Heme-Based Sensors, Exemplified by the Kinase FixL, Are a New Class of Heme Protein with Distinctive Ligand Binding and Autoxidation†

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ABSTRACT: FixL's are chimeric heme protein kinases from symbiotic nitrogen-fixing *Rhizobia.* We have overexpressed three FixL variants in *Escherichia coli. Bradyrhizobium japonicum* FixL, a soluble dimeric protein, is the first full-length FixL to be purified. The other two proteins are soluble truncations of *Rhizobium meliloti* FixL, which is a membrane protein. One contains both heme and kinase domains and is dimeric; the other has only the heme domain and is monomeric. We find that all the FixL's bind oxygen and carbon monoxide non-cooperatively, with very low affinities due entirely to slow association rates. FixL P_{50}'s for oxygen are 17–76 mmHg. FixL's may sense nitric oxide and carbon monoxide in addition to oxygen, especially at the low oxygen pressures encountered *in vivo.* Autoxidation rates are about 50 times faster than that of sperm whale myoglobin. The carbon monoxide affinity of FixL's is about 300 times lower than that of myoglobin, resulting in the unusually low values of 7.5–17 for the partition constant, $M = P_{50}(O_2)/P_{50}(CO)$, between carbon monoxide and oxygen. Met-FixL's have their Soret absorption maximum at 395 nm instead of the typical 408 nm and a steep hydroxymet transition at pH ≥ 9.3; these properties indicate a pentacoordinated high-spin ferric heme and suggest a sterically hindered hydrophobic heme pocket lacking a distal (E7) histidine. FixL is the first member of a new class of heme proteins, the heme-based sensors, distinct from the oxygen carriers and electron transporters. We expect that some of the novel properties of FixL will be characteristic of the class.

Rhizobium meliloti FixL is a signal-transducing membrane protein that shuts down nitrogen fixation in response to oxygen (David et al., 1988). It is homologous to a diverse family of environmental sensors, ubiquitous in prokaryotes and recently also found in eukaryotes (Nixon et al., 1986; David et al., 1988; Chang et al., 1993). These proteins are modular; they consist of a nonconserved, usually N-terminal sensor domain that detects a specific environmental signal and a homologous transmitter domain with the kinase activity [reviewed by Stock et al. (1989) and Parkinson and Kofoid (1992)]. A genetically engineered soluble FixL, called FixL*, proved to be a kinase and heme protein with the characteristic oxy and deoxy spectra of Hb[1] (Gilles-Gonzalez et al., 1991). On the basis of homology, the transmitter domain is in the C-terminal two-thirds of FixL*; the N-terminal one-third is the sensor (Figure 1). FixL* kinase activity under nitrogen is about 7 times faster than in air (Gilles-Gonzalez et al., 1993). Deoxy-FixL*, the active form, transfers a phosphoryl group to the transcriptional activator FixJ, which enhances FixJ's activity and induces the expression of critical nitrogen fixation genes (Gilles-Gonzalez et al., 1991; Gilles-Gonzalez & Gonzalez, 1993; Agron et al., 1993).

We examined three FixL's that we overexpressed in *Escherichia coli* and purified (Figure 1). Two are new truncations of *R. meliloti* FixL; the other is the first FixL to be isolated from a different species and the first full-length FixL to be purified. All three are heme proteins. They are (1) RmFixLT, a more precise truncation of *R. meliloti* FixL that differs from FixL* only in the first 10 amino acids; (2) RmFixLH, a truncation containing only the sensor domain of *R. meliloti* FixL; and (3) BjFixL, a FixL from *Bradyrhizobium japonicum* that is soluble in its native form.

FixL's have no homology to known heme proteins (Vinogradov et al., 1993), and the structure of the heme binding pocket of FixL is still unknown. On the other hand, much is known about the relationship between structure, function, and spectral properties of a great variety of natural and genetically engineered Hb's (Perutz, 1989). We have tried to infer the nature of the heme environment of FixL's and to clarify the biological function of FixL's by comparing their ligand binding and absorption spectra to those of Hb's of known structure. FixL's bind oxygen non-cooperatively, and they differ dramatically from typical globins in their association rates with oxygen and carbon monoxide, susceptibility to autoxidation, and affinity of their ferric form for water.

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[1] Abbreviations: BjFixL, *Bradyrhizobium japonicum* FixL; RmFixLT, *Rhizobium meliloti* FixL truncation with heme and kinase domains; RmFixLH, *Rhizobium meliloti* FixL truncation containing only the heme domain; Mb, myoglobin; Hb, hemoglobin; HbA, human adult hemoglobin; R, relaxed; $P(O_2)$, partial pressure of oxygen; P_{50}, partial pressure of a gaseous ligand at which the hemes are 50% saturated. The alphanumeric code (e.g., E7) refers to the positions of amino acids in helices and turns of myoglobin and hemoglobin (Perutz, 1970). The notation for myoglobin mutants is as follows: a one-letter code for the original amino acid followed by the alphanumeric code for the mutated position in parentheses, followed by a one-letter code for the replacing amino acid; e.g., H(E7)→V refers to the E7 position mutated from histidine to valine.

