Ligand and Oxidation-State Specific Regulation of the Heme-Based Oxygen Sensor FixL from *Sinorhizobium meliloti*†

Jason R. Tuckerman, Gonzalo Gonzalez, Elhadji M. Dioum, and Marie-Alda Gilles-Gonzalez*

Departments of Biochemistry, Plant Biology, and the Plant Biotechnology Center, The Ohio State University, 1060 Carmack Road, Columbus, Ohio 43210-1002

Received February 4, 2002; Revised Manuscript Received March 15, 2002

ABSTRACT: Phosphorylation of the transcription factor *Rm*FixJ is the key step in the hypoxic induction of *Sinorhizobium meliloti* nitrogen fixation genes. Oxygen regulates this process by binding reversibly to *Rm*FixL, a heme protein kinase whose deoxy form catalyzes the phosphoryl transfer from ATP to *Rm*FixJ. Here we present the first quantitative measure of the extent by which various heme ligands inhibit the turnover of *Rm*FixJ to phospho-*Rm*FixJ. We also quantitate the inhibition by ligands of the reaction of *Rm*FixL with ATP, in the absence of *Rm*FixJ, to form phospho-*Rm*FixL, i.e., the "autophosphorylation". Phospho-*Rm*FixL formed from autophosphorylation will transfer its phosphoryl group to *Rm*FixJ in an oxygen-independent "phosphotransfer." Here we show that the mode of substrate presentation, i.e., simultaneous versus sequential, influences the regulation of phosphoryl transfer by heme status. Inhibition factors for O_2, CO, NO, CN^-, and imidazole in the presence of *Rm*FixJ are drastically different from the inhibition of autophosphorylation by the same ligands. Oxidation of the heme iron in unliganded *Rm*FixL is known to have no effect on either of the sequential reactions; yet oxidation causes a 100-fold decrease in *Rm*FixJ turnover when ATP and *Rm*FixJ are presented simultaneously. The profound difference between the regulation of isolated *Rm*FixL versus the complex of *Rm*FixL with *Rm*FixJ shows that interaction of a response regulator with its histidine−kinase partner need not be limited to the enzymatic regions of the histidine kinase, but can extend also to its sensory domain.

In *Sinorhizobium meliloti*, the two-component regulatory system consisting of the *Rm*FixL[1] and *Rm*FixJ proteins ensures that induction of nitrogen fixation (*nif, fix*) genes begins only after the symbiotic bacteria in a developing nodule detect an internal O_2 concentration of $\leq 50\ \mu M$ (*1*, *2*). *Rm*FixL is a dimeric heme protein kinase whose deoxy form catalyzes transfer of a phosphoryl group from ATP to a regulatory partner, the transcription factor *Rm*FixJ (*3*). Phosphorylation of *Rm*FixJ enables this DNA-binding protein to dimerize and induce the expression of the *nifA* and *fixK* genes, both of which encode transcription factors (*4*−*6*). The end result is cascade of expression of more than 20 *S. meliloti nif* and *fix* genes (*7*).

The kinase domain in *Rm*FixL belongs to the large family of two-component regulatory system histidine kinases (*8*, *9*). Such kinases can autophosphorylate at a conserved histidine (H285 in *Rm*FixL) when provided with ATP. Heme regulation of this autophosphorylation in *Rm*FixL is accomplished by reversible binding of ligands such as O_2 to a sensory heme-binding domain immediately preceding the kinase domain (*1*, *10*−*13*). The FixL heme-binding domain is a prototypical PAS domain with an alpha-beta three-dimensional fold and about 130 residues (*14*). Although a PAS fold is unusual for a heme protein, this fold is characteristic of a broad class of sensory proteins that occur in all three kingdoms of life (*15*). As is typical of heme proteins, only the ferrous form of *Rm*FixL can bind O_2 or CO, and only the ferric form can bind CN^- or imidazole (*16*−*19*).

Previously, phosphorylation of *Rm*FixJ was thought to proceed by a "ping-pong, bi-bi" mechanism in which dimeric *Rm*FixL reacts with ATP in a regulated slow step to form phospho-*Rm*FixL, and then phospho-*Rm*FixL reacts with *Rm*FixJ in a faster unregulated step to form phospho-*Rm*FixJ (*10*).

Scheme 1: "Ping-Pong, Bi-Bi" or Sequential Introduction of Substrates

Step 1:	*Rm*FixL$_2$ + 2 ATP	↔	P-*Rm*FixL$_2$ + 2 ADP
Step 2:	P-*Rm*FixL$_2$ + 2 *Rm*FixJ	↔	*Rm*FixL$_2$ + P-*Rm*FixJ$_2$

Studies of the regulation of the first step of this reaction showed met-*Rm*FixL to have the same reactivity toward ATP

† This work was supported by NIH Grant HL 64038 to M.-A. Gilles-Gonzalez.

* Corresponding author: Tel. (614) 688−3303; Fax (614) 688−3302; E-mail: gilles-gonzalez.1@osu.edu.

[1] Abbreviations: *Rm*FixL, *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) FixL; *Rm*FixLT, a soluble *Rm*FixL containing the heme-binding domain followed by a histidine-protein-kinase domain; in this work *Rm*FixL and *Rm*FixLT are used interchangeably; *Rm*FixLH, *Rm*FixL heme-binding domain; *Rm*FixJ, *S. meliloti* FixJ; *Rm*FixLJ, a complex of *Rm*FixL and *Rm*FixJ; *Bj*FixL, *Bradyrhizobium japonicum* FixL; *Bj*FixLH, *B. japonicum* FixL heme-binding domain; PAS, sensory domain with an alpha-beta fold named after the eukaryotic proteins *P*eriod, *A*rnt, and *S*imple-minded; heme-PAS, a heme-binding PAS domain such as the FixL heme-binding domain; *R*, relaxed state of hemoglobin; *T*, tense state of hemoglobin; deoxy, Fe^{II} without ligand; Oxy, $Fe^{II}O_2$; carbonmonoxy, $Fe^{II}CO$; nitrosyl, $Fe^{II}NO$; met, Fe^{III} without ligand; cyanomet, $Fe^{III}CN^-$; imidazolemet, Fe^{III}Imidazole; fluoromet, $Fe^{III}F^-$.

