r | *Biochemistry* 2013, 52, 456−465

Table 1. Ligand Binding Parameters for *Re*FixL[a]

	O_2				CO		imidazole	cyanide
	k_{on} (μM^{-1} s^{-1})	k_{off}[d] (s^{-1})	K_d (μM)	k_{ox} (min^{-1})	k_{on} (μM^{-1} s^{-1})	K_d (μM)	k_{on} (mM^{-1} s^{-1})	k_{on} (mM^{-1} s^{-1})
*Re*FixL	0.098	86	738	0.0118	0.003	8.0	3.755	0.0101
ΔPAS1 *Re*FixL	0.12	17	93	0.0074		2.6		
D573N *Re*FixL			423	0.0122				
*Bj*FixL[b]	0.14	20	142	0.0450	0.008	2.1[c]	4.9[d]	0.027[g]
*Bj*FixL(heme)[f]	0.30	10	33		0.016			
SW Mb[e]	14	16	1.2	10×10^{-4}	0.5	0.037	0.13	0.32

[a]Imidazole and cyanide binding experiments were conducted with the ferric (met) form, while the initial state of the heme was the ferrous form in all others. [b]From ref 7; intercept of the linear fitting of k_{obs} vs oxygen concentration. [c]From ref 14. [d]From ref 18. [e]Autoxidation at 37 °C, data from ref 28. [f]From ref 23. [g]Data for the FixL heme domain.

and to explore the importance of its PAS1 and C-terminal regions. To these ends, we purified the wild-type protein and prepared and purified a mutant (ΔPAS1 *Re*FixL) lacking the N-terminal PAS1 domain and another (D573N *Re*FixL) lacking the phosphoryl accepting residue in the C-terminal region.

The *Re*FixL primary sequence lacked identifiable transmembrane segments, and as expected, this protein and the corresponding mutants were highly soluble when purified from *E. coli* cells overexpressing the corresponding genes. No tagging strategy was necessary to obtain proteins that were >90% pure. The results of analytical gel-filtration analyses on the met (active) and cyanomet (inactive) forms were consistent with a homodimeric structure for both, indicating that the regulatory conformational changes of *Re*FixL do not involve changes in quaternary structure. Like wild-type *Re*FixL, the ΔPAS1 *Re*FixL and D573N *Re*FixL mutants were homodimeric, as assessed by analytical gel filtration. A similar behavior has been reported for other FixL proteins, and so far, no change in quaternary structure has been noted during the activation or inactivation of a FixL.

A FixL with Exceptionally Low Oxygen Affinity. *Re*FixL was initially reported to be hemeless, but this was based on the failure to stain in a gel and probably due to the loss of heme during electrophoresis.[19] We isolated *Re*FixL as a holoprotein and found it to contain one heme *b* cofactor per monomer and to behave generally like a FixL, albeit with some distinctive heme properties. Ligand binding by the ferric form was similar to that of other FixLs (cyanide and imidazole) (Table 1).[20,21] An unhindered and flexible heme pocket was indicated by a relatively rapid binding of imidazole to the ferric form.[20] In addition, the heme pocket is likely to be apolar as suggested by a slow cyanide association, which is usually limited by the need to deprotonate this ligand.[5]

After being exposed to air (256 μM O_2), ferrous *Re*FixL exhibited a Soret band that was broad with an absorption maximum around 432 nm (not shown), indicating poor saturation with O_2, later discovered to be 26%. Thus, the absorption of *Re*FixL in air was the first indication that the O_2 affinity of this protein would be even lower than that of *Bj*FixL (K_d of 142 μM for O_2 binding), which had, until then, been the FixL with the lowest known O_2 affinity.[7] Even in pure O_2 (1250 μM O_2) ferrous *Re*FixL was not fully saturated (63%); the spectra showed a broad Soret band at 422 nm and a shallow trough between the α- and β-bands in the 500−600 nm region (Figure 2A). By contrast, pure oxy spectra, like those of oxy-*Rm*FixL in O_2, show a sharp Soret peak around 418 nm and well-defined α- and β-bands. As an example, examine the spectrum of oxy-ΔPAS1 *Re*FixL in pure O_2, which corresponds to 93% saturation (Figure 2B).

