Nonsteric factors dominate binding of nitric oxide, azide, imidazole, cyanide, and fluoride to the Rhizobial heme-based oxygen sensor FixL

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Background: The FixL protein is a heme-based sensor. Binding of oxygen to a unique heme domain inhibits a kinase domain of the type found in two-component regulators. Oxygen association is slow, but the dissociation rate is comparable to that of myoglobins. We have probed the size and chemistry of the FixL heme pocket by measuring the affinities, on rates and off rates for a wide variety of ferric heme ligands. Cyanide, but not fluoride, regulates the kinase activity. To examine how the sensory heme domain interacts with the kinase, we asked how the presence of the kinase domain affects ligand binding.

Results: The affinities of ferric FixL for heme ligands follow the same trend as their pK_a values: cyanide > 4-methyl imidazole > imidazole > fluoride > azide >> thiocyanate. The association rates follow the reverse trend. Striking differences from myoglobin include a 6-fold greater affinity for, and faster binding to, the bulky ligand imidazole, a 14-fold faster on rate for nitric oxide, a 2 800-fold lower affinity for azide, and a complete failure to bind thiocyanate. The presence of the kinase domain does not alter the affinity or binding kinetics of the high-spin ligand fluoride, but affects the off rates of other ligands. The EPR spectrum shows a characteristic pentacoordinate nitrosyl heme, indicating that the Fe–His bond in FixL is strained.

Conclusions: The importance of ligand deprotonation to the on rates and the fact that large ligands bind readily indicate that the heme pocket is open and apolar. Ligand basicity strongly influences the strength of binding. The destabilization of inhibitory ligands by the presence of the kinase domain is consistent with a 'load' imposed by coupling to the inactivating mechanism.

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Introduction

The FixL protein of *Rhizobia* is a heme-based oxygen sensor that regulates the expression of nitrogen fixation genes. This protein contains a carboxy-terminal kinase domain of ~240 amino acids that is homologous to the sensor kinases of the two-component regulatory systems of *Rhizobia* [1]. In the primary structure, a heme binding region of ~150 amino acids, unrelated to any known hemoproteins, directly precedes the kinase [2]. On binding of oxygen, this heme domain reversibly inhibits autophosphorylation of the kinase domain [3]. If autophosphorylation is not inhibited the phosphoryl group is transferred from phospho-FixL to a transcription factor, which then activates expression at the *nif* and *fix* promoters [4,5]. The regulation of the enzymatic activity of FixL is not limited to oxygen; what is essential is the switch of the heme iron to low spin on binding a strong field ligand [6].

FixL is an unusually simple sensor kinase, as it has a complete sensing mechanism directly attached to its kinase domain. The fact that a heme moiety is at the heart of the signaling mechanism in this protein presents a unique opportunity to examine how a signal is received and transduced to the kinase domain, the most elusive aspect of the signal transduction mechanism in sensor kinases. Unlike most two-component systems identified, the chemical nature of the input signal for FixL and the step of the phosphoryl cycle that it regulates are known [3]. Apart from its importance for the study of protein–protein interactions during signal transduction, FixL also offers a chance to study the movement of ions in and out of a new type of heme pocket that has evolved to sense, rather than carry, heme ligands.

The three-dimensional structure of the FixL heme pocket and the identity of the residue closest to the bound ligand (the distal residue) are not yet known. Based on the FixL absorption spectra, Perutz and colleagues [7] have concluded that ferric FixL without added ligand (met-FixL) does not coordinate water [7].

The pentacoordinate high-spin nature of met-FixL has been confirmed by its resonance Raman spectra [8]. Perutz and colleagues have further surmised that the heme iron is linked to the protein via a histidine (the proximal histidine) and that the distal residue is likely to be aliphatic. ^{1}H-NMR spectra of cyanomet-FixL have now established coordination to histidine [9]. Although histidine is the distal residue in the vast majority of oxygen-binding hemoproteins, several lines of evidence point to an aliphatic distal side chain in FixL. First, hemoproteins with polar distal residues almost always have hexacoordinate met-forms, due to stabilization of bound water by hydrogen bonding, while pentacoordinate ferric heme is always found in heme proteins with an aliphatic distal side chain [10,11]. Second, Bertolucci *et al.* [9] have failed to find any distal histidine signals in the ^{1}H-NMR spectra of cyanomet-FixL. Such signals are easily identified for both myoglobins and peroxidases. Finally, there are only three histidines in the FixL heme domain, His138, His192, and His194. Except for His194, whose replacement abolishes heme binding altogether, substitution of the other histidines with glutamine or arginine had no effect on the absorption spectra ([12]; M.A.G.-G., unpublished data). FixL is only 85 % saturated in air, and any change in affinity larger than two-fold would have been noticeable in the absorption spectra. Substitutions of distal histidines usually result in much larger changes [13].

Despite the apparent lack of a distal histidine, the dissociation rates of oxygen and carbon monoxide from FixLs are comparable to those of myoglobins. Furthermore, the low values for M (K_{CO}/K_{O_2}) show that FixL can discriminate against carbon monoxide as well as or better than myoglobins having a distal histidine [7]. This is surprising, since proteins with aliphatic distal sidechains tend to have accelerated rates of oxygen dissociation and poor discrimination against carbon monoxide [13]. FixLs must therefore possess some unknown but very effective means of ligand stabilization and carbon monoxide discrimination. Nevertheless, they have unusually low oxygen affinities due to slow on rates. All of this, and the complete absence of homology to any other hemoproteins, point to a heme pocket that is distinctly different from those of known hemoproteins.

The factors influencing the binding of ligands to heme proteins include steric effects, polarity of the pocket, displacement of water, deprotonation of the ligand, and electrostatic interactions. The relative importance of each factor depends on the ligand as well as the heme pocket. Therefore a comparison of the binding to a variety of ligands is a rich source of information on the properties of the heme pocket and its interaction with the rest of the protein. In the past decade, Olson and collaborators [13–16] have generated amino-acid substitutions in the heme pocket of myoglobins that have greatly increased our understanding of the influences of various structural features on binding of each type of ligand.

