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A Memory of Oxygen Binding Explains the Dose Response of the Heme-Based Sensor FixL[†]

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ABSTRACT: *Bradyrhizobium japonicum* FixL is a modular oxygen sensor that directs adaptations to hypoxia by coupling the status of a heme-binding domain to a histidine-protein kinase activity. The oxygen-bound form is the “off-state”. The unliganded form is the “on-state” active kinase that phosphorylates a transcription factor, FixJ. We have developed methods to optimize the kinase reactions of FixL and measure the turnover rates (k_{cat}) for reactions catalyzed by highly inhibited states of this sensor at constant, precisely known oxygen saturations. The resulting oxygen dose–response curve shows that an in vitro system with FixL and the response regulator FixJ as its only proteins manifests such a sharp ligand response that, when the proportion of deoxy-FixL decreases less than 3-fold, the kinase activity drops over 50-fold, and by the time the deoxy-FixL declines just 8-fold, the activity is inhibited over 1100-fold. This response is entirely reversible and similar to that reported for the in vivo hypoxic induction of FixLJ-regulated genes. FixL binds oxygen noncooperatively. When complexed with FixJ, FixL is dimeric in both oxy and deoxy states. Therefore traditional models involving cooperative binding of ligand or robust allosteric regulation cannot account for the extremely nonlinear kinase response to the heme saturation. This response, however, can be explained by a form of enzyme hysteresis with the simple assumptions that (i) on association of oxygen with the heme, the kinase is rapidly switched off; (ii) after dissociation of oxygen, the kinase remains inhibited longer than the average time that it takes a deoxy-heme to encounter an oxygen molecule at most oxygen saturations.

The FixL protein provides a prototype for signal transduction in response to small molecules, such as diatomic gases (1). In this homodimeric and modular sensor an O₂-detecting heme-binding domain controls a protein-histidine-kinase region (1–3). The unliganded form of FixL transfers the γ -phosphoryl group from ATP to an aspartyl side chain in the transcription factor FixJ, and binding of O₂ to the heme reversibly switches off the FixL kinase activity (3–8). When phosphorylated, FixJ activates the transcription of target genes essential to hypoxic processes, such as respiration in low O₂ and rhizobial nitrogen fixation (9–18). The mechanism of kinase regulation by O₂ is not fully understood, but O₂ is known to inhibit the formation of a reaction intermediate in which the FixL enzyme is phosphorylated (3–6). Binding of O₂ does not reduce the affinity of FixL for either its ATP or FixJ substrate, nor does it affect the oligomeric state of FixL, or cause either FixL or FixJ to become dephosphorylated (5, 6).

As a sensory kinase, FixL is unusual in having a well-known regulatory ligand, well-established kinetic and equilibrium parameters (k_{on} , k_{off} , and K_{d}) for ligand binding, and a measurable kinase response to ligand regulation. The K_{d}

values of 140 μM measured for binding of O₂ to *Bj*FixL¹ and 50 μM for binding to *Rm*FixL are consistent with the O₂ tensions at which these two proteins switch on the genes they control in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*, respectively (19). On the other hand, the noncooperative binding of O₂ to all FixL proteins should cause a very gradual increase in saturation with rising O₂ tensions, and this is inconsistent with the reported sharp physiological responses caused by these proteins (20, 21). In air, *Bj*FixL would be expected to retain over one-third of its activity if the kinase activity were simply proportional to the deoxy fraction of protein. The maximum possible allosteric effect would result if a single O₂ molecule would switch off a FixL dimer, so that “oxy–deoxy” as well as “oxy–oxy” dimers would be inactive. Even if the “deoxy–deoxy” FixL dimers were the only species to remain active, however, *Bj*FixL in air would possess 13% of its anaerobic activity. By contrast to these predictions from ligand binding, in vivo a quite low basal activity is noted for target promoters in air, and a sharp, more than 30-fold rise is observed in the expression of FixLJ-regulated genes as the O₂ tensions drop to near the K_{d} value for binding of O₂ (20, 21).

Measurement of an accurate in vitro dose response has not been possible for FixL until now for mainly two reasons.

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¹ Abbreviations: *Bj*FixL, full-length *Bradyrhizobium japonicum* FixL; *Rm*FixL, *Sinorhizobium meliloti* FixL_{119–505}, also called *Rm*FixL_T; deoxy, Fe^{II} without ligand; oxy, Fe^{II}O₂; carbonmonoxy, Fe^{II}CO; DTT, dithiothreitol.

On exposure to O_2 , ferrous FixL (Fe^{II}) is oxidized rapidly to the ferric (Fe^{III}) state, and this oxidation accelerates at lower saturations with O_2 (19, 22). Second, FixL, like other sensory histidine kinases, naturally possesses a rather low enzymatic activity. The purpose of a metabolic kinase, e.g., phosphofructokinase, is maximal turnover of a substrate, whereas that of a signaling kinase such as FixL is to phosphorylate relatively few molecules of a transcription factor at precisely controlled rates. This feature presented a challenge to measuring the activities of inhibited states, although it agrees with FixL's function as a signaling enzyme. Herein we examine the O_2 dose response of FixL in a well-defined biochemical system *in vitro*, and we present the methods for protecting FixL from oxidation and for optimizing the enzymatic activity that enabled those measurements. We formulate a simple model to explain this response based on measurable characteristics of FixL and only one fitted parameter.

MATERIALS AND METHODS

Gene Expression and Protein Purifications. The procedures for overexpressing the *BjfixL* and *BjfixJ* genes and purifying the corresponding proteins have been described (19).

Preparation of Deoxy-FixL. Homogeneous deoxy-FixL was prepared by reducing a degassed and concentrated stock of the protein with 10 mM DTT for more than 10 min inside an anaerobic chamber (Coy Laboratory Products, Inc.) while monitoring the 350–700 nm absorption with a UV–vis spectrophotometer (Cary 4000, Varian). Subsequent assays of the deoxy state were done in the anaerobic chamber.