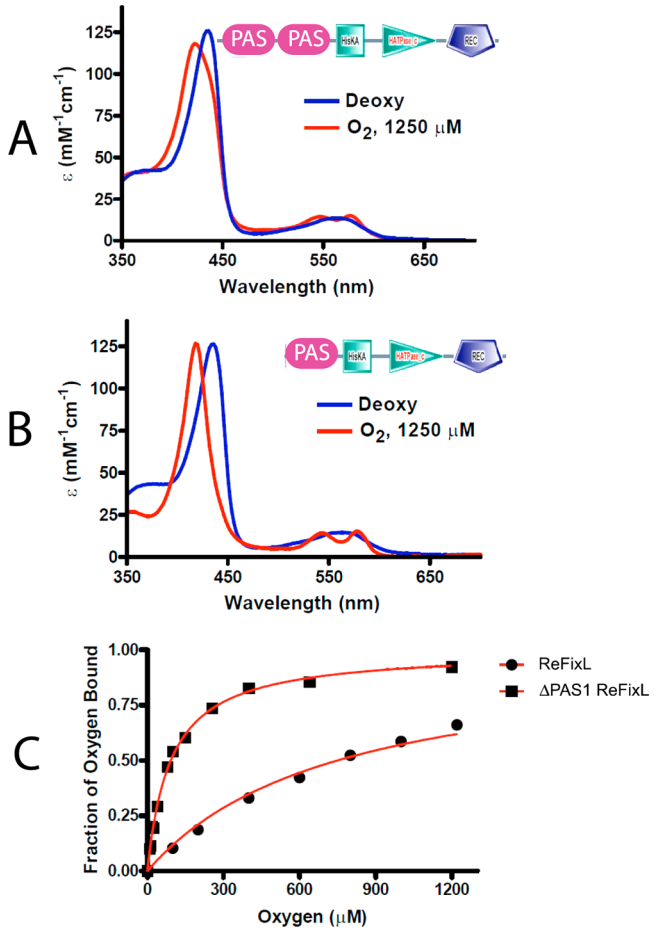


Figure 2. Binding of oxygen to *Re*FixL. The electronic spectra show full-length *Re*FixL (A) and a mutant (ΔPAS1 *Re*FixL) (B) in anaerobic (blue) and oxygen-saturated buffer (red). Direct oxygen titrations were conducted (C) using *Re*FixL (single-binding fit; R^2 = 0.991) and ΔPAS1 *Re*FixL (single-binding fit; R^2 = 0.998) and 25−1220 μM O_2 in 50 mM Tris-HCl buffer (pH 8.0) at 25 °C.

By directly titrating *Re*FixL with O_2 and fitting the data to a single-binding equation (R^2 = 0.991), we directly determined a K_d of 738 ± 29 μM at 25 °C for binding of O_2 (Figure 2C and Table 1). This value was confirmed by stopped-flow measurements of the kinetics of O_2 association, which yielded a k_{on} of 0.098 μM^{-1} s^{-1} and a k_{off} of 86 s^{-1}, based on the plot of k_{obs} versus O_2 concentration (Table 1). Interestingly, the *Re*FixL on rate constant for O_2 binding was in a range typical for FixL proteins, whereas the off rate was faster than for other FixLs (Table 1). This suggests that the rate of O_2 dissociation is an

dx.doi.org/10.1021/bi300991r | *Biochemistry* 2013, 52, 456−465

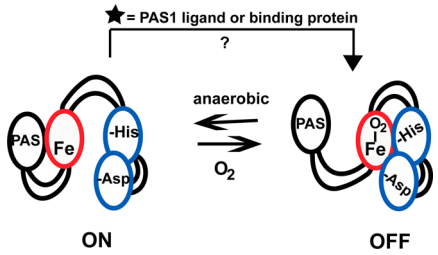
important source of *Re*FixL's low O_2 affinity. This fast off rate cannot simply be explained by the occurrence of a histidine residue (H199) in *Re*FixL where a heme pocket arginine is usually found for other FixLs (e.g., R206 in *Bj*FixL). *Ec*DosP, for example, shows a normal k_{off} and high O_2 affinity (K_d of ~10 μM) despite normally having a histidine residue at the analogous position.[22]

Modulation of Oxygen Affinity and Autoxidation Rate by the PAS1 Domain of *Re*FixL. The *Re*FixL mutants missing the N-terminal PAS domain (ΔPAS1 *Re*FixL) and the phosphorylation site aspartate in the C-terminal receiver domain of unknown function (D573N *Re*FixL) both showed significantly higher O_2 affinities than the wild-type protein. Compared to *Re*FixL, the ΔPAS1 *Re*FixL variant had an 8-fold higher affinity for O_2, and the D573N variant had a 2-fold higher affinity for O_2 (Figure 2B,C and Table 1). This was surprising, given the subtlety of the D573N substitution and the common assumption that the extra N- and C-terminal domains do not interact with the heme-binding pocket to alter its properties. The negative effect of the other protein domains on ligand binding also extended to CO, where a 3-fold higher affinity for CO was noted for ΔPAS1 *Re*FixL compared to that of the wild type (Table 1 and Figure 1 of the Supporting Information).

