Spectroscopic Observation of a FixL Switching Intermediate

Kenton R. Rodgers,* Gudrun S. Lukat-Rodgers, and Lei Tang

Department of Chemistry, Ladd Hall
North Dakota State University
Fargo, North Dakota 58105-5516

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With the discovery of soluble guanylate cyclase (sGC),[1] FixL[2] and CooA,[3] heme-based sensing and signal transduction have become recognized heme protein functions. The heme-containing domains of FixL and CooA and the β subunit of sGC are small-molecule sensors that bind O_2, CO, and NO, respectively, as axial heme ligands.[4] The mechanisms by which ligand binding events are communicated from the heme sensors to the domain or subunit interfaces they regulate are presumed, by analogy to the cooperative hemoglobins, to involve propagation of the ligation-coupled conformational transitions of the hemes and their immediate protein environments.[5]

Oxygen binding or release by the heme in FixL is the first step in the mechanism by which N_2 fixation is regulated in *Rhizobium meliloti*. It has been shown that kinase activity responds to change in spin state of the heme iron.[5b] Recent crystal structures of ligated and unligated heme domains of ferric *Bradyrhizobium japonicum* FixL (*Bj*FixL)[6] reveal the structural differences between high-spin (HS) and low-spin (LS) ferric forms. The heme domains of *Bj*FixL and *R. meliloti* FixL (*Rm*FixL) are highly homologous (50%)[7] and are both members of the PAS family of sensor domains.[8] The PAS fold contains a large cavity lined by two antiparallel β sheets that flank a helix, which contains the proximal His ligand in *Bj*FixL. This cavity can accommodate a variety of chromophores whose conformations are sensitive to environmental conditions and whose conformational transitions can be propagated to other domains or proteins via associated conformational reorganizations of the PAS domain.[9-11] The conformational response of the *Bj*FixL heme domain to inter-

conversion of the heme between its met (5-coordinate, HS) and cyanomet (6-coordinate, LS) forms appears localized to a long (FG) loop at the carboxy terminus of the proximal helix and change in heme planarity.[6] On this basis, it has been suggested that conformational switching of the FG loop is driven by the change in heme planarity.[6] The transient resonance Raman (rR) spectra reported herein provide the first glimpse of how FixL begins its transformation from an inactive to an active state. Frequencies of the proximal Fe−His bond stretch and bending vibrations of peripheral vinyl and propionate groups are reported for heme-CO photolysis products of *Rm*FixL*/LN−CO (*Rm*FixL* is a functional heme-kinase deletion derivative of the full length membrane-bound *Rm*FixL; *Rm*FixLN contains only the heme domain and exhibits no kinase activity[12]). A transient strengthening of the proximal Fe−His bond in these species, relative to their equilibrium deoxy forms, was revealed by an increased Fe−His stretching frequency. Observation of this intermediate suggests that, by themselves, the kinase-active and inactive states of FixL are not sufficient to formulate an accurate model for propagation of conformational free energy between the heme and kinase domains.

Figure 1 shows rR spectra of *Rm*FixL* in its deoxy, CO, and photolyzed states. Blue excitation wavelengths were chosen for optimal enhancement of rR scattering by the ν_{Fe-His} vibration of five coordinate HS ferrous heme.[13] The spectra of deoxyFixL* and the FixL*-CO photolysis product are similar, indicating that a substantial fraction of the hemes are in their equilibrium deoxy conformation. The spectral contribution from equilibrium deoxy-FixL* in the photolyzed FixL*-CO spectrum (Figure 1C) is attributed to its buildup during spectral acquisition. This could be due to the slow second-order recombination ($k_{on} = 1.2 \times 10^4$ $M^{-1} \cdot s^{-1}$)[5a] and/or conformational relaxation of the intermediate during the 3-ns laser flash. In either case, subtraction of the equilibrium deoxyFixL* features (Figure 1C) from the photolyzed spectrum (Figure 1B) yields a difference spectrum (Figure 1D) containing a prominent new band at 218 cm^{-1}. This band cannot be attributed to unphotolyzed or recombined CO adducts, as the low-power, continuously excited spectrum of the CO adduct shows little or no intensity at this frequency (Figure 1A).[14] By virtue of its position and intensity, the 218-cm^{-1} band is assigned to ν_{Fe-His} of a transient HS *Rm*FixL*-CO photoproduct.[15] This transient intermediate is formed after CO photolysis, whereupon it relaxes to equilibrium deoxyFixL*/LN (ν_{Fe-His}, 210 cm^{-1}).[18]