Preparation of Carbonmonoxy-FixL and Measurement of CO Affinity. To prepare carbonmonoxy-FixL, the deoxy protein described above was diluted 10-fold with CO-saturated TKE buffer [50 mM Tris-HCl, 50 mM KCl, 5.0% (v/v) ethylene glycol, pH 8.0] inside an anaerobic chamber, and the 350–700 nm absorption was monitored. For determinations of affinity, the deoxy-FixL in a stoppered cuvette was titrated with 0, 1.2, 2.4, 4.8, 9.5, and 32 μ M CO in TKE buffer, at 25 °C inside the anaerobic chamber. The K_d value for binding of CO under phosphorylation-assay conditions was determined from saturation changes calculated by multiple linear regression analysis of whole spectra.

Stable 12% to 64% O_2 Saturation of Ferrous FixL. Binding of CO to ferrous FixL protects the heme center from oxidation and has little effect on the FixL kinase activity. Taking advantage of these desirable properties of CO, we prepared our fractionally saturated oxy-FixL in O_2 /CO mixtures. Specifically, ferrous FixL was mixed with 24 μ M CO in TKE buffer containing 1.0 mM DTT. Oxygen-saturated TKE buffer was added to this mixture to yield final O_2 concentrations of 200, 600, and 940 μ M, which corresponded to 12%, 28%, and 38% saturation with O_2 , respectively, with the balance consisting principally of carbonmonoxy-FixL. The saturation of FixL with O_2 and CO in these mixtures, verified by multiple linear regression analysis of whole 350–700 nm absorption spectra, agreed with the calculations of the $K_d(CO)$ under assay conditions. An example of such an analysis is shown in Figure 1. No oxidation was detected for over 45 min.

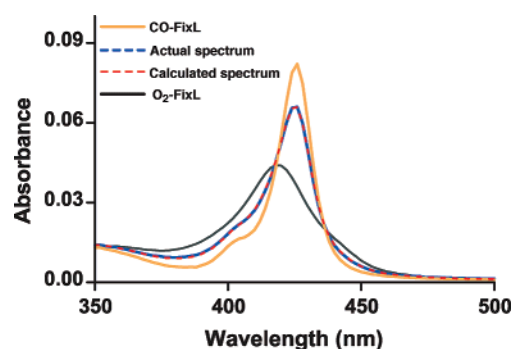


FIGURE 1: Determination of FixL saturation with O_2 in defined mixtures of O_2 and CO at pH 8.0 and 25 °C. Example of the analysis of oxy- and carbonmonoxy-*BjFixL* mixtures that result from adding O_2 and CO to ferrous *BjFixL*. Here, a mixture of 1.2 mM O_2 and 21 μ M CO results in 58% carbonmonoxy-*BjFixL* and 42% oxy-*BjFixL* (true spectrum, blue), as determined from a linear combination of the absorption spectra (calculated spectrum, red) of pure carbonmonoxy-FixL (orange) and pure oxy-FixL (black). In addition to *BjFixL*, the mixture contained 25 μ M *BjFixJ*, 1.0 mM ATP/MgCl₂, 50 μ M MnCl₂, 50 mM Tris-HCl, 50 mM KCl, 5.0% (v/v) ethylene glycol, and 1.0 mM DTT at pH 8.0.

As a validation of our use of O_2 /CO mixtures, we compared the activities of 64% O_2 -saturated *BjFixL* prepared in air to 64% O_2 -saturated *BjFixL* prepared in a background of CO. A slightly slower turnover in the O_2 /CO mixture compared to air (O_2/N_2) could be entirely attributed, within the experimental error, to the slightly lower activity of carbonmonoxy-*BjFixL* compared to the deoxy form (see Results).

Stable Equilibration of Ferrous FixL with 1 atm of O_2 . Deoxy-FixL in TKE buffer containing 10 mM DTT was gently and continuously mixed with pure O_2 . During the phosphorylations in 1 atm of O_2 , a stream of pure O_2 was maintained perpendicularly over the reaction mixture inside a container. From the 350–700 nm absorption spectra routinely collected throughout the reaction, oxidation was insignificant for over 30 min.

Assays of Phosphorylation. These assays determined the rates of conversion of *BjFixJ* to phospho-*BjFixJ* by deoxy-, oxy-, or carbonmonoxy-*BjFixL*, or mixtures of these species. Before and after every assay, the heme state was verified from the 350–700 nm absorption spectra of the reaction mixture to ensure that it was unchanged. The reactions were at 23 °C and contained 1 μ M FixL, 25 μ M FixJ, and 1 mM ATP/MgCl₂ in “phosphorylation buffer” [i.e., unlabeled ATP from Sigma and γ -(³²P)-ATP from Amersham Pharmacia Biotech, of specific activity 0.42 Ci/mmol, in 50 mM Tris-HCl, 50 mM KCl, 5% (v/v) ethylene glycol, 50 μ M MnCl₂, pH 8.0]. Under these conditions, where FixL is complexed with FixJ, the FixL is dimeric in unliganded as well as liganded states. Reactions were begun by introducing the ATP and stopped at 1.0, 2.5, 5.0, 10, 20, and 30 min by mixing 10 μ L aliquots of the reaction mixtures with one-third volume of “stop buffer” [40 mM EDTA, 4% (w/v) sodium dodecyl sulfate, 0.50 M Tris-HCl, 0.20 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 (23). The levels of phosphorylated protein in the dried gels were quantified with a phosphorimager (Bio-Rad Personal Molecular Imager FX).

