Nature of Endogenous Ligand Binding to Heme Iron in Oxygen Sensor FixL

Koji Tamura,*[,†] Hiro Nakamura,[†] Yuji Tanaka,[§] Soichi Oue,[⊥] Koki Tsukamoto,[∥] Masaharu Nomura,[‡] Terumasa Tsuchiya,[#] Shin-ichi Adachi,[†] Satoshi Takahashi,[†] Tetsutaro Iizuka,[†] and Yoshitsugu Shiro[†]

The Institute of Physical and Chemical Research (RIKEN) Wako, Saitama 351-01, Japan
Department of Applied Chemistry, Chuo University Bunkyo-ku, Tokyo 112, Japan
Faculty of Science, Gakushuin University Toshima-ku, Tokyo 170, Japan
College of Science and Technology, Nihon University Chiyoda-ku, Tokyo 101, Japan
Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Ibaraki 305, Japan
Department of Molecular Genetics Medical Research Institute Tokyo Medical and Dental University Bunkyo-ku, Tokyo 113, Japan

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FixL is a protein sensing the O_2 level in plant root nodules to regulate the transcription of nitrogen fixation genes (*nif* and *fix*) *via* a phosphorylation of transcriptional activator, FixJ.[1] *Rhizobium meliloti* FixL with a molecular mass of about 55 kDa consists of three functionally separated domains, i.e., transmembrane, heme-binding, and kinase domains.[2] Binding of O_2 at the heme iron is related to the regulation of the kinase activity, i.e., the kinase is inactive in the O_2-bound (oxy) form of the heme domain, while it is active in the O_2-unbound (deoxy) form. This implies that the conformational change in the heme domain induced by the O_2 dissociation is closely linked to the activation of the kinase domain. However, no structural information has been available so far which provides molecular bases to understand the mechanism of intramolecular signal transduction from the heme to the kinase domain in FixL. In the present study, we characterized the coordination structure of the heme iron of water-soluble *R. meliloti* FixL (FixL* and FixLN)[3] using resonance Raman as well as EPR and EXAFS spectroscopic techniques.

It is well established that resonance Raman spectra of hemoproteins exhibit several totally symmetric modes that are sensitive to the redox state, spin state, and coordination structure of the heme. The useful lines are ν_2, ν_3, and ν_4 that appear between 1560−1590, 1460−1510, and 1350−1380 cm^{-1}, respectively.[4a] Resonance Raman spectra of the O_2-bound form of FixL* (oxy-FixL*) are shown in traces B and C of Figure 1 for the $^{16}O_2$ and the $^{18}O_2$ derivatives, respectively.[5] In the high-frequency region, the ν_4 line (the redox state marker) was observed at 1376 cm^{-1}, similar to those of the ferrous O_2-bound hemoproteins such as oxymyoglobin (Mb-O_2). The frequencies of the ν_2 and ν_3 modes were located at 1577 and 1502 cm^{-1}, respectively, which were slightly lower than the corresponding ones of Mb-O_2 (1583 cm^{-1} for ν_2 and 1505 cm^{-1} for ν_3). Both of the 1577 and 1502 cm^{-1} lines are polarized (data not shown), confirming the assignment that the lines are from the totally symmetric modes. The observation suggested that the porphyrin core of oxy-FixL* is expanded, compared with that of Mb-O_2, since the ν_2 and ν_3 frequencies are linearly correlated with the distances between the porphyrin center to its nitrogen.[6] In the low-frequency region, we identified the stretching vibration of the Fe−O_2 bond on the basis of the difference spectrum between the $^{16}O_2$ and the $^{18}O_2$ derivatives (trace D), where a dispersive pattern was observed around 550 cm^{-1}. The line at 571 cm^{-1} was assigned to the Fe−O_2 stretching mode for the $^{16}O_2$-bound form of FixL*. The frequency is essentially the same as those observed for Mb-O_2 and HbA-O_2,[7] suggesting that the bond strength in the Fe-O_2 moiety in FixL* is similar to those in Mb-O_2 and HbA-O_2.