As well as the processes that normally accompany ligand binding, such as movement of sidechains, protonation, and solvation, FixL has the unique associated step of kinase inactivation. Like any other coupled process, kinase inactivation must profoundly affect ligand binding. For example, deletion of the kinase leads to a 60 % increase in oxygen affinity [7]. In the unliganded FixL, the heme iron is high spin and lies out of the heme plane, toward the proximal histidine. Perutz and colleagues [6] have proposed that the switch to low spin that accompanies binding of oxygen moves the iron atom and its attached histidine toward the plane of the heme, resulting in a 'tense' conformation of the protein. This tense state cannot undergo the conformational changes required for autophosphorylation. The spin-state hypothesis predicts that other ligands that change the spin of the heme iron will inactivate the kinase. Ferric FixL has been shown to be inhibited by cyanide, and cyanomet-FixL is indeed low spin [6,9]. To date, no other mechanism accounts, as the spin-state hypothesis does, for sensing in both oxidation states and equal inactivation by such different ligands as oxygen and cyanide.

The spin-state hypothesis has led to the proposal that oxygen may not be the only physiologically relevant heme ligand that binds to FixL [6]. For example, FixL might shut down nitrogen fixation in response to nitric oxide [7]. This would occur during denitrification, an anaerobic process that runs counter to nitrogen fixation and generates nitric oxide as an intermediate. *Rhizobium meliloti* and *Bradyrhizobium japonicum*, both of which have FixL proteins, can perform denitrification [17].

Here we present the first measurements of binding of nitric oxide, azide, and imidazoles to FixL, as well as more complete and precise measurements of cyanide and fluoride binding than previously reported [6,8]. We compare binding to that of myoglobin and discuss the implications for the unique character of the FixL heme pocket. We also report the effect on heme ligand binding of deleting the kinase and consider what this tells us about interdomain communication.

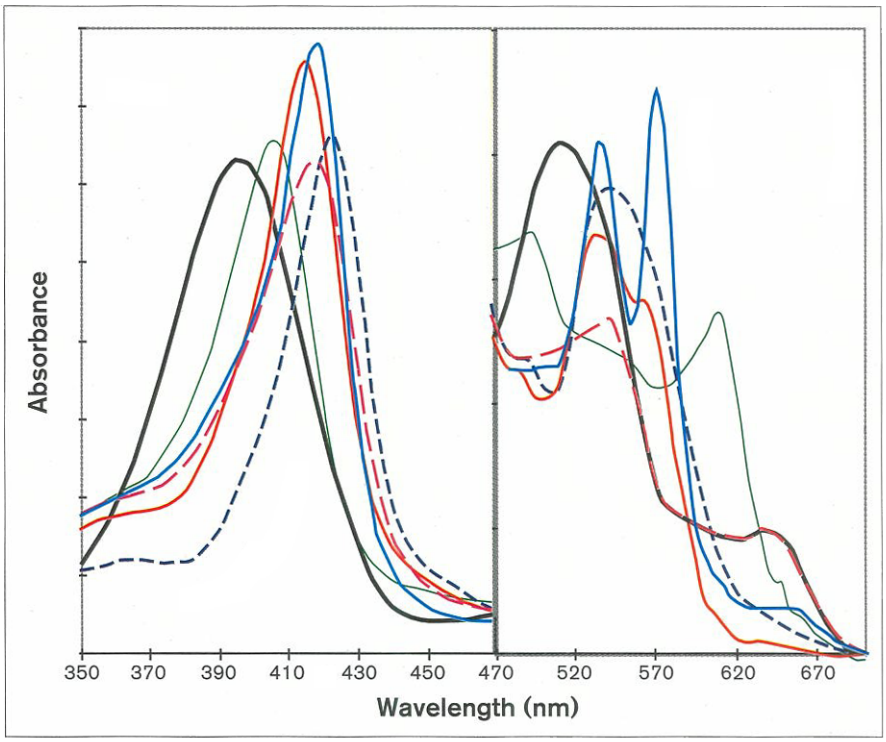
Results

Affinities of *R. meliloti* FixL for various ligands

Figure 1 shows the absorption spectra of met-, azidomet-, imidazole met-, cyanomet-, nitric oxide met- and fluoromet-RmFixLH. RmFixLH is a truncated *R. meliloti* FixL protein, containing the isolated heme domain. These absorption spectra are indistinguishable from those of RmFixLT, which has a kinase domain. The absorption spectra of the FixL derivatives are similar to the corresponding myoglobins, with the exception of the met-form.

Figure 1

Absorption spectra of the met- (black, thick line), azidomet- (magenta, long dashes), cyanomet- (blue, short dashes), fluoromet- (green, thin line), imidazole met- (red), and nitric oxide met-FixL (cyan) of *R. meliloti*. The spectra are shown for derivatives of met-RmFixLH, the isolated heme domain of the *R. meliloti* FixL. There was no noticeable difference between these absorption spectra and the spectra of the derivatives of RmFixLT, which has kinase attached to the heme domain, nor between the spectra of the imidazole and the 4-methyl imidazole derivatives. The absorbance scale is expanded eight-fold for wavelengths above 470 nm, for clarity.