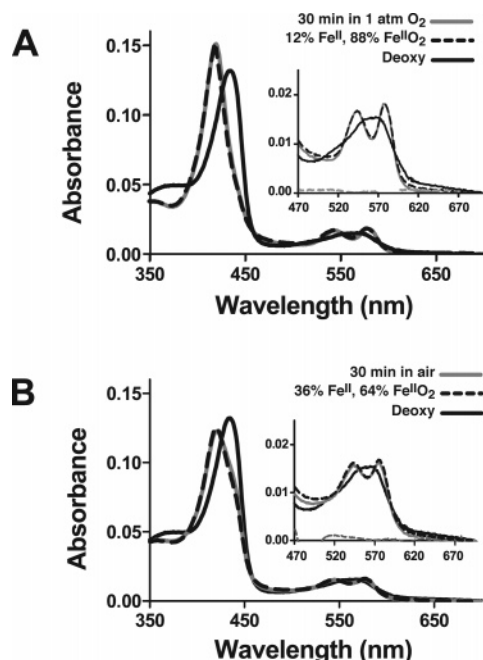


FIGURE 2: Stable saturation of FixL with O_2 in air or pure O_2 under reaction conditions. Panel A shows the absorption spectra of 1.2 μM *Bj*FixL before (solid black curve) and 30 min after (solid gray curve) mixing the deoxy form with an O_2 -saturated buffer having all the reaction components at pH 8.0 and 25 $^{\circ}C$; a synthetic spectrum of 12% deoxy- and 88% oxy-FixL is also shown (dashed black curve). Panel B shows the absorption spectra of 1.2 μM *Bj*FixL before (solid black curve) and after (solid gray curve) mixing the deoxy form with an air-saturated buffer containing the same reaction components; a synthetic spectrum of 36% deoxy and 64% oxy-FixL is also shown (dashed black curve). The insets show a 6-fold expansion of the 470–700 nm region and the difference obtained on subtracting spectra of O_2 -exposed *Bj*FixL recorded at a 30 min interval (dashed gray curve). In addition to *Bj*FixL, the reaction mixtures contained 25 μM *Bj*FixJ, 1.0 mM ATP/MgCl₂, 50 μM MnCl₂, 50 mM Tris-HCl pH 8.0, 50 mM KCl, 5.0% (v/v) ethylene glycol, and 10 mM DTT in vessels open to a continuous reservoir of O_2 in panel A, or air in panel B. Since *Bj*FixL is not saturable at atmospheric pressure but shows spectra identical to *Rm*FixL in partially saturated mixtures, the oxy-*Bj*FixL basis spectrum was estimated by normalizing spectra of pure deoxy-*Bj*FixL with deoxy-*Rm*FixL and converting the deoxy-*Rm*FixL sample to the oxy state. *Rm*FixL is 96% saturated in pure O_2 and therefore serves as an acceptable approximation to the oxy-*Bj*FixL spectrum, with the true O_2 saturations being only slightly lower than those calculated by spectral deconvolution.

RESULTS

Controlling the O_2 Saturation during Activity Measurements. We used two approaches to circumvent the oxidation of FixL during exposure to O_2 . At or above air saturation, a continuous reservoir of gas was supplied together with a mild reducing agent (e.g., 10 mM DTT) to prevent changes in the heme-iron saturation and oxidation state (Figure 2). At O_2 tensions below the air saturation, for which the reactions required sealed atmospheres, oxidation was prevented by substituting carbonmonoxy-*Bj*FixL for deoxy-*Bj*FixL as the active protein fraction. Although CO does slightly inhibit *Bj*FixL, the kinase activity of the ferrous FixL in CO solutions is linear with respect to the saturation with CO. The turnover rate (k_{cat}) is 1.1 min⁻¹ for the carbonmonoxy fraction (70% of the activity of the deoxy form) regardless of the varying saturations of the preparations (Figure 3A).

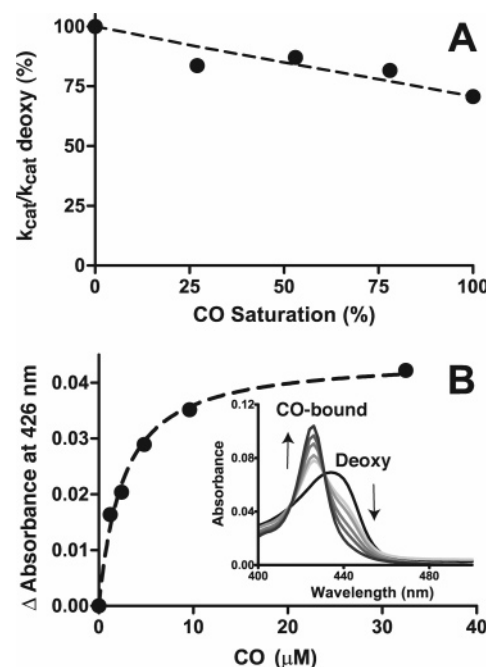


FIGURE 3: Binding of CO to FixL under assay conditions and influence of CO on the kinase activity. Panel A shows the turnover of *Bj*FixJ (25 μM) to phospho-*Bj*FixJ by 0%, 27%, 53%, 78%, and 100% CO-saturated *Bj*FixL (1 μM) at pH 8.0 and 23 $^{\circ}C$. Initial rates were obtained by fitting to a single exponential phosphorylation curves ($R^2 > 0.988$) having 0, 1.0, 2.5, 5.0, 10, 20, and 30 min time points. The reported k_{cat}/k_{cat} deoxy percent (or relative turnover percent) represents these initial rates of turnover divided by the value measured for the deoxy form. Note the linear trend and relatively poor inhibition by CO, with 70% activity remaining for the fully saturated protein. Panel B shows the titration of *Bj*FixL (0.5 μM) with 0, 1.2, 2.4, 4.8, 9.5, and 32 μM CO at pH 8.0 and 25 $^{\circ}C$. The dashed line shows a fit to a quadratic single binding site equation ($R^2 = 0.9927$); the inset shows the 400–500 nm absorption changes on conversion of the deoxy form ($\lambda_{max} = 434$ nm) to the carbonmonoxy form ($\lambda_{max} = 425$ nm). Additional buffer components were 1.0 mM ATP/MgCl₂, 50 μM MnCl₂, 50 mM Tris-HCl pH 8.0, 50 mM KCl, 5.0% (v/v) ethylene glycol, and 10 mM DTT.

Our analysis of FixL in O_2 /CO mixtures therefore treats the carbonmonoxy-FixL as a slightly less active deoxy species.

Verifying a Stable O_2 Saturation throughout a Reaction. The heme-state stability was routinely verified by recording an absorption spectrum at the start of each reaction, “zeroing” the spectrophotometer on this mixture, and collecting difference spectra over the reaction time course (~ 30 min for low-activity states). For example, the absence of anything but noise from the insets to Figure 2 shows that, for *Bj*FixL exposed to air or pure O_2 , no change occurred during at least 30 min in the O_2 concentration, in the O_2 affinity due to reactants or reaction products, or in the oxidation state. Deconvolution of the spectra of *Bj*FixL in the phosphorylation assay mixtures showed this protein to be 64% saturated with O_2 while in air and 88% saturated when in 1 atm of O_2 . These are the saturations expected from the published K_d value for binding of O_2 to *Bj*FixL (19). Although the current study treats *Bj*FixL, we also examined *Rm*FixL and found its affinity for O_2 to be likewise unaffected by the phosphorylation reaction components and products (see Discussion).

