Autophosphorylation and Phosphatase Activities of the Oxygen-sensing Protein FixL of *Rhizobium meliloti* Are Cooperatively Regulated by Oxygen*  

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The FixL and FixJ proteins of *Rhizobium meliloti* control the expression of other *nif* and *fix* genes in response to oxygen levels. FixL is a hemoprotein kinase that senses oxygen availability and responds to the absence of oxygen by activation of its autophosphorylating activity followed by transfer of the phosphate to FixJ. FixJ in turn activates the *nifA* and *fixK* promoters. *In vitro* studies reported here with a soluble truncated version of FixL (FixL*) indicate that, while low oxygen tension specifically increases the autophosphorylating activity of FixL*, the ability of phospho-FixL* to act as a phosphate donor to FixJ is not affected by the presence or absence of oxygen. FixL* is also shown to possess a phosphatase activity that is repressed under anaerobic conditions only when the protein is in the phosphorylated form. A fixL mutant that induces a higher level of *nifA* promoter activity in the presence of *fixJ* in vivo displayed both an increased autophosphorylating activity and a decreased phosphatase activity *in vitro*. These data provide evidence for a role for both autophosphorylation and phosphatase activities of FixL in the mechanism by which oxygen tension within the alfalfa nodule induces expression of bacterial nitrogen fixation genes during symbiosis.

All cells are capable of transferring environmental signals from the outside of the cell to the cytoplasm in order to alter their metabolism in response to environmental changes. *Rhizobium meliloti* processes a wide range of information during its symbiotic association with alfalfa. The development of these bacteria into nitrogen-fixing bacteroids must be coordinated with root cell differentiation in the formation of specialized structures called nodules. During symbiosis, the oxygen tension within a nodule decreases due to bacteroid respiration in the presence of leghemoglobin (1) and to the presence of an oxygen diffusion barrier that prevents the free exchange of oxygen (2). A reduced free oxygen concentration is necessary to prevent inactivation of the oxygen-sensitive enzyme nitrogenase (1), which is responsible for catalyzing the conversion of gaseous nitrogen to ammonia.

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The expression of nitrogen fixation genes appears to be controlled by a regulatory cascade in which reduced oxygen availability is the primary signal (3, 4). A pair of proteins, FixL and FixJ, respond to the lowered oxygen concentration by activating transcription from two key regulatory genes, *nifA* and *fixK*. Transcription of the *nifA* and *fixK* genes is also induced under microaerobic conditions *ex planta* (3, 4). The products of the *nifA* and *fixK* genes are transcriptional activators that promote transcription of all other nitrogen fixation genes (5, 6). FixL and FixJ belong to a family of bacterial two-component signal transduction systems that respond to environmental signals (3). FixL is homologous in its C terminus to a set of proteins that have kinase and/or phosphatase activity and become phosphorylated at histidine residues (sensor class). FixJ is homologous in its N-terminal end to a number of so-called response regulator proteins, most of which are transcriptional activators. Proteins identified as members of the two-component system family can be found in both Gram-positive and Gram-negative bacteria and include NtrB/C (nitrogen regulation), EnvZ/OmpR (osmoregulation), PhoB/R (phosphate regulation), CheA/Y (chemotaxis), VirA/G (virulence), and many others (for reviews see Refs. 7–9). The kinase reaction of proteins studied from the two-component system family proceeds via a phosphorylated sensor intermediate. The phosphate from the sensor protein is transferred to the regulator and may be removed subsequently through the action of sensor phosphatase activity. The environmental signal is thought to exert an effect on the kinase and/or phosphatase activities of the sensor, ultimately controlling the level of phosphorylated regulator protein. Several regulators have been shown to become active transcription factors when phosphorylated by the sensor, including NtrC, OmpR, VirG, and PhoB (for review see Ref. 9).