Although the effect of additional domains without apparent function in sensors has not been systematically explored, a number of results suggest that they might commonly modulate ligand affinity. In particular, the independent heme-binding domains from O_2 sensors typically show higher ligand affinities than the full-length versions. For example, the independent heme-binding domain from *Bj*FixL (*Bj*FixLH) shows a 4-fold higher affinity for O_2 (K_d of 33 μM) compared to full-length *Bj*FixL.[23] Likewise, the *Ec*DosP heme-binding domain (*Ec*DosH) shows a 6-fold higher O_2 affinity (K_d of ~13 μM) than the full-length protein,[22,24] and the *Ec*DosC heme-binding domain shows a 2-fold higher O_2 affinity (K_d of 10 μM) than the full-length version.[22] *Re*FixL has shown the largest change in oxygen affinity modulated by a non-heme-binding PAS domain. Interestingly, this PAS domain with unknown function has unraveled an interesting functional role in ligand binding and signal transduction. These results suggest that regulatory proteins in *R. etli* could well target the PAS1 domain of *Re*FixL as a way to modulate not only the O_2 affinity of this protein but also its signal transduction. However, it is also possible that some small molecule could bind to this PAS domain, promoting a response through domain−domain interactions as well. Nevertheless, this finding points out possible new ways of fine-tuning the binding and signaling response in heme-based sensors through domain−domain contacts (Scheme 1).

Autoxidation Slowed by Removal of the First PAS Domain. *Re*FixL autoxidized in air with a half-life of 55 min (k_{ox} of 0.0127 min^{-1}) (Figure 3A). This oxidation was similar to the rate for the D573N *Re*FixL mutant but slower than those of other known FixL proteins, for which the half-life is typically less than 22 min (Table 1).[7] By contrast, the ΔPAS1 *Re*FixL variant autoxidized with a significantly longer half-life of 94 min (k_{ox} of 0.0074 min^{-1}) (Table 1 and Figure 3B). Studies of the oxidation mechanisms of FixL proteins have showed that the autoxidation rates follow a bell-shaped trend, with a maximal "protection effect" occurring while the proteins are 50% saturated with O_2.[2] Interestingly, ΔPAS1 *Re*FixL showed a slower rate of autoxidation in air than *Bj*FixL (64% saturated in air) despite being similarly saturated with O_2 (73% saturation in air). Interestingly, wild-type *Re*FixL and D573N *Re*FixL are only 26 and 38% saturared in air, respectively. Possibly, those aspects of the heme pocket that confer higher O_2 affinity to ΔPAS1 *Re*FixL also promote changes in the heme pocket

Scheme 1. Model for *Re*FixL Regulation[a]



[a]In this model, we propose the heme-binding domain would interact with the PAS1 domain in the deoxy state (anaerobic condition), relieving its own interaction with other domains (e.g., kinase and receiver). Upon binding to oxygen, the heme domain would experience a conformational change that disrupts PAS1 interaction favoring binding to the kinase and receiver domains and then blocking phosphorylation. Another hypothetical fine-tuning of FixL regulation is proposed, where the PAS1 domain could function by modulating signaling tranduction by disrupting domain−domain interactions upon binding to small molecules or partner proteins.