To ensure that the proportion of oxy-FixL remained constant during the reaction time courses done in mixtures

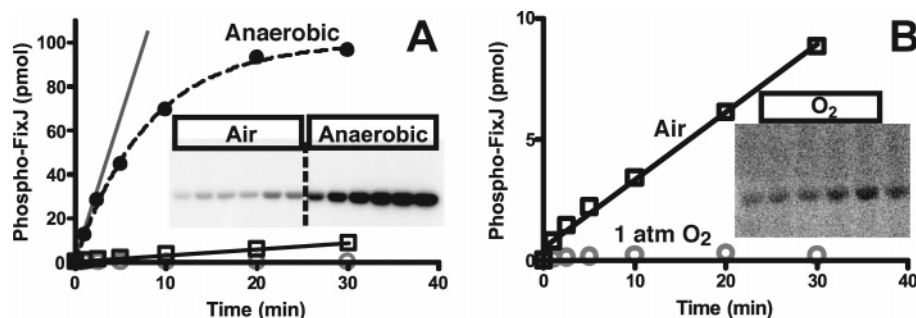


FIGURE 4: Comparison of the activities of FixL under nitrogen, air, and oxygen at pH 8.0 at 23 °C. Reactions of 1 μ M *Bj*FixL with 25 μ M *Bj*FixJ and 1 mM γ -[32 P]-labeled ATP were carried out at 23 °C in a chamber with an atmosphere of pure nitrogen (panel A, closed circles), in air (panels A and B, squares), or in a container supplied with a continuously flowing atmosphere of pure O₂ (panels A and B open circles, and labeled O₂ in panel B). The anaerobic activity was calculated by fitting the whole curve to a first-order kinetics equation and taking the initial rate to be the initial linear phase of the curve (panel A, gray line). For closer inspection of the results in air and pure O₂, a 10-fold expansion of the ordinate is provided together with the autoradiograph of a time course under O₂ (panel B). Additional components of the reaction mixture were 50 mM Tris-HCl, 50 mM KCl, 1.0 mM MgCl₂, 50 μ M MnCl₂, 5.0% (v/v) ethylene glycol, and 10 mM DTT at pH 8.0. Reactions time points were 1.0, 2.5, 5.0, 10, 20, and 30 min. Note that the reactions contained 200 pmol of *Bj*FixJ, one-half of which was phosphorylated at equilibrium (panel A, closed circles). The higher background in the gel of reactions done under O₂ (panel B) results from the requirement of a longer exposure to show the linear phase of reaction for the \sim 1000-fold less active protein.

of O₂ and CO, absorption spectra were collected for the assay mixtures at the start and end of the experiments and compared, as above. The proportions of oxy- and carbonmonoxy-protein in the assay mixtures were quantified by multiple linear regression analyses of whole spectra, as shown in the example in Figure 1. The CO affinity ($K_d = 2.1 \mu$ M) of *Bj*FixL under the enzymatic assay conditions was entirely consistent with the proportions of oxy and carbonmonoxy protein directly observed in defined concentrations of O₂ and CO (Figure 3B).

A Quantitative Measure of FixL Regulation. Here we report the phosphorylation activities of all FixL species as their turnover rates, or k_{cat} .



These rates represent the number of FixJ molecules that one molecule of a specified form of FixL can convert to phospho-FixJ per minute, while the FixL is saturated with both of its substrates, FixJ and ATP. Such an analysis required a large excess of both substrates (25-fold excess of FixJ and 500-fold excess of ATP were supplied), so that the saturation of the enzyme–substrate complex would not change during the part of the reaction time course on which rates were based. All turnover rates were determined at steady state, i.e., when the rate of FixJ phosphorylation matched the rate at which the phospho-FixL intermediate was replenished. For inhibited states of FixL, there can be a lag while the enzyme intermediates are building up. For the most active species, there can be a relatively early deceleration of the reaction rate due to rapid depletion of substrate. Therefore it was essential to collect complete time courses and to base the rates only on the portion of each time course where product was accumulating at a constant rate. For quantitative modeling of enzyme reaction mechanisms, there is no substitute for the turnover rates. To our knowledge, very few sensor kinases have been assayed under conditions that allow their activities to be reported as k_{cat} values.

Quantitative Enzymatic Phosphorylation of FixJ. Under the present assay conditions a k_{cat} of 1.5 min^{−1} was determined for the deoxy-*Bj*FixL, and a k_{cat} of 1.1 min^{−1} for the carbonmonoxy form (Figure 4). In a previous study of

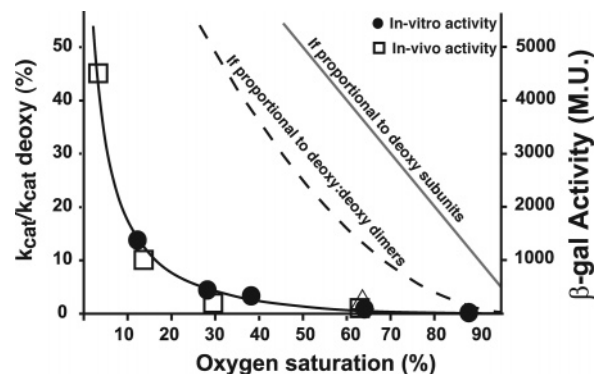


FIGURE 5: Correspondence to the in vitro O₂ regulation data for FixL to models of regulation and previous in vivo results. The relative turnovers (or k_{cat}/k_{cat} deoxy percent) of *Bj*FixJ at 12%, 28%, 38%, and 64% of O₂ saturation in a background of CO, at 64% of O₂ saturation in air, and at 88% of O₂ saturation in 1 atm of O₂ are shown (circles); 64% of O₂ saturation achieved in a background of CO is also shown (triangle), and the zero saturation, i.e., deoxy-FixL, is implied in the figure but not shown. The data from the in vivo induction of a *fixK2::lacZ* fusion by *Bj*FixLJ after 24 h (squares) are from Fischer, Hennecke, and colleagues (20). To compare the in vitro turnover and in vivo β -galactosidase data, the ordinates were adjusted proportionally to the data points around 10–12% of O₂ saturation. The in vitro data fit well to a “memory effect” model with $k_{ia} \sim 0.53 \text{ s}^{-1}$, i.e., $T_{rec} \sim 1.9 \text{ s}$ (solid black curve; see eqs 1 and 1a). This is contrasted to the fits that would be expected if the activity were proportional to the fraction of FixL subunits not bound to O₂ (gray line), or if the activity were proportional to the fraction of FixL dimers not bound to O₂ (dashed curve).

