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O_2-specific regulation of the ferrous heme-based sensor kinase FixL from *Sinorhizobium meliloti* and its aberrant inactivation in the ferric form☆

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Abstract

FixL, a rhizobial heme-based O_2-sensing histidine kinase, catalyzes autophosphorylation in the deoxy form at low O_2 tension, while the kinase activity is inhibited in the case of the O_2-bound form. The present study unambiguously shows that the binding of CO and NO does not significantly inhibit the kinase activity of dithiothreitol (DTT)-reduced ferrous FixL from *Sinorhizobium meliloti*, which is inconsistent with the spin state mechanism previously reported. Kinase inactivation is caused by aberrant disulfide (S–S) bond formation at Cys301 in the ferric homodimer, which explains these contradictory observations. The addition of DTT cleaved the S–S bond, leading to restoration of kinase activity in the ferric form as well as heme reduction, but, sodium hydrosulfite treatment produced the kinase-inactive deoxy form without S–S cleavage. On the basis of these experimental results, it can be concluded that ferrous FixL discriminates O_2 from CO and NO, and signals the O_2-bound state by downregulating the phosphoryl transfer reaction.

Keywords: Heme; Histidine kinase; Oxygen sensor; Signal transduction; Two-component system

Rhizobial FixL, a heme-based O_2 sensor protein, is involved in the regulation of the expression of nitrogen-fixation genes in plant root nodules in response to environmental O_2 levels [1,2]. This protein is one of the sensory histidine kinases of bacterial two-component regulatory systems [3]. The FixL polypeptide contains a sensor domain and a histidine kinase domain [4,5]. The histidine kinase domain, which consists of ~240 amino acids, is homologous in terms of primary structure to other sensory kinases of this superfamily. However, the amino acid sequence of the sensor domain, ~150 amino acid residues, shares no homology with the others because the structures of the sensor domains are highly dependent on the structures of the individual ligands. The sensor domain of FixL contains heme (iron-protoporphyrin) as a ligand binding center. At high O_2 tension, O_2 binds to the ferrous iron of the heme as the sixth ligand, resulting in the inactivation of autokinase activity. In contrast, when the bound O_2 is dissociated from the heme iron in a low O_2 environment, the resulting deoxy FixL is autophosphorylated at an invariant histidine, using ATP as the phosphoryl donor and subsequently transfers the phosphoryl group to FixJ, a transcriptional activator for nitrogen-fixation related genes [6,7].

The issue of how O_2 association/dissociation regulates the autokinase activity of FixL is an important and crucial one. Conformational changes in the sensor domain, induced by O_2 binding, must be transmitted to the kinase domain, in order to downregulate the autokinase

☆ *Abbreviations:* DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FixJ~P, phosphorylated FixJ; FixL~P, phosphorylated FixL; Ni–NTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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activity. To understand the physiological nature of the O_2 sensor protein at the molecular level, spectroscopic studies on ligand binding have been carried out [8–17]. Meanwhile, by examining the kinase activity of ferric FixL complexes with various ligands as well as the ferrous oxy- and deoxy forms, Gilles-Gonzalez and coworkers [18] reported that the electronic spin state of the heme iron is correlated with kinase activity: high spin FixLs are kinase-active and the low spin forms are kinase-inactive. Generally, the iron of a low spin heme is located in the center of the porphyrin plane (in-plane configuration) so that the proximal amino acid ligand becomes tensed. On the other hand, it becomes relaxed when the iron is located out of the porphyrin plane (out-of-plane configuration) in the high spin state. Thus, a spin state mechanism was proposed, in which the movement of the iron into the heme plane induces a shift in the proximal histidine and its associated main chain, thus regulating kinase activity [18].

It has recently been shown that CO and NO react as physiological effectors or cell signaling mediators in living organisms. Since it is well known that diatomic gas molecules such as CO and NO, in addition to O_2, bind to the heme iron centers, it is generally thought that hemoproteins are involved in these gas-sensing systems. Indeed, CooA is a heme-based transcriptional factor that senses environmental levels of CO in *Rhodospirillum rubrum* [19], and mammalian soluble guanylate cyclase generates guanosine $3',5'$-cyclic phosphate when NO binds to the heme domain [20]. In the present study, we carefully re-examined the effect of CO and NO, other natural ligand candidates for ferrous FixL from *Sinorhizobium meliloti*,[3] on kinase activity. To our surprise, although CO- and NO adducts of SmFixL are present in the low spin state, the adducts had a high autokinase activity, suggesting that FixL senses neither CO nor NO. We also found that the kinase activity of DTT-reduced deoxy FixL in our assay system was much higher than that reported by Gilles-Gonzalez group using sodium hydrosulfite ($Na_2S_2O_4$), and that our ferric unliganded (met) form lost the kinase activity. We then examined the effects of DTT and $Na_2S_2O_4$ on the met form of FixL and found out that the oxidation of Cys301 in ferric FixL was involved in the aberrant inactivation and that $Na_2S_2O_4$ did not disperse the kinase-inactive fraction although it efficiently reduced ferric heme to the ferrous form.