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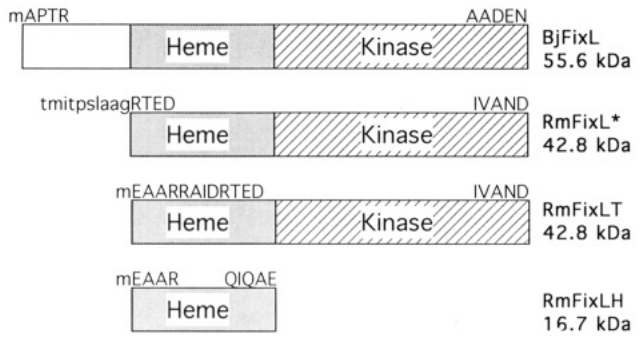

FIGURE 1: Schematic representations of FixL's. *B. japonicum* FixL (BjFixL), *R. meliloti* FixL heme domain (RmFixLH), *R. meliloti* truncated FixL containing heme and kinase domains (RmFixLT), and *R. meliloti* FixL* (RmFixL*), a previously studied truncation (Gilles-Gonzalez et al., 1991; Gilles-Gonzalez & Gonzalez, 1993), are shown. Monomer molecular masses, calculated from the amino acid sequence, are given. Terminal amino acid sequences are at the top of each diagram (David et al., 1988; Anthamatten & Hennecke, 1991); residues shown in lowercase type were introduced during cloning. The hatched kinase regions are highly homologous in sensors belonging to the two-component regulatory systems (Nixon et al., 1986). FixL's also share extensive homology in the stippled heme regions.

MATERIALS AND METHODS

Cloning. DNA manipulations were done according to manufacturers' recommendations and the methods compiled in Sambrook et al. (1989). The *B. japonicum fixL* gene was kindly provided by H. Hennecke on plasmid pRJ7349 (Anthamatten & Hennecke, 1991). The *B. japonicum fixL* gene was prepared from this plasmid in a polymerase chain reaction as a 1.5-kb fragment with an *Nde*I site at its 5′ end, overlapping the start codon, and a *Hin*dIII site at its 3′ end. Polymerase chain reactions were also used to prepare a 1.16-kb DNA fragment encoding RmFixLT and a 0.44-kb fragment encoding RmFixLH from plasmid pMW2 (Weinstein et al., 1992). Primers introduced an ATG start codon that overlaps with an *Nde*I site and added a *Hin*dIII site at the 3′ end. For RmFixLH, stop codons were added to the end of the coding sequence in all three frames.

Each of the three *Nde*I–*Hin*dIII fragments described above was cloned into expression vector pNKT (N. Komiyama and J. Avis, unpublished data). This pUC8-derived vector carries ampicillin resistance (Messing, 1983); the polylinker region is preceded by a *tac* promoter and two tandem ribosome binding sites. The plasmids containing the genes encoding BjFixL, RmFixLT, and RmFixLH are named pBL31, pRT51, and pRH61, respectively. Clones without polymerization error were assembled after verification of the *fixL* sequences by direct double-stranded plasmid sequencing (Sanger et al., 1977; Smith et al., 1979). Each protein was produced from *E. coli* strain TG1 (Gibson, 1984) transformed with the appropriate vector.

Expression and Purification. BjFixL and RmFixLT were expressed from TG1(pBL31) and TG1(pRT51), respectively, after induction with IPTG. The proteins were purified as described for *R. meliloti* FixL* (Gilles-Gonzalez et al., 1991).

RmFixLH was purified as follows. Induction with IPTG and preparation of cleared lysate of TG1(pRH61) were as described for *R. meliloti* FixL* (Gilles-Gonzalez et al., 1991). RmFixLH from a 25–50% saturated ammonium sulfate cut of the cleared lysate was further purified on a Sephacryl S-100 gel filtration column equilibrated in 5 mM Tris-HCl, pH 8.0, and 10 mM β-mercaptoethanol.

All column matrices were from Pharmacia. Throughout the purifications, FixL was kept at <4 °C and was assayed on the basis of 410-nm absorbance. Protein was assayed by the method of Bradford (Bio-Rad dye reagent concentrate), using bovine serum albumin as the standard. Gel filtration columns were calibrated with mixtures of molecular weight markers prior to the runs. A mixture of aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa) was used for Sephacryl S-200 calibration. The Sephacryl S-100 column was calibrated with a mixture of albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25 kDa), and ribonuclease (13.7 kDa).

Spectrophotometric Studies. Spectra (300–700 nm) of samples at 25 °C, in 20 mM phosphate buffer at pH 7, were recorded with an SLM Aminco DW2000 apparatus. Due to the fast autoxidation of FixL's, their met spectra could be obtained by keeping them for a few hours in air at 25 °C. The samples, in stoppered quartz cuvettes, were then deoxygenated with a stream of N_2; deoxy spectra were recorded after reduction with a 2-fold molar excess of sodium dithionite. To obtain oxy-FixL while avoiding autoxidation, one atmosphere of O_2 was layered over the deoxy sample at 5 °C; the solutions were equilibrated by shaking just before the spectra were recorded. CO spectra were obtained from samples equilibrated with 1 atm of CO. Heme content was measured from the protoheme pyridine hemochromogen (Appleby, 1978).