In trace A of Figure 1, the resonance Raman spectrum of FixL* in the deoxy state (deoxy-FixL*) is illustrated. In the high-frequency region, the ν_4 line of deoxy-FixL* was observed at the same position (1355 cm^{-1}) as that of deoxy-Mb, while the ν_2 (1558 cm^{-1}) and ν_3 (1470 cm^{-1}) lines were located in a lower region than the corresponding ones (1562 and 1471 cm^{-1}, respectively) for deoxy-Mb. The frequencies of the ν_2 and ν_3 lines indicate that the heme iron in deoxy-FixL* is in a ferrous high spin and a five-coordination state, but the porphyrin core size is slightly larger than that in deoxy-Mb. In the low-frequency region for deoxy-FixL*, the strong line is present at 209 cm^{-1}, which was undetectable in the spectrum of its O_2-bound form (see trace B). In general, the Fe−N_{His} stretching line is observable in the region from 200 to 230 cm^{-1} for the five-coordinated ferrous hemes having imidazole axial ligand[4b] but not for the six-coordinated ferrous hemes. We could therefore identify this line as that arising from the Fe−N_{His} stretching vibration in deoxy-FixL*. This finding is clearly indicative of coordination of the histidyl imidazole as a fifth ligand of the heme iron in FixL, in good agreement with the site-directed mutagenesis work by Monson *et al.*,[1e] which suggested His194 as a candidate of the heme fifth ligand. It

[†] RIKEN.
[‡] National Laboratory for High Energy Physics.
[§] Chuo University.
[⊥] Gakushuin University.
[∥] Nihon University.
[#] Tokyo Medical and Dental University.
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(3) FixL* is a soluble truncated domain of FixL (MW 46 kDa), in which the transmembrane domain is absent. We constructed the expression system of FixL* in *E. coli* using the FixL cDNA, which was a kind gift from Dr. D. Kahn (CNRS-INRA, France). Plasmid encoding FixL* was obtained as described[1b] except that the Thr128 codon of *fixL* was ligated into the blanted end of *Bam*HI site in pRSETA by Klenow fragment. FixLN is a truncated heme domain of FixL* (MW 19 kDa). Plasmid encoding FixLN was generated from the plasmid encoding FixL* as described.[2] FixL* was prepared from *E. coli* strain JM109(DE3) after induction with IPTG as follows: The supernatant by ultracentrifugation of the lysed cells suspended in buffer A (20 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5% (v/v) glycerol, 10 mM β-mercaptoethanol, and 1 mM PMSF) was applied to a DE52 (Whatman) column. FixL* was eluted with the buffer A containing 300 mM NaCl. It was applied onto a Ni-NTA agarose (QIAGEN) column and eluted with the gradient of imidazole (0−200 mM). Imidazole was removed by dialysis.

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(5) The resonance Raman spectra were measured with a single spectrophotometer (JASCO NR-1800) equipped with a cooled CCD camera (Princeton Instruments). The spectral slit width was about 5 cm^{-1}. The Raman shifts were calibrated on the basis of the spectrum of indene with the absolute accuracy of ±1 cm^{-1}. The excitation lasers were krypton (413.1 nm, Coherent) and helium−cadmium (441.6 nm, Kimmon) lasers, whose powers were adjusted at 10−20 mW at the sample point. The samples (ca. 50 μM) were sealed in a rotating cell (ca. 1000 rpm) and maintained slightly lower than room temperature during the measurements.

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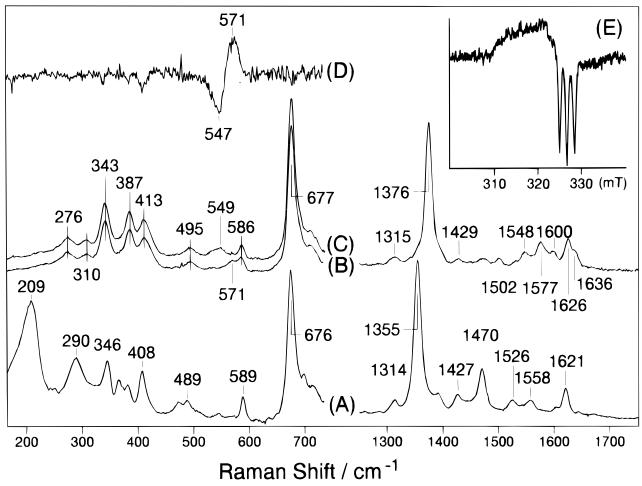

Figure 1. (A) Resonance Raman spectrum of FixL* in the deoxy state. The spectrum in the high-frequency region was excited at 413.1 nm, while that in the low-frequency region was obtained by 441.6 nm excitation: resonance Raman spectra of (B) $^{16}O_2$-bound and (C) $^{18}O_2$-bound FixL* excited at 413.1 nm; (D) difference spectrum between traces B and C; (E) EPR spectrum of the ferrous NO complex of FixLN at 20 K.