10.1021/bi025628w CCC: $22.00
Published on Web 04/17/2002

as deoxy-*Rm*FixL, despite the changed oxidation state of the heme iron (*13*). These studies also showed CN^- to inhibit met-*Rm*FixL analogously to the O_2 inhibition of deoxy-*Rm*FixL. In both cases, the activity relative to the unliganded form corresponded to the amount of *Rm*FixL not saturated with ligand. Thus, *Rm*FixL reactivity toward ATP, although responsive to heme ligands, seemed insensitive to the oxidation state of the heme iron or to the specific ligand bound. To explain these results, the initial event in sensing by *Rm*FixL was hypothesized to be the switch of the iron atom from high-spin to low-spin that is induced by binding of any strong-field ligand (*13*). This ligand-induced transition to low spin is known to cause displacement of the iron atom into the heme plane. In addition, the changes in coordination cause flattening of the porphyrin and repositioning of peripheral groups such as the heme vinyls and propionates.

It is certain that *Rm*FixL and ATP, without any *Rm*FixJ, can react to form phospho-*Rm*FixL (*3*, *10*). It is equally certain that the phospho-*Rm*FixL made in this way can react with *Rm*FixJ, without any ATP, to produce phospho-*Rm*FixJ and regenerate the unphosphorylated *Rm*FixL enzyme. Nonetheless, phosphoryl-transfer Scheme 1 had several unsatisfying aspects. One important shortcoming is that the net rate of phosphoryl transfer implied by the measured rate constants of the separate steps is very slow compared to the rate of hydrolysis of the phospho-*Rm*FixJ produced in these reactions (*10*, *12*). Thus, in a living bacteroid, large quantities of ATP would be continuously hydrolyzed, and little phospho-*Rm*FixJ could be maintained. Another drawback of Scheme 1 is that ATP is now known to react more efficiently with *Rm*FixL if *Rm*FixJ is present, rather than with *Rm*FixL alone, despite the simultaneous transfer of phosphoryl groups to the *Rm*FixJ (*12*). Moreover, this reaction generates phospho-*Rm*FixJ with little loss of the phosphoryl group as free phosphate (*12*). These observations have led to a revised mechanistic model in which turnover of *Rm*FixJ, i.e., all the phosphoryl transfers in this system, takes place within a complex including *Rm*FixL and *Rm*FixJ.

We set out to determine how the presence of *Rm*FixJ influences heme-ligand regulation of this system. The *Rm*FixL enzyme catalyzes the following turnover of *Rm*FixJ and ATP.

$$2\ Rm\text{FixJ} + 2\ \text{ATP} \xleftrightarrow{Rm\text{FixL}_2} \text{P-}Rm\text{FixJ}_2 + 2\ \text{ADP}$$

Studies of the turnover number of *Rm*FixL are inconsistent with Scheme 1 and instead support the following scheme (Scheme 2) (*12*).

Scheme 2: Simultaneous Introduction of Substrates

$$Rm\text{FixL}_2 + Rm\text{FixJ}_2 + 2\ \text{ATP} \leftrightarrow Rm\text{FixL}_2 \bullet Rm\text{FixJ}_2 \bullet 2\ \text{ATP}$$

$$Rm\text{FixL}_2 \bullet Rm\text{FixJ}_2 \bullet 2\ \text{ATP} \leftrightarrow (\text{P-}Rm\text{FixL} \bullet Rm\text{FixJ})_2 + 2\ \text{ADP}$$

$$(\text{P-}Rm\text{FixL} \bullet Rm\text{FixJ})_2 \leftrightarrow Rm\text{FixL}_2 + \text{P-}Rm\text{FixJ}_2$$

A testable prediction that distinguishes the two phosphorylation schemes is that in Scheme 1, regulatory effects of *Rm*FixJ, if any, should only be felt during phosphoryl transfer from phospho-*Rm*FixL to *Rm*FixJ (step 2). Since step 2 has already been shown to be heme-ligand insensitive, then Scheme 1 predicts that regulation of overall turnover should be the same as regulation of autophosphorylation (step 1). In Scheme 2, on the other hand, *Rm*FixJ is always present, raising the possibility that in this scheme regulation by heme ligands could be quite different from regulation of the autophosphorylation carried out in the absence of *Rm*FixJ (Scheme 1, step 1). Here we quantitate for the first time the inhibition imposed by various heme ligands on the sequential (Scheme 1) and the simultaneous (Scheme 2) reactions. We show the impacts of heme-iron oxidation state and ligation to be drastically different for the two reaction schemes. In addition, we provide support for cooperativity with respect to ATP and *Rm*FixJ. This work provides the first evidence of a direct interaction between a response regulator (i.e., *Rm*FixJ) and the signal-receiving domain of a histidine protein kinase, i.e., the heme-binding PAS domain in *Rm*FixL.

EXPERIMENTAL PROCEDURES

Proteins. The *S. meliloti* FixJ protein, *Rm*FixJ, and a soluble *S. meliloti* FixL protein consisting of the heme-binding and kinase domains, *Rm*FixLT, were derived from genes overexpressed in *Escherichia coli.* The proteins were purified as described previously (*3*, *12*). In the experiments described here, *Rm*FixL exists as an equilibrium of monomers and dimers and as a *Rm*FixLJ complex. Therefore, unless otherwise specified, all protein concentrations refer to total monomers in all species per unit volume.

Heme Derivatives of RmFixL. All derivatives were prepared with 1−5 μM *Rm*FixL in 50 mM Tris-HCl, 50 mM KCl, 5% (v/v) glycerol or ethylene glycol, and 0.50 mM $MnCl_2$, pH 8.0, immediately before the phosphorylation reactions. Ligands of ferric heme were equilibrated with the protein for at least 10 min. Cyanomet-*Rm*FixL and imidazolemet-*Rm*FixL were prepared by equilibrating met-*Rm*FixL with 10 mM KCN or 200 mM imidazole, respectively. Deoxy-*Rm*FixL was prepared by reducing an anaerobic met-*Rm*FixL solution with sodium dithionite and removing the dithionite by Sephadex-G25 (Pharmacia) gel filtration of the reduced *Rm*FixL inside of an anoxic glove box (Coy Laboratory Products Inc.). Oxy-, carbonmonoxy-, and nitrosyl-*Rm*FixL were obtained by mixing a deoxy-*Rm*FixL solution containing 10 mM β-mercaptoethanol with air (256 μM O_2), CO-saturated buffer (> 500 μM final), or NO-saturated buffer (>500 μM final).