Autoxidation. The rate of autoxidation was measured at 37 °C on 10–20 μM protein in 75 mM potassium phosphate, pH 7.0, and 0.2 mM EDTA. Ferric FixL was reduced with crystalline sodium dithionite. Oxy-FixL, free of dithionite, was recovered from a small Sephadex G-25 (Pharmacia) column equilibrated with air-saturated 75 mM sodium phosphate, pH 7.0, and 0.2 mM EDTA. Alternatively, an anaerobic solution of met-FixL was reduced with a 2-fold molar excess of dithionite, and oxy-FixL was produced by equilibration of the ice-cooled sample with 1 atm of air or O_2. The oxy-FixL was warmed rapidly to 37 °C, and rates were measured by monitoring absorbance from 350 to 700 nm at intervals of 5–10 min. Both methods of preparation of oxy-FixL gave similar results. All time courses showed clear isosbestic points. Rates were calculated from the absorbance change at 577 nm.

pH Titrations. For each FixL, seven identical aliquots were adjusted to a desired pH and the same final concentration. Titrations were done on 10–20 μM protein in 0.1 M buffer containing 0.2 mM EDTA. Buffers were sodium phosphate for pH 6.5–8.0, glycine NaOH or sodium carbonate/bicarbonate for pH 9.2–10.7, and CAPS at pH 11.1. Visible spectra from 350 to 700 nm were recorded. Absorbance at 605 nm was a measure of transition to the hydroxyl met form.

O_2 Binding at Equilibrium. Binding curves were measured by a continuous method, with a Hemox-Analyzer (TCS Huntington Valley, Southampton, PA), as detailed elsewhere (Kister et al., 1986). Samples were at 25 °C in 50 mM Tris-HCl, pH 7.5, and 0.1 M NaCl containing 20 μg of catalase and 50 μM EDTA. Met-FixL was reduced with sodium dithionite and stripped of the reductant through a small G-25 column equilibrated with aerated buffer in the cold. The P_{50} and the index of cooperativity, n, were calculated from linear regression analysis of points between 80 and 20% O_2 saturation. For comparison, we measured the O_2 affinity of freshly prepared isolated α chains of human Hb in similar conditions but without the addition of dithionite.

CO Binding at Equilibrium. Deoxy-BjFixL was prepared by equilibrating met-BjFixL (3 mL of 20 μM in 13.3 mM phosphate buffer, pH 7) with 1 atm of N_2 at 37 °C in a stoppered tonometer and reducing the protein with sodium

dithionite. The deoxy spectrum was recorded. The sample was then equilibrated for 15 min at room temperature under a stream of 0.010 atm of CO, resulting in a P(CO) of 7.1 mmHg in the tonometer. The spectrum of partially CO-saturated FixL was recorded. The spectrum of 100% CO-saturated FixL was obtained after equilibration with 1 atm of CO at 25 °C. The P_{50}(CO) was estimated from these three spectra.

Ligand Association. The rates of association (k_{on}) of the FixL proteins with CO and O_2 were obtained after flash photolysis with a 10-ns 160-mJ pulse at 532 nm (Quantel YAG laser, France). The heme proteins (10 μM, in 20 mM phosphate buffer, pH 7, at 25 °C in 4-mm cuvettes) were reduced with sodium dithionite and exposed to 1 atm of O_2 or CO before the flash photodissociation. Absorbance at 436 nm was monitored; a low-intensity monochromatic light source was used to avoid photolysis of these samples, which have unusually slow ligand on-rates.

Ligand Dissociation. The CO dissociation rates [k_{off}(CO)] for BjFixL and RmFixLH at 25 °C were measured with a stopped-flow apparatus (Biologic, France) by mixing the sample, equilibrated with 0.1 atm (100 μM) of CO, with 2 mM potassium ferricyanide, resulting in final concentrations of 10 μM free CO and 1.8 mM ferricyanide. Detection was at 425 nm. Control experiments with the deoxy protein and 0.2 mM ferricyanide showed that oxidation is fast (<1 s) compared to CO dissociation kinetics.

Accurate and reproducible measurements of O_2 dissociation rate [$k_{off}(O_2)$] at 25 °C were not possible by O_2/CO replacement with the stopped-flow apparatus, due to incomplete oxygenation of the FixL's, even at 1 atm O_2, and their rapid autoxidation. The $k_{off}(O_2)$ value given in parentheses for RmFixLT was calculated from P_{50} and $k_{on}(O_2)$ values at 25 °C.

The $k_{off}(O_2)$ for BjFixL at 25 °C was measured according to a method described by Astatke et al. (1992) and modified by Kiger et al. (1993). This method allows O_2 binding measurements for low oxygen affinity or easily oxidized non-allosteric hemoproteins. The principle of this technique is to photodissociate a protein sample previously equilibrated with a mixture of O_2 and CO. After photodissociation of the CO, O_2 may rebind to the exposed heme; next O_2 is replaced by CO. If the gas mixture contains nonsaturating O_2, the P_{50} can be estimated. Oxidation is not a problem, as the heme is exposed to O_2 for less than 1 s. For all these studies, detection was at 436 nm.