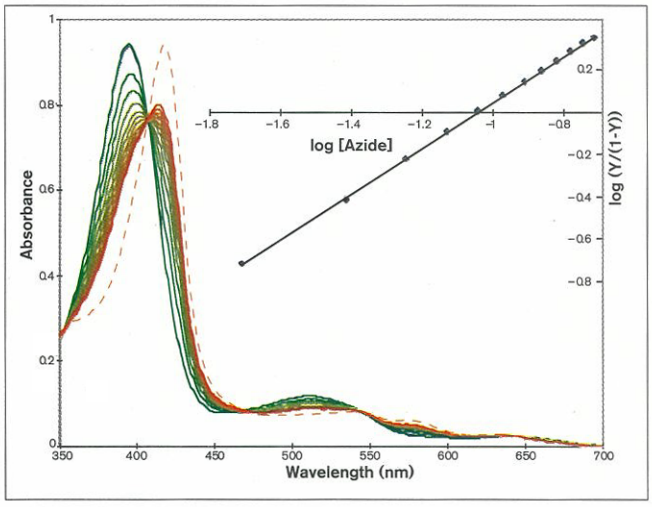


The 11-nm blue-shift in the Soret absorption band, compared to myoglobins, had led us to conclude that the heme iron in met-FixL is pentacoordinate [7]. This is now confirmed by resonance Raman spectroscopy [8]. The affinity of the *R. meliloti* FixL for ligands of ferric heme increases in the following order: cyanide > imidazole > fluoride > azide >> thiocyanate. Specifically, the K_d values of RmFixLH for cyanide, imidazole, fluoride, and azide were 0.0046, 2.0, 16, and 92 mM, respectively, while no binding was detected for thiocyanate even at 1 M. All of these ligands of ferric heme bind noncooperatively to met-FixL (n value = 1).

Azide and thiocyanate binding

The most striking differences between myoglobin and FixL ligand binding occur for azide and thiocyanate. The azide association rate of FixL, like that of *Aplysia* myoglobin, was much too fast to be measured by stopped-flow techniques, especially since the low affinity of FixL for azide rendered it impractical to slow the k_{obs} by reducing the concentration of azide (Table 1). The K_d of FixL for azide is > 2 000 times higher than that of sperm whale myoglobin. Based on the limitations of our stopped-flow instrumentation, we conservatively estimate that the on rate for azide is > 50 000 $M^{-1}s^{-1}$. Figure 2 shows the titration of met-FixL with this ligand. At concentrations of azide of > 200 mM, ionic strength effects on the FixL absorption spectra result in deviations from the isosbestic points. For this reason, the absorption spectrum for fully saturated azidomet-FixL was calculated, as detailed in Materials and methods (Fig. 2, broken line). This spectrum was subsequently found to be remarkably similar to the absorption spectrum of azidomet-hemoglobin.

Figure 2



Binding of azide to *R. meliloti* FixL. The absorption spectra show the conversion of met-RmFixLH (green) to azidomet-RmFixLH (red) on titration with 0–200 mM sodium azide at 25°C, pH 8.0. The broken line represents the fully saturated azidomet-RmFixLH. A Hill plot of the data is at the top right corner.

The difference in thiocyanate affinity is even more dramatic. Thiocyanate will not bind detectably to FixL, even at 10^6-fold excess, regardless of pH (6 to 10), solvent polarity (up to 50 % ethylene glycol or ethanol) or ionic strength, whereas it binds readily to myoglobin (K_d = 10 mM for horse myoglobin).

Binding of imidazole and 4-methyl imidazole

Imidazole binds more strongly to FixL than to myoglobin. The bulkier 4-methyl imidazole binds even more tightly. Specifically, imidazole and 4-methyl imidazole bind to the heme domain of *R. meliloti* FixL with 4- and 12-fold greater affinity, respectively, than their affinities for myoglobin (Table 1). Figure 3 shows the equilibrium titration of met-RmFixLH with imidazole.

We have found that imidazole inhibits the autophosphorylation of RmFixLT; however, we were unable to achieve more than 75 % inactivation, even at saturating concentrations of imidazole. This behavior is unlike that

Table 1

Rates and equilibrium constants for binding of ligands to FixL.

	Protein	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (mM)
Ligands of ferrous heme				
O_2	RmFixLH[a]	2.2×10^5	6.8	0.031
	RmFixLT[a]	2.2×10^5	11	0.050
	BjFixL[a]	1.4×10^5	20	0.14
	SW Mb[b]	1.4×10^7	12	0.00083
	Aplysia Mb[c]	1.5×10^7	70	0.0048
CO	RmFixLH[a]	1.7×10^4	0.083	0.0049
	RmFixLT[a]	1.2×10^4		
	BjFixL[a]	5.0×10^3	0.045	0.010
	SW Mb[b]	5.1×10^5	0.019	0.000037
	Aplysia Mb[c]	5×10^5	0.02	0.00003
Ligands of ferric heme				
NO	BjFixL	1.0×10^6		
	SW Mb[d]	7.0×10^4		
N_3^-	RmFixLH	$>5 \times 10^4$		92
	RmFixLT			280
	Horse Mb[e]			0.1
	SW Mb[e]	2.5×10^4	0.9	0.04
	Aplysia Mb[f]	1.8×10^6	750	0.32
Imidazole	RmFixLH	5.0×10^4	100 (85)g	2.0
	RmFixLT	3.7×10^4	48 (66)g	1.3
	Horse Mb[e]	6.8×10^2	5.1	7.4
	Aplysia Mb[f]	$>1 \times 10^3$	>100	100
4-Methyl imidazole	RmFixLH	8.2×10^3	4.9 (2.5)g	0.60
F^-	RmFixLH	1.8	0.031	16
	RmFixLT	1.9	0.033	18
	Horse Mb[e]	5.3×10^{-1}	0.0093	18
	Aplysia Mb[f]	1.5×10^3	10	10
CN^-	RmFixLH	2.7×10^1	0.00010	0.0046
	RmFixLT	3.1×10^1	0.00015	0.0060
	Horse Mb[e]	4.0×10^2	0.0019	0.0047

Binding of ferric ligands to FixL was at 25 °C, pH 8.0. Association rates were measured by stopped-flow. Equilibrium constants were determined from titrations, except as noted. The dissociation rates of cyanide and fluoride were measured directly by replacement with imidazole (see Materials and methods). The average relative error for our measurements is estimated at ± 15 %. BjFixL is the *Bradyrhizobium japonicum* FixL. Carbon monoxide and oxygen binding to FixL were reported earlier [7].

