The FixL protein of *Rhizobium meliloti* can be separated into a heme-binding oxygen-sensing domain and a functional C-terminal kinase domain

(*fixL* gene/*fixJ* gene/two-component regulatory system)

**ELLEN K. MONSON, MICHAEL WEINSTEIN, GARY S. DITTA, AND DONALD R. HELINSKI**

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92039-0634

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**ABSTRACT**

Transcription of nitrogen fixation (*nif* and *fix*) genes in *Rhizobium meliloti* is induced by a decrease in oxygen concentration. The products of two genes, *fixL* and *fixJ* (FixL and FixJ, respectively) are homologous to a family of bacterial proteins that transduce environmental signals through a common phosphotransfer mechanism [David, M., Daveran, M., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertzig, C., Boistard, P. & Khan, D. (1988) *Cell* 54, 671–683]. FixL, the oxygen sensor, is a membrane protein. It has previously been shown that a soluble derivative of FixL, FixL*, is an oxygen-binding hemo-protein and a kinase that autophosphorylates and also phosphor-ylates FixJ [Gilles-Gonzalez, M. A., Ditta, G. S. & Helinski, D. R. (1991) *Nature (London)* 350, 170–172]. In this work, deletion derivatives of *fixL* were constructed and over-expressed in *Escherichia coli*, and the truncated proteins were purified. We show that a fragment of FixL from amino acid residue 127 to residue 260 binds heme, retains the ability to bind oxygen, and has no detectable kinase activity. A C-terminal fragment of FixL, beginning at residue 260, fails to bind heme but is active as a kinase. We also demonstrate that anaerobiosis results in an enhancement of FixL* autophosphorylation and FixJ phosphorylation activities in *vitro*. Finally, we show that the heme-binding region of FixL is required *in vitro* for oxygen regulation of its kinase activities.

The soil bacterium *Rhizobium meliloti* fixes dinitrogen (N₂) when associated with root nodules formed on its plant host, *Medicago sativa* (alfalfa). The expression of most of the known genes required for nitrogen fixation (*nif* and *fix* genes), including the structural genes for nitrogenase, is induced in response to a decrease in oxygen concentration (1). Induction of *nif* and *fix* gene expression by low oxygen is physiologically relevant because a low-oxygen environment is maintained in root nodules to prevent inactivation of the highly oxygen-sensitive nitrogenase enzyme (2). The genes responsible for sensing and transducing the low-oxygen signal, *fixL* and *fixJ*, encode proteins (FixL and FixJ, respectively) that are homologous to a large family of bacterial proteins involved in signal transduction, the two-component regulatory system proteins (3, 4). Two-component system proteins are involved in a wide range of adaptive responses in bacteria, including nitrogen regulation, osmoregulation, chemotaxis, virulence, phosphate regulation, and sporulation (5, 6). The two components consist of a sensor protein, to which FixL is homologous, and a response regulator protein, to which FixJ is homologous. For several of these systems, genetic and biochemical evidence supports a phosphotransfer model for signal transduction (7). The sensor protein responds to an activating signal by auto-phosphorylating and then transferring the phosphate to its cognate response regulator protein. The phosphorylated response regulator, which is often a transcriptional activator, is then able to activate its target (see refs. 8–10 for reviews on two-component regulatory systems).

A cascade model of *nif* and *fix* gene regulation in *R. meliloti* has been proposed, whereby FixL acts as an oxygen sensor as the initial event in the cascade and transmits this information to FixJ (3). FixJ, which possesses a putative helix-turn–helix DNA-binding motif (3), then activates transcription of the *nifA* and *fixK* genes. The *nifA* and *fixK* gene products, in turn, are transcriptional activators of at least 14 other *nif* and *fix* genes (11–14).

FixL*, the oxygen sensor, is a membrane protein with four transmembrane helices (A. Lois, G.S.D., and D.R.H., unpublished data). Previous work resulted in the purification of a soluble derivative of FixL, FixL*, and the demonstration that FixL* is an oxygen-binding hemoprotein and a kinase capable of catalyzing its own phosphorylation and the phosphor-ylation of purified FixJ (15). The discovery that the oxygen sensor is an oxygen-binding hemoprotein suggested that FixL may act as the direct sensor of oxygen concentration in the *nif/fix* gene activation cascade. Preliminary *in vitro* phosphorylation experiments conducted in our laboratory revealed that higher levels of FixL phosphate and FixJ phosphate are produced in the absence of oxygen (M. A. Gilles-Gonzalez, G.S.D., and D.R.H., unpublished data).