RESULTS

Expression and Purification. RmFixLT and BjFixL eluted from DEAE-Sephacel around 200 mM NaCl, at pH 7.8. This is similar to the elution behavior of *R. meliloti* FixL* (Gilles-Gonzalez et al., 1991). The gel filtration molecular mass estimates of RmFixLT and BjFixL are 91 and 130 kDa, respectively, indicating that they are dimeric. By contrast, we found that RmFixLH is monomeric, with an estimated molecular mass of 21 kDa. Recoveries of >95% pure protein per liter of bacterial culture are 10–12 mg for BjFixL, 6–8 mg for RmFixLT, and 3–4 mg for RmFixLH. Isoelectric focusing verified the purity of our preparations (Figure 2). On the basis of protein and pyridine hemochromogen assays, FixL's contain one iron protoporphyrin IX per protein monomer (Falk, 1964; Appleby, 1978).

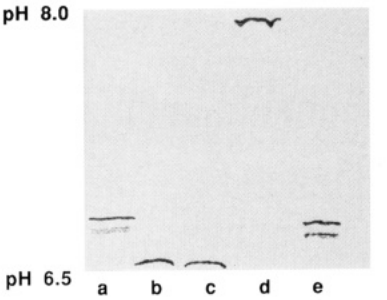

FIGURE 2: Isoelectric focusing of HbA (a, e), RmFixLH (b), RmFixLT (c), and BjFixL (d). The samples were focused in a gradient of pH 6–9 using an LKB system.

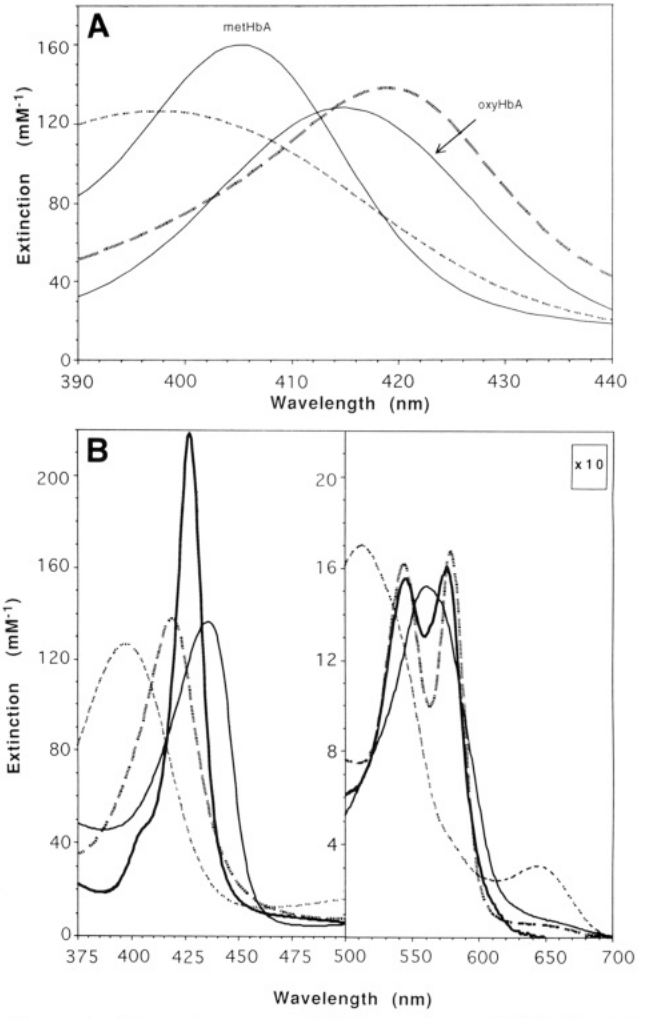

FIGURE 3: Absorption spectra of FixL proteins at pH 7.0. Panel A shows a comparison of the Soret bands of metHbA and oxyHbA (solid lines) to those of met-FixL (thin broken line) and oxy-FixL (thick broken line). Panel B shows the met (thin broken line), oxy (thick broken line), CO (thick solid line), and deoxy (thin solid line) spectra of RmFixLT. The absorption spectra of the *R. meliloti* and *B. japonicum* FixL's are nearly identical, except near 280 nm. Details of sample preparation are given in Materials and Methods.

Spectral Properties. Figure 3 shows the absorption spectra of the FixL's, which are nearly identical at 350–700 nm. Pure ferric BjFixL, RmFixLT, and RmFixLH have absorbance ratios of A_{280nm}/A_{395nm} = 0.69, 0.34, and 0.24, respectively (not shown). The oxy, deoxy, and carbonmonoxy spectra of FixL are similar to those of Hb, but the met spectra are markedly different (Figure 3) (Van Assendelft, 1970; Perutz et al., 1974). Hb, which exists below pH 8 predominantly as the aquomet form with water at the sixth coordination position, has an intense Soret band at 405 nm with an extinction