the activities of different *Bj*FixL states, the k_{cat} values were reported to be 0.43 min^{−1} (26 h^{−1}) for the deoxy state and 0.10 min^{−1} (8.2 h^{−1}) for the carbonmonoxy state (7). The difference is due principally to the use of a *Bj*FixJ substrate in the current experiment and a *Rm*FixJ substrate in the previous study. An additional improvement was achieved by supplying Mg^{II} at physiological levels together with the Mn^{II} used previously. The use of Mg^{II} alone will not substitute for Mn^{II}, and interestingly, the ideal combination of divalent cations for the reaction mimics the reported concentrations of these cations in symbiotic root nodules (24). Under the new conditions, the reaction proceeded until all the FixJ was phosphorylated (Figure 4A, closed circles). To our knowledge, this represents the first instance of the

quantitative enzymatic phosphorylation of a response regulator of the two-component class by its cognate kinase. As shown in Figure 4A, equilibrium was reached when the molar ratio of radiolabeled phosphate to FixJ was about 1:2. This resembles the level of phosphorylation achieved by reacting FixJ with acetyl phosphate and indicates that only one monomer is phosphorylated in the resulting FixJ dimers (25). The phospho-FixJ formed under these reaction conditions was quite stable, and there were no side reactions, such as the generation of free phosphate (5).

An Inhibition That Is Robust and Disproportionate to the Oxygen Saturation. Figure 5 shows the relative activity of FixL as a function of the O₂ saturation, with 100% activity being defined as the k_{cat} measured for 100% deoxy-FixL. The raw phosphorylation data are provided as Supporting Information. The enzymatic data (circles, triangle) do not fit to models that show the relative activity expected if the fraction of active protein were equal to the fraction of deoxy-protein (gray line), and the relative activity expected if the fraction of active protein were equal to the fraction of “deoxy–deoxy” dimers (dashed curve). The data do fit to a simple hysteretic “memory effect model” (solid black curve); they also fit to the published in vivo O₂ response of *B. japonicum* FixL/FixJ (squares). Therefore: (i) a simple in vitro system containing only FixL and its FixJ and ATP substrates can recapitulate the highly nonlinear response of FixL-dependent gene expression in living rhizobia; (ii) of the models shown, the simple memory-effect model best fits the actual O₂ response of FixL.

DISCUSSION

Quantitative Enzymatic Phosphorylation of a Response Regulator by Its Partner Sensor Kinase. We know of no response regulator of the two-component class that has been quantitatively phosphorylated by its cognate sensor kinase, as shown in Figure 4 for FixJ. Apart from providing evidence for quantitative phosphorylation, these data also imply the absence of any competing phosphatase under the reaction conditions. Researchers who need phosphorylated response regulators typically generate them nonenzymatically by reacting these proteins with phosphoryl donors such as acetyl phosphate (26, 27). The phosphorylation of response regulators by sensor kinases is widely believed to be accompanied by a “phosphatase” reaction that removes the phosphoryl group from the protein substrate nearly as fast as the kinase can deliver it, limiting the enzymatic phosphorylation to less than 10% of the total response regulator. Although in the case of FixL there exists an early report of an oxygen-dependent “phosphatase” activity, such an activity was probably due to a phosphatase contamination and has not been reproduced (28). As a rule, a phosphatase activity is never seen in FixL under turnover conditions, unless this protein is either contaminated or partially degraded (5). All of the phosphorylation reactions reported here were verified, by anion-exchange thin-layer chromatography, to generate no free phosphate (5).

Nucleotides Do Not Affect the Oxygen Affinity of FixL. Recently, the O₂ affinity of RmFixL_T in an RmFixL_T/RmFixJ complex was reported to drop to less than a third of its normal value on exposure to 200 μM ATP or ADP, and this perceived effect of nucleotides on the O₂ affinity was