[a][7] measured at pH 7.5, 25 °C, K_d values are calculated.

[b][11,26] pH 7.0 at 20 °C.

[c][24,25] pH 7.0 at 20 °C.

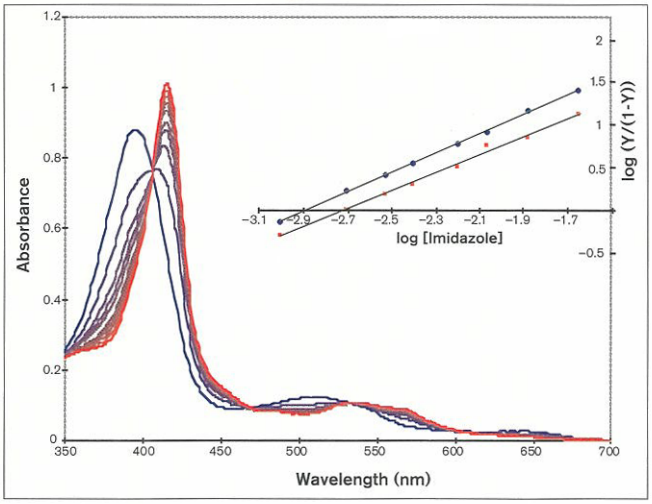
[d][16] pH 7.0 at 20 °C.

[e][23] pH 8.0 at 25 °C.

[f][21] azide at pH 6.0 and 20 °C, imidazole at pH 6 and 20 °C, fluoride at pH 7.0 and 20 °C.

[g]Imidazole's rate constants were so high that direct measurement of k_{off} was impractical. Dissociation rates of imidazole and 4-methyl imidazole were calculated from the measured K_d and k_{on} or from extrapolation (data in parentheses) of the apparent on rates to zero ligand.

Figure 3



The presence of the kinase domain increases the affinity of imidazole for *R. meliloti* FixL. The absorption spectra show the conversion of met-RmFixLT (blue) to imidazole met-RmFixLT (red) on titration with 0–106 mM imidazole at 25 °C, pH 8.0. The Hill plots at the top right corner show that the affinity of met-RmFixLT (blue circles) for imidazole is higher than that of met-RmFixLH (red squares), which lacks the kinase domain.

of cyanide, for which kinase inactivation is directly proportional to the fractional saturation with the ligand [6]. Interestingly, the presence of the kinase domain causes a 50 % increase, not a reduction, in the affinity for imidazole, opposite to what we observed for the effect of kinase deletion on affinity for oxygen [7].

The rate of association of imidazole with RmFixLH is 5.0×10^4 $M^{-1}s^{-1}$, > 70 times faster than its association with myoglobin, with a hyperbolic dependence on the ligand concentration (Table 1, Fig. 4). Nevertheless, each individual time course fit a single exponential (Fig. 4, inset). The bulkier ligand, 4-methyl imidazole, shows an even higher affinity for RmFixLH, largely because its off rate is 20-fold slower than that of imidazole; its on rate is also reduced, but only 6-fold (Table 1).

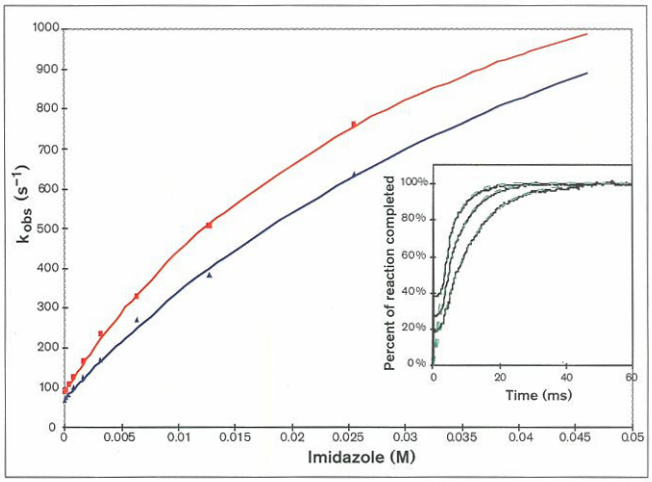
Binding of cyanide

The affinity of met-RmFixLH for cyanide is similar to that of met-myoglobin (Table 1). However, the kinetics are very slow, with on and off rates slowed by 20- and 10-fold, respectively. The presence of the kinase in RmFixLT has little effect on the on rate of cyanide, but accelerates its off rate by 50 %. The first measurements of cyanide binding reported cooperativity [6]. This was an error due to the effects of carbon dioxide, and possibly oxygen, in the buffers [18]. A recent report of cyanide binding constants listed faster off rates [8]. This may be due to the use of a single wavelength to follow the reaction. This approach results in baseline problems arising from changes in the sample over the very long times required for this slow dissociation. The data in Table 1 are based on fitting of entire spectra and are unaffected by the addition of a constant to any spectrum. Our use of imidazole as a replacing ligand gives a very accurate endpoint for the reaction, as there is no residual cyanomet-FixL.