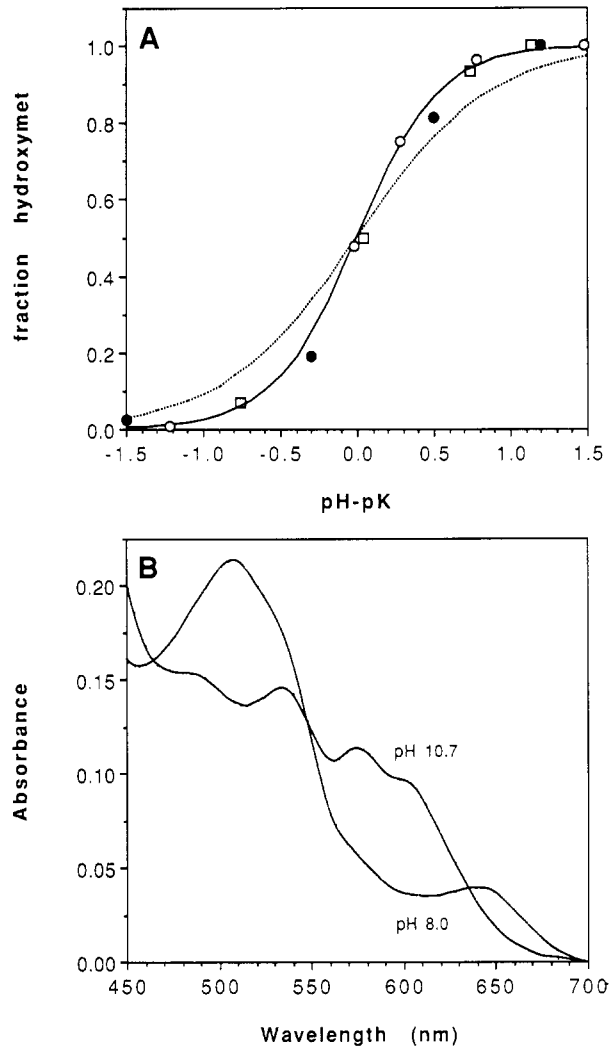

FIGURE 4: pH titration of met-FixL's. Panel A shows the pH titrations of the met forms of BjFixL (□), RmFixLT (●), and RmFixLH (○). The dotted line is the titration curve of sperm whale Mb; the solid line represents an average titration curve of the FixL's. Absorbance at 605 nm was a measure of the hydroxymet form. Panel B shows the absorption spectra of RmFixLT at pH 8.0 and 10.7. Details are in Materials and Methods.

coefficent of 161 mM^{-1} cm^{-1} heme, much higher than that of oxyHbA (131 mM^{-1} cm^{-1}) (Figure 3A). In contrast, the Soret band of met-FixL is blue-shifted to 395 nm with an extinction of 126 mM^{-1} cm^{-1}, lower than that of oxy-FixL; this indicates that met-FixL is pentacoordinated below pH 8 (Shikama & Matsuoka, 1989; Quillin et al., 1993). With increasing pH, the high-spin peak of Hb at 630 nm gradually disappears as the heme-linked water is titrated to a hydroxyl group. FixL's also undergo a transition from the high-spin "acid" spectrum to the low-spin alkaline spectrum, with pK_a's of 9.3 for RmFixLH and RmFixLT and 10 for BjFixL, but this transition is from a five-coordinated to a six-coordinated ferric iron (Figure 4). Hydroxide binds to the heme cooperatively, with Hill parameter n = 1.5.

Autoxidation. Clear isosbestic points occur at 409, 461, 535, and 600 nm for the decay of the oxy-FixL's to their corresponding met-FixL's. The half-lives of the oxy derivatives of BjFixL, RmFixLT, and RmFixLH are 15, 20, and 18 min, respectively, in air at pH 7, 37 °C (Table 1). This represents an autoxidation rate about 50 times faster than that of native sperm whale Mb or its mutant which has the distal histidine replaced by glutamine [H(E7)→Q] but 3–30 times slower than those of other Mb mutants lacking a distal histidine (Quillin et al., 1993). Springer et al. (1989) have reported even faster autoxidation rates, about 50 times those of the FixL's, for the sperm whale Mb mutants H(E7)→Y and H(E7)→D.

Table 1: Autoxidation of FixL Proteins in Air at pH 7, 37 °C

protein	k_{ox} (h^{-1})	half-life (min)	met Soret λ_{max} (nm)	oxy Soret λ_{max} (nm)
BjFixL	2.7	15	395	419
RmFixLT	1.9	22	395	418
RmFixLT[a]	2.1	20	395	418
RmFixLH	2.3	18	395	418
Aplysia Mb[b]			395	418
SW Mb[c]	0.06	720	409	418
SW Mb H(E7)→L[c]	10	4	393	

[a] One atmosphere of O_2 instead of air. [b] Shikama and Matsuoka (1989). [c] Sperm whale myoglobin (Quillin et al., 1993).