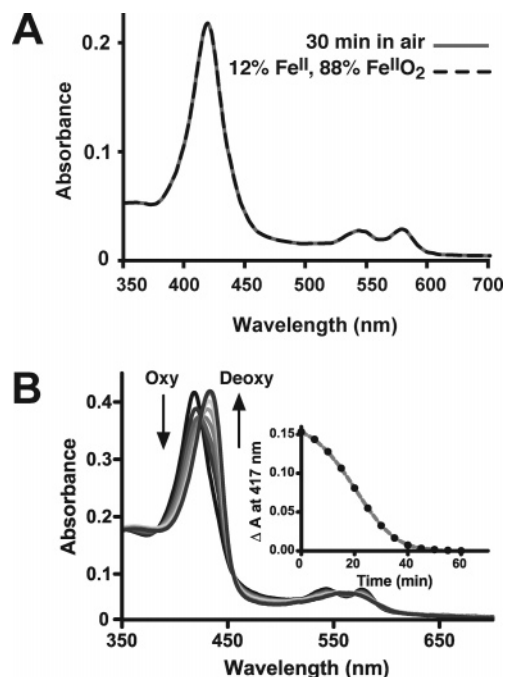


FIGURE 6: Comparison of the RmFixL O₂ saturation when well equilibrated with air, versus when sealed in a cuvette after exposure to air, at pH 8.0 and 25 °C. In panel A, RmFixL in a phosphorylation assay mixture was kept well equilibrated for 30 min in air; the true absorption spectrum (gray) was found to correspond to a linear combination of the absorption spectra of pure deoxy-FixL and pure oxy-RmFixL giving 88% oxy and 12% deoxy (dashed black), as expected from the K_d for binding of O₂. In panel B, a similar mixture of RmFixL in phosphorylation buffer was sealed; the conversion of the oxy form ($\lambda_{\text{max}} = 417 \text{ nm}$) to the deoxy form ($\lambda_{\text{max}} = 434 \text{ nm}$) was clear from the absorption spectra collected over time. The inset shows the relatively rapid rate of the disappearance of the oxy form, as determined from the absorbance difference, ΔA , at 417 nm. In addition to RmFixL, the mixtures contained a 25-fold excess of RmFixJ, 1.0 mM ATP/MgCl₂, 50 μM MnCl₂, 50 mM Tris-HCl, 50 mM KCl, 5.0% (v/v) ethylene glycol, and 10 mM DTT at pH 8.0.

interpreted as a mode of regulation (29). This observation, however, had the more drastic implication that any attempts to measure phosphorylation in air would result in uninterpretable reaction time courses, with the enzyme becoming less oxygenated, and more active, as the reaction progressed. This is clearly not the case. As a rule, a turnover reaction of FixL easily fits to a single rate constant and, as expected, the reaction slows later in the time course, as the substrates are consumed and the products accumulate. Figures 2 and 6A show that the O₂ saturation of FixL, whether from *B. japonicum* or *S. meliloti*, is unchanged by the addition of nucleotides and other reaction components. Since the activity of a heme-based sensor, by definition, depends on its heme state, any activity measurement requires verifying the heme state before and after a reaction. This is done principally to guard against mishaps such as oxidation of the heme or rupture of a seal. Our best guess as to the source of the artifactual effect attributed to nucleotide is that O₂ was consumed by reducing agents in a cuvette poorly equilibrated with gas, leading to O₂ concentrations lower than those assumed. An example of such a case is shown in Figure 6B.

A Sharp In Vitro Oxygen–Dose Response. The proportion of BjFixL remaining in the deoxy state in air is 36%, and in pure O₂ is 12%. If a deoxy-subunit were as active in an aerobic preparation of BjFixL as in an anaerobic preparation,

For any heme protein that binds O₂ noncooperatively, $1/(1 + O_2/K_d)$ is simply the fraction of deoxygenated protein, D .

$$Y_A = (D) \frac{k_{ia}/k_{on}[O_2]}{(1 + k_{ia}/k_{on}[O_2])} \quad (1)$$

A relevant property for the current model is the relative population of oxy-state protein molecules in the reaction mixtures; this can be measured directly by deconvoluting the UV–visible spectra of the mixtures into their pure components. Errors in measuring the O₂ concentrations were avoided by eliminating the O₂ concentration from eq 1 and deriving an expression for activity purely as a function of the O₂ saturation, which could be precisely measured. If we substitute $k_{off}(1 - Y)/Y$ for $k_{on}[O_2]$ in eq 1, we obtain

$$Y_A = D \frac{k_{ia}Y}{(k_{off}D + k_{ia}Y)} \quad (2)$$

This is the equation that is fit to the data in Figure 5 (solid black curve). One possible way to get an intuitive sense for the memory effect model is to recast this model in terms of the fraction of an average molecule's time that is spent in each of the available states. Although every individual FixL cycles through all of the available states (oxy, inactive-deoxy, then active-deoxy) every few seconds, the total number of molecules in each state will remain constant as long as the O₂ concentration is stable. The average time for the recovery of activity after O₂ dissociates, T_{rec} , is the reciprocal of reactivation rate, i.e., $1/k_{ia}$. Similarly, $1/k_{on}[O_2]$ gives T_{deoxy} , the average lifetime of a deoxy state before rebinding O₂ at a particular O₂ concentration. Therefore, an alternative form of eq 1 in terms of state lifetimes rather than rates is

$$Y_A = (D) \frac{T_{deoxy}/T_{rec}}{(1 + T_{deoxy}/T_{rec})} \quad (1a)$$

The recovery time T_{rec} is a property of the kinase and is independent of the O₂ saturation, whereas the T_{deoxy} will shorten as the O₂ concentration increases. Therefore the T_{deoxy}/T_{rec} ratio depends on both the saturation and binding characteristics of a particular ligand.

Carbon monoxide binds slowly to *Bj*FixL ($k_{on} = 0.005 \mu\text{M}^{-1} \text{s}^{-1}$), which becomes half-saturated in $2 \mu\text{M}$ CO (Figure 3). When the heme is half-saturated, the lifetime of a deoxy state equals the lifetime of a carbonmonoxy state. For CO, this lifetime is 100 s. This is about 50-fold longer than the estimated T_{rec} of 1.9 s. Therefore there should essentially be no deoxy molecules in the process of recovery. If, for a wide range of ligand saturations, T_{rec} is short compared to the lifetime of a deoxy state ($T_{rec} \ll T_{deoxy}$), then $Y_A \sim D$ in eq 1a, and the activity simply becomes proportional to the protein fraction in the deoxy state. This is why the modest inhibition caused by CO binding is linear with respect to the CO saturation and shows no sign of a memory effect (Figure 3A).

Oxygen binds to *Bj*FixL at a rate of $0.14 \mu\text{M}^{-1} \text{s}^{-1}$, and the protein becomes half-saturated in $140 \mu\text{M}$ O₂ (19). So at half-saturation, the T_{deoxy} is only 0.051 s and about 37 times shorter than the T_{rec} . Therefore very few deoxy-FixL molecules manage to regain activity before rebinding O₂. Since $T_{deoxy} < T_{rec}$ for most O₂ saturations, very little of the

time that a FixL molecule spends in the deoxy state will be spent as a productive kinase if even a small amount of O₂ is present. In air, out of any 100 *Bj*FixL, 64 will be oxy. Of the remaining 36 deoxy, 34 will be recovering and only two will be active, resulting in only 2% of the maximum activity. Equation 1 predicts a rapid drop in Y_A with a rise in the O₂ concentration, and this matches the observed FixL dose response in vitro and in vivo (Figure 5). Note that when FixL is returned to anaerobic conditions, all the deoxy-FixL molecules will relax back to the active state within seconds, and 100% activity will be restored.