Binding of fluoride

Although the affinities of the truncated FixL proteins for fluoride are very similar to that of myoglobin, on and off rates are significantly faster (off rate three times faster, on rate four times faster) (Table 1). Comparison of RmFixLT to RmFixLH shows that the presence of the kinase has no significant effect on either the affinities or the kinetics of binding to fluoride. The independently measured k_{on}, k_{off}, and K_d values in Table 1 are in agreement. Ionic strength has no effect on these values over the range of fluoride concentrations used (see Materials and methods). Rodgers and his colleagues have reported a much higher affinity, based not on equilibrium measurements but calculated from their off rate of $(4.14 \pm 0.06) \times 10^{-2}$ s^{-1} and on rate of 5.61 ± 0.47 M^{-1} s^{-1} for RmFixLH [8]. No values were given for RmFixLT. The discrepancies between our measurements and theirs are due primarily to the differences in the mixing times. Their measurements are based on hand mixing. Given an off rate corresponding to a half life of 17 s, the mixing time was a significant

Figure 4



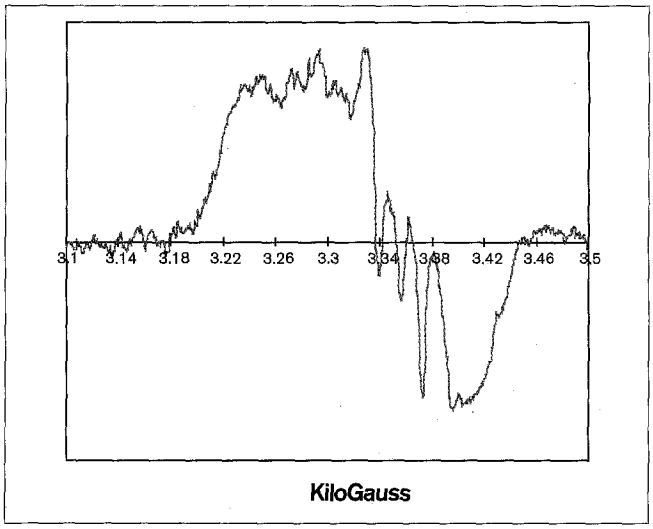
Rates of association of imidazole with *R. meliloti* FixL. This figure illustrates the hyperbolic dependence of imidazole binding to RmFixLH (red squares) and RmFixLT (blue triangles). The rate of imidazole association with RmFixLT is slower than its association with RmFixLH (see also Fig. 3). The limiting velocities are 1600 and 1900 s^{-1} for RmFixLH and RmFixLT, respectively, at 25 °C, pH 8.0. Half of the limiting velocity is reached at 35 and 61 mM imidazole for RmFixLH and RmFixLT, respectively. The inset at the bottom right corner shows the raw kinetic traces obtained with 0.80, 1.6, and 6.4 mM imidazole and the fit of each trace to a curve describing a single exponential (green line).

fraction of the time required for reaction. For the association rate measurements, hand mixing would only be feasible at very low concentrations of fluoride. Given the low affinity for fluoride, this implies very small absorbance changes and an error larger than that estimated. The fluoride on and off rates reported in Table 1 were determined by stopped-flow methods, with a dead time of 2 ms; the dissociation rates are based on imidazole replacement of a series of five fluoride concentrations (see Materials and methods).

Binding of nitric oxide and coordination state of nitrosyl heme

The association rate of nitric oxide with met-BjFixL (the FixL protein from *B. japonicum*, which was used because the binding of NO to the truncated *R. meliloti* proteins is too fast to measure by stopped flow) is 1.0 x 10^6 M^{-1} s^{-1}; this is 14-fold faster than the on rate for sperm whale myoglobin [16] (Table 1). The electron paramagnetic resonance (EPR) spectrum of the Fe^{II} nitric oxide derivative of hemoproteins reveals whether the heme iron is pentacoordinate, hexacoordinate, or found in a mixture of these states. The finding that the nitrosyl heme is pentacoordinate is diagnostic for strain in the Fe–His bond. The classic three-line ^{14}N nitrosyl-heme signature at 3.33–3.4 kiloGauss in the spectrum of RmFixLT in Figure 5 indicates that as much as 50% of the protein has pentacoordinate nitrosyl heme.

Figure 5



EPR spectrum of Fe^{II} nitric oxide RmFixLT. Presence of the classic three-line ^{14}N nitrosyl-heme signature at 3.33–3.4 kiloGauss indicates that as much as 50 % of the signal is from pentacoordinate nitrosyl heme. The EPR parameters were: microwave frequency, 9.44955 GHz; microwave power, 10 mW; modulation amplitude, 5 Gauss; temperature, 150 K.

Discussion

Heme–kinase interactions

The spin-state mechanism implies that motion of the iron atom into the heme plane changes the structure on the proximal side of the heme. Any work done to inactivate the kinase constitutes a 'load' on the in-plane movement of the heme iron that accompanies binding of strong-field ligands. The observation that the EPR spectrum of Fe^{II} nitric oxide RmFixLT contains a significant contribution due to pentacoordinate nitrosyl heme shows that the Fe–His bond is indeed strained (Fig. 5). Binding of nitric oxide weakens the Fe–His bond and ruptures it if it is sufficiently strained. In FixL, the strain is only just sufficient to begin rupturing the bond, leading to incomplete rupture, instead of the essentially complete disruption observed for the α-subunits of hemoglobin bound to nitric oxide. This effect of nitric oxide on ferrous FixL would not be evident in protein preparations of poor quality. The strain on the Fe–His bond in FixL would certainly reduce the affinity of RmFixLT for strong-field ligands compared to RmFixLH. The effects on the on rate would not be as straightforward, since ligand binding is a multi-step process, and only the final bond formation would be affected by loading. The off rates should be accelerated by strain, since the energy required to break the Fe–ligand bond is offset by the relaxation from a tense state on release of the ligand. These effects of proximal side loading would be greatest for strong-field ligands (e.g., cyanide, imidazole, oxygen), intermediate for mixed-spin ligands (e.g., azide), and nonexistent for weak-field ligands (e.g., fluoride). The results for oxygen, cyanide, and fluoride are consistent with these predictions. The kinase domain had no detectable effect on binding of fluoride and accelerated the off rates of cyanide and oxygen by about 50% (Table 1).