FixL from *R. meliloti* is a membrane-bound hemoprotein kinase (10, 34). The kinase activity of a soluble derivative of FixL is regulated by oxygen *in vitro* (11). The sensing domain of FixL carrying the heme moiety is localized between amino acids 127 and 260 and is required for oxygen regulation of the kinase activity (11). As expected from its homology to other two-component sensors, the kinase domain of FixL was localized to the C-terminal half of the protein (11). FixL can transfer phosphate to the regulator FixJ, which, once phosphorylated, is thought to become an active transcription factor capable of inducing transcription from the *nifA* and *fixK* promoters. Control of the steady-state level of regulator-phosphate in response to an environmental signal could theoretically be exerted at any or all of the following reactions: sensor autophosphorylation, transfer of sensor phosphate to regulator, or sensor phosphatase activity toward regulator...
phosphatase. Additionally, some regulators possess an auto-
phosphorylating activity which could itself be modified in re-
response to an environmental cue, through the action of the
sensor.

The present study is directed at more fully understanding the
signal transduction mechanism mediated by the FixLJ system in response to oxygen. Experiments are carried out in order to identify the various reactions that control the level of phosphorylated FixL and to determine which reactions are regulated by oxygen. Using a soluble, truncated version of FixL, we first established conditions under which oxygen regulation is most pronounced. Both kinase reactions (auto-
phosphorylation and phosphotransfer) were tested separately for evidence of oxygen regulation. We also examined FixL for phosphatase activity and for regulation of this activity by oxygen. We report here that FixL has both kinase and phosphatase activity, and demonstrate that both of these activities are regulated in vitro in response to oxygen availability. Finally, the properties of an up-mutant of FixL were exam-
ine. It was shown that the increased activity of this FixL mutant in vivo can be explained by both an increased phosphor-
ylating activity and a decreased phosphatase activity.

MATERIALS AND METHODS

Bacterial Strains—All strains used in this work have been described previously. Rml102F34 is the wild-type strain of R. meliloti (12); T219 is derived from Rml102F34 and contains an unspecified deletion of the fixL and fixJ genes (13). Escherichia coli strains used were L250 (p δlac-1α XIII) (14), T1B1 (p δlac-1α XIII) (15) and TG1 (p δlac-1α XIII) (16).

Media and Growth Conditions—Rhizobia were grown in either TY (4) or YMB (17) media. E. coli strains were grown in LB broth (GIBCO/BRL) or on MacConkey lactose agar (Difco).

Mutant Isolation—fixL362 was isolated following N-methyl-N'-
nitro-N-nitrosoguanidine mutagenesis of plasmid pMW2.1 Plasmid
pMW2 expresses FixL and FixJ from the lac promoter (18). TB1 cells carrying pMW2 appear white when plated on MacConkey lactose plates. However, TG1 cells carrying pMW2, a plasmid that expresses only the FixJ protein, are red on MacConkey media (18). After NTC mutagenesis of plasmid pMW2, mutant plasmids isolated from colonies which appear red on MacConkey were selected for further analysis. The fixL gene of one such mutant, fixL362, was sequenced and found to contain a point mutation in codon 362 that results in a substitution of alanine for valine in the C-terminal half of the FixL protein. Double-stranded sequencing was carried out using Sequenase 2.0 (U.S. Biochemical Corp.) according to manufacturer's specifications.

Plasmids—pMW2 (18), pCHK57 (4) and pGD311 (18) have been described elsewhere. pGD31, pGD1502, and pGD344 plasmids are cotointegrates of a pUC-derived plasmid and pCHK57, which carries a nA/lacZ fusion (4). pGD344 is the control plasmid consisting of pUC9 as a cotegrate with pCHK57. pGD311 is identical to pGD344 except for containing wild-type fixL and fixJ genes. pGD1502 is identical to pGD311 except that it contains the fixL362 gene in place of the wild-type fixL gene. The fixL362 gene was isolated and initially characterized by Marie Gilles-Gonzalez.

FixL purification—FixL, FixL* and FixJ were purified according to the procedure described in Weinsteint et al. (18).

Autophosphorylation and Transfer Assays—Autophosphorylation assays were carried out with 3 μg of FixL* in 15-μl volume of reaction buffer (50 mM Tris, pH 8.0, 50 mM KCl, 0.8 mM MgCl2, 0.8 mM ATP, 1.2 mM CaCl2, 1 mM dithiothreitol, 5% glycerol, and 10 μCi of γ-[32P]ATP (6,000 Ci/mmol) for the indicated times.