Biochemical Implications. During the mid-1960s, Brian R. Rabin proposed that slow conformational transitions occurring during enzyme reactions might cause apparent cooperative behavior, even in a monomer (30). Soon thereafter, Carl Frieden called this behavior an “enzyme hysteresis” and defined it as a slow response of the enzyme activity to fast changes in a ligand concentration (31). Many important metabolic enzymes fit the category of hysteretic enzymes, with the conversion times from one kinetic form to another ranging from seconds to minutes (32). Variations in enzyme hysteresis deriving from an assortment of changes result in many fascinating behaviors (31, 32). The slow response might be a conformational change induced by a substrate, product, or noncompetitive inhibitor; it might be an isomerization; it might be the displacement of a tightly bound ligand by another that differently affects the activity; or it might be a change in the kinetics of substrate binding and the affinity for substrate. The memory-effect model predicts that FixL would remain inactive several seconds after a large instantaneous drop in the O₂ concentration, and therefore FixL represents a special case of a hysteretic enzyme. This is shown schematically in Figure 7. The general equations governing hysteresis are quite complex. For FixL, however, it is straightforward to measure relevant equilibrium and kinetic parameters and to show that many of the considerations that can greatly complicate hysteretic models do not apply. For example, the single-exponential binding of O₂ to full-length *Bj*FixL and a *Bj*FixLH truncation lacking the enzymatic domain justify the assumption that $k_{on}(O_2)$ is the same for both active and inactive deoxy. The deoxy and oxy forms of FixL bind ATP and FixJ equally well (6). Indeed, FixL may well be the most comprehensible and accessible hysteretic enzyme for systematic study. To our knowledge, FixL provides the first example of a hysteretic sensory kinase.

Structural Implications. The existence of an inactive-deoxy state of FixL is an inescapable consequence of the simple arithmetic comparison of the deoxy-protein fraction to the active-protein fraction. The former is directly measurable spectroscopically; the latter is directly measurable by enzyme assays. The inactivation is completely reversible. The inactive-deoxy state does not exist when the protein is entirely anaerobic. The hysteretic “memory-effect model” represents one way to account for the observed populations of active- and inactive-deoxy states at different O₂ saturations. This simple model depends only on hypotheses regarding the behaviors of these states and neither contains nor needs any assumptions about their structures.

The hysteresis associated with the memory effect derives from a slow response of the kinase to rapid changes in the heme-binding domain. Consequently, the kinetics of the

memory effect should not be confused with the kinetics of ligand binding, or with spectroscopic signals deriving directly from the heme state. The reactivation of the kinase after O₂ dissociation occurs after the spectroscopically observable changes in the heme and its immediate environs are complete. Such changes of the kinase would have no obvious spectral signatures. Since an inactive-deoxy state results from the kinase and heme-binding regions impinging on each other, such a state would not be expected to exist in either domain in isolation. In principle, the inactive-deoxy state might be “trapped” in a crystal by rapid freezing, but this is not feasible, given that no one has yet succeeded in crystallizing a complete FixL.

Physiological Implications. For *S. meliloti* living symbiotically with *Medicago sativa* (alfalfa), the levels of expression of a *RmFixLJ*-regulated *fixK::lacZ* gene fusion surge approximately 100-fold over an O₂ concentration range that should linearly change the FixL saturation from 84% to 32% (21). For *B. japonicum* grown vegetatively, a 30-fold surge is observed in the expression of a *fixK2::lacZ* on switching from air to 0.5% O₂ for 24 h (20). Under these conditions, the saturation of *BjFixL* with O₂ should drop from 64% to 14%, resulting in only a 2.4-fold increase in the deoxy-fraction of this sensor. In the absence of an in vitro dose-response profile for any FixL protein, it has been tempting to imagine that other elements might be needed to sharpen the in vivo dose response of FixLJ-regulated genes. The FixT protein, for example, is a distant FixJ homologue that inhibits phosphorylation of FixL, probably by blocking its active site (18, 33). FixT has been entertained as an element in *S. meliloti* for reducing the background of aerobic FixL activity. This seemed unlikely, however, given that FixT's action is entirely independent of O₂, and an analogous protein has not been found in *B. japonicum* where a steep O₂ response also occurs. Our results show that the simple O₂-sensing system consisting of FixL and FixJ is sufficient to account for the observed nonlinear response to O₂ (Figure 5).

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SUPPORTING INFORMATION AVAILABLE

A figure showing autoradiographs of gels with the raw data corresponding to Figure 5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