A proximal side strain energy of about 240 calories mol^{-1} would result in the observed decrease in the affinity of RmFixLT for cyanide, compared to RmFixLH. For comparison, the proximal side tension of the T-state in hemoglobin results in a 3.3-fold decrease in the affinity for cyanide, corresponding to 700 calories mol^{-1} [19]. This reduced strain in FixL, compared to hemoglobin, is reflected both in the smaller effect on ligand affinities and in the incomplete rupture of the Fe–His bond on binding of nitric oxide [19,20] (Fig. 5). Although these strain energies are small, the precedent of hemoglobin establishes that energies of this size are sufficient to bring about profound changes in the properties of a molecule with relatively little dependence on the precise nature of the heme ligand.

Ligands also cause structural changes on the distal side, but these are highly dependent on the specific nature of the ligand. For particular ligands, such distal-side changes could assist or interfere with triggering the conformational change that inactivates the kinase. For example, the affinity of RmFixLT for imidazole is higher, not lower, than that of RmFixLH. This suggests an additional steric

barrier and stabilizing factor in RmFixLT that is not present in the isolated heme domain. This type of interaction is not unusual in hemoproteins. In most myoglobins, the distal histidine hinders the entry of ligand but stabilizes the ligand by hydrogen bonding once it is bound. Alternatively, imidazole, a bulky ligand that binds perpendicularly to the heme, may perturb regions of the heme pocket unaffected by smaller ligands. Even in *Aplysia* myoglobin, whose heme pocket is comparatively open, imidazole binding significantly alters a portion of the structure known as the CD region, which is unaffected by cyanide, azide, or fluoride binding [21]. Analogous alterations of parts of the FixL structure not normally perturbed by ligand binding might interfere with the kinase inactivation machinery. This could account for both the incomplete inactivation of kinase by imidazole and a resultant diminution of proximal side loading. In the absence of proximal loading, other protein–protein interactions that are normally minor could dominate the effect of kinase on imidazole binding. The reduction in affinity for RmFixLT relative to RmFixLH is greatest for azide, although azide binding results in mixed-spin and presumably intermediate loading effects. This indicates the contribution of other factors besides loading, including possible differences in the distal side of the heme pockets of the two proteins. These differences between RmFixLT and RmFixLH are not necessarily relevant to regulation and may simply be the result of truncation.

Effect of the distal residue in FixL

Absorption spectra, ^{1}H-NMR spectra, and mutagenesis data suggest very strongly that FixL has an aliphatic residue in place of the distal histidine usually present in myoglobins [7,9,12]. The important role of the distal histidine in mammalian myoglobins, and the dramatic effects of mutagenizing this residue, might lead one to attribute FixL's unusual ligand-binding characteristics to its distal residue. A large number of mutagenesis and structural studies have established at least three roles for the distal histidine in myoglobin: it is a steric barrier, it increases the polarity of the heme pocket, and it hydrogen bonds to polar ligands, increasing the affinity. The relative importance of each of these effects depends on the ligand. On the other hand, similar studies show that hemoproteins that function naturally without a distal histidine satisfy the function of this residue in alternative ways and need not resemble mutagenized sperm-whale myoglobin [21,22]. FixL's ligand-binding behavior has as little resemblance to myoglobins with alternative distal residues as it does to typical myoglobins (Table 1). *Aplysia* myoglobin has a distal valine, yet FixL differs more from *Aplysia* myoglobin in its imidazole binding than it does from sperm whale myoglobin [21,23]. The affinity of *Aplysia* myoglobin for azide is over 300-fold higher than that of RmFixLH [21]. Even for oxygen, while *Aplysia* myoglobin shows a somewhat reduced affinity, this is due to increased off rates, whereas in FixL it is the on rates that are dramatically slower [7,24,25]. Replacement of the distal histidine does lead to a remarkable reduction in the affinity of mammalian myoglobins for azide, but the lowest affinity mutant of this kind, HisE7→Ile, in which the distal sidechain is not coordinated to heme, still binds azide 15-fold more strongly than FixL [14]. For cyanide, where the polarity of the heme pocket is more important, replacement of the distal histidine in myoglobin by an aliphatic sidechain leads to affinities 20- to 80-fold weaker than the wild type [15]. Yet FixL's affinity for cyanide is very similar to that of myoglobins. Ligand binding is a function of the entire heme pocket, and the roles and relative importance of particular residues must be established independently for FixL.

Role of steric effects

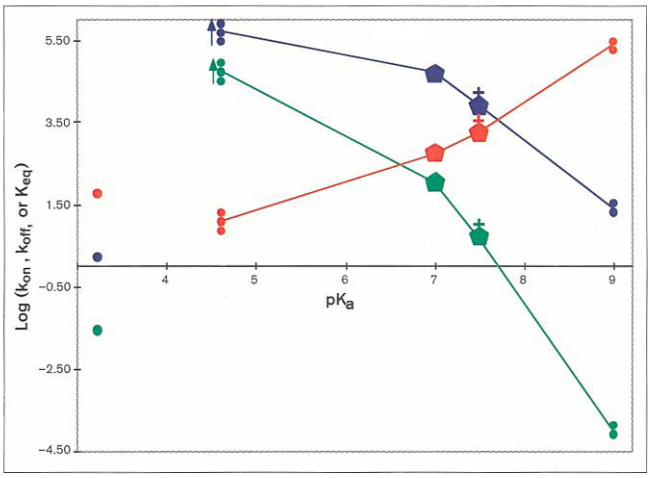
The high affinity and very rapid binding of imidazole make it clear that steric hindrance near the heme iron is not a significant factor for binding of ligands to FixL. This bulky ligand binds at a rate of 5.0×10^4 M^{-1} s^{-1}, over 70 times faster than to myoglobin, with affinities more than three-fold higher (Table 1). In fact, RmFixLH binds 4-methyl-imidazole, which is even bulkier, three times more strongly than imidazole. The hyperbolic dependence of imidazole binding shows that the rate-limiting step, even for these large ligands, is not entry into the heme pocket (Fig. 4). Rather, some concentration-independent step following entry into the pocket, possibly bond formation, is rate limiting. This limiting rate is similar for both imidazoles and for both RmFixLH and RmFixLT.

