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Role of conserved F_α-helix residues in the native fold and stability of the kinase-inhibited oxy state of the oxygen-sensing FixL protein from *Sinorhizobium meliloti*

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ABSTRACT

The oxygen-sensing FixL protein from *Sinorhizobium meliloti* is part of the heme-PAS family of gas sensors that regulate many important signal transduction pathways in a wide variety of organisms. We examined the role of the conserved F_α-9 arginine 200 and several other conserved residues on the proximal F_α-helix in the heme domain of *Sm*FixL* using site-directed mutagenesis in conjunction with UV–visible, EPR, and resonance Raman spectroscopy. The F_α-helix variants R200A, E, Q, H, Y197A, and D195A were expressed at reasonable levels and purified to homogeneity. The R200I and Y201A variants did not express in observable quantities. Tyrosine 201 is crucial for forming the native protein fold of *Sm*FixL* while Y197 and R200 are important for stabilizing the kinase-inhibited oxy state. Our results show a clear correlation between H-bond donor ability of the F_α-9 side chain and the rate of heme autoxidation. This trend in conjunction with crystal structures of liganded *Bj*FixL heme domains, show that H-bonding between the conserved F_α-9 arginine and the heme-6-propionate group contributes to the kinetic stability of the kinase-inactivated, oxy state of *Sm*FixL*.

Introduction

An exciting new class of signaling proteins called the heme-based gas sensor proteins that sense NO, CO, or O_2 and regulate many important biological processes such as blood pressure, gene transcription, and chemotaxis by initiating the appropriate signaling cascades have been discovered in all kingdoms of life over the past two decades [1–6]. The dimeric FixL proteins from *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) and *Bradyrhizobium japonicum* are members of the heme-PAS family of gas sensing proteins [1,7,8]. These proteins, which include the bacterial protein *Ax*PDEA1 that regulates cellulose synthesis, *Escherichia coli* DOS, and mammalian NPAS2 that controls circadian rhythm in some mammals, [1,7,8] are found in a wide variety of organisms. Based on a growing body of physical, biochemical, and molecular biological data, an increasingly detailed understanding of the mechanisms by which these important regulatory proteins transduce the free energies of interaction with their respective gases and propagate their signals is beginning to emerge [1–8].

*Sm*FixL comprises a membrane anchor domain, a heme-containing PAS domain that senses oxygen and a histidine kinase domain that, in conjunction with its response regulator FixJ, regulates nitrogen fixation and micro aerobic respiration in the symbiotic root nodules of legumes [9–21]. While *Sm*FixL is absolutely required for nitrogen fixation and micro aerobic respiration in *S. meliloti*, its homolog *Bj*FixL from *B. japonicum* controls anaerobic denitrification and micro aerobic respiration [9–21]. Under atmospheric conditions the heme-PAS domain of *Sm*FixL binds oxygen to form an oxy complex that inhibits its kinase domain, thereby preventing *Sm*FixJ phosphorylation and transcriptional activation of the genes responsible for nitrogen fixation [9–21]. However, under the hypoxic conditions that develop during symbiosis, *Sm*FixL–O_2 dissociates to yield the deoxy or unliganded state, whereupon the kinase domain is activated. The cognate transcriptional activator, *Sm*FixJ is then phosphorylated, up regulating the

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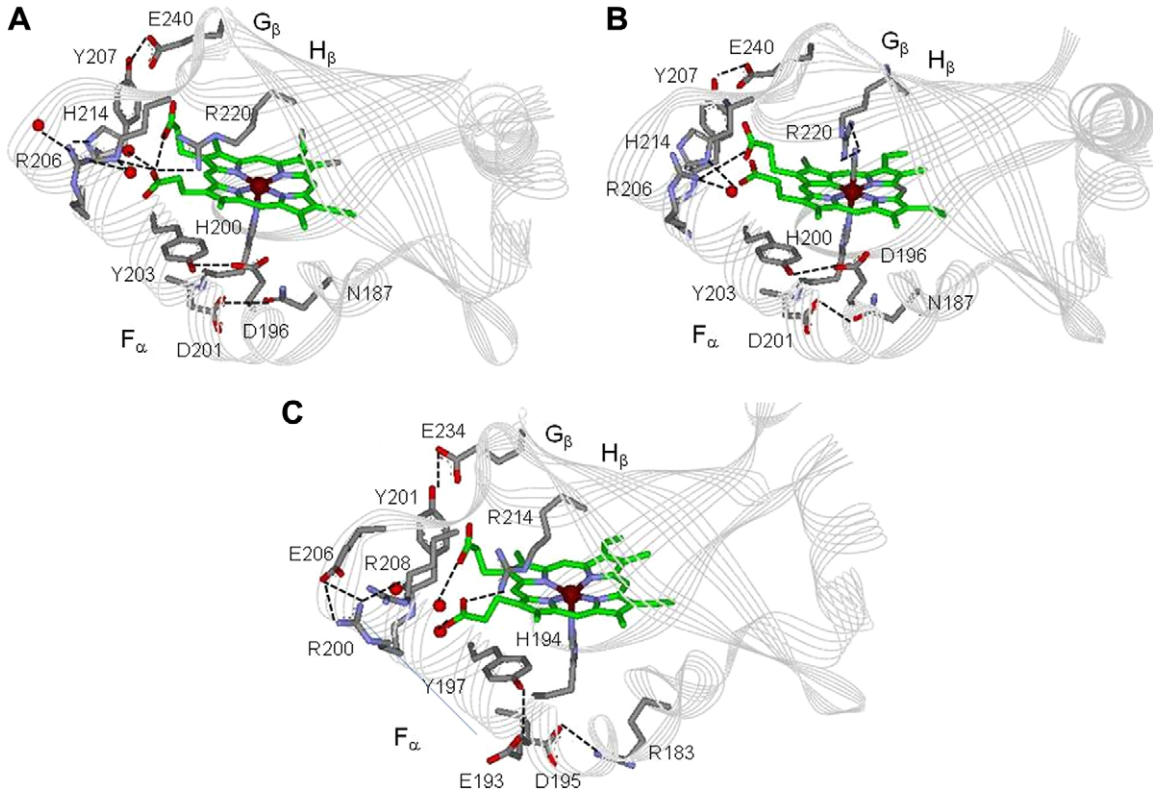