For the phosphotransfer reactions, the autophosphorylation assay was first carried out for 1 h at 25 °C under anaerobic conditions. The 32P-labeled FixL* phosphate was then separated from ATP by gel filtration chromatography on Sephadex G-25 with FixL buffer (20 mM Tris, pH 7.5, 125 mM NaCl, 5% glycerol, 10 mM β-mercaptoethanol and 0.015% Triton X-100. Purified FixL-phosphate (0.15 μM) was then incubated with FixJ (0.6 μM) in autophosphorylation reaction buffer without ATP.

Phosphatase Assay—An affinity column to bind FixL was made by coupling FixL antiserum to cyanogen bromide activated Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) according to manufacturer's specifications. To isolate 32P-labeled FixL-phosphate, FixL362 (4 μg) was incubated with FixJ (6 μg) in the autophosphorylation reaction buffer at 25 °C under anaerobic conditions for 1 h. Nucleotides were removed from the reaction mixture by gel filtration through Sephadex G-25. The labeled proteins were then passed three consecutive times through a 150-μl FixL, affinity column in FixL buffer. The phosphatase assay was carried out in autophosphorylation reaction buffer by incubating 32P-labeled FixJ-phosphate (0.6 μM) with FixL* (0.3 μM) or with FixL* that had been preincubated with unlabeled ATP under anaerobic conditions for 1 h at 25 °C in order to generate FixL-phosphate. Anaerobic conditions were established by placing tubes containing the reaction mix in a desiccator jar under house vacuum for 4 min. The jar was filled with nitrogen gas, and the evacuation was repeated twice. All subsequent manipulations of the deoxygenated samples were done under a stream of nitrogen.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was run according to Laemmli (19) using 4% stacking and 8% separating gels. Gels containing labeled proteins were dried and exposed to x-ray films. For quantitation, autoradiograms were scanned using a laser densitometer and the data were processed using software from the Ambis Corporation (San Diego, CA).

RESULTS

Temperature-dependent Kinetics of Autophosphorylation—

Previous work from our laboratory has shown that the kinase activity of a soluble, truncated version of FixL (FixL*) is regulated by oxygen in vitro. A 2-3-fold enhancement of FixL* autophosphorylation was observed at 25 °C under anaerobic conditions (11). In order to develop conditions where oxygen might have a greater effect on the activity of FixL*, the effects of temperature on FixL autophosphorylation were examined. It can be seen in Fig. 1 that varying the temperature of the reaction (15, 25, and 30 °C) had no significant effect on FixL* autophosphorylation under anaerobic conditions. However, in the presence of oxygen, the rate of autophosphorylation, was substantially less at lower temperature. Consequently, at 15 °C there is a 12-15-fold higher rate of FixL* autophosphorylation under anaerobic conditions than under aerobic conditions.

To determine whether the observed temperature effect resulted from temperature-dependent changes in oxygen solubility in the reaction mixture, we compared the rate of FixL* phosphorylation in response to oxygen. Reactions mixtures contained 3 μg of FixL* and were carried out under "Materials and Methods" at the specified temperature with γ-32P]ATP under aerobic (filled symbols) and anaerobic (open symbols) conditions. At the indicated times, reactions were terminated with SDS, FixL-phosphate was separated by polyacrylamide gel electrophoresis and detected by autoradiography. Quantitation was done using a laser densitometer. Triangles, squares, and circles denote reactions carried out at 30, 25, and 15 °C, respectively.
autophosphorylation at 25 °C under 100% oxygen conditions. No significant difference in autophosphorylation was observed (data not shown), indicating that the decreased activity of FixL* at 15 °C under aerobic conditions is not due to an increase in the oxygen available to FixL* at this temperature. This result also indicates that 21% O₂ is sufficient to maximally repress FixL* kinase activity. Spectral data also support the idea that FixL* is fully oxygenated at 25 °C in air (data not shown).

**Effect of Oxygen on FixJ Phosphorylation**—When FixL is incubated with FixJ in the presence of labeled ATP at 15 °C, the rate of accumulation of FixJ-phosphate increases 15-20-fold under anaerobic conditions relative to aerobic conditions (Fig. 2). These data confirm previous results (11), and together, the data clearly demonstrate that oxygen availability can regulate the level of FixJ-phosphate in a coupled reaction where both sensor and regulator are present.