Phosphorylations. In general, the reactions contained various additions of *Rm*FixL, *Rm*FixJ, and ATP in "phosphorylation buffer" [50 mM Tris-HCl, 50 mM KCl, 0.5 mM $MnCl_2$, and 5% (v/v) ethylene glycol, pH 8.0]. For all reactions, the oxidation states and liganded states of proteins were verified from their 250−700 nm absorption spectra (ATI Unicam UV4 UV−Vis spectrometer) not to change during the reactions. Reactions were stopped by mixing aliquots (20 μL) of reaction mixtures with one-third volume of "stop buffer" [4 mM EDTA, 4% (w/v) sodium dodecyl sulfate, 0.5 M Tris-HCl, 0.2 M NaCl, 50% (v/v) glycerol, and 2% (v/v) β-mercaptoethanol, pH 6.8]. The products were electrophoresed on 15% (w/v) polyacrylamide gels (*20*). The levels of phosphorylated protein in the dried gels were quantitated with a phosphorimager (Molecular Dynamics).

For the autophosphorylation reactions, *Rm*FixL (5 μM, unless otherwise indicated) was preincubated 5 min at 23 °C in phosphorylation buffer. Reactions were begun by

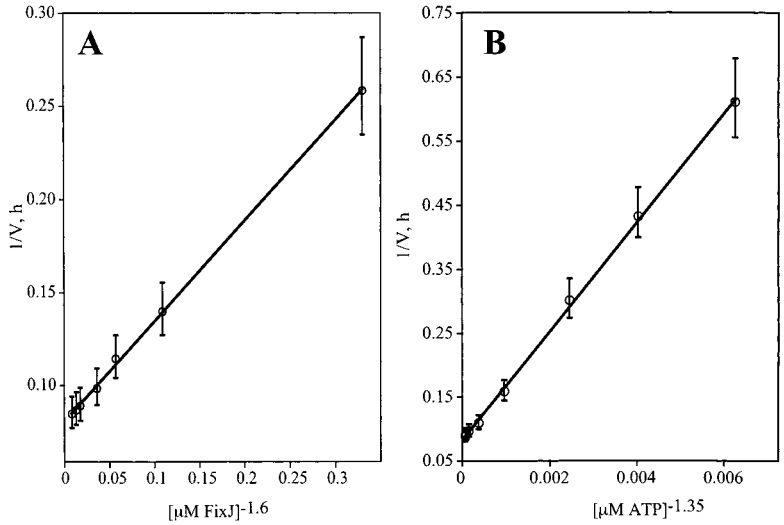

FIGURE 1: Modified Lineweaver−Burke plots showing the influence of *Rm*FixJ and ATP concentrations on the turnover of *Rm*FixJ to phospho-*Rm*FixJ. Each point represents an initial rate of *Rm*FixJ phosphorylation per mole of *Rm*FixL at pH 8.0 and 23 °C. In part A, the concentration of *Rm*FixJ was varied in reactions having 0.5 μM *Rm*FixL and 1 mM ATP. In part B, the concentration of ATP was varied in reactions having 30 μM *Rm*FixJ and 1 μM *Rm*FixL. In addition to *Rm*FixL, *Rm*FixJ, and ATP, all reactions contained 50 mM Tris-HCl, 50 mM KCl, 0.5 mM $MnCl_2$, 5% (v/v) ethylene glycol, pH 8.0. Initial rates were determined from the slope of a linear plot of phospho-*Rm*FixJ produced versus time, containing at least three points and having an R^2 value of ~0.99. Additional details are under Experimental Procedures.

introducing 0.2 mM γ-[^{32}P]-ATP (unlabeled ATP from Roche and γ-[^{32}P]-labeled ATP from Amersham Pharmacia Biotech) at 23 °C. Aliquots containing approximately 2 μCi (~ 5×10^6 cpm) were removed at various times and mixed with stop buffer, and the reaction products were electrophoresed and quantitated as described above.

For the turnover assays, the reaction mixtures contained 1−5 μM *Rm*FixL and *Rm*FixJ:*Rm*FixL ratios ≥ 25:1, in phosphorylation buffer, unless otherwise specified. Reactions were started by mixing the solution of protein with a solution of γ-[^{32}P]-ATP at 23 °C. Unless otherwise specified, the ATP concentration was 1 mM. At the indicated times, aliquots (~ (2.5−5) $\times 10^6$ cpm) were withdrawn from the reaction and mixed with stop buffer, and the reaction products were electrophoresed and quantitated as described above. For standard reactions done with *Rm*FixJ/*Rm*FixL ratios ≥ 25:1, the ^{32}P-*Rm*FixJ accounted for > 95% of all the radiolabeled protein.

Oxygen Association and Dissociation. Deoxy-*Rm*FixL was prepared by reducing a solution of met-*Rm*FixL with 4 mM sodium dithionine and then removing the dithionite with a Sephadex-G25 desalting column preequilibrated with anaerobic phosphorylation buffer. Oxy-*Rm*FixL was prepared by equilibrating the deoxy-*Rm*FixL with 1 atm O_2 at 4 °C. All rates were followed at 25 °C for final proteins concentrations of 3 μM *Rm*FixL or 3 μM *Rm*FixL mixed with 35 μM *Rm*FixJ in phosphorylation buffer. Measurements were done with a stopped-flow spectrometer (Applied Photophysics Ltd., Leatherhead U.K.) at the wavelength of maximum difference between the starting and final species. Each apparent rate was measured at least three times. To measure the association rates, deoxy-*Rm*FixL was mixed with 30−150 μM O_2, and O_2 association was followed by the decreased absorbance at 438 nm. The association rate constants were calculated from a linear plot of k_{obs} vs ligand concentration. Dissociation of O_2 was followed by the increased absorbance at 438 nm after mixing oxy-*Rm*FixL with 2 mM sodium dithionite. The dithionine consumes any dissociated O_2 essentially instantaneously.