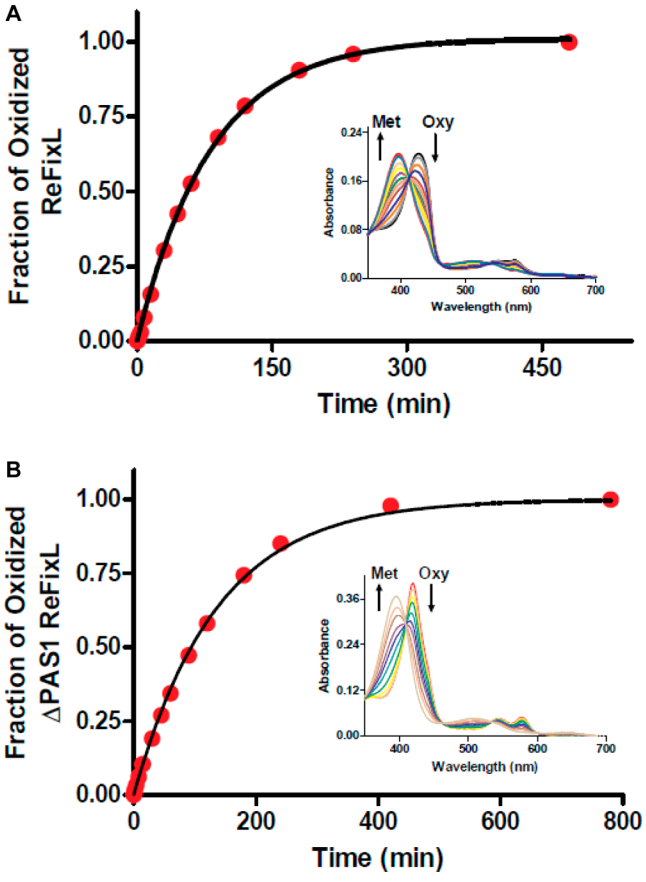


Figure 3. Autoxidation rate of *Re*FixL. Wild-type *Re*FixL (A) and ΔPAS1 *Re*FixL (B) were reduced inside an anaerobic glovebag with a 2-fold excess of dithionite, cleared of dithionite by a gel exclusion column, and maintained in 50 mM Tris buffer (pH 8.0). The protein was diluted 100-fold with air-saturated buffer and the oxidation rate monitored at 25 °C. The inset shows spectral changes to the met form from the oxidation in air.

dx.doi.org/10.1021/bi300991r | *Biochemistry* 2013, 52, 456−465

microenvironment that protect the iron atom from autoxidation. Autoxidation is relatively poorly understood, although it occurs in all O_2-binding heme proteins and interferes with their function. This area merits further investigation for *Re*FixL.

Signal Transduction by *Re*FixL. The phosphorylation of the FixJ transcription factor by the FixL protein kinase is a typical enzymatic reaction in which ATP initially reacts with the FixL to yield a phospho-His intermediate before the transfer of the phosphoryl group to an aspartate in the FixJ substrate. This reaction and its regulation by ligands are typically monitored from the turnover of FixJ to phospho-FixJ.[16,25] Such a strategy is not possible for *Re*FixL, because the histidine and aspartate residues (H280 and D573) for the phosphoryl transfers reside in the same molecule in a 1:1 ratio. To explore *Re*FixL signal transduction, we measured the total phosphorylation and examined the products.

An initial investigation of the effect of divalent cations on the reaction led us to adopt a divalent metal mixture of 50 μM Mn^{2+} and 1 mM Mg^{2+} for the experiments, as was previously done for the *Bj*FixL–*Bj*FixJ complex.[14] Specifically, under anaerobic conditions, the *Re*FixL was more rapidly phosphorylated in Mn^{2+} than in Mg^{2+}, and under aerobic conditions, a significantly higher yield of phosphorylation was promoted by Mn^{2+} than by Mg^{2+} (Figure 4A,B). This could have been because the phospho-histidine intermediate was more efficiently generated in Mn^{2+}. The kinase activity of *Re*FixL was strongly switched off by exposure to air (Figure 4A,B). On the other hand, despite complete saturation with NO or CO, regulation was poor with NO (68% activity remaining) and nil with CO (Figure 2 of the Supporting Information).

The inhibition by O_2 was fully reversible and relieved by addition of dithionite as an O_2 scrub (Figure 4C).[26] The strong inhibition of the kinase activity despite the relatively weak binding of O_2 implies that a much larger fraction of the *Re*FixL was inhibited than was bound to O_2. In other words, much of the protein in the "deoxy state" had an inhibited kinase. This phenomenon, initially observed for the *Bj*FixL–*Bj*FixJ complex, has been explained as a hysteresis event and an oxygen memory effect on the kinase.[14] In *Bj*FixL, virtually 100% of the molecules were inhibited despite 30% of the heme being in the deoxy state. In *Re*FixL, which has a lower O_2 affinity, this phenomenon is even more pronounced, suggesting that *Re*FixL exploits the memory effect to greater advantage in promoting efficient regulation.

We estimated the levels of histidine and aspartate phosphorylation at equilibrium by exploiting the acid lability of phospho-histidine and base lability of phospho-aspartate modifications (Figure 4D). Specifically, after strong base treatment of the anaerobically phosphorylated *Re*FixL, the residual level of phosphorylation was ~25%, suggesting that 75% of the phosphorylation had been on the aspartate (Figure 4D). Likewise, after treatment with heat and 0.10 M HCl, the residual level of phosphorylation was 75–80%, suggesting that 20–25% of the phosphorylation had been on the histidine (Figure 4D). As an additional control for histidine dephosphorylation, we examined the acid treatment of phospho-*Bj*FixL and found this protein to maintain less than 10% of its phosphorylation (Figure 4D).