Materials and methods

Sample preparation. Recombinant 6xHis SmFixL and site-directed mutants thereof were overexpressed in the *Escherichia coli* strain JM109 [21]. Cell-free supernatants from JM109 harboring pHEL2-Sm*fixLJ* and its derivatives were obtained by two passages through a French press cell (1000 kg/cm^2) and subsequent centrifugation of the homogenate at 75,000*g* for 1 h, followed by Ni–NTA agarose (Qiagen) chromatography with a linear gradient elution of 20–300 mM imidazole in 50 mM potassium phosphate buffer, pH7.5, containing 150 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The FixL fractions were applied to a DEAE–Sepharose FF (Amersham–Pharmacia) column and eluted with a linear gradient of 0–500 mM Na_2SO_4 in 20 mM Tris–H_2SO_4 buffer, pH 8.0, containing 10 mM β-mercaptoethanol and 5% glycerol. The highly purified FixL was dialyzed against 20 mM Tris–H_2SO_4 buffer, pH 8.0, containing 10 mM β-mercaptoethanol and 5% glycerol, and concentrated with Centriprep (Amicon) and Centrisart I (Sartorius). The samples were stored at −20 °C in the presence of 50% glycerol. To prepare the ferric met form, the addition of β-mercaptoethanol was omitted in the purification procedure, or the purified sample was completely oxidized by treatment with 2 mM potassium ferricyanide, followed by dialysis to remove ferri/ferrocyanide ions. Recombinant FixJ was also purified by the same procedure.

FixL* was produced in JM109 with a pUC19-based expression plasmid containing the same N-terminal sequence joint as that of pGG820 previously described [2]. FixL*, partially purified with DEAE, was applied to a Phenyl-650S hydrophobic interaction column equilibrated with 20 mM Tris–H_2SO_4, pH 8.0, 10 mM β-mercaptoethanol, 5% glycerol, and 400 mM $(NH_4)_2SO_4$, and eluted by a linear inverse gradient of $(NH_4)_2SO_4$. After dialysis against 20 mM Tris–H_2SO_4, pH 8.0, 10 mM β-mercaptoethanol, and 5% glycerol, the sample was further subjected to a Macro-Prep ceramic hydroxyapatite type I 20 μm (Bio-Rad) column equilibrated with the dialysis buffer and eluted with a linear gradient of 0–50 mM potassium phosphate, pH 8.0, in the buffer.

Kinase activity of FixL. FixL samples were diluted in a kinase buffer (50 mM Tris–HCl, pH 7.8, 50 mM KCl, and 0.2 mM $MnCl_2$) at a final concentration of 5.6 μM. When FixL was fully reduced to the ferrous deoxy form, DTT and sodium hydrosulfite were added to the protein solution at final concentrations of 10 and 1 mM, respectively, under a stream of N_2 gas. Oxygen-bound FixL was prepared by exposure to air. To prepare the CO-bound form, the deoxy FixL sample in the kinase was flushed with CO gas. The NO-bound form was obtained by the addition of a few crystals of *S*-nitroso-*N*-acetylpenicillamine to the reduced form. The preparation of each form was spectroscopically confirmed.

The oxy form was subjected to the kinase assay under the air, and the deoxy-, CO-, and NO-bound forms were transferred into an N_2-filled glove box for the kinase assay. The kinase reaction was initiated by the addition of 1/9 volume of 2 mM [γ-^{32}P]ATP (~0.09 MBq/nmol) at 23 °C and terminated in an SDS–PAGE sample buffer (65 mM Tris–HCl, pH 6.8, 3% SDS, 10% glycerol, 1 mM Na–EDTA, and 0.05% BPB). When required, FixJ protein was added to the reaction buffer at a final concentration of 10 μM. Aliquots of 30 pmol FixL or 60 pmol FixJ were applied to each lane of SDS–PAGE gel and phosphorylation levels of the protein bands were determined as previously described [22].