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(14) The Raman excitation wavelength used to obtain the continuous-wave FixL*-CO spectrum in Figure 1A (441.6 nm) was different from that used to record the transient spectrum in Figure 1B (435.7 nm), as it was not possible to obtain a spectrum of FixL*-CO using pulsed excitation. To avoid introducing subtraction artifacts due to different excitation wavelengths, the FixL*-CO contributions were not subtracted from the spectrum of the intermediate in Figure 1D.

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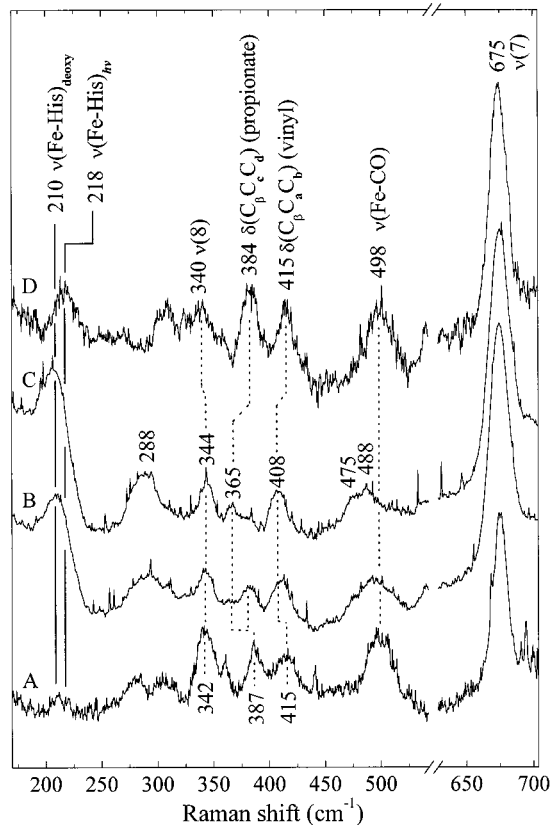(17) Hu, S.; Smith, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1996,** *118,* 12638–12646.

Figure 1. Low-frequency resonance Raman spectra of 250 μM deoxy-FixL*, FixL*-CO, and its photolysis products acquired at room temperature in 50 mM Tris buffer at pH 7.8. (A) FixL*-CO spectrum recorded with 200 μW of CW 441.6-nm excitation. (B) FixL*-CO, 435.7-nm pulsed excitation; pulse repetition rate = 50 Hz, pulse energy = 0.3 mJ/pulse. (C) Authentic deoxyFixL* recorded with 435.7-nm pulsed excitation, pulse repetition rate = 50 Hz, pulse energy = 0.3 mJ/pulse. (D) 2(B−C) to subtract the equilibrium deoxyFixL* contribution from B. In obtaining D, the 210-cm^{-1} band of the equilibrium deoxyFixL* spectrum (C) was used as an indicator of when the equilibrium deoxyFixL* contributions had been removed.

The heme-CO features in Figure 1B and 1D arise from unphotolyzed or recombined FixL*-CO. Bands characteristic of the heme-CO adduct are also present in the high-frequency photolyzed FixL*-CO spectra (ν_3, 1494 cm^{-1}; ν_4, 1370 cm^{-1}).