The Receiver Aspartate Assists in the Regulation and Phosphorylation of the Histidine. Compared to wild-type *Re*FixL, under aerobic conditions the D573N variant of *Re*FixL was better saturated with O_2. Nevertheless, this protein dramatically lacked O_2 regulation of the histidine phosphor-

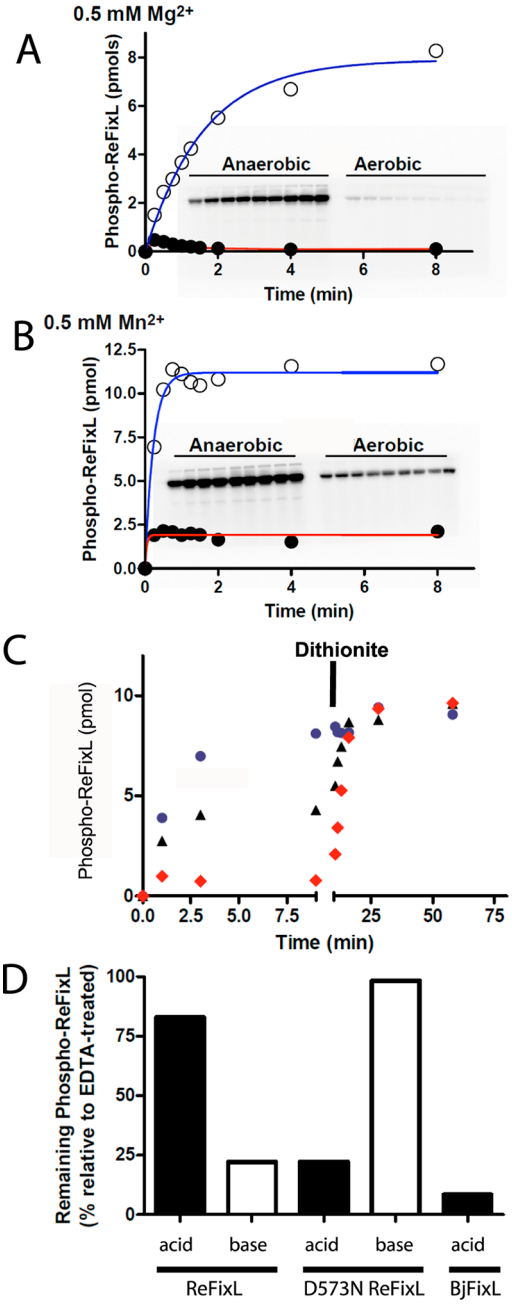


Figure 4. Oxygen regulation and divalent metal response for *Re*FixL. Phosphorylation reactions were conducted with 5 μM *Re*FixL in phosphorylation buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 5% (v/v) ethylene glycol] including 0.5 mM $MgCl_2$ (A) or 0.5 mM $MnCl_2$ (B), 1 mM DTT, and 0.5 mM ATP (unlabeled ATP from Roche and [γ-^{32}P]ATP, with a specific activity of 0.21 Ci/mmol); these reactions were conducted under air-saturated (●) and anaerobic (○) conditions for 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 4.00, and 8.00 min at 23 °C. (C) Phosphorylation reactions were conducted in buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 5% (v/v) ethylene glycol], including 1 mM $MgCl_2$, 50 μM $MnCl_2$, and 0.5 mM ATP (unlabeled ATP from Roche and [γ-^{32}P]ATP, with a specific activity of 0.21 Ci/mmol), and followed for 9 min. Then 500 μM dithionite was added to samples with 240 μM (red diamonds), 60 μM (black triangles), or 0 μM O_2 (blue circles), and the reaction was monitored for an additional 1 h. (D) Base and acid stabilities are compared for phospho-*Re*FixL, phospho-D573N *Re*FixL, and phospho-*Bj*FixL. Samples were loaded onto SDS–PAGE gels.

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ylation (autokinase switch off) (Figure 5). Additionally, the much slower autokinase rate for this mutant suggests that the

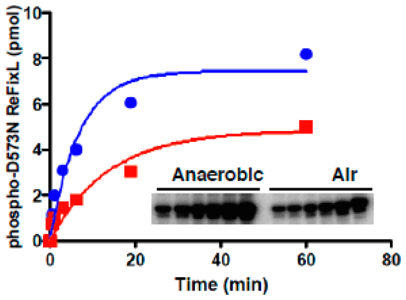

Figure 5. Oxygen regulation of *Re*FixL upon disabling of the aspartate residue. Phosphorylation reactions were conducted with 5 μM D573N *Re*FixL in phosphorylation buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 5% (v/v) ethylene glycol] including 1 mM $MgCl_2$, 50 μM $MnCl_2$, 1 mM DTT, and 0.5 mM ATP (unlabeled ATP from Roche and [γ-^{32}P]ATP, with a specific activity of 0.21 Ci/mmol) in air (squares) and under anaerobic conditions (circles), and followed for 0.5, 1.0, 3.0, 6.0, 20, and 60 min.