In this study, we were interested in identifying the regions of FixL required for heme binding and kinase activity. We show that the heme- and oxygen-binding region of FixL is separable from a functional C-terminal kinase domain. In addition, we demonstrate that anaerobiosis results in a substantial enhancement of FixL* autophosphorylation and FixJ phosphorylation *in vitro* and that the heme-binding region is required for this oxygen regulation of kinase activity.

**MATERIALS AND METHODS**

**Bacterial Strains.** *Escherichia coli* MC1061 [araD139 (ara-leu)7696 Δ(lac)174 galU galK hsdR strA](16) was used for plasmid constructions. *E. coli* TG1 [Δ(lac–proAB) supE thi (F' traD36 lacF proA proB lacZAM15)] (17) or BL21(F' hsdS gal) (18) was used for overexpression of *fixL* and *fixJ*.

**Plasmids.** DNA manipulations were carried out using standard procedures (19). Restriction enzymes were purchased from Bethesda Research Laboratories and used according to the supplier’s directions. pEM plasmids are derivatives of the FixL*–overproducing plasmid pGG820 (15). pEM100, pEM110, and pEM130 (Fig. 1) express FixL derivatives with the same N-terminal fusion to LacZ as FixL*. pEM10, the precursor to plasmid pEM100, was constructed by excising the 1.6-kilobase (kb) *HincII–XhoI* fragment from pGG8 (15).

Abbreviation: IPTG, isopropyl β-thiogalactoside.
filling in the 5' overhangs with the Klenow fragment of DNA polymerase and dNTPs and ligating with T4 DNA ligase. The 1.88-kb *Eco*RI fragment from pMW2 (15), containing the 3' region of *fixL* and *fixJ*, was then cloned in the *Eco*RI site of pEM10 to generate pEM100. pEM130 was constructed by excising the 0.71-kb *Xho I*-containing fragment from pGG820, filling in the 5' overhangs, and religating. pEM11, the precursor to plasmid pEM110, was constructed by removing the 1.50-kb *Cla I*-containing fragment from pGG820, filling in the 5' overhangs, and religating. The 1.88-kb *Eco*RI fragment of pMW2 was inserted into the regenerated *Eco*RI site in pEM110 to produce pEM110.

**Purification of FixLC and FixJ**. FixLC was purified from *E. coli* strain TG1 carrying pEM100 and the *nifA::lacZ* reporter plasmid pCHK57 (1). Three liters of cells were grown at 37°C in Luria broth (LB; ref. 19) supplemented with penicillin-G at 250 μg/ml and tetracycline at 15 μg/ml. When the *OD*₂₅₀ reached 0.5, isopropyl β-thiogalactoside (IPTG) was added to 250 μM and cells were grown 4-5 additional hours. All subsequent procedures were performed at 4°C. Cells were harvested, washed, and resuspended in 50 ml of buffer A [20 mM Tris-HCl, pH 7.8/100 mM NaCl/5% (vol/vol) glycerol/10 mM 2-mercaptoethanol]. Cells were lysed by sonication and membranes were pelleted by centrifugation at 200,000 × g for 30 min. The supernatant was applied to a 2.5 × 9 cm DEAE-Sepharose (Pharmacia) column equilibrated with buffer A. Proteins were eluted with a 300-ml linear gradient from 0.1 to 0.3 M NaCl in buffer A. Fractions containing FixLC and FixJ were eluted at approximately 170 mM NaCl. Pooled fractions were precipitated with 40% saturated ammonium sulfate, resuspended in buffer B [10 mM Na₂HPO₄, pH 7.8/100 mM NaCl/5% (vol/vol) glycerol/10 mM 2-mercaptoethanol], and applied to a 1.6 × 40 cm Sephacryl S-200 (Pharmacia) column equilibrated with buffer B. Fractions containing FixLC and FixJ were pooled and applied to a 100 ml gradient from 10 to 150 mM Na₂HPO₄, pH 7.8, in buffer B. FixLC is eluted below 50 mM Na₂HPO₄ and FixJ is eluted between 50 and 100 mM Na₂HPO₄. FixLC or FixJ fractions were pooled, concentrated by ultrafiltration, and dialyzed overnight against two changes of buffer C [buffer A with 20% (vol/vol) glycerol]. Purified proteins were stored at -70°C.