RESULTS

The RmFixL-Catalyzed Turnover of RmFixJ to Phospho-RmFixJ: Dependence on ATP and RmFixJ Concentrations. For 1 μM *Rm*FixL, the rate of conversion of *Rm*FixJ to phospho-*Rm*FixJ increased with the *Rm*FixJ concentration according to a modified Lineweaver−Burke equation $1/V = 0.54(1/[RmFixJ]^{1.6}) + 0.080$ (Figure 1A). This gives a K_m value for *Rm*FixJ of 3.3 μM and a V_{max} value of 12 μmol of phospho-*Rm*FixJ per μmol of *Rm*FixL per hour. The rate of conversion of *Rm*FixJ to phospho-*Rm*FixJ increased with the ATP concentration according to the equation $1/V = 4.7(1/[ATP]^{1.35}) + 0.0045$ (Figure 1B). This gives a K_m value for ATP of 170 μM and a V_{max} value of 12 μmol of phospho-*Rm*FixJ per μmol of *Rm*FixL per hour. In the equations, the exponents of 1.6 for *Rm*FixJ and 1.35 for ATP indicate cooperativity with respect to both substrates, with at least two interacting sites for both the ATP and the *Rm*FixJ. The data from three repetitions of these experiments were within 10% of each other.

Reaction of Isolated RmFixL with ATP. Table 1 summarizes the inhibitory effect of heme ligands on the reaction of *Rm*FixL with ATP in the absence of *Rm*FixJ.

autophosphorylation:

$$Rm\text{FixL}_2 + 2\ \text{ATP} \leftrightarrow \text{P-}Rm\text{FixL}_2 + 2\ \text{ADP}$$

In this reaction, *Rm*FixL functions as a reactant rather than a catalyst, and its phosphorylation is measured. In previous studies, unliganded ferrous and ferric *Rm*FixL were shown to have the same reactivity toward ATP (*13*). In addition-,binding of CN^- to ferric *Rm*FixL or binding of O_2 to ferrous *Rm*FixL appeared to abolish this reaction (*10*, *13*). Here inhibition factors are quantitated for the first time for O_2, CO, NO, CN^-, and imidazole (Table 1). An important finding

Table 1: Inhibition of Autophosphorylation and Turnover Reactions by Ligands[a]

	autophosphorylation[b]		turnover[c]	
*Rm*FixL heme status	k_{ph} (% h^{-1})	I_{ph}	k_j (h^{-1})	I_j
deoxy (Fe^{II})	33	1	12	1
oxy ($Fe^{II}O_2$)[d]	2.2	15	<0.1	>100
carbonmonoxy ($Fe^{II}CO$)	6.6	5	4.5	2.7
nitrosyl ($Fe^{II}NO$)	15.7	2.1	5.2	2.3
Met (Fe^{III})	33	1	0.12	1
cyanomet ($Fe^{III}CN^-$)	2.2	15	0.06	2
imidazolemet (Fe^{III}Imid)	<0.4	>75	0	
fluoromet ($Fe^{III}F^-$)	33	1	0.12	1

[a] Each value represents the average of at least three measurements, with a deviation of less than ± 15%. Preparations of liganded forms of *Rm*FixL and the experimental conditions for both the autophosphorylation and the turnover reactions were as described under Experimental Procedures. [b] The rate constant k_{ph} is the initial rate of autophosphorylation. I_{ph} is the inhibition factor for *Rm*FixL autophosphorylation, obtained by dividing the activity of the unliganded form by the activity of the liganded form of *Rm*FixL, for the same oxidation state. [c] The rate constant k_j is the number of *Rm*FixJ molecules phosphorylated by one molecule of *Rm*FixJ per hour. I_j is the inhibition factor for *Rm*FixJ turnover, obtained by dividing the activity of the unliganded form by the activity of the liganded form of *Rm*FixL, for the same oxidation state. [d] In air, *Rm*FixL is 90% oxy and 10% deoxy. For oxy-*Rm*FixL, the value of k_{ph} was calculated by subtracting the contribution of deoxy-*Rm*FixL (3.6% h^{-1}) from the observed phosphorylation rate of *Rm*FixL in air (5.9% h^{-1}).

from these new measurements is that although all strong-field ligands inhibit autophosphorylation, they do not all do so equally well. For example, CN^- and O_2 both inhibited autophosphorylation 15-fold, but CO only decreased this activity 5-fold, and NO only halved the reaction rate. Imidazole was the most potent inhibitor of autophosphorylation, and activity was fully restored to protein rid of the imidazole by dialysis.

RmFixJ Turnover. In the turnover reaction, *Rm*FixL functions as a catalyst, and phosphorylation of *Rm*FixJ is measured. The regulation of this reaction provides a valuable contrast to the control of the autophosphorylation discussed above.

turnover:

$$2\ Rm\text{FixJ} + 2\ \text{ATP} \xleftrightarrow{RmFixL_2} \text{P-}Rm\text{FixJ}_2 + 2\ \text{ADP}$$

In the turnover reaction, we found deoxy-*Rm*FixL to be 100-fold more active than met-*Rm*FixL (Table 1). This is very remarkable given that (i) there is no difference in the reactivities of isolated deoxy- and met-*Rm*FixL toward ATP, and (ii) there is no discernible difference in the three-dimensional structures of the heme-binding domains of *S. meliloti* deoxy- and met-*Rm*FixL (*21*). Turnover was specifically responsive to O_2, with O_2 inhibiting more than 100-fold, and other heme ligands being only slightly inhibitory.

RmFixL Monomer−Dimer Equilibria. For several histidine kinases of the two-component class, autophosphorylation has been shown to consist of a transphosphorylation between the partner monomers in these homodimeric proteins (*22*−*25*). A simple mechanism for inhibiting *Rm*FixL autophosphorylation would be for heme ligands to alter the equilibrium dissociation constant for dimerization. We found that although *Rm*FixL autophosphorylation requires dimerization,