Fig. 1. The crystal structures of (A) the deoxy kinase-active state of *Bj*FixLH (1XJ3), (B) the kinase-inhibited cyanomet state of *Bj*FixLH (1LT0) and (C) the deoxy kinase-active state of *Sm*FixLH (1EW0). Potential H-bonding interactions are shown as dashes (---) and water molecules are shown in red. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

nitrogen fixation genes *nifA* and *fixK* [9–21]. Thus FixL is an ideal target for studying the relationship between the heme-PAS sensing domain and the kinase effector domain.

Biochemical, biophysical, and crystallographic studies of the liganded and unliganded forms of the truncated heme-PAS domain of *Bj*FixL (*Bj*FixLH)[1] and the unliganded states of *Sm*FixLH have revealed a complex network of non-covalent interactions that are altered by ligand binding [22–27]. The most significant structural rearrangements in *Bj*FixLH occurred in the flexible FG loop region between the proximal F_α-helix and the distal G_β-strand that line the heme pocket in heme-PAS domains [22–27]. Based on these and other observations it was proposed that structural changes in the FG loop of the heme-PAS domain are propagated to the kinase domain [22–28]. Recent studies of *Bj*FixL and *Sm*FixL have focused on the roles of conserved arginine residues in the FG loop region of the heme-PAS domain in oxygen sensing [29–33]. The distal Gβ−2 arginine (R220) of *Bj*FixLH has been shown to exchange its H-bond donor interaction with bound O_2 or CN^- in the kinase-inhibited oxy and cyanomet states for an H-bond donor interaction with the heme-7-propionate in the kinase-active deoxy and met states (Fig. 1) [23–25]. Substitution of alanine or other amino acids for the G_β-2 R220 in *Bj*FixLH caused a substantial decrease in the O_2 affinity. However, the kinase activity was not dramatically altered in the R220A *Bj*FixL variant, suggesting that, while this residue is important for oxygen selectivity, it is not solely responsible for oxygen sensing and signal transduction in *Bj*FixL [29–31].

Another conserved arginine residue at position F_α-9 was shown to change orientations to become a hydrogen-bond donor to the heme-6-propionate upon forming the kinase-inhibited oxy and cyanomet states of *Bj*FixLH (Fig. 1) [23–25]. This F_α-9 arginine is conserved among the FixL proteins but can also be found as a histidine in the broader heme-PAS family of proteins [24]. Recent studies of a proximal R206A variant found that the kinase inhibition was significantly impaired in the liganded state [32]. This single activity measurement led to the proposal that the conserved F_α-9 arginine is involved in the regulation of kinase activity in *Bj*FixL. The crystal structure of R206A *Bj*FixLH also had significant changes in the conformations of the nearby histidine 214 and heme-7-propionate residues indicating that the heme propionate periphery was altered in this variant [32]. In a recent study of *Sm*FixL, the conserved G_β-2 arginine was changed to alanine (R214A in *Sm*FixL) and had a much greater affect on oxygen affinity, autoxidation rate, and kinase activity than changing the F_α-9 arginine to an alanine residue (R200A in *Sm*FixL) [33]. Whether there are differences in the role of these conserved arginine residues in *Sm*FixL and *Bj*FixL is not yet clear. It remains to be determined whether the structural changes observed in the crystal structures of the unliganded and liganded

[1] *Abbreviations used: Sm, Sinorhizobium meliloti*; *Bj, Bradyrhizobium japonicum*; *Ec*DOS, *E. coli* direct oxygen sensor; *Ax*PDEA1, *Acetobacter xylinum* phosphodiesterase 1; PAS, sensory domain with an α-β fold named after the eukaryotic proteins period, aromatic nuclear transporter (arnt,) and simple minded; Heme-PAS, heme-binding PAS domain; *Sm*FixL$^*_{127-505}$, truncated FixL from *S. meliloti* with the heme and kinase domains but without the N-terminal, transmembrane helices (instead there is an N-terminal extension: N-TMITPSLAAG-R(127)-505-C); *Bj*FixLH, the truncated heme domain-only form of *Bj*FixL without the kinase domain; *Sm*FixLH, the truncated heme domain-only form of *Sm*FixL without the kinase domain; *Ec*DOSH, the truncated heme domain-only form of *Ec*DOS without the phophodiesterase domain; LB, Luria Broth; EPR, electron paramagnetic resonance; rR, resonance Raman; PCR, polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HS, high spin; LS, low spin.