**Purification of FixL*. FixL* was purified from *E. coli* strain TG1 bearing pGG820 and pCHK57. DEAE-Sepharose chromatography and ammonium sulfate precipitation were done essentially as described (15). Sephacryl S-200 chromatography, hydroxyapatite chromatography, and dialysis were conducted as described for FixLC.

**Purification of FixLN**. FixLN was purified from *E. coli* strain BL21 carrying pEM130 and pCHK57. DEAE-Sepharose chromatography was carried out as described for FixLC except FixLN fails to bind this matrix. Effluent fractions containing the purest FixLN were pooled and precipitated with 58% saturated ammonium sulfate. Sephacryl S-200 and hydroxyapatite chromatography were done as described for FixLC except that FixLN fails to bind to hydroxyapatite. Effluent fractions containing FixLN were pooled, concentrated, and dialyzed as for FixLC.

**SDS/Gel Electrophoresis**. Proteins were separated on discontinuous SDS/12% polyacrylamide gels and visualized by staining with Coomassie brilliant blue (Sigma) as described (20).

**Gel Heme Assays**. High-pH, discontinuous, nondenaturing, 8% polyacrylamide gels were run as described (20). Crude extracts of IPTG-induced cells bearing the appropriate plasmid (see Fig. 2 legend) were made as described for FixLC. Cells also contained plasmid pCHK57 (1), which is not likely to affect the expression of the fixL derivatives or of *fixJ*. Gels were stained for heme by using o-dianisidine (Sigma) as described (21).

**Phosphorylation Assays**. Aerobic phosphorylation assays at 22°C were conducted as described (15) except for the addition of 1.2 mM CaCl₂ and the use of 0.8 mM MgCl₂ and 0.8 mM [γ-³²P]ATP (1667 cpm/pmol). Reaction mixtures contained 2 μM FixLC or 2 μM FixL*, and, where included,
RESULTS

Construction and Expression of FixL* Deletions. To investigate the domain structure of R. meliloti FixL, three deletion derivatives of FixL* were constructed from plasmid pGG820 (Fig. 1) and overexpressed in E. coli. The deletion junctions were chosen on the basis of convenient restriction enzyme sites located near the boundary between the conserved and nonconserved regions upon comparison with other two-component system sensors. Crude extracts of IPTG-induced E. coli cells bearing each of the deletion constructs were analyzed by SDS/PAGE followed by Western blotting [using antiserum produced against FixL* (15)] to confirm that each construct produced a FixL species of the appropriate size. Each of the constructs also overexpressed FixJ. The levels of expression of the FixL* deletions varied from approximately 0.5% to 10% of total cellular protein, depending on the particular deletion derivative.

Heme Analysis of FixL* Deletions. Crude extracts were made from E. coli cells bearing each of the deletion constructs, and the extracts were assayed for a heme-containing protein by using a nondenaturing gel heme assay (Fig. 2). To ensure that the FixL derivatives migrated into the high pH nondenaturing gel, side-by-side Western blots were done (data not shown). As expected, a crude extract bearing FixL* stained positively for heme (lane 2), while extracts bearing the vector alone (pUC9) or FixJ alone (pMW5) showed no reaction with the heme stain (lanes 7 and 8, respectively). Of the three extracts bearing deletion proteins FixLN, FixLC, or FixLNC, only the FixLN extract (lane 3) gave a positive reaction with the heme stain. We attribute the appearance of multiple heme-staining bands in lanes 2 and 3 to different oligomeric forms of FixL* and FixLN, to the association of these derivatives with another protein in the extract, perhaps FixJ, or possibly to breakdown products. Heme-staining bands were not detected when the extracts were analyzed on SDS gels, in either the presence or the absence of 2-mercaptoethanol, suggesting that the heme is noncovalently bound.