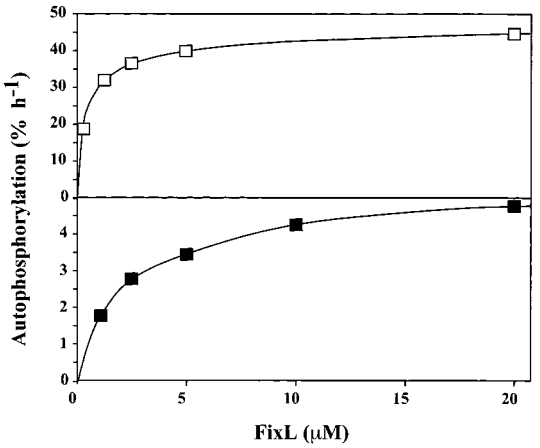

FIGURE 2: Contribution of *Rm*FixL monomer−dimer equilibria to the regulation of autophosphorylation by heme ligands. Initial rates of met- (Fe^{III}, open squares) and cyanomet- ($Fe^{III}CN^-$, closed squares) autophosphorylation were measured as a function of *Rm*FixL concentration at pH 8.0 and 23 °C. Each point represents an initial rate, determined from the slope of a linear plot of phospho-*Rm*FixL produced versus time, containing at least four points and having an R^2 value of ~ 0.99. The curves represent a fit of the data to the equation specified under Results. The reaction mixtures contained *Rm*FixL in 50 mM Tris-HCl, 50 mM KCl, 0.2 mM ATP, 0.5 mM $MnCl_2$, 5% (v/v) ethylene glycol, pH 8.0. The preparation of cyanomet-*Rm*FixL and additional experimental details are described under Experimental Procedures.

heme ligands have only a minor effect on monomer−dimer equilibria (Figure 2). We measured the *Rm*FixL concentration dependence of the autophosphorylation reaction. The expression for the fraction of total protein existing in the dimer form can be easily derived. Let L_t be the total concentration of *Rm*FixL if all the protein were monomeric, and m be the fraction of total protein actually existing as the monomer. Then $[L] = mL_t$, the dimer concentration $[L_2] = [(1 - m)/2]L_t$, and the equilibrium constant for dimer dissociation is given by

$$K_d = \frac{[L]^2}{[L_2]} = \frac{(mL_t)^2}{\left(\frac{1-m}{2}\right)L_t} = L_t\frac{2m^2}{1-m}$$

This can be rearranged to the quadratic equation

$$\frac{1}{m^2} - m - 2\frac{L_t}{K_d} = 0$$

Solving the quadratic equation for $1/m$ yields:

$$\frac{1}{m} = \frac{1 + \sqrt{1 + 8\frac{L_t}{K_d}}}{2}$$

Since the fraction of protein in the dimeric form is simply $1 - m$, the observed rate of reaction for phosphorylation that is dependent on dimerization has the form:

$$k_{obs} = v_{max}\left(1 - \frac{2}{\sqrt{1 + 8\frac{[L]}{K_d}}}\right)$$

where v_{max} is the rate at infinite concentration, (when all

protein is dimerized), and K_d is the apparent dimerization dissociation constant for *Rm*FixL.

The nonlinearity of the dependence of autophosphorylation rate on overall *Rm*FixL concentration, at very low *Rm*FixL concentrations, is fully accounted for by a model in which dimeric, but not monomeric, *Rm*FixL can autophosphorylate, and the equilibrium dissociation for FixL dimers is 0.1 μM.

We tested for contributions from a mechanism in which inhibitory heme ligands alter *Rm*FixL monomer−dimer equilibria by comparing the protein concentration dependence of the autophosphorylation of inhibited forms of *Rm*FixL to that of active forms. The results in Figure 2 indicate a dimer dissociation constant of 0.51 μM for met-*Rm*FixL and 3.8 μM for cyanomet-*Rm*FixL. At the experimental concentration of ~5 μM, met-*Rm*FixL would be 80% dimerized, whereas cyanomet-*Rm*FixL would be 54% dimerized. Therefore, if dimerization were a dominant factor in regulation, then the observed inhibition by cyanide would be 54/84, and cyanomet-*Rm*FixL would have 65% of the activity of met-*Rm*FixL. Since the observed activity of cyanomet-*Rm*FixL is only about 7% of the activity of met-*Rm*FixL (Figure 2, Table 1), the effect of ligands on monomer−dimer equilibria makes only a minor contribution to the regulation of autophosphorylation under our experimental conditions. These results are also inconsistent with formation of large aggregates as part of a regulatory mechanism (*26*, *27*).

The equilibrium dimerization constant calculated for *Rm*FixL falls within the range of K_d values (0.05−0.4 μM) reported for histidine−kinase dimerizations and is probably reasonably close to the true value (*28*, *29*). We must emphasize, however, that this experiment is not offered as a precise quantitative measurement of the dimerization constant. It serves merely to establish two qualitative results. First, at very low *Rm*FixL concentrations, dimerization equilibria have a small effect on autophosphorylation activity. Second, at normal experimental concentrations of *Rm*FixL the effects of ligands on dimerization do not have a major impact on autophosphorylation. We must also emphasize that these dimerization equilibria do not apply to the physiologically relevant *Rm*FixLJ complex but apply only to the autophosphorylation reaction that has been the basis of FixL work until now.

Influence of RmFixJ on the Ligand Affinity of RmFixL. The dramatic effects of *Rm*FixJ on *Rm*FixL regulation were not accompanied by changes in the affinity of *Rm*FixL for heme ligands. Whether or not a 10-fold excess of *Rm*FixJ was added to *Rm*FixL in phosphorylation buffer, the association and dissociation rate constants for binding of O_2 to *Rm*FixL were 0.23 ± 0.02 μM^{-1} s^{-1} and 7.2 ± 0.4 s^{-1}, respectively. An independent direct verification of O_2 affinity showed ferrous *Rm*FixL, with and without *Rm*FixJ, to be 38% saturated in 1% O_2 (99% N_2). These results indicate that the effect of *Rm*FixJ on O_2 affinity, if any, was less than 10%.

DISCUSSION

General Considerations for Enzymology of Signaling Enzymes. Signaling enzymes have distinct characteristics that pose unique challenges in the study of their enzymatic parameters. For a meaningful value of turnover number, it is necessary to carry out reactions with a substantial excess of all substrates over enzyme, preferably substrate concentrations that are large compared to the K_m values for those substrates. These high concentrations ensure that the enzyme is measured at its maximum velocity, and that, at least for early reaction times, the substrate concentrations remain essentially constant. For typical metabolic two-substrate enzymes**,** the conditions that allow for quantitative studies are happily compatible with physiological conditions. This is not at all the case for enzymes that function in signaling instead of metabolism. In vivo, the response-regulator protein substrates of the histidine−protein kinases exist in very low abundance, comparable to the concentration of the enzyme itself. In addition, the amount of "product" that a signaling molecule needs to generate to satisfy its function is vastly lower than required of metabolic enzymes. This is especially true if the product only needs to titrate a single DNA molecule to regulate gene expression. Thus, we may find units of turnover that are in hundreds per hour, rather than millions per second. To determine ligand regulation of individual steps of signal-transducing phosphorylations, it becomes necessary to measure these reactions for active and inhibited forms under conditions that are nonphysiological. The challenge then becomes the modeling of physiological processes using these fundamental rate constants.