Two other lines of evidence exist that suggest that FixL has a distal pocket more capacious than that of myoglobin. First, there are the faster entry and exit rates for azide, nitric oxide, and fluoride. Second, the ν_{Fe-CO} stretch of 497 cm^{-1} in the resonance Raman spectra of carbon monoxy-FixL suggests that carbon monoxide binds perpendicularly to the heme plane without nearby polar interactions (Fan, B., Rousseau, D., G.G. and M.A.G.-G., unpublished data). Some factor other than steric hindrance must account for the low affinities for carbon monoxide and oxygen (Table 1) [7].

Importance of ligand basicity

If the heme pocket of FixL offers no significant steric barriers to ligand entry and exit, what then is the basis for ligand discrimination? One important clue is the proportionality between the logs of the affinity constants and the pK_a values of the ligands that are basic (Fig. 6). This trend would imply that heme ligands with $pK_a < 3.5$ would have $K_d > 10$ M, and thus no detectable binding. Thiocyanate, which is the conjugate base of a very strong acid, fits this prediction. The reverse trend is apparent for both the on and off rates of all these ligands, except fluoride. We propose that, for basic heme ligands, the rate of bond

Figure 6



Correlation of the pK_a values of ligands of ferric heme with the association rates, dissociation rates, and affinities of *R. meliloti* FixL for those ligands. Increasing pK_a values of the ligands enhance the affinities (red line), while decelerating both the k_{on} (blue line) and k_{off} (green line). The symbols for the ligands are: azide (three circles), fluoride (one circle), imidazole (pentagon), 4-methyl imidazole (pentagon and cross), and cyanide (two circles). Note the absence of any obvious relationship between the number of atoms in the ligand and the binding parameters. All measurements were at 25 °C, pH 8.0.

formation dominates the kinetics of binding to met-FixL. We further propose that the deprotonation within the heme pocket is the rate-determining step. Fluoride is an exception to these trends. This may be due to special kinetic barriers deriving from the small size of fluoride and its highly hydrated state in solution.

For myoglobin, no simple trends exist between the basicity of the ligands and their kinetic and thermodynamic parameters. Ligand binding to myoglobin is complicated by the steric and stabilizing effects of the distal histidine. For myoglobin, as for FixL, the deprotonation step is a factor. Indeed, for binding of HCN, this appears to be the principal factor [14,15]. But the more crowded myoglobin pocket increases the relative importance of steric factors, while the less polar FixL pocket augments the importance of deprotonation.

Significance

The importance and widespread existence of two-component regulatory systems begs for a complete understanding of their signal transduction events. Although knowledge is rapidly accumulating for various steps, such as autophosphorylation, phosphoryl transfer, and dephosphorylation, the initial signaling step is relatively unexplored. FixL is an excellent model for this, as signal binding and the subsequent kinase inactivation can be easily followed. This can be used to elucidate the mechanism of signal transmission to the kinase. We show here that, in FixL, binding of ferric ligands such as cyanide, imidazole, 4-methyl imidazole, azide, and thiocyanate is dominated by the pK_a values of these ligands and not by their bulk. Moreover, the rate-limiting step in the binding of a large ligand such as imidazole is not entry into the pocket but a subsequent step, possibly bond formation. Therefore the low affinity for oxygen is not due to crowding of the heme pocket.

Correlation of the on rates with ligand pK_a values implies that deprotonation is rate-limiting. We infer a relatively apolar heme pocket. The combination of strong binding of imidazole and no binding of thiocyanate is sufficiently unusual to serve as a diagnostic for heme-based sensors, which have no recognizable heme-binding motifs. This work extends our understanding of binding of ligands to hemoproteins and the movements of small molecules in and out of proteins in general. One possible application of this knowledge is the design of hemoproteins that will be appropriate as blood substitutes. Of all ligands, only fluoride, which does not alter spin, is unaffected in its affinity, on rate and off rate by removal of the kinase domain. This observation, together with the strained Fe–His bond revealed by EPR spectroscopy, provide further support for a spin-state-mediated regulation of the kinase.

Materials and methods

Protein purification

The *R. meliloti* truncated protein RmFixLT and the FixL heme domain RmFixLH were overproduced in *Escherichia coli* strain TG1 carrying plasmid pRT51 and pRH61, respectively [7]. The proteins were purified as described previously. Purity was assessed by gel electrophoresis and the ratio of the absorbance at 280 nm to that at 395 nm.

Autophosphorylation

Assays of the autophosphorylation of met-RmFixLT were performed as previously described, except that imidazole (0, 2, 4, 8, 20, and 40 mM) was included in the reaction mixture [3].

Association and dissociation rate constants

The rates of binding of all ferric heme ligands except nitric oxide were measured at the Ohio State University (laboratory of M.A.G.-G.) with an Applied Photophysics SX17MV reaction analyzer (Leatherhead, UK) having a dead time of 2 ms. The rates of nitric oxide binding were measured at the Albert Einstein College of Medicine (laboratory of J.B.W.) with a High Tech SF-61mx stopped-flow system (Salisbury, UK). All reactions had 2–8 μM RmFixLT or RmFixLH in 20 mM Tris-HCl (pH 8.0) at 25°C. Ligand solutions were prepared in 20 mM Tris-HCl (pH 8.0). Fluoride concentrations were 0.50–128 mM. Ionic strength was not a factor over this range; there was less than 10 % variation in the k_{app} measured with 20 mM fluoride in NaCl solutions ranging from 0 to 200 mM. Imidazole and cyanide concentrations ranged from 0.11 to 51.2 mM. The cyanide solutions were prepared fresh with little headspace. The apparent rate constants (k_{obs}) were obtained by following absorption versus time at the wavelength of maximum difference between the liganded and unliganded met-species. This value was 424 nm for cyanide, 408 nm for fluoride, and 418 nm for imidazole, as obtained from the difference spectra using an ATI Unicam UV4 UV–Vis spectrophotometer. Association rate constants (k_{on}) were

obtained from the linear region of k_{obs} versus ligand concentration. For every ligand concentration, a minimum of three k_{obs} were averaged, each of which fit a single exponential. Each k_{on} was calculated from the slope of k_{obs} versus concentration, for 6–10 ligand concentrations spanning a wide range. In the case of the imidazoles, whose rates have a hyperbolic concentration dependence, k_{on} was calculated from the linear portion of the curve. All r^2 values for these lines were > 0.99.