- Gilles-Gonzalez, M. A., and Gonzalez, G. (2005) Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses, *J. Inorg. Biochem.* 99, 1–22.
- Gilles-Gonzalez, M. A., Ditta, G. S., and Helinski, D. R. (1991) A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*, *Nature* 350, 170–172.
- Gilles-Gonzalez, M. A., and Gonzalez, G. (1993) Regulation of the kinase activity of heme protein FixL from the two-component system FixL/FixJ of *Rhizobium meliloti*, *J. Biol. Chem.* 268, 16293–16297.
- Tuckerman, J. R., Gonzalez, G., Dioum, E. M., and Gilles-Gonzalez, M. A. (2002) Ligand and oxidation-state specific regulation of the heme-based oxygen sensor FixL from *Sinorhizobium meliloti*, *Biochemistry* 41, 6170–6177.
- Tuckerman, J. R., Gonzalez, G., and Gilles-Gonzalez, M. A. (2001) Complexation precedes phosphorylation for two-component regulatory system FixL/FixJ of *Sinorhizobium meliloti*, *J. Mol. Biol.* 308, 449–455.
- Sousa, E. H., Gonzalez, G., and Gilles-Gonzalez, M. A. (2005) Oxygen blocks the reaction of the FixL-FixJ complex with ATP but does not influence binding of FixJ or ATP to FixL, *Biochemistry* 44, 15359–15365.
- Dunham, C. M., Dioum, E. M., Tuckerman, J. R., Gonzalez, G., Scott, W. G., and Gilles-Gonzalez, M. A. (2003) A distal arginine in oxygen-sensing heme-PAS domains is essential to ligand binding, signal transduction, and structure, *Biochemistry* 42, 7701–7708.
- Gilles-Gonzalez, M. A., Caceres, A. I., Sousa, E. H., Tomchick, D. R., Brautigam, C., Gonzalez, C., and Machius, M. (2006) A Proximal Arginine R206 Participates in Switching of the *Bradyrhizobium japonicum* FixL Oxygen Sensor, *J. Mol. Biol.* 360, 80–89.
- Fischer, H. M. (1994) Genetic regulation of nitrogen fixation in rhizobia, *Microbiol. Rev.* 58, 352–386.
- Dixon, R., and Kahn, D. (2004) Genetic regulation of biological nitrogen fixation, *Nat. Rev. Microbiol.* 2, 621–631.
- Brencic, A., and Winans, S. C. (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria, *Microbiol. Mol. Biol. Rev.* 69, 155–194.
- Virts, E. L., Stanfield, S. W., Helinski, D. R., and Ditta, G. S. (1988) Common regulatory elements control symbiotic and microaerobic induction of *nifA* in *Rhizobium meliloti*, *Proc. Natl. Acad. Sci. U.S.A.* 85, 3062–3065.
- David, M., Davaeran, M. L., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P., and Kahn, D. (1988) Cascade regulation of *nif* gene expression in *Rhizobium meliloti*, *Cell* 54, 671–683.
- Anthamatten, D., and Hennecke, H. (1991) The regulatory status of the *fixL*- and *fixJ*-like genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*, *Mol. Gen. Genet.* 225, 38–48.
- Mesa, S., Bedmar, E. J., Chanfon, A., Hennecke, H., and Fischer, H. M. (2003) *Bradyrhizobium japonicum* NnrR, a denitrification regulator, expands the FixLJ-FixK2 regulatory cascade, *J. Bacteriol.* 185, 3978–3982.
- Lopez, O., Morera, C., Miranda-Rios, J., Girard, L., Romero, D., and Soberon, M. (2001) Regulation of gene expression in response to oxygen in *Rhizobium etli*: role of FnrN in *fixNOQP* expression and in symbiotic nitrogen fixation, *J. Bacteriol.* 183, 6999–7006.
- Boesten, B., and Priefer, U. B. (2004) The C-terminal receiver domain of the *Rhizobium leguminosarum* bv. *viciae* FixL protein is required for free-living microaerobic induction of the *fnrN* promoter, *Microbiol.* 150, 3703–3713.
- Crosson, S., McGrath, P. T., Stephens, C., McAdams, H. H., and Shapiro, L. (2005) Conserved modular design of an oxygen sensory/signaling network with species-specific output, *Proc. Natl. Acad. Sci. U.S.A.* 102, 8018–8023.
- Gilles-Gonzalez, M. A., Gonzalez, G., Perutz, M. F., Kiger, L., Marden, M. C., and Poyart, C. (1994) Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand binding and autoxidation, *Biochemistry* 33, 8067–8073.
- Sciotti, M. A., Chanfon, A., Hennecke, H., and Fischer, H. M. (2003) Disparate oxygen responsiveness of two regulatory cascades that control expression of symbiotic genes in *Bradyrhizobium japonicum*, *J. Bacteriol.* 185, 5639–5642.
- Soupe, E., Foussard, M., Boistard, P., Truchet, G., and Batut, J. (1995) Oxygen as a key developmental regulator of *Rhizobium meliloti* N₂-fixation gene expression within the alfalfa root nodule, *Proc. Natl. Acad. Sci. U.S.A.* 92, 3759–3763.
- Gonzalez, G., Gilles-Gonzalez, M. A., Rybak-Akimova, E. V., Buchalova, M., and Busch, D. H. (1998) Mechanisms of autoxidation of the oxygen sensor FixL and Aplysia myoglobin: implications for oxygen-binding heme proteins, *Biochemistry* 37, 10188–10194.

23. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227, 680–685.
24. Becana, M., and Klucas, R. V. (1992) Transition metals in legume root nodules: iron-dependent free radical production increases during nodule senescence, *Proc. Natl. Acad. Sci. U.S.A.* 89, 8958–8962.
25. Da Re, S., Schumacher, J., Rousseau, P., Fourment, J., Ebel, C., and Kahn, D. (1999) Phosphorylation-induced dimerization of the FixJ receiver domain, *Mol. Microbiol.* 34, 504–511.
26. Lukat, G. S., McCleary, W. R., Stock, A. M., and Stock, J. B. (1992) Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors, *Proc. Natl. Acad. Sci. U.S.A.* 89, 718–722.
27. Mayover, T. L., Halkides, C. J., and Stewart, R. C. (1999) Kinetic characterization of CheY phosphorylation reactions: comparison of P-CheA and small-molecule phosphodonors, *Biochemistry* 38, 2259–2271.
28. Lois, A. F., Weinstein, M., Ditta, G. S., and Helinski, D. R. (1993) Autophosphorylation and phosphatase activities of the oxygen-sensing protein FixL of *Rhizobium meliloti* are coordinately regulated by oxygen, *J. Biol. Chem.* 268, 4370–4375.
29. Nakamura, H., Kumita, H., Imai, K., Iizuka, T., and Shiro, Y. (2004) ADP reduces the oxygen-binding affinity of a sensory histidine kinase, FixL: the possibility of an enhanced reciprocating kinase reaction, *Proc. Natl. Acad. Sci. U.S.A.* 101, 2742–2746.
30. Rabin, B. R. (1967) Co-operative effects in enzyme catalysis: a possible kinetic model based on substrate-induced conformation isomerization, *Biochem. J.* 102, 22C–23C.
31. Frieden, C. (1970) Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept, *J. Biol. Chem.* 245, 5788–5799.
32. Frieden, C. (1979) Slow transitions and hysteretic behavior in enzymes, *Annu. Rev. Biochem.* 48, 471–489.
33. Garnerone, A. M., Cabanes, D., Foussard, M., Boistard, P., and Batut, J. (1999) Inhibition of the FixL sensor kinase by the FixT protein in *Sinorhizobium meliloti*, *J. Biol. Chem.* 274, 32500–32506.

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