Further examination of the difference spectrum reveals that no rR intensity attributable to propionate and vinyl bending vibrations[17] is observed at frequencies characteristic of equilibrium deoxyFixL*.[18] The absence of bands at these frequencies is consistent with an unrelaxed heme periphery and suggests that the nonbonded contacts between these groups and the putative regulatory FG loop have not yet responded to the change in ligation state. This combination of Fe−His and peripheral heme

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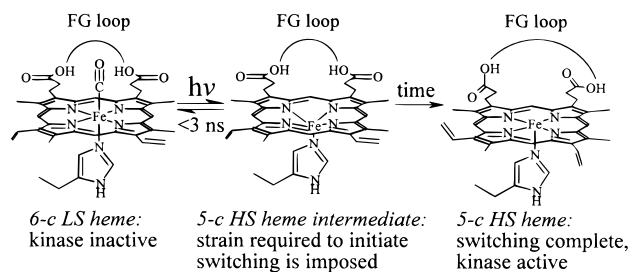(20) Scheidt, R. W., Gouterman, M., Lever A. B. P., Gray H. B., Eds. *Iron Porphyrins* Part One; Addison-Wesley: Reading, Massuchusetts, 1983; pp 89−140.
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Scheme 1

6-c LS heme: kinase inactive

5-c HS heme intermediate: strain required to initiate switching is imposed

5-c HS heme: switching complete, kinase active

frequencies suggests that, even though the Fe−His unit has responded to photolysis within the 3-ns laser pulse, the heme periphery, which makes van der Waals contacts with the hydrophobic interior of the PAS fold (vinyl groups) and H-bonding contacts with the hydrophillic FG loop[6] (propionate groups), has not undergone its ligation-coupled conformational transition. The analogously generated FixLN difference spectrum reveals the same upshifted ν_{Fe-His} band (data not shown). Hence, the conformational properties of the intermediate appear to be independent of any interactions between heme and kinase domains.

The mechanistic implications of this switching intermediate provide an insightful compliment to the crystal structures. Two properties of LS→HS heme conversions argue for a compressed Fe−His bond in the 218-cm^{-1} intermediate. First, since photolysis of heme−CO adducts elicits ultrafast conversion from LS to HS ferrous hemes,[19] it is reasonable to expect that the intermediate *Rm*FixL species observed here is HS. This is corroborated by the absence of a LS ν_3 band after subtraction of the heme-CO contribution from the high-frequency spectrum (single ν_3, 1476 cm^{-1}; ν_4, 1353 cm^{-1}). Second, Fe−N$_{pyrrole}$[20] and Fe−His[21] bonds are generally longer in HS hemes than in their LS analogues (for Hb[21a] and HbCO[21b] Δr_{Fe-His} = 0.086 Å (α) and 0.068 Å (β)). Badger's rule[22] yields an estimated 0.07-Å difference in Fe−His bond length corresponding to the 8-cm^{-1} difference in ν_{Fe-His} reported here. This difference is close to that between LS R-state HbCO[21b] and HS T-state deoxyHb[21a] and between *Rm*FixLT-CO and deoxy *Rm*FixLT (0.03 Å),[23] suggesting that the ferrous *Rm*FixL intermediate has an Fe−His bond length similar to that of the equilibrium CO form. Hence, the model illustrated in Scheme 1 is proposed. The spin state change occurring upon ligand loss leaves a ferrous HS heme with a LS-like Fe−His bond length. In this model, transient Fe−His bond compression provides the energy required to drive the protein conformational motion thought to be necessary for signal transmission. Although the Fe−His bond lengths in the ferric HS and LS *Bj*FixL heme domains are the same,[6] the transient 218-cm^{-1} ν_{Fe-His} band reported here for *Rm*FixL is consistent with the Fe−His bond being stronger, and likely shorter, in the intermediate than in the equilibrium deoxyFixL*/LN. This behavior is similar to that observed in the Hb−CO,[16a,b] cyctochrome c oxidase−CO,[16c,d] and sGC−CO[16e] photoproducts, wherein the proximal Fe−His bonds are strengthened in response to the loss of CO.[16] Hence, FixL appears similar to other heme proteins whose heme spin state and coordination number are coupled to functionally relevant conformational reorganizations. Moreover, given these data and the direct correlation between heme−His distance and kinase activity,[23] it is reasonable to hypothesize that FixL's ability to generate transient strain in its Fe−His bond provides one means of propagating conformational motion to its kinase domain in response to changes in O$_2$ tension.