Dissociation rates

Dissociation rates were measured by replacement with imidazole. The same rates were obtained whether the appearance of imidazole met-FixL or the disappearance of the derivative was measured. Depending on the rates, the change in absorbance was followed with either a stopped-flow or a standard UV–Vis spectrophotometer. The rapid association rates with imidazole and its high affinity for FixL make it an ideal replacement ligand.

Replacement of a ligand (X) by imidazole (I) is described by the following equation:

$$\text{FixL–X} + \text{I} \underset{k'_x}{\overset{k_x}{\rightleftharpoons}} \text{FixL} + \text{X} + \text{I} \underset{k_I}{\overset{k'_I}{\rightleftharpoons}} \text{FixL–I} + \text{X} \qquad (1)$$

If ligand concentrations are kept high, the amount of unliganded protein is very small and constant. Therefore the rate of increase of the replacing ligand is equal to the rate of decrease of ligand X, i.e. $d\text{FixL–I}/dt = -d\text{FixL–X}/dt$. Under these conditions, the observed replacement rate, k_{obs}, is described by equation 2 [23]:

$$k_{obs} = \frac{k_X k'_I[I] + k_I k'_X[X]}{k'_X[X] + k'_I[I]} \qquad (2)$$

The rate of binding to 50 mM imidazole is much faster than binding to a wide range of concentrations of cyanide or fluoride. Since $k'_I[I] >> k'_X[X]$, the first term in the denominator is negligible compared to the second term, and equation 2 can be simplified to equation 3, below.

$$k_{obs} = (k_I k'_X / k'_I[I])[X] + k_X \qquad (3)$$

Therefore a plot of k_{obs} versus [X] gives k_X as the y intercept.

To measure the dissociation of fluoride, protein solutions (4 μM) equilibrated with 30, 40, 50, and 100 mM fluoride were mixed 1:1 in a stoped-flow spectrophotometer with 100 mM imidazole in 20 mM Tris-HCl (pH 8.0). To measure the dissociation of cyanide, a small concentrated sample of cyanomet-FixL was first run through a gel filtration column rapidly (< 1 min) to remove all unbound cyanide. The eluate was brought to an imidazole concentration of 50 mM by addition of a concentrated stock, and the absorption spectra were recorded periodically. The first spectrum was 100 % cyanomet. Entire spectra were deconvoluted into cyano- and imidazolic components by multiple linear regression, and the rates were calculated from the change in the fraction of the imidazole derivative over time. The use of imidazole following dilution allows us to observe complete replacement of cyanide without using extreme dilutions, which cause artefactual effects with RmFixLT. Because the slope term in equation 3 is essentially zero under these conditions, a single cyanide concentration suffices to measure the dissociation rates.

Kinetics of nitric oxide binding

Nitric oxide reacts rapidly with traces of oxygen. Therefore great care was taken to exclude oxygen. All solutions, in 20 mM Tris-HCl (pH 8.0), were deoxygenated by thorough sparging with helium. A saturated solution of nitric oxide was prepared by bubbling nitric oxide that had been washed through 1.0 M KOH. This stock solution of 571 μM, corrected for the vapor pressure of water at 25°C, was diluted to 286, 228, 114, 57, and 29 μM. These dilutions were mixed 1:1 in a stopped-flow spectrophotometer with degassed 5.0 μM met-BjFixL. Each k_{obs} was the average of three or more measurements.

Affinities

Met-FixL (2–8 μM) was titrated with small increments of ligand. Clear isosbestics were observed in the 350–700 nm region of the absorption spectra. Entire spectra were used for K_d determination. The absorption spectra were decomposed by multiple linear regression analysis into the proportions of the liganded and unliganded species. The fraction of the liganded species was taken as the saturation. Values of n and of the K_d were obtained from Hill plots.

Absorption spectrum of azidomet-FixL

We estimate that 2 M azide would be required to achieve 95 % saturation of RmFixLH. The effects of elevated ionic strengths on the absorption spectrum of FixL made it difficult to record an azidomet-FixL spectrum. The azidomet-FixL spectrum in Figure 2 (dotted line) was calculated as follows. A Y value was calculated for the most saturated azidomet-RmFixLH solution (200 mM azide) giving the identical isosbestic points as solutions have less azide. Then an estimated Y value was chosen that minimized the r^2 value for the Hill plot. Once this value (0.69 for RmFixLH) was assessed, a fully saturated azidomet-RmFixLH spectrum could be extrapolated from it.

EPR spectrum

To prepare the nitric oxide derivative of ferrous RmFixLT, 200 μl of nitric oxide-saturated 50 mM Tris-Cl, pH 8.0 was added to an equal volume of dithionite reduced RmFixLT in an EPR tube flushed with O_2-free argon and sealed with a rubber septum. Spectra were obtained on a Bruker ER 300 Spectrometer equipped with a ER035M Gaussmeter and a Hewlett-Packard 5352B microwave frequency counter (Laboratory of R.H.). To improve the signal-to-noise ratio, 30 scans of 40 s each were accumulated. The EPR parameters were: microwave frequency, 9.44955 GHz; microwave power, 10 mW; modulation amplitude, 5 Gauss; temperature 150 K.

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