Purification and Spectral Properties of the Heme-Binding Fragment, FixLN. FixLN was purified to greater than 90% purity (Fig. 3). Purified FixLN is orange-red, similar to purified FixL*, and shows a heme-staining band with the nondenaturing gel heme assay (Fig. 2, lane 6). The concentration of the FixLN protein calculated from the absorbance at 417 nm by using the extinction coefficient at 417 nm determined for FixL* (15) agrees with the protein concentration determined by using the BCA protein assay (Pierce). This suggests that, like FixL*, FixLN contains one heme per monomer. The different mobilities observed between purified FixLN and FixJ in crude extracts (Fig. 2, lanes 3 and 6) may be the result of the 200-fold difference in amounts of total protein loaded (200 μg in lane 3 versus 1 μg in lane 6) or the presence of another protein that associates with FixLN in the crude extract. Purified FixLN and FixJ in crude extracts have the same mobility on SDS gels, suggesting that proteolysis is not responsible for the difference in mobility observed on nondenaturing gels.

Absorption spectra of oxy-FixLN and deoxy-FixLN were compared to the spectra of FixL* (Fig. 4A and B). Despite the deletion of nearly 30 kDa of the FixL* protein, the spectrum of oxygenated FixLN corresponds very closely to that of FixL*. The absorption maxima for oxy-FixLN (417, 542, and 576 nm) are almost identical to those of oxy-FixL* (417, 542.5, and 576.5 nm [Fig. 4C]). The peak shapes are also very similar. Upon deoxygenation, FixL* and FixLN exhibit similar spectral shifts. These results demonstrate that FixLN not only binds heme, it also retains the ability to bind oxygen. The only notable difference between the FixL* and FixLN spectra occurs in the upper region of the deoxygenated spectra. While the absorption maximum of deoxy-FixLN in the Soret region (433.5 nm) is nearly identical to that of deoxy-FixL* (434 nm), the deoxy-FixLN peak at 558.5 nm shifts to the left relative to the peak for FixL* at 565 nm. A shift in absorption maximum of similar magnitude between FixL* and FixLN was observed in two separate preparations of FixLN and two separate preparations of FixL*. This peak shift suggests that the greatest difference in heme environment between FixLN and FixL* occurs in the deoxygenated state. The absorption maxima for oxygenated and deoxygenated hemoglobin (22) are shown for comparison (Fig. 4C).

FixL Kinase Activity Resides in the C Terminus. Crude extracts bearing each of the FixL* deletion constructs were assayed for in vitro phosphorylation activity. Of the three protein deletions, FixLN, FixLC, and FixLNC, only FixLC gave detectable kinase activity in crude extracts (data not shown). FixLC was purified to greater than 90% purity (Fig. 3), and the protein solution had neither color nor absorbance at 417 nm, confirming that FixLC indeed lacks heme. Purified FixLC is active for autophosphorylation and the phosphorylation of FixJ (Fig. 5). However, the autophosphorylation and FixJ phosphorylation specific activities of FixLC were consistently observed to be approximately 1/3 to 1/4 (based on densitometric scans of autoradiograms) of those of FixL* under aerobic conditions (see Discussion).
low oxygen conditions (M. A. Gilles-Gonzalez, G. S. D., and D. R. H., unpublished data). We confirmed these results by demonstrating that after reaction times of 5 and 15 min, FixL* exhibits approximately a 3-fold enhancement of autophosphorylation (Fig. 5A) and a 4- to 8-fold enhancement of FixJ phosphorylation (Fig. 5B) under anaerobic relative to aerobic conditions (based on densitometric scans of autoradiograms). In addition, while FixLC, like FixL*, autophosphorylates and catalyzes the phosphorylation of FixJ (Fig. 5), it fails to show any change of these activities in response to oxygen. This suggests that the enhancement of FixL* autophosphorylation and FixJ phosphorylation activities under low-oxygen conditions requires the heme-binding region of the protein. Purified FixLN exhibits no detectable autophosphorylation or FixJ kinase activities in either the presence or the absence of oxygen. FixJ alone shows no kinase activity (15).