Shortcomings of the Ping-Pong Bi-Bi Scheme for Phosphoryl Transfer in the RmFixL/RmFixJ System. If the *Rm*FixL enzyme is given its two substrates, ATP and *Rm*FixJ, this enzyme forms the products ADP and phospho-*Rm*FixJ (*10*). Until now, this phosphoryl transfer has been treated as a classical "ping-pong bi-bi" enzymatic reaction, in which the first substrate, ATP, covalently modifies the *Rm*FixL enzyme by transferring a chemical group, and subsequently the second substrate, *Rm*FixJ, displaces the group from the enzyme, restoring the active form of the enzyme. In such a reaction scheme, the two substrates react with the enzyme independently and without ever encountering or influencing each other. By introducing ATP first and then completely removing the unreacted ATP by gel filtration before introducing *Rm*FixJ, we can force *Rm*FixL to react by a ping-pong bi-bi mechanism. Under those conditions, *Rm*FixL is clearly capable of the net transfer of a phosphoryl group from ATP to *Rm*FixJ (*10*, *11*). Since both substrates are never present simultaneously, ping-pong bi-bi is the only permitted mechanism. However, this forced ping-pong bi-bi reaction is very inefficient. For example, in a coordinated mixture of *Rm*FixL, *Rm*FixJ, and ATP the net phosphoryl transfer to *Rm*FixJ is vastly more efficient than the sum of the separate reaction steps, with rate constants orders of magnitude larger (*12*). Therefore, it appeared that, unless they are artificially prevented from doing so, the two substrates of *Rm*FixL work closely together. Our current results show that not only the rates, but also the regulation of the reactions, depend on the mode of substrate presentation. For example, although neither step of the forced ping-pong bi-bi reaction in Scheme 1 was responsive to the oxidation state of the *Rm*FixL heme iron, the turnover reaction was strongly sensitive to oxidation state (Table 1). Although the phosphoryl transfer to *Rm*FixJ in Scheme 1, step 2 was unregulated, the heme regulation of overall turnover was different from the regulated ping-pong bi-bi step in Scheme 1, step 1. Those observations imply that *Rm*FixJ, previously thought to associate only with the *Rm*FixL kinase domain, also interacts with the *Rm*FixL heme-binding domain.

Ligand Specificity of the Reaction of Isolated RmFixL with ATP. We quantitated for the first time the residual activities of several liganded *Rm*FixL species with ATP (Table 1). Interestingly, even with saturating concentrations of ligand, there was a broad range of inhibition. Imidazole was the most potent inhibitor of the reaction of *Rm*FixL with ATP. In practice, the activity of the imidazolemet-*Rm*FixL was too low to be precisely measured, and the inhibition factor was estimated as being greater than 75-fold. Nonetheless, inhibition by imidazole was reversible, with full activity being restored after removal of this ligand by dialysis. Cyanide and O_2 were intermediate, with a 15-fold inhibition. Nitric oxide was a poor regulator, with an inhibition factor of only 2-fold.

O_2-Specific Regulation of RmFixJ Turnover. A special factor is clearly at work in the O_2 inhibition of phosphorylation. For regulation of *Rm*FixJ turnover, O_2 was by far the most potent inhibitor (Table 1). Indeed, oxy-*Rm*FixL activity was so feeble that an inhibition factor could only be estimated as being greater than 100. The protein was verified to be ferrous from its optical spectra, and full activity could be restored by removing O_2 with a stream of nitrogen.

Oxidation-State Specific Regulation of RmFixJ Turnover. In the absence of heme ligands, oxidation of the *Rm*FixL heme iron from Fe^{II} to Fe^{III} did not influence either the first or the second phosphorylation step of the sequential scheme (Scheme 1, Table 1). If the overall *Rm*FixL-catalyzed transfer of a phosphoryl group from ATP to *Rm*FixJ were a simple combination of these two oxidation-state independent processes, the overall transfer reaction would also be oxidation-state independent. Surprisingly, the turnover of *Rm*FixJ was very sensitive to the oxidation state of the *Rm*FixL heme iron (Table 1). With *Rm*FixJ and ATP at saturating concentrations, the rate of *Rm*FixJ turnover to phospho-*Rm*FixJ dropped more than 100-fold on oxidation of deoxy-*Rm*FixL to met-*Rm*FixL. This effect was entirely reversible, with a 100-fold rise in activity being observed on reduction of the met-*Rm*FixL back to deoxy-*Rm*FixL. Since *Rm*FixL is always ferrous in vivo, a redox switch is unlikely to have physiological relevance. Nonetheless, any proposed mechanism must account for this feature.

Implications of the Measured Inhibition Factors for Structural Models. The essence of a heme-based sensor is to regulate an activity in response to heme ligands. Therefore, a logical and necessary first step in understanding such a sensor is to quantitate its response to ligands. After characterizing this response, one can attempt to devise structural models to account for the observed sensitivity. For FixL, the order of events has been reversed. Structural models have sought to explain ligand sensitivities that have, for the most part, been assumed rather than measured. In some instances, ligand responses have been proposed based solely on structural models and not on any actual measurements of activity. Rather than offer additional models, below we critique the existing structural models on the basis of their success in accounting for fundamental, directly observed characteristics of this sensing system.

The Spin-State Hypothesis. The first structural model proposed for sensing by FixL was the "spin-state" hypothesis (*13*). This hypothesis was based on the following observations:

(i) Quantitative measurements of autophosphorylation showed both high-spin unliganded forms of FixL (met and deoxy), as well as the high-spin liganded form fluoro-FixL, to have the same activity.

(ii) Qualitative measurements of the autophosphorylation of cyanomet-FixL, imidazolemet-FixL, and carbonmonoxy-FixL showed these low-spin forms to be inhibited.