Gel filtration chromatography. The FixL samples (1 ml) were applied to a Superdex 75 pg column (1.6 × 36.5 cm; Amersham Biosciences) equilibrated 50 mM Tris–H_2SO_4, pH 7.5, 100 mM Na_2SO_4, and 5 mM Na–EDTA at a flow rate of 1 ml/min. FixL fraction was detected by measuring the absorbance at 420 nm for the ferrous form or at 400 nm for the ferric form.

Results

Autokinase and FixJ-phosphorylation activities of ligand-bound ferrous FixLs

To address the possibility that other gaseous molecules, in addition to O_2, function as physiological

[3] *Sinorhizobium meliloti* was formerly called *Rhizobium meliloti* and its FixL protein was designated RmFixL. Throughout this study, however, SmFixL was used for RmFixL.

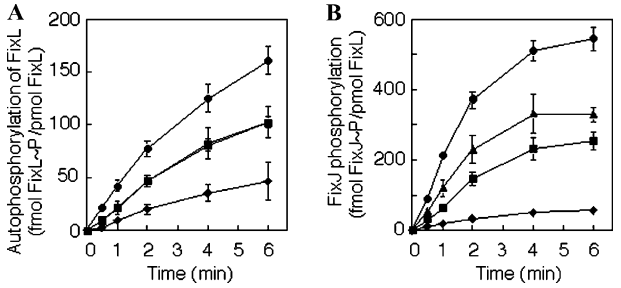

Fig. 1. Kinase activities of DTT-reduced ferrous SmFixL derivatives. (A) Autophosphorylation and (B) FixJ-phosphorylation. 6xHis-tag FixL was purified in the presence of 10 mM β-mercaptoethanol and diluted in the kinase buffer containing 10 mM DTT. ●, deoxy; ◆, oxy; ▲, CO; ■, NO complexes. Autophosphorylation of CO- (■) and NO (▲) forms was almost overlapped in panel A. Deoxy-, CO-, and NO forms were uniformly prepared but the oxy form contains the 15% deoxy form.

effectors, we first examined the effect of CO and NO on FixL kinase activity in our standard kinase assay conditions containing 10 mM DTT. DTT was observed to reduce the heme iron of FixL without the rapid consumption of oxygen, and therefore the deoxy and oxy forms could be obtained under anaerobic and aerobic conditions, respectively. The optical absorption spectra of DTT-reduced ligand-free (deoxy), O_2-bound (oxy), CO-bound, and NO-bound forms of FixL (data not shown) were completely identical to previously reported data using $Na_2S_2O_4$-reduced FixL [8,10].

Fig. 1 shows the kinase activities of the ferrous complexes of the SmFixL samples. As shown in Fig. 1A, the autokinase activity of deoxy FixL is high (circles), while it is inhibited when O_2 is bound to the heme iron (diamonds). The residual activity of the oxy FixL sample appears to be derived from a small amount of deoxy form due to its oxygen affinity of ~50 μM [8]. The concomitant phosphoryl transfer to FixJ is also shown in Fig. 1B. The O_2-linked kinase regulation observed here is in agreement with previously reported data for Sm- and BjFixLs [6,7,18]. However, the CO-bound form of SmFixL, which is in a typical low spin state, exhibited a significantly high activity (triangles), compared to that of the oxy form. The ferrous NO-bound complex of SmFixL, another low spin form, was autophosphorylated to the same extent (squares). Consequently, both the CO- and NO-complexes catalyzed FixJ-phosphorylation at significantly high rates (triangles and squares in Fig. 1B). These results are inconsistent with those of a previous study that reported a correlation of kinase activity with heme spin state [18], but partly agree with their latest data, obtained using SmFixL [23].

Effects of DTT and $Na_2S_2O_4$ on the autokinase activity

We noted that the autokinase activity of deoxy FixL in our assay (~50 fmol FixL~P/pmol FixL/min) (Fig. 1A) was much higher than that reported by Gilles-Gonzalez group (2–6 fmol FixL~P/pmol FixL/min) [7,18,23]. In their study, the deoxy form was prepared by deoxygenation under N_2 in the presence of 10 mM β-mercaptoethanol [7] or by reduction of the ferric met form with $Na_2S_2O_4$ [18,23], while our assay conditions a priori included 10 mM DTT as a reductant. In order to understand the nature of these contradictory observations, we prepared the ferric met form and examined the effects of DTT and $Na_2S_2O_4$ on the autokinase activity of met SmFixL.