**FixLN C** Lacks Heme-Binding and Kinase Activities. Because FixLN C showed neither heme-binding activity nor kinase activity in crude extracts, it was not further purified. We expected FixLN C to lack kinase activity because the deletion removes a considerable portion of the region of FixL that is conserved among two-component sensors, including the putative phosphorylation site (8, 10). The fact that we fail to see a heme interaction with FixLN C could indicate that amino acids 127–212 are insufficient for heme binding. However, it is possible that the C-terminal amino acids that are fused to residue 212 prevent a heme interaction that would otherwise occur within residues 127–212 of FixL.

**DISCUSSION**

We have demonstrated that a soluble derivative of FixL, FixL*, can be separated into two distinct domains. A region of FixL from residue 127 to residue 260 binds heme, functions in oxygen binding, and has no detectable kinase activity. The C-terminal region of FixL, beginning at residue 260 and including the entire region of homology with two-component system sensors, retains autophosphorylation and FixJ kinase activities but fails to bind heme. The localization of kinase functions to the C-terminal region has been well documented among other sensor homologues, including VirA (23, 24), EnvZ (25–27), and Phor (28).

We also present data showing that oxygen concentration regulates the in vitro phosphorylation activities of FixL*, resulting in the generation of higher levels of FixL* phosphate and FixJ phosphate in the absence of oxygen. This suggests that FixL directly senses oxygen concentration and is responsible for initiating the signal transduction cascade resulting in nif and fix gene expression. It also suggests that FixJ phosphate is the active form for transcriptional activation, because increased FixJ phosphate under low-oxygen conditions in vitro correlates with microaerobic activation of the nifA and fixK promoters in vivo (29). Deletion of the heme-binding domain results in a FixL derivative, FixLC, that is active for autophosphorylation and FixJ phosphorylation, but which no longer regulates these activities in response to oxygen. Our data are consistent with a three-domain model for FixL. The N-terminal domain consists of four transmembrane helices (A. Lois, G. S. D., and D. R. H., unpublished data) that have been deleted from the FixL derivative discussed in this paper. The central domain of FixL binds heme and serves a regulatory function where the oxygenation state of the heme moiety controls the activity of the third FixL domain, the C-terminal kinase domain.

It is not clear why removal of the oxygen-sensing heme domain results in a FixL derivative, FixLC, that shows reduced autophosphorylation activity relative to FixL*, under both aerobic and anaerobic conditions. Possibly, the deleted region contains residues essential for optimum autophosphorylation activity. Alternatively, FixLC or FixLC phosphate may be less stable than FixL* or FixL* phosphate, respectively.

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**Fig. 4.** Absorption spectra of FixL* (——) and FixLN (——). (A1) Oxy-FixL* and oxy-FixLN, 350–500 nm. (A2) Oxy-FixL* and oxy-FixLN, 500–650 nm. Note expanded vertical scale relative to A1. (B1) Deoxy-FixL* and deoxy-FixLN, 350–500 nm. (B2) Deoxy-FixL* and deoxy-FixLN, 500–650 nm. (C) Absorption maxima for oxygenated and deoxygenated derivatives of FixL*, FixLN, and hemoglobin (Hb). Spectra were measured in 20 mM Tris-HCl, pH 7.8/100 or 125 mM NaCl/20% glycerol/10 mM 2-mercaptoethanol. Deoxygenation was achieved by the addition of a crystal of sodium dithionite to a 1-ml sample.

**Oxygen Regulation of FixL Kinase Activity Requires the Heme-Binding Domain.** The kinase activities of purified FixLC were compared to those of FixL* in the presence and absence of oxygen (Fig. 5). Preliminary work in our laboratory showed that FixL* kinase activities are enhanced under

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**Fig. 5.** Aerobic and anaerobic phosphorylation of FixL* and FixLC in vitro. (A) Autophosphorylation of FixL* and FixLC. Lanes 1–4, 2 μM FixLC; lanes 5–8, 2 μM FixL*; lanes 1, 3, 5, and 7, aerobic; lanes 2, 4, 6, and 8, anaerobic; lanes 1, 2, 5, and 6, 5 min; lanes 3, 4, 7, and 8, 15 min. (B) Phosphorylation of FixJ by FixL* and FixLC. Lanes are as described for A except that each reaction mixture included 4 μM FixJ.