receiver D573 residue is able to assist the initial histidyl phosphorylation with ATP. Actually, this behavior was earlier observed for the *Rm*FixL–*Rm*FixJ complex, where it was shown that complex formation precedes phosphorylation and was essential for improving the autokinase and phospho-transfer reaction.[23] Those results indicated that a likely physiological FixL–FixJ complex would indeed function, assisting each domain of these proteins in achieving optimal autokinase and phospho-transfer rates as well as a maximal equilibrium level of phosphorylation. In another study using D54N FixJ, which lacks the phospho-acceptor aspartate residue, a remarkable autokinase rate increase was measured along with a lack of oxygen regulation.[26] Here, we determined a quite fast overall phosphorylation rate for wild-type *Re*FixL, which was strongly slowed upon removal of the aspartate residue supporting this assistance. Overall, these data agree with previous demonstrations that FixJ accelerates the FixL autokinase and phosphoryl transfer, with O_2 regulation having its maximal impact on the latter reaction.[26]

Removal of the First PAS Domain Clearly Inactivated the Kinase Activity but Did Not Prevent Phosphorylation in Trans by Wild-Type *Re*FixL. To compare signal transduction by *Re*FixL and ΔPAS1 *Re*FixL despite their vastly different O_2 affinities, we turned to their met forms, because they can be fully saturated with cyanide, and met-*Re*FixL was demonstrably active and could be regulated by cyanide binding. Compared to the wild type, met-ΔPAS1 *Re*FixL showed little phosphorylation activity (<1% of that of the wild type) (Figure 6A). The vanishingly low activity of ΔPAS1 *Re*FixL responded poorly to cyanide (Figure 6B,C).

To investigate whether the phosphorylation site histidyl and aspartyl residues in ΔPAS1 *Re*FixL could be phosphorylated, we mixed this variant with a 2-fold excess of prephosphorylated wild-type *Re*FixL. This mixture resulted in the efficient phosphorylation of ΔPAS1 *Re*FixL, with concomitant loss of wild-type *Re*FixL phosphorylation (Figure 6D). Specifically, phospho-*Re*FixL maintained ~45% of the phosphorylation and transferred ~55% of the phosphoryl groups to ΔPAS1 *Re*FixL, a level of ΔPAS1 *Re*FixL phosphorylation that could not be achieved by a direct reaction with ATP. A similar phosphoryl transfer was achieved when ΔPAS1 *Re*FixL was mixed with phospho-D573N *Re*FixL; in this case, D573N *Re*FixL maintained 38% of the phosphoryl groups and transferred 62% to ΔPAS1 *Re*FixL (Figure 6D). Compared to the transfer of the phosphoryl group from the wild type to ΔPAS1 *Re*FixL, the transfer from phospho-D573N *Re*FixL to ΔPAS1 *Re*FixL was much faster. Transfer from phospho-D573N *Re*FixL was complete in less than 30 s, as opposed to the 20 min required for the transfer from *Re*FixL to the deletion variant (Figure 6). Overall, these data indicate deletion of the first PAS domain did not prevent phosphorylation from taking place by trans-phosphorylation but clearly turned off its own phosphorylation reaction.

CONCLUSIONS

By examining a multidomain hybrid FixL protein, we were able to observe that a PAS domain without known functionality strongly impacts O_2 binding and signal transduction. Excision of this domain disrupted signaling, possibly by locking the protein in an off state, despite the catalytic domain remaining intact and fully capable of being phosphorylated (Scheme 1). Additionally, the removal of the PAS1 domain caused quite interesting changes in the heme properties. To the best of our knowledge, strong changes in the O_2 affinity of FixL proteins have so far been reported only for residue substitutions conducted within their heme-binding domain. Though previous studies comparing the isolated heme-binding domains to the full-length proteins have hinted at such long-range effects on ligand affinity, these effects have not been investigated until now. Our observations support the notion that changes in domain–domain interactions might serve as triggers for signal transduction. Indeed, by promoting interdomain interaction, we are able to cause major changes in the ligand affinity and signal transduction of *Re*FixL, at long range. By constraining a heme cofactor, for example, one should be able to alter its geometry and the polar interactions of its propionate groups, which have been shown to affect ligand binding.[16] A possible competition of the PAS domains for the kinase, depending on physiological conditions, would help to explain how diverse sensing domains might alternately couple to a single functional domain in some proteins. Additionally, our studies suggest that interacting proteins could switch off the *Re*FixL activity or enhance its oxygen affinity by binding to the PAS1 domain. However, we can not discard the possibility that the PAS1 domain could also be regulated upon binding to endogenous small molecules (Scheme 1).