It was surprising to find that met FixL exhibited little activity (triangles in Fig. 2A), and furthermore, the kinase activity of $Na_2S_2O_4$-reduced deoxy FixL still remained low although it immediately reduced the heme (squares). However, DTT treatment yielded the kinase-active deoxy form with the slow reduction of ferric heme to the ferrous form in the presence of N_2 (circles). Although the FixL proteins used in this study contain the 6xHis tag in front of the sensor domain, these results were not affected by the 6xHis-tag modification and the same results were obtained with our FixL* preparation (Fig. 2B).

Both DTT and $Na_2S_2O_4$ reduced the heme group, giving deoxy FixL, however, only DTT treatment restored the kinase activity. It is possible that DTT could cleave the disulfide bond (see the next section), resulting in the restoration of kinase activity, whereas the met and $Na_2S_2O_4$-reduced FixLs remain as the covalently bound dimer or are present in an oligomeric form. We then examined this possibility using gel filtration column chromatography.

As shown in Fig. 3B, DTT-treated FixL migrated as a dimer at a high protein concentration (initial concentration of 20 μM), and the monomer fraction appeared at a low concentration (initial concentration of 0.5 μM), which is in agreement with the established fact that FixL, as well as other sensory histidine kinases, is present as an equilibrium mixture of monomer and homodimer, and functions in the non-covalently bound

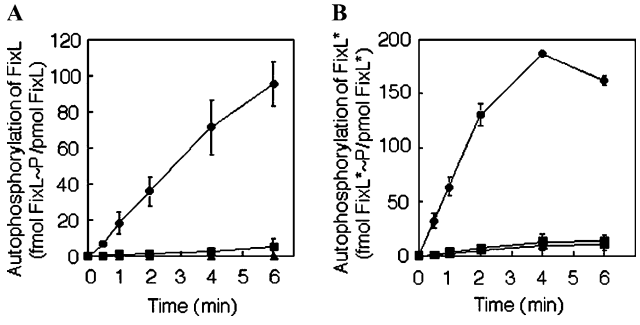

Fig. 2. Effects of DTT and $Na_2S_2O_4$ on the autokinase activity of ferric met SmFixL. (A) 6xHis-tag FixL and (B) FixL*. Met FixL (▲) was completely reduced with 10 mM DTT (●) or 1 mM $Na_2S_2O_4$ (■) to prepare the deoxy form.

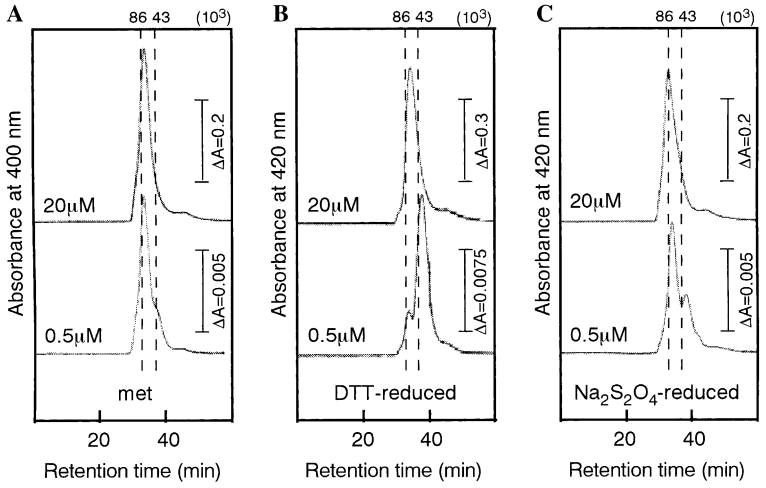

Fig. 3. Gel filtration analysis of FixL*. Met- (A), DTT-reduced- (B), and $Na_2S_2O_4$ (C) forms were subjected to the superdex G-75 column chromatography. One millilitre aliquots of 20 μM FixL* (upper) and 0.5 μM FixL* (lower) were applied.

homodimeric form [24]. On the other hand, a large fraction of met FixL was present in dimeric form even at a low protein concentration (A), and it is noteworthy that $Na_2S_2O_4$ does not eliminate the dimer fraction although it reduces heme (C).