**Effect of Oxygen on the Phosphotransfer Reaction**—To test the effect of oxygen on the phosphotransfer activity of phospho-FixL, we isolated ³²P-labeled FixL-phosphate. FixL-phosphate is extremely stable, consistent with data obtained for other two component kinases (9). Up to 85% of the protein is phosphorylated under the autophosphorylating conditions described under “Materials and Methods” and as determined from measurements of the specific activity of FixL*-phosphate. As shown in Fig. 3, at 15 °C the labeled phosphoryl group from FixL-phosphate is efficiently transferred to FixJ, but the rate of phosphotransfer is not affected by changes in oxygen concentration. Oxygen similarly has no effect on the rate of transfer when the reaction is carried out at 25 °C (data not shown).

FixL* Phosphatase Activity—To determine whether FixL* has phosphatase activity, we purified ³²P-labeled FixL-phosphate. The half-life of the phosphoryl group in FixJ-phosphate is about 4 h at 25 °C under our assay conditions. This relatively long half-life facilitated the isolation of FixJ-phosphate for use in subsequent experiments. When FixJ-phosphate is incubated with FixL* at 25 °C in a 1:2 molar ratio, the half-life of the phosphoryl group decreases to about 5 min (Fig. 4A). Oxygen has little or no effect on the initial rate of dephosphorylation, although some oxygen responsiveness is seen at later reaction times (see discussion below). At 15 °C, FixL* also displays a phosphatase activity (data not shown).

Although several sensor proteins from the two component family require nucleoside triphosphate as a cofactor to display phosphatase activity, we observed no ATP requirement for the phosphatase activity of FixL*. Equivalent rates of FixJ-phosphate dephosphorylation were seen both aerobically and anaerobically in the presence or absence of ATP added at the start of the reaction (data not shown). Surprisingly, however, prior incubation of FixL* with ATP before the addition of FixJ-phosphate caused a substantial loss of phosphatase activity under anaerobic, but not aerobic, conditions (Fig. 4B). Under aerobic conditions, the phosphatase activity of FixL*-phosphate was completely equivalent to that of FixL*. When a non-hydrolyzable ATP analog, [γ-S]ATP, was used instead of ATP, the phosphatase activity was fully active under anaerobic conditions. This indicates that FixL*-phosphate, formed during preincubation with ATP, has significantly less phosphatase activity under anaerobic conditions, but is nevertheless fully competent as a phosphatase under aerobic conditions. In Fig. 4A, it can be seen that there is a slight, but

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2 M. Weinstein, unpublished data.
reproducible, effect of oxygen on phosphatase activity at later reaction times for unphosphorylated FixL*. This may indicate some degree of phosphatase regulation for unphosphorylated FixL*. It is also possible, however, that a small fraction of FixL* is in the phosphorylated form when the enzyme is isolated from cells and this accounts for the oxygen effect. As noted previously, FixL*-phosphate is extremely stable and only 85% of FixL* is autophosphorylated in the presence of \([\gamma-32P]ATP\) as measured by the incorporation of \([32P]\)phosphate.

Characterization of a Mutant FixL Protein with Increased Activity in Vivo—The results from the \textit{in vitro} studies presented above indicate that both the kinase and phosphatase activities of FixL play key roles in the regulation of the level of FixL-phosphate in \textit{in vivo} in response to oxygen. Additional support for this conclusion comes from an examination of the \textit{in vitro} properties of mutations in FixL that promote increased transcription \textit{in vivo} from the \textit{nifA} promoter. A number of mutants of the fixL gene were identified by expressing \textit{E. coli} cells that also carried the reporter plasmid pCHK57 (p\textit{nifA::lacZ}) as described under "Materials and Methods." One of the mutants (fixL362) was tested in \textit{R. meliloti} and was found to increase \textit{nifA} transcription 5–8-fold above wild-type levels under aerobic conditions (Table I). A small increase in \textit{nifA} expression was also seen for the mutant under anaerobic conditions. Sequence analysis of the fixL mutant identified the mutation in the C-terminal half of the protein at residue 362 (Ala \textrightarrow{} Val).