A mechanistic model for domain–domain regulation called helix swap was earlier proposed to explain how a heme-based sensor could work.[1] In that model, a sensing domain upon ligand binding would trigger an equilibrium change on domain–domain interactions leading to an off or on state, through a conecting helix (Scheme 1). Some interesting examples supporting this model that could better explain the modularity observed for PAS proteins have been reported.[13,27] A well-conserved α-helical structure has been identified linking these domains and might be involved in the modularity and coupling of the PAS domain to effector domains. This was shown by creating a chimera using a light-regulated photoreceptor YtvA and oxygen-regulated *Bj*FixL proteins. In that work, their PAS-sensing domains were swapped and their signaling was reasonably alternated.[13] Additionally, a deletion of the first PAS domain of *Bj*FixL, which has similarities to *Re*FixL, has significantly modified signaling transduction, increasing the activity of the off state while lowering the activity of the on

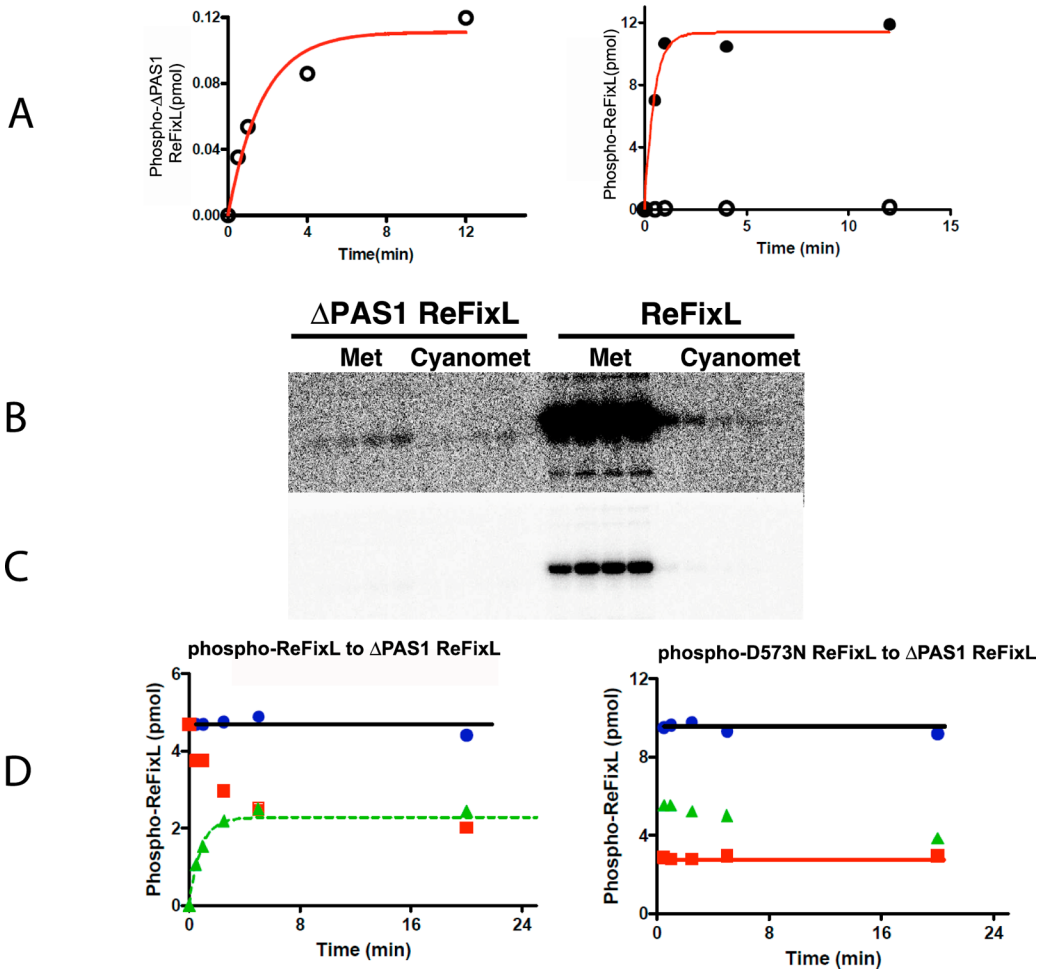

Figure 6. Impact of the PAS1 domain on *Re*FixL signal transduction. Phosphorylation reactions were conducted with 5 μM met-*Re*FixL (●) or met-ΔPAS1 *Re*FixL (○) in phosphorylation buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 5% (v/v) ethylene glycol] including 1 mM $MgCl_2$, 50 μM $MnCl_2$ and 0.5 mM ATP (unlabeled ATP from Roche and [γ-^{32}P]ATP, with a specific activity of 0.21 Ci/mmol); the total amount of *Re*FixL loaded on gels corresponds to 37 pmol. Cyanide-bound forms of these protein samples were prepared by adding 1 mM KCN and verifying binding by electronic spectroscopy before the reactions. The products of reactions of the met and cyanomet forms of *Re*FixL and ΔPAS1 *Re*FixL were electrophoresed and autoradiographed with quantification of the phosphorylated proteins *Re*FixL (●) and ΔPAS *Re*FixL (○) (A) and gels under overexposure (B) and regular exposure (C). (D) Prephosphorylated wild-type *Re*FixL and D573N *Re*FixL were prepared as described in Materials and Methods and used after removal of the ATP, where the phospho-*Re*FixL or phospho-D573N *Re*FixL was mixed with a 2-fold molar excess of ΔPAS1 *Re*FixL in aerobic phosphorylation buffer containing divalent metals (1 mM $MgCl_2$ or 50 μM $MnCl_2$). In the quantification, the control reaction is that of wild-type *Re*FixL without addition of the ΔPAS1 mutant (circles), the amount remaining in wild-type phospho-*Re*FixL after mixing with the deletion mutant (squares), and the amount transferred to ΔPAS1 *Re*FixL (triangles) (left); similar measurements are shown for the transfer from phospho-D573N *Re*FixL: control with phospho-D573N FixL alone (circles), amount remaining in phospho-D573N *Re*FixL after transfer (squares), amount transferred to ΔPAS1 *Re*FixL (triangles) (right).

state.[13] Because of the fact that the heme-based sensor and other regulatory proteins are multidomains, where many of these domains do not have a known function, it is possible they share a common mechanistic feature. This could help to reconcile the puzzle of how a sensing domain can be combined into so many output functional domains.