further demonstrated that the Fe−N_{His} stretching frequency is located much lower in deoxy-FixL* (209 cm^{-1}) than those in deoxy-Mb (220 cm^{-1}) and deoxy-HbA (221 cm^{-1} in the R state and 215 cm^{-1} in the T state),[8] showing that the Fe−N_{His} bond in deoxy-FixL* is weaker than those in deoxy-Mb and deoxy-HbA. In other words, the Fe−N_{His} bond in FixL* is under tension more than that even in deoxy-HbA in the T state.

We obtained three interesting features of the iron coordination structure for oxy- and deoxy-FixL*; as compared with Mb, the Fe−O_2 bond strength is similar, while the Fe−N_{His} bond in the deoxy state is weaker, and the porphyrin central core is expanded in both oxy and deoxy states. These structural features were also manifested in FixLN, the truncated heme domain of FixL*, since resonance Raman spectra obtained from the FixLN in the deoxy and oxy states are identical to the corresponding ones of FixL* (data not shown). Further support for the weak Fe−N_{His} bond was provided from the EPR spectrum of the ferrous NO complex of FixLN at 20 K (trace E of Figure 1), where three lines were obtained at the g_z absorption ($g_z = 2.0$, $A_N =$ 17 G).[9] This EPR feature is the same as that of the NO complex of the HbA α-chain in the T state, in which the NO-bound iron is in a five-coordination, indicating that the Fe−N_{His} bond in deoxy-FixLN is weak enough to be cleaved upon the NO coordination in the frozen state. In addition, we found by EXAFS measurement that the Fe−N_{His} bond distance in deoxy-FixLN (2.14 Å) is significantly longer than that in deoxy-Mb (2.10 Å).[10] The results from these different measurements, i.e. resonance Raman, EPR, and EXAFS, are well consistent with each other.

Our present findings remind us of the weak Fe−N_{His} bond (ν(Fe−N_{His}) = 203 cm^{-1}) in Hb from Mollusc *Scapharca inaequivalvis*, which is exerted by a steric interaction of the Fe-His moiety with Phe97. This interaction is significantly involved in cooperativity of this Hb.[12] On the other hand, we know that the conformational change caused by a change in the Fe−N_{His} bond plays a crucial role in the intramolecular signal transduction in HbA and guanylate cyclase.[13] The resonance Raman spectra of the cyanomet valency-hybrid Hb and the Fe−Co hybrid Hb revealed the weak Fe−N_{His} bond (ν(Fe−N_{His}) = 206−207 cm^{-1}) in their α-subunit,[14] which is closely involved in an allosteric effect of the molecule. From these facts, it is likely to suggest for FixL that the weak Fe−N_{His} bond might be related to the mechanism in the activation/inactivation of the kinase domain (catalytic site) upon the O_2 dissociation/association in the heme domain (regulatory site).

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(9) The EPR spectrum was obtained with a JEOL JES-RE 1X spectrometer equipped with an Air Products model LTR-3 Heli-tran cryostat system, in which the temperature was monitored with a Scientific Instruments series 5500 temperature indicator/controller. The FixL* concentration was about 100 μM in 20 mM sodium phosphate buffer (pH 5.9). Conditions of the measurement were frequency = 9.135 GHz, modulation frequency = 100 kHz, and modulation width = 0.1 mT. Its ferrous NO complex was prepared by reduction of the ferric FixLN with $Na_2S_2O_4$ in the presence of $NaNO_2$. Immediately after the preparation, the sample was frozen in liquid N_2. The optical absorption spectrum of the ferrous NO complex of FixLN at room temperature showed the six-coordination of the heme iron, with concomitant mixing of the five-coordination. However, the five-coordinated iron was predominant and the signal derived from the six-coordinated iron could hardly be seen in the frozen state at 20 K, as was manifested in the characteristic EPR feature.

(10) The fluoresence EXAFS spectrum for deoxy-FixLN (∼1 mM) was measured using monochromatized synchrotron radiation at the BL 12C of the Photon Factory in the National Laboratory for High Energy Physics (Tsukuba, Japan). The data were analyzed using EXCURV92 (Daresbury Laboratory).[11] Raw data and details for the data analysis for FixLN and FixL* in several oxidation/ligation states will be described elsewhere.

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