A soluble deletion derivative of the FixL362 protein, identical to FixL* except for the Ala-Val mutation (FixL*362), was purified. As shown in Fig. 5A, the mutant protein retains its regulatory properties but has an increased rate of auto-phosphorylation both aerobically and anaerobically when compared to the wild-type protein. The rate of FixL*362 phosphotransfer to FixJ under aerobic conditions is slowed more than the wild type (Fig. 5B). Interestingly, the mutation also affects the phosphatase activity of the protein. As shown in Fig. 5C, FixL*362 catalyzes the dephosphorylation of FixJ-phosphate under aerobic conditions at a much lower rate than the wild-type protein. FixL*362-phosphate also has reduced phosphatase activity (data not shown).

**DISCUSSION**

A soluble derivative of the sensor FixL (FixL*) was used to study oxygen regulation of the phosphorylation and dephosphorylation properties of this enzyme. This derivative, although missing amino acids of the N terminus of FixL, exhibits oxygen regulation in the heterologous host \textit{E. coli}. We show here that FixL* is a bifunctional protein with both kinase and phosphatase activities, and that, as with other two-component systems, phosphorylation of the response regulator proceeds via a phosphorylated intermediate. From previous data, it was known that FixL* becomes phosphorylated when incubated with ATP \textit{in vitro} (10) and that reduced oxygen tension increases FixL* autophosphorylation by a factor of 2–3 (11). In this work we show that the effect of oxygen on autophosphorylation is substantially enhanced when the reaction is carried out at reduced temperature. At 15 °C, the rate of FixL* phosphorylation is 12–15-fold higher anaerobically than aerobically. Significantly, the primary effect of reduced temperature is to increase repression of FixL* autophosphorylation by oxygen. There is little or no effect of temperature on FixL* autophosphorylation in the absence of oxygen. It is worth noting that the effect of temperature on these experiments is unlikely to be due to changes in oxygen solubility in \textit{H}_2\textit{O} (1.3-fold increase in dissolved oxygen concentration at 15 versus 30 °C, see Ref. 20). FixL* appears to be fully saturated with oxygen at 25 °C as determined by spectral analysis and by the fact that the rate of autophosphorylation in 100% oxygen was not significantly less than that observed in air. A more likely explanation for the tem-
Oxygen has no effect on the rate of phosphate transfer from FixL-phosphate to FixJ at either 15 or 25 °C. Since relatively little FixL-phosphate accumulates when both sensor and regulator are incubated with ATP, it is likely that transfer is more rapid than autophosphorylation. A similar conclusion has been reached for other two-component system proteins including, EnvZ, NtrB, and CheA (21-24). The transfer reaction, therefore, would not be expected to be regulated by oxygen since the regulatory step in metabolic pathways is most likely to be the slowest step in the pathway.

FixL* also contains a FixJ-phosphate dephosphorylating activity that does not require adenosine nucleotide as cofactor (ATP or ADP). This is in contrast to the results obtained for NtrB and EnvZ (25, 26) where a nucleotide cofactor is necessary for phosphatase activity. The phosphatase activity of FixL* is not significantly regulated by the presence or absence of oxygen. However, the phosphatase activity of FixL*-phosphate is very responsive to the oxygen concentration. When FixL*-phosphate is preformed and a steady-state level maintained with ATP under anaerobic conditions, phosphatase activity is almost completely eliminated. When oxygen is present, the phosphatase activity observed for FixL*-phosphate is fully equivalent to that observed for FixL*, indicating that FixL*-phosphate is inherently as good a phosphatase as FixL*.

Together these observations suggest a direct role for oxygen in regulating FixL*-phosphate phosphatase activity. Oxygen regulation of the phosphatase activity exhibited by the phosensor provides a sensitive means whereby the cell can rapidly reduce the steady-state level of FixJ-phosphate if the oxygen concentration rises. This may have particular relevance for the FixJ system given the relatively long half-life of FixJ-phosphate (4 h). The possibility exists, however, that other two-component sensors function in a similar way.