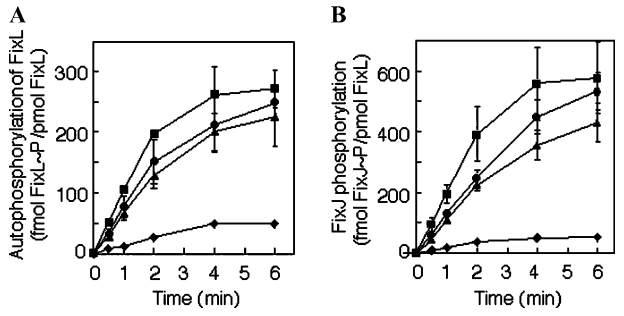

Fig. 4. Kinase activities of the 6xHis SmFixL Cys301Ala mutant. (A) Autophosphorylation and (B) FixJ-phosphorylation. Ferric met form (▲) of the Cys301Ala mutant was prepared and reduced with 10 mM DTT (●) or 1 mM $Na_2S_2O_4$ (■). Oxy form (◆) was prepared from the DTT-reduced from in the presence of air. Cys7 derived from 6xHis-tag vector [21] and Cys301 of SmFixL were substituted with Leu and Ala, respectively.

Oxidation of Cys301 is involved in the inactivation of autokinase acivity of SmFixL

SmFixL contains a single cysteine residue at position 301. Since this cysteine may be involved in aberrant kinase inactivation, we constructed a C301A mutant and examined its kinase activities (Fig. 4). As expected, the met (triangles), DTT-reduced (circles), and $Na_2S_2O_4$-reduced (squares) forms all exhibited the maximal autokinase and FixJ-phosphorylation activities, while the re-binding of O_2 to the DTT-reduced form inhibited them (diamonds). Met (Fig. 5A) and $Na_2S_2O_4$-reduced (Fig. 5C) samples consistently eluted as a monomer similar to the DTT-reduced form (Fig. 5B) at low protein concentrations in a gel filtration column. Similar results were obtained with C301M and C301S mutants (data not shown).

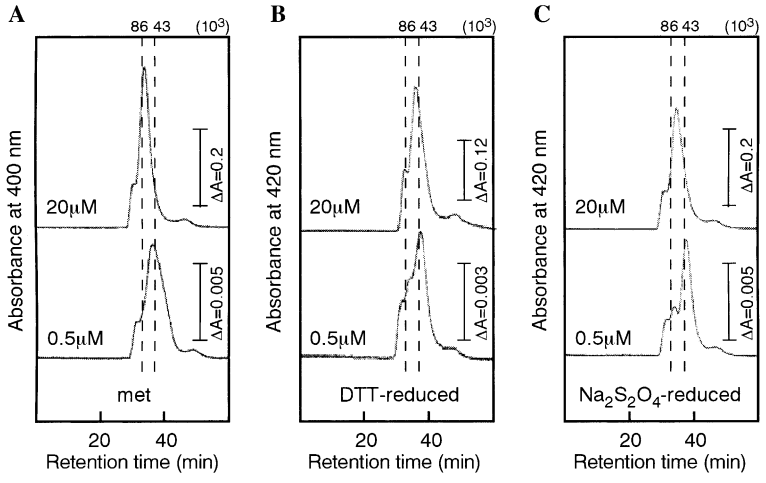

Fig. 5. Gel filtration analysis of the 6xHis SmFixL Cys301Ala mutant. Assay conditions were the same as those of Fig. 3.

S–S bond formation seems to be irrelevant to its function in the cell, since Cys301 in SmFixL is not conserved among other rhizobial counterparts in *Azorhizobium caulinodans*, *Bradyrhizobium japonicum*, and *Mesorhizobium loti*. Thus, we conclude that the decrease or loss of the kinase activity of wild-type SmFixL in the in vitro oxidized state results from aberrant inactivation caused by S–S bond formation at Cys301.

Discussion

Inactivation of kinase activity in the ferric state and the effects of DTT and $Na_2S_2O_4$ on the activity

Gilles-Gonzalez and co-workers previously reported a correlation between kinase activity and heme electronic spin state that the histidine kinase of FixL, in which the heme (sensor) domain is in the high spin states (deoxy-, met-, and F-bound forms) was active, while that in the low spin states (O_2-, CO-, NO-, and CN-bound forms) was inactive [18]. The initial aim of the present work was to re-examine again the possibility that CO and NO react as a surrogate of O_2 by inhibiting kinase activity because their experimental data were not presented. Unexpectedly, it was found that their ferrous deoxy SmFixL displayed an extremely low autokinase activity per FixL molecule compared to ours and that our met sample lost its autokinase activity. The present study demonstrates that S–S bond formation at Cys301 causes aberrant kinase inactivation in the oxidized state, and that DTT treatment restores kinase activity by cleavage of the S–S bond, not only reduces heme whereas the $Na_2S_2O_4$-reduced sample contains the kinase-inactive S–S bonded dimer. Assuming that the phospho-acceptable core domain containing His285 might form a homodimer consisting of a helical bundle structure similar to EnvZ [25], two Cys301 residues can be located in the loop region of each monomer and in close proximity to each other. Thus, S–S bond formation between these cysteine residues is apparently possible, resulting in the formation of an unnatural and inactive FixL homodimer.