Recently, Zamorano-Sanchez et al. identified a putative response regulator, FxkR, as the missing link for *Re*FixL, where no FixJ-like regulator was found. This transcription factor would be the final phospho-acceptor and would regulate gene expression.[7] Genetic experiments have shown that the *R. etli fixL* gene regulates the microaerobic expression of fixKf, which is involved in activating the expression of symbiotic cbb_3-type heme-copper oxidases and other proteins needed by the bacteria before the expression of the nitrogenase subunits.[6] This bacterium presents two other oxygen sensors, NifA and FnrN, that are responsible for the modulation of other gene expression.[8] To understand the actual role of *Re*FixL, we would need to conduct an oxygen dose−response experiment to address its specific role during the process of symbiosis. A quite linear decrease in oxygen concentration from 250 to ≤1 μM was measured in nodules upon symbiotic association between *S. meliloti* and the root, which was correlated to stages involved in this process.[9] On the basis of that, we can notice these bacteria need to coordinate adaptation toward a significantly large range of oxygen concentrations. Therefore, it is not uncommon for them to have more than one oxygen regulator. In the case of *R. etli*, it might be using this triad, FixL, NifA, and FnrN, to respond to an oxygen gradient coordinating activation of a particular gene. Unfortunately, this has not been shown in a bacteroid and would require an oxygen dose−response experiment followed by gene regulation. However, oxygen

dx.doi.org/10.1021/bi300991r | *Biochemistry* 2013, 52, 456−465

affinity could provide hints about the range of their response to oxygen. In *M. tuberculosis*, two oxygen sensors, DevS and DosT, have been suggested to function by sensing different levels of oxygen, which was first implied on the basis of their differences in oxygen affinity and later observed to promote differences in genetic responses upon decreases in the level of oxygen.[3,10] Thus, *Re*FixL could serve as an early alert for a decrease in the O_2 levels, because this sensor can respond sharply to minor changes in O_2 concentration at relatively high O_2 levels. Remarkably, the low O_2 affinity of *Re*FixL does not compromise regulation, reinforcing the notion that a ligand memory effect might be a common feature of FixL proteins and possibly also other sensor kinases.

■ ASSOCIATED CONTENT

Supporting Information

Measurements of carbon monoxide binding to wild-type and ΔPAS1 *Re*FixL (Figure 1) and ligand regulation of *Re*FixL (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E.H.S.S.: telephone, 55-85-3366-9938; fax, 55-85-3366-9938; e-mail, eduardohss@dqoi.ufc.br. M.-A.G.-G.: telephone, (214) 648-9438; fax, (214) 648-8856; e-mail, marie-alda.gilles-gonzalez@utsouthwestern.edu.

Funding

We acknowledge financial support from Welch Foundation Grant I-1575.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

*Re*FixL, full-length *R. etli* FixL; ΔPAS1 *Re*FixL, truncated *R. etli* FixL; D573N *Re*FixL, full-length *R. etli* FixL in which aspartate 573 has been substituted with asparagine; *Bj*FixL, *B. japonicum* FixL; *Rm*FixL, *S. meliloti* FixL; deoxy, Fe^{II} form; oxy, $Fe^{II}O_2$ form; carbonmonoxy, $Fe^{II}CO$ form; nitrosyl, $Fe^{II}NO$; met, Fe^{III} form; cyanomet, $Fe^{III}CN$ form; DTT, dithiothreitol.

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