On the basis of these observations, the flattening of the heme induced by a switch of the iron atom to low spin was proposed to inactivate FixL reversibly, and the out-of-plane distortions of the heme in high-spin forms were proposed to permit enzymatic activity. The trigger for the spin-state induced conformational change was envisioned to be the movement of the iron atom into the heme plane, and a concurrent movement of the proximal histidine, in a manner analogous to the spin-induced conformational change that triggers the *T* to *R* transition in hemoglobin (*30*).

Accurate and precise quantitation of the enzymatic activities of inhibited forms of FixL have required greater sensitivity than the measurement of active forms. It is only now that we can see that although all low-spin forms are indeed inhibited, they are not all inhibited to the same extent. The current quantitations of autophosphorylation in Table 1 fully confirm the qualitative observations that formed the basis of the spin-state hypothesis (*13*). On the other hand, those measurements also show that the spin-state hypothesis is insufficient to account for the wide difference in inhibition factors for different ligands. Any new structural model for FixL sensing should provide for at least two levels of inhibition: one that results in a modest (2- to 5-fold) inhibition by all heme ligands except fluoride, and another that causes the enhanced inhibition by O_2 and CN^-. Oxygen and CN^- both result in highly polar iron−histidine bonds. For oxy-*Bj*FixL, the well-conserved G_β-2 arginine (R220) breaks a salt bridge to a heme propionate and moves into the heme pocket to form a hydrogen bond to the bound O_2. Since no hydrogen bond would form to apolar ligands, a similar movement by the G_β-2 arginine in (R214) *Rm*FixL could very plausibly serve as the basis for enhanced inhibition by O_2 and CN^-.

Other Hypotheses about Ligand-Induced Conformational Changes in FixL. The crystal structures of oxy-*Bj*FixLH and cyanomet-*Bj*FixLH have shown a roughly 2-Å movement of a loop, i.e., the FG loop, relative to its position in the unliganded ferric form met-*Bj*FixLH (*14*, *31*). On the basis of this movement, a "loop-displacement hypothesis" has been proposed for FixL regulation. Although the details of how ligands trigger this putative "off" conformation have been viewed as the most controversial aspect of this hypothesis, it is vastly more worthwhile to consider the more fundamental question of whether this change in the structures of crystallized heme-binding domains is in fact related to an inhibition of kinase activity. At least three limitations of the available structural models must be kept in mind. First, all structures have been restricted to the isolated heme-binding domains. Second, all FixL derivatives for crystal structure determination have been prepared from crystals of the unliganded ferric forms. Third, not all unliganded and liganded derivatives are obtainable for the most appropriate comparisons. Currently, there are crystal structures for presumed "on" states of *Rm*FixLH (met and deoxy), but there is not yet a structure for any liganded "off" state of this

domain (*21*). Consequently, the nature of the conformational change induced by ligands in *Rm*FixLH is unknown. For ferric *Bj*FixLH, structures are known for the presumed "on" (met) as well as "off" states (cyanomet and imidazolemet) (*14*, *30*). On the other hand, for ferrous *Bj*FixLH, a structure is available only for an "off" state (oxy-*Bj*FixLH) but not the corresponding deoxy-*Bj*FixLH "on" state (*30*). Consequently, the regulatory conformational changes of ferrous *Bj*FixLH have had to be inferred from comparison of a ferrous "off" state (oxy) to a ferric "on" states (met).

At least four lines of evidence indicate that the structures of isolated heme-binding domains do not adequately reflect the ligand-induced conformational changes in a FixL/FixJ complex or even in intact FixL.

(i) The structures of isolated heme-binding domains differ from those of the corresponding domain in intact FixL. For example, the crystal structure of NO-bound ferrous *Bj*FixLH shows no sign of a putative "off" conformation (*30*). Nevertheless, direct measurements of activity show that NO inhibits *Bj*FixL autophosphorylation to approximately the same extent as reported here for *Rm*FixL (unpublished results of J. Tuckerman and M.-A. Gilles-Gonzalez).

(ii) In full-length FixL, the kinase domain has been reported to constrain the distal side of the heme. Comparison of the resonance Raman spectra of carbonmonoxy forms of *Rm*FixLH and *Rm*FixL indicate that the kinase domain in the latter restricts movement of the distal side of the heme (*27*). If so, then removal of the kinase domain must permit conformations of the heme-binding domain that are normally inaccessible to the full-length protein.

(iii) A ligand-induced movement, shown to occur in solution, cannot be observed in structures of *Rm*FixLH or *Bj*FixLH. The crystal structure of *Rm*FixLH has shown that a helix in this domain, i.e., helix II, contains the only contacts between the partner monomers in a dimer of heme-binding domains (*21*). *Rm*FixLH crystallizes as a dimer, and the deoxy form is dimeric in solution (*21*, *32*). In contrast *Bj*FixLH, which lacks helix II, crystallizes as a monomer, and all forms of *Bj*FixLH are monomeric in solution (*14*, *16*). If, as reported, deoxy-*Rm*FixLH is dimeric and oxy-*Rm*FixLH is monomeric, then helix II must undergo a significant conformational change when O_2 binds to the domain. Changes in helix II are impossible to observe in liganded *Bj*FixLH structures because they lack the peptide that forms this helix. Those conformational changes cannot be observed for *Rm*FixLH because liganded structures cannot be prepared from the crystals. If a significant movement of helix II must accompany binding of inhibitory ligands, then constraint of this helix from movement by the crystal lattice would interfere with ligand binding. Removal of the helix would facilitate binding of ligands, but it would also abolish key features of the conformational change.

(iv) Presence of FixJ alters the structure of the heme-PAS in FixL. The profound effect of *Rm*FixJ on sensing of heme status by *Rm*FixL leads to the inevitable conclusion that in the *Rm*FixLJ complex the structure of the heme-binding domain must differ significantly from its structure in *Rm*FixL without *Rm*FixJ (Table 1). Therefore, even a structure of a complete FixL, with its kinase domain, would not reveal all of the conformational changes near the heme.