Oxygen and Carbon Monoxide Binding. FixL's bind oxygen non-cooperatively (Hill parameter n = 1.0) and have lower oxygen affinities than most natural Mb's or relaxed Hb (Table 2). Their P_{50}'s are 40–150 times the 0.3–0.5 mmHg typical of Mb, HbA (R), or its free α and β subunits. This unusually low oxygen affinity is due to slow oxygen association rates; the dissociation rates are comparable to those of globins (Table 2). BjFixL has a P_{50} of 76 mmHg, a value much higher than for the *R. meliloti* FixL's. The presence of a kinase domain in RmFixLT increases P_{50} to 27 mmHg from the 17 mmHg of RmFixLH. The rates of association of FixL with carbon monoxide are even more unusual: BjFixL has a rate 100 times slower than that of Mb and over 1000 times slower than that of HbA (R) (Table 2). The carbon monoxide dissociation rates are similar to those of Hb.

DISCUSSION

Typical metMb has water bound at the sixth coordination position of the ferric heme iron at low pH. Quillin et al. (1993) have compared the visible spectra and three-dimensional structures of a variety of Mb mutants. They found that hexacoordinated ferric heme iron has its Soret absorption maximum at 407–410 nm, while pentacoordinated metMb has it blue-shifted to 390–396 nm. Distal histidine, glutamine, or other hydrogen-bonding residues stabilize the heme-linked water. Hydrophobic distal residues, like phenylalanine, valine, or leucine, prevent binding of water to the sixth coordination site and result in a pentacoordinated ferric iron. A distal threonine results in partial occupancy of water and an intermediate spectral behavior. Below pH 9, the Soret absorptions of the met-FixL's are characteristic of a high-spin pentacoordinated heme iron (Figure 3A; Table 1). While in Mb or HbA the transition at high pH is from a water at the sixth coordination position to a hydroxyl, the FixL transition is a more abrupt one from a pentacoordinated to a hexacoordinated heme iron (Figure 4). The cooperativity of hydroxide binding, even in the monomeric RmFixLH, suggests that the affinity of the heme toward hydroxide is linked to deprotonation or hydroxide binding at another site in the monomer. The transition pH's of 9.3–10 for FixL's and ~8.5 for human HbA correspond to a free energy of hydroxymet formation that is 1.3–2.2 kcal mol^{-1} more positive in FixL's.

Despite the difficulties we encountered due to the high autoxidation rates of the FixL's, the oxygen affinities derived from our equilibrium measurements agree with those calcu-

Table 2: Ligand Binding Parameters of FixL Proteins at 25 °C[a]

ligand	protein	P_{50} (mmHg)	K ($\times 10^{-4}$ M^{-1})	k_{on} ($\times 10^{-4}$ M^{-1} s^{-1})	k_{off} (s^{-1})	M^b
O_2	BjFixL	76	0.73	14.5	20	17
	RmFixLT	27	2.0	21.7	(11)[c]	
	RmFixLH	17	3.2	21.7	6.8	7.5
	Aplysia Mb[d]	2.65	21	1500	70	100
	SW Mb[e]	0.48	120	1400	12	23
	SW Mb H (E7)→L[e]	23	2.4	9800	4100	48 000
	human HbA (R)	0.3	80	4000	50	300
CO	BjFixL	4.3	10	0.5	0.045	
	RmFixLT			1.2		
	RmFixLH	2.2	20	1.7	0.083	
	Aplysia Mb[d]	0.02	3000	50	0.02	
	SW Mb[e]	0.028	2700	51	0.019	
	SW Mb H(E7)→L[e]	0.0007	110 000	2600	0.024	
	human HbA (R)	0.001	50 000	600	0.013	

[a] Details in Materials and Methods. [b] Values calculated from $P_{50}(O_2)/P_{50}(CO)$. [c] This value is calculated from K and k_{on}. [d] Wittenberg et al. (1965, 1972). [e] Sperm whale myoglobin (Springer et al., 1989; Quillin et al., 1993).

Table 3: FixL Oxygenated at Various Oxygen Pressures

environment	$P(O_2)$ (mmHg)	% oxy-BjFixL	% oxy-RmFixLT	% oxy-RmFixLH
air	152	67	85	90
microaerobic culture	20	21	43	54
nodule	0.010	0.013	0.037	0.059

lated from our rate constants. They show that the oxygen affinities of FixL's are lower than those of almost all natural oxygen carriers. Even in air, RmFixLT contains 15% of the deoxy form, which is sufficient to account for the residual kinase activity we had observed (Gilles-Gonzalez et al., 1993). FixL-dependent induction of nitrogen fixation genes in free-living wild-type *Rhizobia* occurs even at partial pressures of oxygen as high as 20 mmHg (Ditta et al., 1987). Such induction is consistent with the high P_{50} of RmFixLT (Table 3) and suggests that this truncated FixL, *in vitro,* is a good model for membrane-bound wild-type FixL in *R. meliloti.* RmFixLT would be 99.96% deoxygenated and therefore have maximal kinase activity in nitrogen-fixing nodules, where the partial pressure of oxygen is about 10 μmHg (Table 3) (Appleby, 1969; Wittenberg et al., 1972). The oxygen affinity of RmFixLH is 60% greater than that of RmFixLT, perhaps because RmFixLH consists solely of a monomeric heme domain, while RmFixLT is a dimer containing heme and kinase domains in each subunit and is therefore probably more rigid (Table 2; Figure 1).