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FixLC is also less active than FixL* with respect to FixJ phosphorylation, both aerobically and anaerobically. A reduction in FixJ phosphorylation could be the result of FixLC’s lower autophosphorylation activity. Additionally, it is possible that FixL possesses an oxygen-regulated FixJ phosphatase activity. If the latter were true, the final level of FixJ phosphatase would be the net result of competing kinase and phosphatase activities. It is relevant that in vivo experiments show that FixL sends both a positive signal to FixJ under microaerobic conditions and a negative signal to FixJ under aerobic conditions (29). As suggested previously (29), one explanation for this data is that FixL has both FixJ kinase and FixJ phosphatase activities. Other two-component sensor homologues, including NtrB (7, 30) and EnvZ (26, 31), possess response regulator phosphatase activity, and in the case of NtrB this activity is regulated by the PII protein (7, 30), which is responsible for transmitting information regarding cellular nitrogen status to NtrB.

FixL* retains sensing function in vitro despite the deletion of the N-terminal transmembrane regions. However, it has recently been shown that the membrane-spanning regions are essential in vivo for normal levels of sensing and activation of FixJ (A. Lois, G.S.D., and D.R.H., unpublished data). When all four transmembrane regions are deleted, the FixL-dependent microaerobic activation of the nifA promoter is significantly reduced, although it is still oxygen dependent. It may be that the 3-fold increase in FixL* autophosphorylation and the 4- to 8-fold increase in FixL*-mediated FixJ phosphorylation that we observe in vitro represents a significantly reduced oxygen effect as compared with the wild-type protein. Maximal FixL function may require interaction with other membrane proteins or lipids.

It is perhaps surprising that deletion of nearly 30 kDa from FixL* results in a heme-binding fragment (FixLN) that retains the ability to bind heme and to interact with oxygen. The absorption spectra of FixLN and FixL* are very similar in both the presence and the absence of oxygen. The only significant difference between the FixLN and FixL* spectra occurs in the deoxygenated state, where the FixL* peak at 565 nm shifts to 538.5 nm for FixLN. Since this shift indicates a change in the heme environment, and because a significant difference between FixL* and FixLN occurs only in the deoxygenated state, the interaction of the heme domain with the C terminus may occur predominantly under low-oxygen (activating) conditions.

The absorption spectra of FixL* (15) and FixLN are very similar to the absorption spectra of hemoglobin, both in the presence and in the absence of oxygen, suggesting that the heme moieties of FixL and hemoglobin are in similar environments. In hemoglobin, histidine-F8, the proximal histidine, is bound to the fifth coordination position of the heme iron (32). Histidine-E7, the distal histidine, influences the binding of ligands (i.e., oxygen, CO, and others) to the sixth coordination position of the heme iron (32). This suggests that histidines may play an important role with respect to FixL heme and oxygen binding. Furthermore, based on conservation with other two-component system sensors, the likely site of FixL autophosphorylation is histidine-285. Thus, it is possible that histidines could be directly involved in communication between the heme domain and the kinase domain. It is noteworthy that four histidine residues in R. meliloti FixL are conserved in the FixL proteins encoded by the fixL homologues cloned from Bradyrhizobium japonicum (33) and Azorhizobium caulinodans (34). Two of these histidines, corresponding to positions 138 and 194 of R. meliloti FixL, are located in the heme-binding region of FixL (residues 127–260). It has not yet been established whether the B.

japonicum or the A. caulinodans homologues encode heme-proteins, but in vivo experiments show that both are involved in regulating transcription in response to oxygen (35, 36).

Thus we have demonstrated a sensing system that exhibits a response to its environmental signal in vitro and consists solely of a sensor homologue and a response regulator homologue of bacterial two-component regulatory system proteins. It is now necessary to determine precisely how the levels of FixL phosphatase and FixJ phosphatase are regulated by the oxygen signal and how these levels control expression of R. meliloti nif and fix genes.

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