A New Role for RmFixJ in O_2 Signal Transduction. In the sequential phosphoryl transfer scheme, the only step involving *Rm*FixJ, i.e., the phosphoryl transfer from phospho-*Rm*FixL to *Rm*FixJ (Scheme 1, step 2), shows no sign of heme-ligand dependence. In the alternative scheme, both phosphoryl transfers (Scheme 2, steps 2 and 3) occur in the presence of *Rm*FixJ, allowing the possibility that *Rm*FixJ could influence its own rate of phosphorylation as well as the rate of *Rm*FixL phosphorylation. It seems unlikely that the phosphoryl transfer to *Rm*FixJ would be ligand dependent in Scheme 2 but not in Scheme 1. We therefore favor the theory that the dramatic impact of *Rm*FixJ on regulation by heme ligands reflects a ligand-dependent effect of *Rm*FixJ on the rate of reaction of *Rm*FixL with ATP. Our results showing that binding of O_2 is unchanged in *Rm*FixLJ compared to *Rm*FixL imply that, in the *Rm*FixLJ complex, *Rm*FixJ perturbs the structure of the heme-binding domain of *Rm*FixL in a way that affects regulation but not the affinity for heme ligands. Interestingly, a more obvious case of a sensing complex already exists in nature as the FixL protein from *Rhizobium leguminosarum (33)*. The *R. leguminosarum* FixL normally contains, fused within the same polypeptide, all FixL domains plus the phosphorylatable "receiver" domain that is usually in FixJ proteins.

REFERENCES

1. Gilles-Gonzalez, M. A. (2001) *IUBMB Life 51*, 165−173.
2. Soupene, E., Foussard, M., Boistard, P., Truchet, G., and Batut, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3759−3763.
3. Gilles-Gonzalez, M. A., Ditta, G. S., and Helinski, D. R. (1991) *Nature 350*, 170−172.
4. Agron, P. G., Ditta, G. S. and Helinski, D. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3506−3510.
5. Reyrat, J. M., David, M., Blonski, C., Boistard, P., and Batut, J. (1993) *J. Bacteriol. 175*, 6867−6872.
6. Galinier, A., Garnerone, A.-M., Reyrat, J.-M., Kahn, D., Batut, J., and Boistard (1994) *J. Biol. Chem. 269*, 23784−23789.
7. David, M., Daveran, M.-L., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P., and Kahn, D. (1988) *Cell 54*, 671−683.
8. Parkinson, J. S., and Kofoid, E. C. (1992) *Annu. Rev. Genet. 26*, 71−112.
9. Aizawa, S.-I., Harwood, C. S., and Kadner, R. J. (2000) *J. Bacteriol. 182*, 1459−1471.
10. Gilles-Gonzalez, M. A. and Gonzalez, G. (1993) *J. Biol. Chem. 268*, 16293−16297.
11. Lois, A. F., Weinstein, M., Ditta, G. S., and Helinski, D. R. (1993) *J. Biol. Chem. 268*, 4370−4375.
12. Tuckerman, J. R., Gonzalez, G., and Gilles-Gonzalez, M. A. (2001) *J. Mol. Biol. 308*, 449−455.
13. Gilles-Gonzalez, M. A., Gonzalez, G., and Perutz, M. F. (1995) *Biochemistry 34*, 232−236.
14. Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 15177−15182.
15. Taylor, B. L. and Zhulin, I. B. (1999) *Microbiol. Mol. Biol. Rev. 63*, 479−506.
16. Gilles-Gonzalez, M. A., Gonzalez, G., Perutz, M. F., Kiger, L., Marden, M. C., and Poyart, C. (1994) *Biochemistry 33*, 8067−8073.
17. Winkler, W. C., Gonzalez, G., Wittenberg, J. B., Hille, R., Dakappagari, N., Jacob, A., Gonzalez, L. A., and Gilles-Gonzalez, M. A. (1996) *Chem. Biol. 3*, 841−850.
18. Mansy, S. S., Olson, J. S., Gonzalez, G., and Gilles-Gonzalez, M. A. (1998) *Biochemistry 37*, 12452−12457.
19. Bertolucci, C., Ming, L.-J., Gonzalez, G., and Gilles-Gonzalez, M. A. (1996) *Chem. Biol. 3*, 561−566.
20. Laemmli, U. K. (1970) *Nature 227*, 680−685.

21. Miyatake, H., Masahiro, M., Park, S.-Y., Adachi, S.-I., Tamura, K., Nakamura, H., Nakamura, K., Tsuchiya, T., Iizuka, T., and Shiro, Y. (2000) *J. Mol. Biol. 301*, 415−431.
22. Yang, Y. and Inouye, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11057−11061.
23. Ninfa, E. G., Atkinson, M. R., Kamberov, E. S., and Ninfa, A. J. (1993) *J. Bacteriol. 175*, 7024−7032.
24. Swanson, R. V., Bourret, R. B., and Simon, M. I. (1993) *Mol. Microbiol. 8*, 435−441.
25. Jourlin. C., Ansaldi, M., and Mejean, V. (1997) *J. Mol. Biol. 267*, 770−777.
26. Miyatake, H., Kanai, M., Adachi, S., Nakamura, H., Tamura, K., Tanida, H., Tsuchiya, T., Iizuka, T., and Shiro, Y. (1999) *Acta Crystallogr. D 55*, 1215−1218.
27. Miyatake, H., Masahiro, M., Adachi, S.-I., Nakamura, H., Tamura, K., Iizuka, T., Shiro, Y., Strange, R. W., and Hasnain, S. S. (1999) *J. Biol. Chem. 274*, 23176−23184.
28. Surette, M. G., Levit, M., Liu, Y., Lukat, G., Ninfa, E. G., Ninfa, A., and Stock, J. B. (1996) *J. Biol. Chem. 271*, 939−945.
29. Stewart, R. C., VanBruggen, R., Ellefson, D. D., and Wolfe, A. J. (1998) *Biochemistry 37*, 12269−12279.
30. Perutz, M. F., Sanders, J. K, Chenery, D. H., Noble, R. W., Pennelly, R. R., Fung, L. W., Ho, C., Giannini, I., Porschke, D., and Winkler, H. (1978) *Biochemistry 17*, 3640−3652.
31. Gong W., Hao B., and Chan M. K. (2000) *Biochemistry 39*, 3955−3962.
32. Rodgers, K. R., Tang, L., Lukat-Rodgers, G. S., and Wengenack, N. L. (2001) *Biochemistry 40*, 12932−12942.
33. Patschkowski, T., Schluter, A., and Priefer, U. B. (1996) *Mol. Microbiol. 21*, 267−280.

BI025628W