The kinase and heme domains of *B. japonicum* and *R. meliloti* FixL's are highly homologous, but not their N-terminal domains (Figure 1) (Anthamatten & Hennecke, 1991). The N-terminal domain of *R. meliloti* FixL contains several transmembrane segments, while the BjFixL N-terminal domain has none (Lois et al., 1993). BjFixL is essential for nitrogen fixation in *B. japonicum,* but its regulatory target is unknown; unlike *R. meliloti* FixL, it does not regulate nitrogenase production (Anthamatten & Hennecke, 1991). The coordinated expression of more than 20 gene products is required for nitrogen fixation. FixL's would begin to function as kinases at relatively high oxygen pressure, possibly inducing genes involved in the transition to and from the nearly anaerobic nodule environment (Table 2). The *fixNOPQ* gene cluster, which encodes the alternative oxidase complex of *B. japonicum* required to support bacterial respiration in nodules, is an excellent candidate (Preisig et al., 1993). FixL proteins may sense other heme ligands in addition to oxygen. In the nearly anaerobic environment of nodules, oxygen binding would not interfere with the detection of other ferrous heme ligands such as nitric oxide or carbon monoxide, even at very low concentrations.

The low oxygen affinity of FixL's is due entirely to a very slow association rate (Table 2). On the other hand, Mb mutants that lack heme-linked water in their met forms, due to replacement of the distal histidine by either leucine, valine, or phenylalanine, have off-rates for oxygen accelerated 300-, 460- and 700-fold, respectively. Surprisingly, the on-rates are also accelerated, although this is only by 6, 7, and 5 times, respectively. The replacements all increased the affinity for carbon monoxide, due to rises in on-rates outweighing smaller rises in off-rates. Their on-rates are raised probably because a water molecule, hydrogen-bonded to the distal histidine (but not bound to the ferrous iron) in native deoxyMb, presents a kinetic barrier to oxygen binding; this water molecule is absent when the distal histidine has been replaced by a hydrophobic residue. All these replacements in Mb raised the value of M between 400- and 1000-fold (Quillin et al., 1993), whereas FixL has a very low M. In *Aplysia limacina* Mb, the distal histidine is replaced by a valine, so that its ferric form also lacks a heme-linked water molecule; but unlike FixL, it has a rather high oxygen affinity due to a high on-rate and a slow off-rate (Table 2) (Conti et al., 1993; Wittenberg et al., 1965, 1972). The former is due to ready access to its open heme pocket, and the latter, to the ability of its arginine E10 to swing into the heme pocket and donate hydrogen bonds to electronegative heme ligands.

There is no model that explains the low affinities for both oxygen and carbon monoxide, caused almost entirely by slow on-rates. In their extensive experiments on the effects of proximal and distal hindrance in the synthetic picket fence heme complex, Collman, Gibson, and their colleagues (1983) found that steric hindrance on either side of the heme increased the off-rates for oxygen but left the on-rates almost unchanged. Proximal steric hindrance slowed the on-rates for carbon monoxide and accelerated the off-rates about equally. Distal hindrance slowed the on-rate for carbon monoxide and left the off-rate unchanged. This did lower M, but even the lowest value is still 100 times higher than that of FixL (Collman et al., 1983).

Autoxidation of Hb is often attributed to dissociation of superoxide ion (O_2^-) from oxyHb. Interesting new light on the mechanism that prevails in the absence of a distal hydrogen bond donor to the bound oxygen has emerged from a study of the autoxidation of the iron cyclidenes pictured in Figure 5A. Dickerson et al. synthesized a series of such bridged cyclidenes in which R1 is an aliphatic chain varying in length