It is noteworthy that FixL* is the only protein thus far reported from the two-component system family that can, in the absence of other proteins, sense a specific environmental signal in vitro and respond to it by modulating both kinase and phosphatase activities. The kinase, phosphatase, and sensing activities in the chemotaxis system for example are encoded in different polypeptides: CheA, CheZ, and receptor, respectively. Ninfa et al. (27) demonstrated, with purified components from Salmonella typhimurium, an increase in CheA autophosphorylation in response to aspartate using a reconstituted system that also contained aspartate receptor and CheW. The rate of autophosphorylation of CheA from E. coli increases upon addition of CheW (28). Whether the phosphatase activity of the CheZ protein is also regulated during chemotaxis is not yet known. In vitro regulation of the kinase activity of other sensor proteins like VirA and EnvZ has not been reported with purified proteins. It is possible that the truncated versions that were used in these studies may have lost their sensing or regulating capabilities (25). The sensing domain of the membrane bound FixL protein (heme binding region) has been localized to the cytoplasmic portion of the protein (11, 34). FixL* lacks the transmembrane domain, suggesting that membrane attachment is not an absolute requirement for oxygen sensing (34).

Aside from CheA, another protein of the two-component family that has been shown to have regulated kinase/phosphatase activity is NtrB. The phosphatase activity of the NtrB protein appears to be regulated in response to the nitrogen status of the cell through the P4 protein (29). It is possible that the only regulation in response to nitrogen status is at the level of the NtrB phosphatase activity (9).

Our studies with a mutant FixL protein that causes increased FixJ-dependent transcription of nifA in vivo yielded interesting results. The mutation was localized to the C-terminal half of the protein at residue 362 (Ala → Val) in a region of the protein that appears to be poorly conserved among two-component kinases (7, 9). A soluble derivative of FixL362 displays an increased rate of autophosphorylation in response to oxygen limitation, and a marked decrease in the rate of dephosphorylation of FixJ-phosphate when compared to the soluble derivative of the wild-type FixL protein. It is interesting that a single point mutation modifies two opposing activities of the FixL protein in a reciprocal manner. Since the phosphatase and kinase activities are both regulated by oxygen, it is possible that the mutation affects the interaction between the sensing (heme) and kinase-phosphatase domains.

On the basis of the properties of wild-type and mutant FixL protein, we propose the following reactions (Fig. 6) for the regulation of the level of FixJ-phosphate. At atmospheric oxygen tension, some FixL molecules may become phosphorylated if the concentration of ATP within the cell is sufficiently high. The phosphate can then be transferred to FixJ but is subsequently removed through the action of the FixL phosphatase activity, keeping the level of FixJ-phosphate low. A decrease in the oxygen tension causes an increase in the level of FixL-phosphate and a concomitant decrease of the phosphatase activity. The net effect is an increase in the level of FixJ-phosphate and subsequent activation of the nifA promoter. The phosphatase activities of FixL and FixJ-phosphate serve to maintain a very tight control over FixJ-phosphate levels. FixJ-phosphate will accumulate only when a sufficient amount of FixJ is phosphorylated and when oxygen tension is reduced. In addition, the FixL phosphatase activity conceivably can prevent the accumulation of FixJ-phosphate levels as a result of phosphorylation of FixJ by heterologous sensors (cross-talk) or by residual oxygen-insensitive FixL-kinase activity. Phosphatase activity also provides a way for the system to efficiently revert to its original state if the oxygen concentration rises again. Conditions which favor kinase activity suppress phosphatase activity and vice versa.

It is likely that the level of FixJ-phosphate regulates expression from the nifA promoter. Phosphorylation of several transcriptional activators that belong to the family of two component regulatory systems, including NtrC, OmpR, and VirG, increases their effectiveness to induce transcription in vitro from the target promoters (21, 24, 30, 31). Also, the
DNA binding ability of NtrC and OmpR is stimulated severalfold after phosphorylation (38, 32).

According to the model in Fig. 6, FixL possesses both positive and negative regulatory activities. There is genetic and biochemical evidence that a negative activity exists not only during regulation in the FixL1 system (18, 33) but also in the Omp, Ntr, and Pho systems (9), suggesting that both kinase and phosphatase activities may be essential for optimal functioning of a number of two-component regulatory systems.

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