In previous reports, FixL samples were obtained in the presence [7,23] or absence of 10 mM β-mercaptoethanol [18], and the ferrous forms were prepared by treatment with $Na_2S_2O_4$ and subsequent desalting chromatography, instead of DTT treatment [7,23]. Therefore, their samples possibly contained the kinase-inactive fraction, the contents of which can vary, depending on the sample preparation. Based on data obtained using these samples, they discussed the slow velocity for the activity of the deoxy form from the viewpoint of a signal transducing function. Furthermore, they reported that an interaction with FixJ is dependent on the oxidation state of the heme, based on the observation that the rate of FixJ-phosphorylation of ferric SmFixL is 100 times slower than that of the deoxy form [23], which is inconsistent with our data for the C301A mutant. However, it is evident that the results of the FixL kinase activities regarding several oxidation/ligation/spin states of the heme iron will be misleading as long as they contain the aberrant inactive form, although the ferric SmFixL is intrinsically active (Fig. 4). $Na_2S_2O_4$ is very often used to reduce the heme of hemoproteins, but attention needs to be paid to the effects of protein amino acid residues when dealing with enzymatic activities.

Oxygen-dependent downregulation on autokinase and FixJ-phosphorylation activities of ferrous FixL

Using the DTT-reduced and fully active FixL, we unambiguously showed that CO- and NO adducts were rather active in terms of autophosphorylation and FixJ-phosphorylation. The finding implies that ferrous FixL is able to discriminate O_2 from CO and NO, and physiologically senses only O_2. This also rules out the correlation between the spin state and kinase activity and the accompanying spin state mechanism [18], although it agrees, in part, with their latest biochemical data [23].

Oxygen-dependent regulation seems to be undertaken by changes in the heme-distal portion of the sensor domain rather than those in the proximal domain. An earlier crystallographic study of the met and metCN^- complexes of the BjFixL heme domain concluded that the rearrangement of the hydrogen bonds between the heme 6,7-propionate and the F/G loop region of the distal side is caused by a flattening of the heme plane upon ligand binding [26]. However, flattening of the heme itself is not necessarily required for the observed downregulation, since the heme plane of the CO complex, which is kinase-active, is flat, similar to the O_2-bound form [16].

It is most likely that changes in the F/G loop upon ligand binding may be involved in ligand descrimination and subsequent kinase regulation. In the heme distal pocket of FixL, the hydrophobic side chains Ile209 and Ile210 in the F/G loop, and those of Leu230 and Val232 in the H β-sheet (numberings in SmFixL) are densely packed [26,27]. When NO binds to the iron, the positions of these side chains are subtly changed to allow the ligand accommodation [28] and this might also be the case for the CO-bound form. In contrast, O_2 binding to the heme iron appears to directly or indirectly affect the distal hydrophobic residues because the Ile209 and Ile210 mutants of Sm FixL exhibited constitutive upregulation of the kinase activity upon O_2 binding [29]. Perutz and co-workers [30] also independently suggested that the "hydrophobic triad" in the heme distal portion could be involved in the O_2-sensing action. Indeed, the

most recent crystallographic data of the oxy BjFixL sensor domain by Chan and co-workers [28,31] showed that a conserved Arg220 residue (homologous to Arg214 in SmFixL) at the end of the distal F/G loop swings into the hydrophobic distal pocket and is hydrogen-bonded with the bound oxygen instead of heme propionate 7, whereas the NO- and CO-bound forms do not contain such a rearrangement. These structural differences are consistent with the present biochemical data.

In Hb and Mb, the steric hindrance generated by the exposure of the bound ligands to the distal residues leads to discrimination between O_2 and CO in terms of the modulation of the affinity for the ligands [32]. In the case of FixL, hydrogen-bonding, steric and/or hydrophobic interactions of the bound O_2 with the distal residues modulate not only the stability of the bound ligand, but the kinase activity as well, via an intramolecular signal transduction from the sensor domain to the kinase domain.

Acknowledgments

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