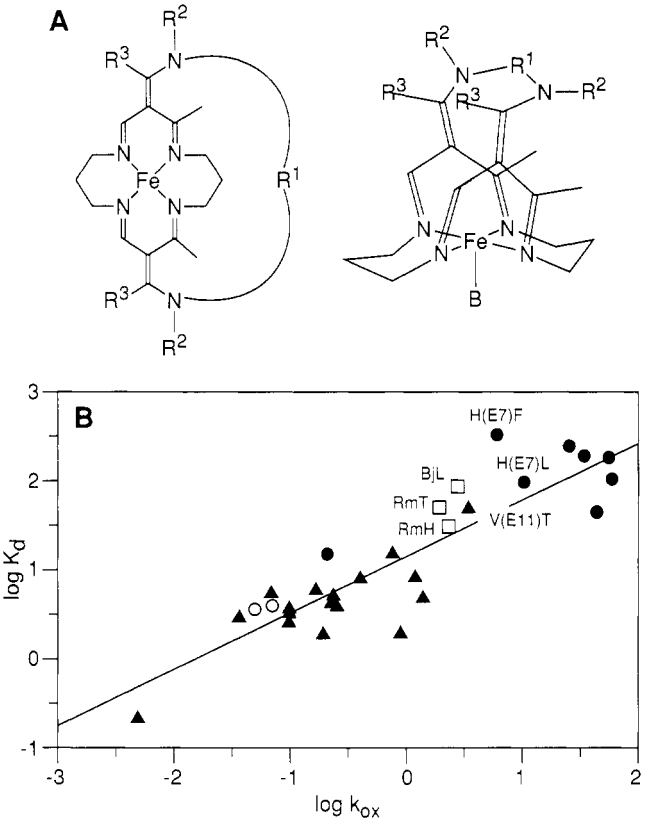

FIGURE 5: Relationship of FixL autoxidation to synthetic iron(II) cyclidene models and myoglobin variants. Panel A shows the structure of the bridged cyclidene [$R^1 = (CH_2)_4$] that most closely resembles FixL in its autoxidation (Dickerson et al., 1993). Panel B is a comparison of the autoxidation rates (h^{-1}) and oxygen equilibrium dissociation constants (μM) for FixL's and for myoglobins from sperm whale and pig: FixL's (□), wild-type Mb (○), histidine (E7) Mb mutants (●), other mutations of highly conserved Mb residues (▲). This plot is based on Figure 7 of Brantley et al. (1993) and Mb data from Tables II–V of the same article. BjFixL, RmFixLT, and RmFixLH are shortened to BjL, RmT, and RmH, respectively.

from $(CH_2)_3$ to $(CH_2)_5$ and above (Dickerson et al., 1993). $(CH_2)_3$ does not bind either O_2 or CO; $(CH_2)_4$ combines with the two gases with low affinity. Thereafter, the affinity rises with increasing length of the aliphatic chain. The oxidation rate of $(CH_2)_3$ is fast and follows simple first-order dependence on $P(O_2)$, while according to the superoxide dissociation mechanism it should not autoxidize at all. The authors argue that such first-order dependence is consistent only with an "outer shell mechanism", i.e., electrons being transferred from iron to oxygen colliding with the outer shell of the complex. The autoxidation rate of $(CH_2)_4$ reaches saturation at $P(O_2)$ > 60 mmHg, when oxygen combining with and protecting the ferrous iron competes successfully with electron transfer to oxygen. $(CH_2)_5$ shows a steep rise in autoxidation rate up to $P(O_2)$ = 10 mmHg and then a steep fall, with the rate approaching zero near $P(O_2)$ = 100 mmHg where protection by the bound oxygen dominates.

The mechanism of Dickerson et al. predicts that autoxidation rate will be inversely related to oxygen affinity, since the deoxy form is the reactive species. It further predicts that heme pockets in which absence of hydrogen bonding disfavors occupancy by water will show the autoxidation dependence on $P(O_2)$ of the type described above for $(CH_2)_4$-bridged cyclidenes. The approximately linear relationship between the log of the autoxidation rate and the log of the oxygen dissociation constant of Mb mutants, observed by Brantley et al. (1993), supports the Dickerson model. FixL fits very well on this plot (Figure 5B). When the distal histidine in myoglobin is replaced by a valine, its autoxidation rate varies with the partial pressure of oxygen in much the same way as that of the $(CH_2)_4$-bridged cyclidene (Brantley et al., 1993; Quillin et al., 1993). RmFixLT, which resembles the H(E7)→V mutant, in the absence of a water molecule linked to its ferric iron, has the same elevated autoxidation rate in air as at 1 atm of oxygen, again fitting this model (Table 1). The rates of autoxidation of FixL's and the bridged cyclidene that does not bind oxygen are comparable: 1.9–2.7 versus 0.9 h^{-1}.

There is little evolutionary pressure to evolve resistance to oxidation in the extremely reducing, nearly anaerobic environment of a nitrogen-fixing nodule. Despite the high autoxidation rates of FixL's (Table 1), even mildly reducing conditions protect their heme irons from oxidation. In 10 mM β-mercaptoethanol, in air, ferric *R. meliloti* FixL is reduced to oxy-FixL and does not autoxidize even after two days at room temperature. There is therefore no need for reductases of the kind present in red cells and muscle tissue.

What can we infer about the heme environment? The absorption spectra of deoxy-, oxy-, carbonmonoxy-, and met-FixL leave no doubt that the proximal heme ligand is a histidine, since heme proteins with cysteine, methionine, or tyrosine as proximal ligands show very different absorption spectra. The very low oxygen affinity and the absence of a water molecule at the sixth coordination position of the ferric heme are indicative of nonpolar residues on the distal side of the heme. The low association rates for both oxygen and carbon monoxide suggest that these residues hinder ligand binding. There is one highly conserved histidine in RmFixLH that is likely to be the proximal ligand. In the myoglobin sequence, the axial ligands are separated by 20–30 residues. Either of two phenylalanines that are about this distance from the conserved histidine might be the distal residue.

FixL proteins are the only known members of a third class of heme proteins, which we are calling the heme-based sensors; they are distinct from oxygen carriers or electron transporters. As such, the uniqueness of their properties is not surprising. We predict that other heme-based sensors exist, which sense not only oxygen but other heme ligands such as nitric oxide, and that these sensors will share some of the novel characteristics of FixL. Likely candidates include the endothelial nitric oxide sensors that regulate blood pressure and the sensors that regulate hypoxia-induced genes such as the erythropoeitin gene and the Fos and Jun protooncogenes (Blanchard et al., 1992; Webster et al., 1993; Wang & Semenza, 1993).

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